Cytoskeletal organization of axons in vertebrates and invertebrates

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eTOC: Five decades of ultrastructural studies reveal microtubule bundles as the essential common architectural element of vertebrate and invertebrate axons

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Abstract

Maintaining axons for an organism's lifetime requires a cytoskeleton that is robust, but also flexible to adapt to mechanical challenges and to support plastic changes of axon morphology. Furthermore, cytoskeletal organization has to adapt to axons of dramatically different dimensions, and to their compartment-specific requirements in the axon initial segment, in the axon shaft, at synapses or in growth cones. To understand how the cytoskeleton caters for these different demands, this review summarizes five decades of electron microscopic studies. It focuses on the organization of microtubules and neurofilaments in axon shafts in both vertebrate and invertebrate neurons, as well as the axon initial segments of vertebrate motor and interneurons. Findings from these ultrastructural studies are being interpreted here on the basis of our contemporary molecular understanding. They strongly suggest that axon architecture in animals as diverse as arthropods and vertebrates, are all dependent on loosely cross-linked bundles of microtubules running all along axons, with only minor roles played by neurofilaments.

Introduction

Axons are the cable-like processes of neurons that conduct nerve impulses and transmit this information at synapses to other cells. Axons can be up to a meter long in humans and potentially up to 30 m long in blue whales (Smith, 2009). Axons are delicate structures, subject to enormous mechanical challenges imposed from the outside (e.g. bending and stretching through body movement) and the inside (e.g. axonal transport of large cargoes). In spite of
these challenges, most axons cannot be replaced throughout an organism’s lifetime, and mammals lose up to 40% of their axon mass towards high age (Calkins, 2013; Marner et al., 2003). Combatting such deterioration requires active maintenance mechanisms; understanding them will provide new strategies to address the problem of axon loss in aging and disease (Hahn et al., 2019). To this end, we need detailed knowledge of axonal architecture.

An important property to be considered is that neurons and their axons can display substantial variations. For example, axon calibers can vary enormously between distinct neuron types (details below): the need to minimize space and energy demand favors thin fibers, whereas the need to send information at high velocity and/or spike frequencies requires large diameter fibers (Friede et al., 1984; Perge et al., 2009; Perge et al., 2012). Furthermore, neurons can be of different polarity (multipolar, unipolar, or pseudo-monopolar), depending on their animal species of origin and their functions as motor-, inter-, or sensory neurons (Fig.1A); whether axon architecture needs to be adapted to these different morphologies remains an open question.

Neurons usually display lengthy axon shafts (light blue in Fig. 1A) with a range of common features including organelles, continuous networks of smooth ER (endoplasmatic reticulum), and an extensive cytoskeleton (Hahn et al., 2019). The axonal cytoskeleton of invertebrates and vertebrates alike shows conserved features, such as central longitudinal F-actin trails and cortical F-actin rings arranged by spectrins into a periodic pattern (He et al., 2016; Leterrier et al., 2017a; Xu et al., 2013). Of these, the actin rings seem to have important roles in regulating axonal stiffness (Krieg et al., 2017), axon diameter (Costa et al., 2018; Costa et al., 2020) and microtubule (MT) polymerization (Qu et al., 2017). MTs of various lengths are organized into longitudinal parallel bundles that run all-along axons, often accompanied by intermediate filaments (neurofilaments, NFs). MTs have a key role as the essential highways for axonal transport, as structural backbones of axons, and as the source of dynamic MTs that can contribute to morphogenetic processes such as axon branching (Hahn et al., 2019). But do MTs perform these functions based on common mechanistic or organizational principles that apply to the wide range of neuron types and axon diameters across the animal kingdom? To which degree can MT functions be replaced by NFs, which are abundant in some organisms and completely absent in others (Peter and Stick, 2015)?

To address these questions, modern super-resolution imaging techniques provide us with powerful strategies (Leterrier et al., 2017a), but there are clear limitations. For example, resolving closely spaced MTs in axonal bundles remains a challenge (Mikhaylova et al., 2015; Tas et al., 2017). Therefore, electron microscopy (EM) analyses remain pivotal. However, the bulk of existing EM studies (performed mainly on axons of vertebrates, to a lesser degree on invertebrates; see below) dates back to the final three decades of the last century. Those studies were interpreted with the very limited molecular and mechanistic knowledge existing at the time. This review will therefore revisit those publications, and interpret classical structural findings in light of state-of-the-art molecular understanding. First, it will focus on axon initial segments (AIS; a specialized zone of the proximal axon in vertebrate motor- and interneurons; Fig.1B). It will then take a comparative look at the organization of MTs and NFs in the shafts of small and large caliber axons of vertebrate and invertebrate neurons to address whether and how different species of origin or size differences correlate with different concepts of axon architecture.

The AIS in vertebrate motor- and interneurons: its structure and molecular composition
To uphold the polarity of vertebrate inter- and motoneurons, components specific to the somato-dendritic compartment have to be prevented from entering the axon (Fig.1Ai). This is thought to involve a staggered filter system including the axon hillock (referred to as the pre-axonal exclusion zone, PAEZ; Farias et al., 2015; details in Fig.1B) and the highly specialized AIS (see below); even the remainder of the axon shaft appears to display filter properties, as illustrated by MAP2 which advances only poorly into the axon shaft if the AIS is abrogated (Jenkins et al., 2015).

The AIS is a specialization of vertebrate inter- and motoneurons which is tens of micrometers long and up to a few micrometers wide (Tab.S3), characterized by at least two prominent ultrastructural features reported consistently across different neuron types and animal species: parallel MT fascicles, and a conspicuous electron-dense undercoat of the plasmalemma (Tab.S3; Figs.1B and 2A-D). According to EM studies of Mitral cells in the mouse olfactory bulb, MT fascicles are not fully established until the end of embryogenesis and the dense undercoat not until days after birth (Hinds, 1973 #10090).

These AIS-specific structural features correlate with reports of proteins enriched at the AIS, usually appearing after axons have formed; they include the large cortical scaffold proteins AnkG (ankyrin-G) and non-erythrocytic β4-spectrin, filament-forming septins, the MT-binding ring finger protein TRIM46 (tripartite motif-containing 46), the MT-binding factor EB3 (end-binding protein 3), the dynein regulator Ndel1 (neurodevelopment protein 1 like 1), the actin regulator Mical3 (Molecule Interacting with CasL), cell adhesion molecules such as Neurofascin-186, and voltage-gated sodium channels and potassium channels; many of these components, as well as myosin 2 activity, were shown to contribute to AIR formation and maintenance (Berger et al., 2018; Freal et al., 2019; Hamdan et al., 2020; Hedstrom et al., 2008; Jenkins et al., 2015; Kuijpers et al., 2016; Leterrier et al., 2017b; Salzer, 2019; Sobotzik et al., 2009). Several of these proteins can be clearly assigned to the two ultrastructural specializations:

Fasciculated MTs are connected via ~25 nm long electron-dense lateral bridges, thus forming sheath-like arrangements (curved arrows in Fig.3C) that appear like pearls on a string when analyzed in axonal cross sections (Fig.2A-D). The sizes of these fascicles are extremely variable, as illustrated by examples in Fig. 2 A-D that show between 2 and 23 MTs per fascicle (see also Palay et al., 1968). The electron-dense lateral bridges contain and require TRIM46 (Fig.3B) which is widely expressed in the nervous system (Harterink et al., 2019; van Beuningen et al., 2015). Also the MT-MT cross-linker MTCL1 (microtubule cross-linking factor 1) was shown to promote MT fascicle alignment; however, its role is restricted to the AIS of Purkinje cells (Satake et al., 2017), leaving open how MTCL1 functionally relates to TRIM46 which is likewise present in this cell type (van Beuningen et al., 2015).

The structure of the electron-dense undercoat was described in a model (Fig.3A; Chan-Palay, 1972) that agrees with various independent TEM (transmission EM) studies (Conradi, 1969; Kosaka, 1980a; Kosaka, 1980b; Nakajima, 1974). In its radial dimension, the TEM model matches well with a model based on super-resolution microscopy of AIS-enriched proteins (Fig.3B; Leterrier et al., 2015). However, in the longitudinal dimension, both models diverge: the TEM model proposes a repetitive pattern with a periodicity of 60-80 nm (Fig.3A; arrow head in Fig.5b of Nakajima, 1974); in contrast, numerous super-resolution analyses have built a convincing case for a periodicity of ~190 nm, which I could not find reflected in any TEM reports (Fig.3B; Huang and Rasband, 2018; Jacko et al., 2018; Jones and Svitkina, 2016; Leterrier, 2018; Leterrier et al., 2017a; Schützer et al., 2019; Sohn et al., 2019). To explain this mismatch,
refined EM studies will be required.

Functional roles of the AIS of vertebrate motor- and interneurons

Already 50 years ago, the MT fascicles at the AIS were discussed to be required for "protoplasmic streaming into the axon", and the dense undercoats for the "production of the electrical signal" (Palay et al., 1968). Today we know that AIS specializations help to filter somato-dendritic components (preventing them from diffusing or being transported into axons), and establish the spike initiation zone (SIZ) where action potentials are initiated:

Firstly, the AIS serves as a diffusion barrier for non-axonal lipids and transmembrane proteins (Kobayashi et al., 1992; Nakada et al., 2003; Winckler et al., 1999), potentially requiring components of the dense undercoat such as cortical actin rings, AnkG and ß4-spectrin (Albrecht et al., 2016; Machnicka et al., 2014; reviewed in Huang and Rasband, 2018).

Secondly, the filtering of transported somato-dendritic cargo at the AIS was proposed to involve actin patches or polarized actin networks, which are regulated by Mical3 and mediate the reversal of somato-dendritic cargo transport via the actin-based motor protein myosin-Va (Al-Bassam et al., 2012; Balasanyan et al., 2017; Hamdan et al., 2020; Lewis et al., 2009; Watanabe et al., 2012). This said, other studies have questioned the existence of adequate actin networks at the AIS (Jones et al., 2014; Jones and Svitkina, 2016). Alternatively or in addition, MT-based mechanisms were proposed that involve the selective activation of dynein-mediated retrograde transport through Ndel1 (Huang and Rasband, 2018; Leterrier, 2018; Kuijpers et al., 2016; Ye et al., 2020). Notably, both actin- and MT-based filtering mechanisms seem to involve a tug-of-war between motor proteins that pull in opposite directions, potentially strong enough to cause MT disorganization if thrown out of balance (Kuijpers et al., 2016). Such mechanical forces likely require a more stable architecture, potentially explaining why MTs arrange into tight fascicles at the AIS.

Furthermore, it has been shown that MTs at the AIS are anchored to the axonal surface through binding via EB3 to the tail domains of AnkG (Fig.3B; Freal et al., 2019; Leterrier, 2018; Vassilopoulos et al., 2019); this interaction is essential for MT fasciculation (Sobotzik et al., 2009). Furthermore, the dynein regulator Ndel1 binds to the AnkG tail (asterisks in Fig.3B), and this interaction is essential for AIS filter functions (Ye et al., 2020). In agreement with these functional studies, EM analyses suggest that a fraction of MTs are in contact with the undercoat (Fig.3C). However, many MT fascicles are positioned centrally in the axon, clearly out of reach of AnkG (Fig.3D). This raises the question as to whether only peripheral MT fascicles are involved in transport filtering and whether central MT fascicles drift freely in the axoplasm or are linked up via inconspicuous linkers that ultimately depend on AnkG.

Thirdly, the AIS is a spike initiation zone (SIZ), meaning it triggers action potentials as the integrated outcome of supra-threshold depolarizations propagating from the somato-dendritic compartment (Kole and Stuart, 2012). This function requires a well-balanced enrichment of voltage-gated sodium and potassium channels at the AIS, which are likewise anchored via AnkG (NaV and Kv in Fig. 3B; Akin et al., 2015; Hefting et al., 2020). Importantly, neurons can fine-tune their excitability by changing the length of the AIS, the composition and posttranslational modification of ion channels, or the distance from the soma - through mechanisms that involve calcium/calciuneurin, MT stabilization and Myosin II (Berger et al., 2018; Bolós et al., 2019; Evans et al., 2015; Evans et al., 2017; Grubb and Burrone, 2010; Hatch et al., 2017; Kuba et al., 2014; Kuba et al., 2010; Kuba et al., 2015).
As a further means to regulate excitability, the AISs of various neuron types harbor synaptic contacts, in their majority inhibitory input synapses (Fig.2C,E; Tab.S3). Their formation can be mediated by AnkG-dependent recruitment of the adhesion factor neurofascin-186 (Nfas in Fig.3B; Ango et al., 04; Kriebel et al., 2011) and is regulated by synaptic activity (Pan-Vazquez et al., 2020). As listed in Tab. S1, many AIS descriptions highlight the occurrence of varicose spines of variable lengths and shapes (Fig.2E,F; see also Sobotzik et al., 2009) which seem to lack MTs and sometimes harbor more than one synapse. Beneath input synapses or spines the dense undercoat is consistently reported to be interrupted (Fig.2C) and many authors mention the presence of cisternal organelles (Tab.S3) that bear some resemblance with the spine apparatus (i.e. stacks of ER required for synaptic plasticity; Deller et al., 2003).

Do AIS features apply to other neuron classes?
In accordance with the polarity of vertebrate motor- and interneurons, the AIS is both a filter zone and a SIZ (Fig.1Ai). A similar combined compartment might be expected in the initial axon segment of arthropod sensory neurons (which have similar polarity; Fig.1Avi), but not in vertebrate sensory neurons or arthropod motor- and interneurons (Fig.1Aii and iii).

Unfortunately, only few data are available for those neuron classes (legend of Fig.1A; Smaranadache-Wellmann, 2016). For example, sensory neurons of vertebrates (e.g. dorsal root/DRG or trigeminal ganglion neurons) form a stem axon which then bifurcates into a central and peripheral axon arm (Fig.1Aii; Nascimento et al., 2018). Stem axons display fasciculated MTs comparable to the AIS, whereas the electron-dense undercoat is absent (Fig. 2G; Bird and Lieberman, 1976; Ishikawa et al., 1980; Peach, 1975; Zelená, 1971). Only one report mentions an extremely short (2-4 µm) stretch of membrane with an electron-dense undercoat (Zenker and Högl, 1976).

Consistent with the presence of MT fascicles and similar to the AIS, the stem axon displays TRIM46 and, proximal to it, MAP2 (Fig. 1Ai versus ii; Gumy et al., 2017; van Beuningen et al., 2015). These proteins may represent a staggered PAEZ/AIS-like filter system (see above), since MAP2 is required to block somatic organelles from entering DRG axons; in addition MAP2 selectively activates kinesin-mediated transport, in line with a recently proposed ‘MAP code’ for the regulation of polarized MT-based traffic (Gumy et al., 2017; Monroy et al., 2020); these roles of MAP2 demonstrated at stem axons may similarly apply to the axon hillock. Outside the stem axon, vertebrate sensory neurons likely have further filter zones (Fig.1Aii), but little is known at present.

Consistent with the lack of the electron-dense undercoat (Fig. 2F), stem axons do not show enrichment of AnkG, β4-spectrin or sodium channels (Fig. 1Ai versus Aii; Gumy et al., 2017). This suggests that MT fascicles can form independently of AnkG, and that stem axons have no SIZ functionality. Instead, electrophysiological studies have shown that the SIZ of DRGs is located in the peripheral axon, slightly removed from the dendritic compartment (Fig.1Aiii); similar to the AIS, this peripheral SIZ is dynamic and moves closer to the dendrites upon sensitization (Goldstein et al., 2019). Whether it displays AnkG expression and/or an electron-dense undercoat remains to be seen.

Nodes of Ranvier (NoR) are the small unmyelinated segments along myelinated axons required for saltatory conduction (Fig.1Ai). NoRs display AIS-like undercoats (15-25 nm thick, 5-10 nm separated from plasmalemma) but no obvious MT fasciculation (Fig. 2E; Conradi and Skoglund, 1969; Waxman, 1972; Elfvin, 1961; Kosaka, 1980b; Peters, 1966; Reles and Friede, 1991;
Tsukita and Ishikawa, 1981; p.104 and 260ff. in Peters et al., 1991). Only the most proximal
NoRs in DRG neurons seem to display MT fascicles, potentially due to spill-over of cross-linkers
from the stem axon (Nakazawa and Ishikawa, 1995). Ethanololic phosphotungstic acid (an
anionic heavy metal complex known to label basic proteins at synaptic densities; Jones, 1993)
was reported to label the undercoats at both NoR and AIS suggesting molecular commonalities
(Sloper and Powell, 1973; Sloper and Powell 1979). We now know that NoRs lack TRIM-46
consistent with the absence of MT fascication (van Beuningen et al., 2015), but express
characteristic AIS proteins regulating excitability, including AnkG, β4-spectrin, neurofascin-186,
Nr-CAM and high levels of NaV (Fig.1Ai; Kordeli et al., 1995; Yang et al., 2007; Dzhashiashvili
et al., 2007). A further site where AIS-like undercoats were shown to correlate with the
expression of at least AnkG are the ‘hot spots’ in dendrites of the rat olfactory bulb (Kosaka et
al., 2008).

Taken together, key features coinciding at the AIS can occur in separation in other neuron types
or compartments. Therefore, using the term AIS for neurons other than vertebrate motor- or
interneurons might be misleading; filter zones, diffusion barriers, or SIZs are expected to be
distributed differently in neurons of other polarity (Fig.1A), and whether their molecular
mechanisms match those at the AIS remains to be seen.

The numbers and lengths of axonal MTs vary as a function of neuron type and organism

The axon shaft is the longest neuronal compartment and can display enormous structural
variations (Tab.4). With regard to axon length, it was originally believed that all axonal MTs
extend from the soma. This was supported by studies in rat sensory and motor fibers showing
that MT numbers of the main axon are equal to the added numbers of its daughter neurites, as
if MTs at branch points merely decide whether to continue into one or the other branch (e.g.
Weiss and Mayr, 1971a). This view was disputed by other findings: for example, in certain rat
and crayfish motorneurons the sum of cross-sectional areas of daughter branches was found to
increase up to ~10-fold, accompanied by substantial increases in MT counts (Nadelhaft, 1974;
Zenker and Hohberg, 1973). This clearly indicated that new MTs arise within the axon, and we
now know that nucleation, the seeding of new MTs, occurs in the axon shaft (Baas and Ahmad,
1992; Sanchez-Huertas et al., 2016; Stiess et al., 2010). Anchoring mechanisms that orient
nucleation events towards the axon tip (Cunha-Ferreira et al., 2018) ensure that MTs have a
consistent parallel polarity in axons (Baas et al., 1988; Baas and Lin, 2011; Yau et al., 2016).

Little data is available describing the actual lengths of MTs in axons. From serial TEM analyses
of fairly short axon fragments a range of MT lengths was estimated: 430 μm / 97 μm (frog,
olfactory axons in the olfactory nerve / olfactory bulb; Burton, 1987), 108 μm (rat, cultured
sensory nerves; Bray and Bunge, 1981), 513-757 μm / 374 μm (mouse, internodal / nodal
regions of sensory saphenous nerve; Tsukita and Ishikawa, 1981) and 9-23 μm / ~5 μm / ~6.5
μm (C. elegans, mechanosensory axons / axons of GABAergic dorsal D-type neurons / other
axons; Chalfie and Thomson, 1979; Kurup et al., 2015). In agreement with the EM data for C.
elegans, advanced live imaging strategies reported ~6 μm long MTs in axons of DA9
motorneurons (Yoge et al., 2016). Serial TEM analyses in both C. elegans and rat also
reported the direct observation of very short MTs (1-4 μm), which might represent MT fragments
undergoing transport (Baas, 2002; Baas et al., 2006). Notably, developing rat hippocampal
neurons in culture overwhelmingly showed short fragments (range: 0.05-40 μm, average: 4 ± 5
μm; Yu and Baas, 1994), which might reflect high MT dynamics at the axon growth stage.
The length of MTs potentially impacts axonal transport dynamics (Yogev et al., 2016). But likely more important is the number and density of MTs which, according to mathematical modelling, correlates with transport volume (Wortman et al., 2014). This has been most convincingly studied in dorsal root ganglion neurons (Fig. 1Aii) where the peripheral axon branch has a larger caliber, displays more MTs and has a higher transport rate than is observed in the central branch of the same neurons (reviewed in Nascimento et al., 2018).

Across the animal kingdom, axon diameters were reported to be in the range of 0.1-30 μm in vertebrates, 0.1 to >100 μm in arthropods (giant axons of some spiders, crustaceans, and cockroaches; Castel et al., 1976; Foelix and Troyer, 1980; Nadelhaft, 1974), and up to a millimeter in squid (as the result of axonal fusion of 300-1500 neurons; Young, 1936; Brown and Lasek, 1990). For axons of vertebrates and arthropods, MT numbers per profile area range between 1 and >250,000 MTs and their densities between 2 and ~100 MTs/μm² (Tab. S4), with a reported outliers far above 100 (Fadić et al., 1985).

In light of these enormous ranges, the question arises as to whether the architectural challenges posed by different axon diameters require specific solutions or can be accommodated by common organizational concepts.

In small caliber axon shafts of vertebrate and invertebrates, MTs are spaced by MAPs

Small-caliber axons (<300 nm diameter) can be found in most species (Fig. 4A,B,G,H), for example in the olfactory bulb or the molecular layer of the cerebellum of vertebrates (Andres, 1965a; Harada et al., 1994; Kreutzberg and Gross, 1977; Peters et al., 1991), peripheral nerves and the ventral nerve cord of insects (Egger et al., 1997; Stephan et al., 2015), or nerve tracts of *C. elegans* (Chalfie and Thomson, 1979; Chalfie and Thomson, 1982). In these examples, MTs are distributed across the axonal profile; they are spaced from each other but appear only loosely cross-linked. Accordingly, conventional TEM strategies do not reveal conspicuous MT-MT connections as observed at the AIS. However, linkers clearly exist and become visible upon use of other strategies. For example, lanthanum is a trivalent cation that penetrates cell membranes if applied during fixation and severely enhances contrast of many subcellular structures upon TEM analysis (Leeson, 1982 #10475; Shaklai, 1982 #10476). In lanthanum-stained axons, MTs display irregular coating and fuzzy connections become visible between them (Burton and Fernandez, 1973; Lane and Treherne, 1970; Ochs and Burton, 1980). In classical freeze-etch preparations, nerves were detergent-extracted, quick-frozen, mechanically fractured to expose the inside, etched via freeze-drying and then rotary-shadowed with platinum/carbon to produce a replica for analysis. Freeze-etch preparations clearly revealed 4-6 nm thick MT-MT cross-bridges (Chen et al., 1992; Harada et al., 1994; Hirokawa, 1982; Hirokawa, 1991).

Although nearest-neighbor MT-MT distances are not constant, their means show reproducible values that differ between neuron types and species (e.g. Fig. 4A,F,G): MTs are spaced on average ~22 nm in parallel fibers of the mouse cerebellum (Chen et al., 1992), ~110 nm in *Drosophila* (Egger et al., 1997; Stephan et al., 2015), and 9.8 nm in *C. elegans* (Krieg et al., 2017). In all these cases, loss-of-function studies have revealed specific MT-associated proteins (MAPs) as MT-MT cross-linker candidates: loss of the *Drosophila* MAP1b homologue Futsch caused a reduction in nearest-neighbor values from ~110 to ~70 nm (Bettencourt da Cruz et al., 2005; Stephan et al., 2015), loss of ptl-1 (the *C. elegans* tau homologue) a reduction from 9.8 to 6.7 nm (Krieg et al., 2017), and loss of murine Mapt/tau abolished the majority of cross-bridges.
in freeze-etch preparations (Harada et al., 1994).

Differences in MT spacing become meaningful when comparing the longest isoforms of three MAPs (all data taken from ensembl.org): ptl-1 (458 aa) is shorter than tau (749 aa), whereas Futsch is dramatically longer (5495 aa). Futsch has a large repeat-rich central domain and its terminal regions show homology with the N- and C-terminal parts of human MAP1B, excluding however the two designated MT-binding domains (Fig. S1; Hummel et al., 2000; Villarroel-Campos and Gonzalez-Billault, 2014). It remains therefore to be seen whether Futsch achieves direct or indirect MT-association through its terminal homology regions, which could mean that a single molecule could act as a MT-MT cross-linker.

In contrast, tau proteins binding to adjacent MTs were proposed to dimerize via their N-termini (Rosenberg et al., 2008) - and the N-termini are clearly longer in tau (N-term ~450) than in ptl-1 (N-term ~300). A more recent model for tau-mediated MT-MT cross-linkage is less static and allows tau to diffuse (Méphon-Gaspard et al., 2016), consistent with observations that tau has a very short dwell time on MTs (Janning et al., 2014; Samsonov et al., 2004). Such loose binding dynamics might be one explanations for why MTs are not firmly interlinked into fascicles or hexagonal patterns but are free to detach and 'diffuse' into empty areas of the axonal profile. This said, at high expression levels MAPs can very well induce hexagonal MT patterns (Chen et al., 1992), suggesting that MAP levels are well controlled in axons.

Surprisingly, the loss of specific MAPs (see above) did not cause a complete collapse of MTs onto each other. This may be explained by repulsion due to the high negative surface charge of MTs lattices, but more likely through the expression of potentially redundant MAPs, as reviewed elsewhere (Bodakuntla et al., 2019; Burgoyne, 1991; Hahn et al., 2019; Hirokawa, 1991; Lee and Brandt, 1992; Matus, 1991). As a further complication, MAPs may change their affinities for MTs as a function of posttranslational tubulin modifications or tubulin isotypes. For example, poly-glutamylation regulates binding of tau to MTs (Boucher et al., 1994). Furthermore, the average MT-MT distance of 9.8 nm in C. elegans (see above) was measured in mechanosensory neurons expressing mec-7 β-tubulin; upon mec7 deficiency, the same mechanosensory axons form MTs of smaller diameter which display a reduced spacing of 4 nm (Chalfie and Thomson, 1982; Krieg et al., 2017), suggesting that MTs with different tubulin isotypes are cross-linked differently.

In arthropods, the shafts of small and large caliber axons form identical MT networks

The organizational principle described for small caliber axons appears to be maintained in large diameter axons of crustaceans, insects, or spiders: MTs are distributed across the axon profile and, where data are given, nearest-neighbor distances are typically 100-200 nm (Fig.4G,H; Bettencourt da Cruz et al., 2005; Burton and Fernandez, 1973; Egger et al., 1997; Foelix and Hebets, 2001; Foelix and Troyer, 1980; King and Wyman, 1980; Lane and Abbott, 1975; Nadelhaft, 1974; Smith et al., 1977; Stephan et al., 2015; Viancour et al., 1987; Warren, 1984).

In crustaceans, lanthanum staining revealed "axoplasmic matrix proteins" in the form of 5-10 nm thick MT-MT cross-bridges (Burton and Fernandez, 1973; Ochs and Burton, 1980). Upon extraction with glycerol-sucrose-DMSO solution, MT-MT distances were seen to collapse and the extracted protein was of high weight and heat-stable, as is typical of MAPs (Warren, 1984).

An anti-neurofilament antibody (clone NN18) stains the cross-bridges and detects a 600 kDa protein with homology to MAP1B (Weaver and Viancour, 1991; Weaver and Viancour, 1992; but see Correa et al., 2004).
These data match very well with those for the *Drosophila* Futsch protein: like observed in crustaceans, Futsch spaces MTs at ~110 nm (see above; Stephan et al., 2015), displays MAP1B homology, and its central domain is composed of 60 repeats with intermediate filament homology (Hummel et al., 2000). In support of this notion, arthropod genomes including crayfish (asterisk in Fig.S2) tend to contain Futsch-like genes, which display homology with Futsch in exactly those N- and C-terminal regions that are also homologous to human MAP1B (Fig.S1).

In addition to MT-MT linkers, cross-bridges from MTs to the cortex were also reported in crayfish (Burton and Fernandez, 1973). Extrapolating from work in *Drosophila*, these might be large ankyrins. Thus, long isoforms of cortex-associated Ankyrin2 joins forces with Futsch to form MT-MT networks that help to expand and maintain axon calibers in *Drosophila* (Stephan et al., 2015). Also in *C. elegans*, large ankyrins seem to anchor MTs along the axon shaft (He et al., 2019 Preprint), and large AnkB isoforms appear to perform similar functions along axon shafts in mouse (Yang et al., 2019), consistent with reports of ~100 nm long filaments linking the plasmalemma to MTs in freeze-etch preparations of frog axons (Hirokawa, 1982). For a discussion of further candidates involved in surface anchorage of MTs see Hahn et al. (2019).

Taken together, different arthropod species seem to use a consistent strategy of MT-MT cross-linkage across a wide range of axon diameters (~0.1 to ~60µm). Although Futsch-like proteins seem the most prominent players, further MAPs such as Tau or Enscnsin, or even type 6 kinesins might contribute to these networks (Hahn, 2016; Monroy et al., 2018).

**In vertebrates, NFs contribute to caliber enlargement of axons**

A major difference between axons of arthropods and vertebrates is the presence of neurofilaments (NFs). Cyttoplasmic intermediate filaments are clearly absent in arthropods (Peter and Stick, 2015), whereas NFs are highly abundant in large caliber axons of vertebrates where they are spaced out and distributed in a similar manner as described for MTs (Fig.4D,E; Hirokawa, 1991; Peters et al., 1991).

NFs of vertebrates are 10 nm thick, highly stretch-resistant intermediate filaments composed of a chain of hetero-oligomeric subunits (Fig.S2). Each subunit contains a mix of type IV proteins encoded by different genes (Fig.S2A; Herrmann and Aebi, 2016; Kornreich et al., 2015; Perrot et al., 2008), usually including a light weight (α-internexin, 66 kDa or NF-L, 62 kDa), medium weight (NF-M, 103 kDa), and heavy weight (NF-H, 117 kDa) protein, from now referred to as α, L, M, H. These proteins can co-assemble with type III peripherin (Yuan et al., 2012). In small unmyelinated fibers, peripherin even seems to be the predominant NF component (Lariviere et al., 2002). However, in large myelinated fibers neither loss nor gain of peripherin have revealed major impacts on NF formation - although long-term overexpression triggers ALS (amyotrophic lateral sclerosis)-like axonopathy (Beaulieu et al., 1999; Lariviere et al., 2002).

NFs promote large axon diameters, as is clearly demonstrated by axon caliber reduction in NF-deficient mouse and quail models or human patients (Eyer and Peterson, 1994; Sakaguchi et al., 1993; Yamasaki et al., 1991; Yum et al., 2009). H, M, L, and α seem to contribute to different degrees: calibers of large axons were strongly reduced and NFs virtually absent in L− mice, quail and humans, whereas both parameters were only moderately affected in M− mice, and marginally in H− and α− mice (Beaulieu et al., 1999; Elder et al., 1998; Jacomy et al., 1999; Levasseur et al., 1999; Ohara et al., 1993; Yamasaki et al., 1992; Yum et al., 2009; Zhu et al., 1997; Zhu et al., 1998). However, masked redundant roles of H and α became apparent in H−
M\textsuperscript{+} or α\textsuperscript{+} L\textsuperscript{+} double-mutant mice, which displayed complete or next to complete NF depletion (Jacomy et al., 1999; Levavasseur et al., 1999). Importantly, NF proteins have to be present in proportional amounts: overexpression of M or H (but not L) caused axon caliber reduction, even further enhanced upon MH co-expression; in contrast, LM or LH co-expressions caused caliber increases (Xu et al., 1996).

Consistent with the knock-out data, systematic tail domain deletions (\textsuperscript{tail\textendash}) in transgenic mice revealed a reduction in cross-bridge numbers in axons of H\textsuperscript{tail\textendash} or M\textsuperscript{tail\textendash} mice, but only for M\textsuperscript{tail\textendash} this resulted in narrower axons and slightly shorter NF spacing (39 nm); in M\textsuperscript{tail\textendash} H\textsuperscript{tail\textendash} double-mutant mice NF-NF side-bridges were absent and axon diameters were further reduced (Garcia et al., 2003; Rao et al., 2002), lending further support to the idea of redundancy between M and H. Surprisingly, M\textsuperscript{tail\textendash} H\textsuperscript{tail\textendash} filaments still ran in parallel to the axon axis and maintained a spacing of ~30 nm, likely mediated by the shorter tail domains of L and/or α (Kornreich et al., 2015). Consistent with these findings, application of osmotic shock induced by high sucrose in axons or polyethylene glycol \textit{in vitro} (likely abolishing charge-dependent extensions in particular of H and M tails), caused a severe reduction in NF spacing but not a total collapse (Friede and Samorajski, 1970; Kornreich et al., 2015).

Therefore, in contrast to modular MT-MT coupling which depends on separately encoded MAPs, NF-NF linkage and spacing is a function of the filaments themselves and can be regulated through phosphorylation. It appears that their cross-linking affinities are low enough so that NF-NF networks can undergo dynamic changes, as was similarly discussed for MT-MT networks.

In large caliber axon shafts of vertebrates, MTs intermingle with NFs. NFs tend to be sparse in small axons. However, with increasing diameters NF numbers rise dramatically, much faster than MT numbers, and may eventually outnumber MTs by an order of
magnitude (Tab.S4; Fig.4A-E; Alfei et al., 1991; Alvarez et al., 1982; Malbouisson et al., 1985; Pannese et al., 1981). This trend applies to myelinated and unmyelinated axons (Friede and Samorajski, 1970; Smith et al., 1970), although some exceptional reports find constant MT densities across different caliber ranges (Elder et al., 1998; Zenker and Hohberg, 1973), and NF:MT ratios in the extraordinarily large giant axons of squid seem to be as low as 2:1 (Fath and Lasek, 1988). However, in general MT densities in large caliber axons of vertebrates tend to be lower than in arthropods due to the presence/absence of NFs; for example, MT densities in axons of lamprey are about an eighth compared to cockroach axons of similar diameter (Tab. S4).

Images and descriptions of small versus large caliber axons across vertebrate species suggest fundamental differences in MT distribution (Nakazawa and Ishikawa, 1995; Peters et al., 1991; Raine et al., 1971; Tanner et al., 1998; Tsukita and Ishikawa, 1981 and references listed in Tab. S4). In small fibers (Fig.4A-C), MT-MT and NF-NF networks tend to segregate, which may be a function of their composition (e.g. predominance of peripherin; see above) and/or of their numbers. With increasing NF numbers there is a clear impact on MT organization (Fig.4D,E): patches of MT-MT networks become rarer and smaller, and often single MTs seem to 'dissolve' into the surrounding 'sea' of NF-NF networks, thus spreading out at low densities across axonal profiles.

To act as a 'solvent', NFs would have to be able to cross-link to MTs, and this seems to be the case: in non-neuronal cells, the cell-junctional scaffold protein plectin was described as a cross-linker between MTs and the intermediate filament vimentin (Svitkina et al., 1996); in axons, MT-NF cross-bridges have also been described (Hirokawa, 1991; Rice et al., 1980; Schnapp and Reese, 1982). Such cross-bridges were suggested to be MAPs or MAP-like factors (e.g. tau, MAP2, MAP6) or even motor proteins (Leterrier et al., 1982; Miyata et al., 1986; Perrot et al., 2008). Unlike the regular side-arms of NFs, MT-NF linkers in axons were described to display random periodicity, and their shapes were often bifurcating with a length of 54.7 nm, which was distinct from the spacing of MT-MT (49.6 nm) or NF-NF (40.61 nm) cross-bridges (Monaco et al., 1989; Tsukita et al., 1982).

NFs are proposed to form "hydrogel networks" (Kornreich et al., 2015) and to behave like "inert 'molecules' in a dilute solution" that "are relatively free to move apart from each other and to fill the available space within the axon" (Price et al., 1988). This would mean that also MTs dissolved in these networks are fairly free to drift, ideal to accommodate dynamic space requirements, for example when voluminous cargoes are transported through an axonal region.

In support of this idea, serial TEM analyses of axons reveal loose parallel arrangements with MTs changing their relative positions in consecutive axon segments (Fig.1A; Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). Furthermore, MTs often cluster around mitochondria (Friede and Samorajski, 1970; Hirokawa, 1982; Malbouisson et al., 1985; Pannese et al., 1981; Raine et al., 1971; Smith et al., 1975; Smith et al., 1977; Tsukita and Ishikawa, 1981). This suggests that MTs can be attracted towards mitochondria, likely mediated by MT-mitochondrial linkers that have a higher affinity than MT-MT or MT-NF linkers. These could be MT-associated motor protein complexes for mitochondria that are being transported (Hirokawa et al., 2010), or the cross-linker protein syntaphilin known to dock or anchor stationary mitochondria to MTs (Kang et al., 2008; Lin et al., 2017). The same logic may apply to occasional groups of narrowly cross-bridged MT fascicles positioned within NF-rich axon shafts of lamprey neurons (Smith et al., 1970; Smith et al., 1975).
In the absence of NFs, MTs form functional structural networks

Surprisingly, removal of the highly abundant NF-NF networks from axons is not a lethal condition, although up to 20% of axons can be lost and axon calibers and their conduction velocities are significantly reduced (Beaulieu et al., 1999; Bocquet et al., 2009; Eyer and Peterson, 1994; Jacomy et al., 1999; Kriz et al., 2000; Sakaguchi et al., 1993; Yamasaki et al., 1991; Yum et al., 2009; Zhu et al., 1997). In the absence of NFs, the densities of MTs increase up to 10-fold, but they are well organized and look very similar to those of small caliber vertebrate axons, or axons of arthropod neurons: MTs are distributed across the axonal profile and seem to maintain an average nearest-neighbor spacing from each other (Fig.4I versus A-C,F-H).

In lower motorneurons of rat, MAPs localize to MTs scattered throughout the axonal profile (Papassozomenos et al., 1985). This suggests that the typical MT-regulating machinery is readily available so that MTs can interact in meaningful ways even when dissolved into NF-NF networks. Such a scenario could explain why loss-of-function of the MT-binding and -regulating spectraplakin dystonin causes severe disorganization of axonal MTs regardless of whether NFs are present or absent (Bernier and Kothary, 1998; Dalpe et al., 1998; Eyer et al., 1998; Voelzmann et al., 2017), and that even loss of its fly homologue Short stop causes the same phenotype (Alves-Silva et al., 2012; Sánchez-Soriano et al., 2009). This said, NF depletion might impact on MTs. For example, NFs were reported to regulate MT stability and/or polymerization (Bocquet et al., 2009; Kurup et al., 2018 and references therein).

This might explain why the number of MTs per axon was doubled in H-/- single mutant mice although NF numbers and axon diameters were hardly affected in these animals (Jacomy et al., 1999; Zhu et al., 1998). In contrast, there seems to be no upregulation of MT numbers in NF-depleted quiver quail axons when calculating increases in MT density against the reduction in cross-sectional area (Yamasaki et al., 1991).

Regardless of whether MT numbers change, NF-depletion provides unique opportunities to study the 'default' MT-MT networks in large caliber axons. For example, nearest-neighbor relationships of MTs are impossible to assess in larger axons where MTs are dissolved into NF-NF networks. Depletion of NFs would therefore provide a condition where true spacing could be assessed, thus providing improved strategies to study MAP functions in these neurons.

An efficient experimental strategy is provided by the injection of the toxins 2,5-hexanedione or IDPN (β,β'-iminodipropionitrile). Apart from affecting the proximo-distal NF distribution in axons (see above), these toxins trigger a dramatic segregation of the axonal cytoskeleton: within hours or days, a core of MT-MT networks forms, surrounded by a halo of NF-NF networks (Fig.4J; Griffin et al., 1983a; Griffin et al., 1983b; Papassozomenos et al., 1981; Parhad et al., 1987).

Importantly, the central MT-MT networks were shown to form MAP1A/B immuno-reactive crossbridges in sciatic nerve (Hirokawa et al., 1985) and to overlap with MAP staining in the ventral root (Papassozomenos et al., 1985), suggesting that MTs brought their MT-regulating machinery into this core when segregating away from NFs.

The underlying mechanisms of this segregation process remain hypothetical. A mathematical model has been proposed stating that cytoskeletal segregation is caused by a reduction in slow MT-based transport of NFs, thus shifting the bias towards other transported cargoes that may cross-link MTs and zip them together (Xue et al., 2015). Similar mechanisms might explain comparable segregations that occur in a number of axonal pathologies although with certain variations: for example in giant axonal neuropathy, MT-MT networks do not form at the core but...
in the axon periphery (Asbury et al., 1972; Berg et al., 1972; further references in Xue et al., 2015).

Conclusions and final remarks

This review aimed to understand concepts of axonal architecture by integrating knowledge from over 50 years of study across a range of animal models, including also arthropods that have developed their axonal organization in the absence of NFs. From this I propose that loose MT-MT networks are a fundamental common element of axons. In these networks, MTs are spaced out and only loosely connected by MAPs, thus allowing for flexible rearrangement, for example to permit and support the passing of large transported cargoes. The main exception to this rule are fasciculated MTs at the AIS or in stem axons where tight coupling might represent a structural feature to resist the mechanical forces caused by a tug-of-war between antagonistic motor activities.

As discussed elsewhere (Hahn et al., 2019), essential architectural properties of axons depend on stable MTs and a stiff cortex. In contrast, the addition of NF-NF networks appears to have its major purpose in expanding axon calibers, thus improving conduction velocity and spike frequency (Friede et al., 1984; Perge et al., 2009; Perge et al., 2012). However, NFs seem to make astonishingly little contribution to axonal integrity and sustainability. NFs might improve elastic properties of axons, but arthropods clearly demonstrate that caliber enlargements can be achieved solely through increased MT numbers. In large caliber axons of arthropods, most MTs seem to serve a mere structural purpose with only a specialized subset used for axonal transport (Miller et al., 1987). This might explain why it was possible during evolution to replace a fraction of the axonal MTs by NFs in other animal groups.

If MT networks are indeed the key element of the longitudinal axonal cytoskeleton, it makes sense that MT-regulating mechanisms are functional in small and large caliber axons of vertebrates alike. Accordingly, MTs seem to be equipped with their regulatory tool box even when submerged into the vast NF-NF networks, enabling them to interact in meaningful ways and to rapidly form intact MT-MT networks when cytoskeletal segregation occurs after 2,5-hexanedione or IDPN application.

I hope that the descriptions throughout this review have made clear how much information and understanding can be obtained from the retrospective study of classical EM work, which has become easily accessible thanks to the efforts of most journals to systematically digitize their back issues. There is wealth of information for many other contemporary lines of investigation, such as detailed descriptions of MT behaviors during axon branching (Yu et al., 1994), of the ultrastructure of growth cones, dendrites, and synapses (Peters et al., 1991), or the description of curious phenomena such as impressive packages of ER-derived tubular structures found in certain axons (Andres, 1965b; Peters et al., 1991). Axonal MTs of species as diverse as cockroach, lamprey, frog, toad, chick, mouse and rats were reported to contain luminal material in form of a ~4 nm thick central dot or filament (Andres, 1965a; Burton, 1984; Burton, 1987; Gonatas and Robbins, 1965; Lane and Treherne, 1970; Nixon et al., 1994; Rodríguez Echandía et al., 1968; Smith et al., 1970; Smith et al., 1975; Wuerker and Palay, 1969), matching recent reports of MTs with incorporated actin filaments or MAP6/STOP (stable tubule only peptide; Cuveillier et al., 2020; Paul et al., 2019 Preprint) - of which the latter could help to explain long-term cold resistance of axonal MTs observed in vivo (Delphin et al., 2012; Pannese et al., 1982). Furthermore, classical studies revealed the presence of post-translationally modified
subdomains in MT lattices (Baas and Ahmad, 1992; Baas and Black, 1990; Baas and Joshi, 1992), which start finding their explanations in current models of MT stability (Baas et al., 2016).

In conclusion, I hope that this review will help to build bridges to the past, to capitalize on that valuable treasure trove which can complement and often even instruct our work with modern techniques in meaningful ways. But it should also have become clear that existing descriptions only scratch the surface of what we need to understand. Classical EM techniques had clear limitations with respect to efficiency and the use of fixation and staining methods that might not always represent axonal structure in all accuracy. New techniques such as large volume EM or cryo-electron tomography (Kubota et al., 2018; Xu et al., 2017; Chakraborty et al., 2020) provide ever improving means to drive the discovery processes towards good understanding of axon structure, which will eventually have important implications for work on neuronal pathologies.

Acknowledgements

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Abbreviations

AIS, axon initial segment
ALS, amyotrophic lateral sclerosis
Ank, ankyrin
DRG, dorsal root ganglion
EB, end-binding protein
EM, electron microscopy
ER, endoplasmatic reticulum
IDPN, ß,ß'-iminodipropionitrile
Kv, voltage-gated potassium channel
MAG, myelin-associated glycoprotein
MAP, microtubule-associated protein
Mical, molecule Interacting with CasL
MT, microtubule
MTCL, microtubule cross-linking factor
NaV, voltage-gated sodium channel
NF, neurofilament
Ndel, neurodevelopment protein 1 like
NoR, node of Ranvier
PAEZ, pre-axonal exclusion zone
SIZ, spike initiation zone
STOP, stable tubule only peptide
TEM, transmission electron microscopy
References


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A. Prokop - Axonal microtubules


A. Prokop - Axonal microtubules


Figures

**Fig. 1.** Axonal compartments in different neuron types of vertebrates and arthropods. **AI, Aiv** Vertebrate motor/interneurons (here shown with myelin sheath, green) and arthropod sensory neurons are bi- or multipolar; they can be expected to follow similar organizational principles, with transport of somatic, dendritic, and axonal materials (gray, red, blue arrows, respectively) being decided mostly through exclusion mechanisms that reject somatic/dendritic materials (red(gray T)s at the base of axons and axonal materials at the base of dendrites (blue T); note that in sensory neurons of Drosophila also somatic materials are excluded from dendrites (gray T; Rolls, 2011). The axonal exclusion zone in the AIS of vertebrate motor/interneurons coincides with the spike initiation zone (SIZ/orange circle; yellow arrow indicates direction of potential propagation), but little is known about this in arthropod sensory neurons. **Aii** Vertebrate sensory neurons (e.g. dorsal root or trigeminal ganglion cells) are pseudo-monopolar (Nascimento et al., 2018). They are known to have an SIZ in the distal peripheral axon (Goldstein et al., 2019) and to filter somatic materials in the stem axon (gray T; Gumy et al., 2017); hypothetical filter zones should include barriers to dendritic materials at the entry point to the central axon (red T) and to axonal materials at the base of the peripheral dendrites (gray T). **Aiii** Arthropod motor/interneurons are unipolar (Sánchez-Soriano et al., 2005); their axonal/dendritic filter zones are likely at the dendrite base (blue T), and the SIZ in the axon slightly peripheral to the dendrites (Burrows, 1996; Günay et al., 2015); the cell body is positioned away from that site and known to retain somatic materials, thus requiring a specific filter zone (gray T; Cohen, 1967). Circular insets show cross-sections of different axonal compartments of vertebrate neurons (compare Fig. 2 and 4) illustrating the known MT distribution (small green circles; MTF, MT fascicles) and presence/absence of an electron dense plasmalemma undercoat (DUC); the red text lists certain proteins present/absent at AISs and stem axons (see main text). Further abbreviations: AIS, axon initial segment; AnkG, ankyrin-G; β4-Spec, β4-spectrin; dendr, dendrite; Nav, voltage-gated sodium channels; Nfas, Neurofascin-186. Note that some neurons lack axons entirely (e.g. amacrine cells in the retina, granule cells in the olfactory bulb; Peters et al., 1991), whereas other axons can exist without their cell bodies (reported for ~200 µm long parasitic wasps; Poliov, 2012). **B** Soma and proximal axon of a typical vertebrate motor/interneuron: in the cell body, MTs (green) are diffusely arranged; the axon hillock forms a transition zone where MTs are funneled towards the axon and somato-dendritic organelles including Golgi stacks and rough ER are prevented from entering the axon (thus forming a pre-axonal exclusion zone, PAEZ; Farias et al., 2015; Peters et al., 1991), whereas smooth ER networks continue all along axons (Burton and Laveri, 1985; Gonzalez and Couve, 2014; Tsukita and Ishikawa, 1976); in the axon initial segment (AIS) MTs are fasciculated (see Fig. 2) and the plasmalemma forms an electron-dense undercoat; in the axon shaft, MTs form loose parallel arrangements (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981); * note that the centrosome has been reported to lose it MT nucleation function in neurons (Stiess et al., 2010).

**Fig. 2.** Ultrastructural features at the AIS, stem axon, and nodes of Ranvier. **A** AIS of a giant cell of Deiters in the rat lateral vestibular nucleus (Palay et al., 1968); **B** AIS of a goldfish Purkinje cell (Matsumura and Kohno, 1991); **C** AIS of a small pyramidal neuron of the rat cerebral cortex (Peters et al., 1968) bearing an input synapse (IS) which is symmetric (thin postsynaptic undercoat) with flat pleiotropic vesicles; **D** AIS of a rat Purkinje cell (Chan-Palay,
1972); E) reconstruction from a TEM series of a pyramidal cell AIS with numerous presynaptic boutons and small spines in the rat hippocampus (redrawn from Kosaka, 1980); F) initial unmyelinated portion (IP; proximal part of which is the AIS; see Tab.S3) of a ruffled cell in the frog olfactory bulb with numerous pre- and post-synaptic spines (redrawn from Kosaka and Hama, 1979); G) stem axon of a dorsal root ganglion cell in rat (Nakazawa and Ishikawa, 1995); H) node of Ranvier in the midbrain of rat (Peters, 1966). Note that neurofilaments are sparse in AISs and that MTs are not fasciculated in nodes of Ranvier. Best guesses were made with respect to structures drawn; scales in most original photos were given as magnifications (unfortunately meaningless in PDF format) and were estimated here by measuring MT diameters; suggested black scale bar for A-D, G and H is 500 nm. Publications providing further examples of stem axons or nodes of Ranvier are provided in the main text; for further AIS descriptions see Tab.S3.

Fig. 3. Dimensions at the AIS. Descriptions of the three-layered organization of the dense undercoat at the AIS viewed in longitudinal axon sections, either at the ultrastructural (A; modified from Chan-Palay, 1972) or super-resolution level (B; modified from Leterrier et al., 2015): the continuous dense layer may reflect spectrins (spec) separated about 8 nm from the plasma membrane in both models; the tufted triangles composed of ~5 nm thick filaments likely correspond to the tails of AnkG (reported to be 4-7.5 nm thick also in platinum replica EM; Jones et al., 2014), with the position of their C-tail domains peaking at ~35 nm beneath the membrane (black arrow head in B), although a maximal stretch of ~139 nm was reported (yellow arrow head; Jones et al., 2014); asterisks on the AnkG C-tail indicate the Ndel1 binding site (Ye et al., 2020); granules in the translucent layer in A have currently no molecular explanation, although a sub-fraction of them might represent actin rings; for explanations regarding voltage-gated sodium/potassium channels (Kv/Nav) or Neurofascin-186 (Nfas) see main text. C) Original micrograph of a longitudinal axon section showing a MT in contact with the tufted layer of the dense undercoat (black arrow heads; modified from Palay et al., 1968), suggesting that some MTs are in reach of AnkG; further MT bundles (curved arrows) appear to be out of reach even of the extended AnkG C-tail. D) AIS cross-section of a giant cell of Deiters in the rat lateral vestibular nucleus (Palay et al., 1968; same as in Fig.2A): the stippled/dashed lines indicate 35/150 nm distance from the plasma membrane (i.e. the peak/maximal distances of the AnkG C-tail shown in B) illustrating that many MT bundles are unlikely to be directly anchored to the axonal cortex.

Fig. 4 MT and NF distributions in axon shafts of neurons in mammals, C. elegans and arthropods. Images represent axons in different animals, either wild-type or genetically/pharmacologically manipulated, as indicated; they were drawn from: A) Andres, 1965a, B) Bray and Bunge, 1981, C) Nixon et al., 1994, D) Zenker and Hohberg, 1973, E) Eyer and Peterson, 1994, F) Chalfie and Thomson, 1979, G) Egger et al., 1997, H) Ochs and Burton, 1980, I) Eyer and Peterson, 1994, J) Papasozomenos et al., 1981. Best guesses were made with respect to structures shown. Abbreviations: IDPN, β,β'-iminodipropionitrile; NFH-lacZ, lac-Z knock-in into the heavy polypeptide NF gene. Scales in most original photos were given as magnifications (unfortunately meaningless in PDF format) and were estimated here by measuring MT diameters; suggested scale bar: 500 nm.
**Supplementary Figures**

**Fig. S1.** Homology of *Drosophila* Futsch to human MAP1B and Futsch-like genes in other invertebrates. Human MAP1B is shown at the top; borders of functional domains were taken from the 9 amino acid shorter rat gene (Villarroel-Campos and Gonzalez-Billault, 2014); MBD, MT-binding domain; ABD, actin-binding domain; MTA, MT assembly-helping domain. The *Drosophila* Futsch gene is shown below, indicating the homologous regions shared with human MAP1B. The bars further below indicate homologies of other invertebrate Futsch-like genes, mapped onto the *Drosophila* Futsch gene; asterisk indicates the crayfish homolog mentioned in the main text. All sequences were blasted against the *Drosophila melanogaster* genome using the blastp 2.2.18 tool provided in FlyBase (Altschul et al., 1997; Marygold et al., 2016). In all cases, Futsch was identified as the only stringent homolog, with the areas of homology all coinciding in the same N- and C-terminal regions as indicated in the figure. Sequences used:

- Human MAP1B-201: ensembl.org gene ENST00000296755.12
- *Drosophila melanogaster* Futsch: ensembl.org gene FBpp0300235
- *Procambarus clarkii*: extracted by Lililans Calvo-González from non-assembled genomic sequence kindly provided by Yanhe Li (Shi et al., 2018)
- *Aedes aegypti*: VectorBase gene AAEL009847-RE
- *Tricholium castaneum*: ensembl.org gene TC001001_001
- *Cryptotermes secundus*: NCBI gene XP_023712736.1
- *(Lito)penaeus vannamei*: NCBI gene XP_027226604.1
- *Hyalella azteca*: NCBI gene XP_018026291.1
- *Daphnia magna*: ensembl.org gene APZ42_014156
- *Stegodyphus mimosarum*: ensembl.org gene X975_00566
- *Tetranychus urticae*: NCBI gene XP_015781552.1
- *Ixodes scapularis*: VectorBase gene ISCW009767-RA
- *Strigamia maritima*: ensembl.org gene SMAR006539-RA
- *Strongylocentrotus purpuratus*: ensembl.org gene SP-MAP1APH

**Fig. S2.** Composition of neurofilaments. **A)** Usually, NF-M or NF-H form parallel hetero-dimers with NF-L which, in turn, arrange into anti-parallel hetero-tetramers through partial overlap of their coiled domains, explaining the lack of polarity in NFs. **B)** 8 tetramers assemble into a unit, with the tail domains sticking out further aided by phosphorylation of the M- and H-tails (tail domains of the NF-proteins with stippled outlines are left out for clarity). **C)** 3D view of the same unit as shown in B, now displaying all tail domains; note that inside-out orientation of tetramers is randomized since it seems unknown whether this property follows a strict order in real filaments. **D)** Proposed dimensions in assembled filaments where units are compacted and annealed end-to-end. Information for this model was taken from: Janmey et al., 2003; Kornreich et al., 2015; Szaro and Strong, 2010.

**Tab. S3:** Ultrastructural reports of AISs in different neuron types. 1\(^{st}\) column: the described neuron types; 2\(^{nd}\)/3\(^{rd}\) column: diameters/lengths of AISs (where mentioned); 4\(^{th}\) column: properties of AISs; apart from periglomerular cells, all AISs display dense undercoats (DUC)
and MT fascicles (MTF); many AISs display cisternae (C) or cisternal organs (CO) that tend to be close to synaptic contacts or synaptic spine-like processes (SLP); according to Peters et al. (1991; pp.152 and 176), type I/asymmetric synapses with round clear vesicles are considered excitatory, type II/symmetric synapses with flat or pleiotropic clear vesicles inhibitory (abbreviations: IS, OS, RS, NS: input, output, reciprocal, no synapses; -1/2: asymmetric/symmetric synapse; -FV, -PV, -RV: flat, pleiotropic, round vesicles); axons of ruffed cells display an unconventional initial unmyelinated portion (IP; Fig.2F), of which only only the most proximal part displays all AIS features. 5th column: source.

Tab. S4. Axonal MT and NF numbers as reported in the literature. Data were extracted from graphs and/or texts of the various publications; they indicate highest and lowest values as a crude representation of data ranges, whereas more specific analyses and correlations could not be considered here. Values without brackets were taken from those publications, whereas data in brackets were calculated in retrospect on the basis of those data. Footnote and abbreviations: (1) reduced by organelle area (~15%); n.d., not determined.
A rat, lateral vestibular nucleus, Deiters cell AIS

B goldfish, Purkinje cell AIS

C rat, cerebral cortex, small pyramidal neuron AIS

D rat, Purkinje cell AIS

E rat hippocampus, pyramidal cell

F goldfish, ruffed cell

G rat, DRG cell, stem axon

H rat, midbrain of rat, node of Ranvier

Fig. 2 Prokop
Fig. 3 Prokop
Drosophila, ventral nerve cord

rat cultured sensory nerve

rat accessory nerve, A-α-motor fibre

mouse, ventral root

mouse, optic nerve

C. elegans, mechanosensory axons

crayfish, ventral nerve cord

rat, olfactory bulb

mouse, ventral root

NFH-lacZ transgenic mouse, ventral root

Fig. 4 Prokop
antiparallel hetero-tetramer

uncompacted neurofilament unit

compact neurofilament

Fig. S2 Prokop
<table>
<thead>
<tr>
<th>neuron type</th>
<th>Ø [µm]</th>
<th>L [µm]</th>
<th>AIS properties</th>
<th>Reference</th>
</tr>
</thead>
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<td>hippocampal dentate granule cells</td>
<td>~1</td>
<td>-</td>
<td>CO, many IS-2-FV, occasional SLP</td>
<td>Kosaka, 1983</td>
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<td>0.8-3.3</td>
<td>31-54</td>
<td>CO; IS-2 (2-19); pyramidal cell: occasional SLP; one AIS arising from a dendrite</td>
<td>Sloper and Powell, 1979</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>CO; IS-2-FV; frequent SLP</td>
<td>Somogyi et al., 1982</td>
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<tr>
<td>pyramidal neurons, rat hippocampus</td>
<td>0.8-2</td>
<td>20-65</td>
<td>CO, IS-2-FV (25-143), SLP (0-26)</td>
<td>Kosaka, 1980a</td>
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<tr>
<td>small pyramidal neurons, rat cerebral cortex</td>
<td>0.6-1.2</td>
<td>-</td>
<td>CO, IS-RV (?; see Fig. 2C)</td>
<td>Peters et al., 1968</td>
</tr>
<tr>
<td>pyramidal neurons, rat pyriform cortex</td>
<td>0.5-3</td>
<td>-</td>
<td>CO, IS-2-FV; frequent SLP</td>
<td>Westrum, 1970</td>
</tr>
<tr>
<td>pyramidal neurons, cat somatic sensory cortex</td>
<td>-</td>
<td>-</td>
<td>C, IS-2-FV</td>
<td>Jones and Powell, 1969</td>
</tr>
<tr>
<td>motor/somatosensory cortex, rhesus monkeys</td>
<td>-</td>
<td>24-54</td>
<td>CO, IS-2, SLP</td>
<td>Sloper and Powell, 1973</td>
</tr>
<tr>
<td>various neuron types, rat</td>
<td>-</td>
<td>-</td>
<td>Purkinje cell: NS; pyramidal cell: many IS</td>
<td>Palay et al., 1968</td>
</tr>
<tr>
<td>Purkinje cells, frog</td>
<td>~1.3</td>
<td>-</td>
<td>-</td>
<td>Kohno, 1964</td>
</tr>
<tr>
<td>Purkinje cells, rat;</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Chal-Palay, 1972</td>
</tr>
<tr>
<td>giant cells of Deiters, rat vestibular nucleus</td>
<td>~2</td>
<td>27</td>
<td>proximal IS</td>
<td>Sotelo and Palay, 1968</td>
</tr>
<tr>
<td>cartwheel neurons, rat dorsal cochlear nucleus</td>
<td>0.6-1.5</td>
<td>35</td>
<td>rare proximal IS</td>
<td>Wouterlood and Mugnaini, 1984</td>
</tr>
<tr>
<td>cat olivary neurons</td>
<td>0.9-1.3</td>
<td>33-40</td>
<td>23-36 IS: IS-2-PV (2/3), IS-1-RV (1/3)</td>
<td>De Zeeuw et al., 1990</td>
</tr>
<tr>
<td>I- &amp; P-cells, monkey lateral geniculate nucleus</td>
<td>-</td>
<td>-</td>
<td>I-cell AISs: may arise from dendrites, with OS-FV in varicosities</td>
<td>Hamori et al., 1978</td>
</tr>
<tr>
<td>mitral cells in mouse olfactory bulb</td>
<td>-</td>
<td>-</td>
<td>timeline of structural AIS development</td>
<td>Hinds and Ruffett, 1973</td>
</tr>
<tr>
<td>Mitral cells, goldfish olfactory bulb</td>
<td>-</td>
<td>-</td>
<td>IS-2 (0-3), OS-1 (20-38), RS (14-20)</td>
<td>Kosaka and Hama, 1979a</td>
</tr>
<tr>
<td>Mitral cells; cat olfactory bulb</td>
<td>-</td>
<td>-</td>
<td>OS-1, IS-2, RS</td>
<td>Willey, 1973</td>
</tr>
<tr>
<td>Mitral cells, rat olfactory bulb</td>
<td>-</td>
<td>-</td>
<td>NS; AIS full of scattered vesicles</td>
<td>Price and Powell, 1970</td>
</tr>
<tr>
<td>short-axon, periglomerular &amp; tufted cells, rat olfactory bulb</td>
<td>-</td>
<td>-</td>
<td>C, IS-2-FV; periglomerular cell: AIS lacks MTF &amp; DUC</td>
<td>Pinching and Powell, 1971</td>
</tr>
<tr>
<td>ruffed cell in goldfish olfactory bulb</td>
<td>0.8-1.5</td>
<td>10-20 (Pt.1)</td>
<td>~100µm long IP with 10-20 SLP &amp; 30-105 synapses (OS-1-RV; IS-2-PV); Pt.1: SLP, DUC, MTF, Pt.2: DUC, SLP, no MTF; Pt.3: SLP, no DUC, no MTF</td>
<td>Kosaka and Hama, 1979b; Kosaka, 1980b</td>
</tr>
<tr>
<td>cat Clarke nucleus neurons/dorsal spino-cerebellar tract</td>
<td>1.7-4.2</td>
<td>28</td>
<td>no S</td>
<td>Saito, 1972</td>
</tr>
<tr>
<td>motoneurons, cat spinal cord</td>
<td>2.9-4.2</td>
<td>23-38</td>
<td>no S</td>
<td>Conradi, 1969</td>
</tr>
<tr>
<td>motoneurons, cat spinal cord</td>
<td>1.3-2.8</td>
<td>19-31</td>
<td>compares AIS of newborn vs. adult; newborn: IS-FV/RV (15-19), SLP; adult: NS, no SLP</td>
<td>Conradi and Skoglund, 1969</td>
</tr>
<tr>
<td>motoneurons, cat spinal cord</td>
<td>≥3.3</td>
<td>66.7</td>
<td>on AIS analysed: 3 IS-FV</td>
<td>Kojima and Saito, 1970</td>
</tr>
<tr>
<td>motoneurons, cat spinal cord</td>
<td>-</td>
<td>40</td>
<td>IS only on proximal part</td>
<td>Poritsky, 1969</td>
</tr>
<tr>
<td>cat caudate nucleus</td>
<td>-</td>
<td>-</td>
<td>AIS can arise from dendrites; CO; SLP, many IS-2-FV/PV</td>
<td>Kemp and Powell, 1971a; Kemp and Powell, 1971b</td>
</tr>
<tr>
<td>Mauthner cell, goldfish</td>
<td>10</td>
<td>50</td>
<td>IS-RV?</td>
<td>Nakajima, 1974</td>
</tr>
<tr>
<td>axon profile area [µm²]</td>
<td>axon diameter [µm]</td>
<td>MT no.</td>
<td>MTs/µm²</td>
<td>NF no.</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>lizard, DRGs</td>
<td>&lt;0.12 - &gt;4</td>
<td>(0.39 - 2.3)</td>
<td>4.4 - 130</td>
<td>7 - 95</td>
</tr>
<tr>
<td>cat, DRGs</td>
<td>&lt;0.1 - ~10</td>
<td>(0.36 - 3.6)</td>
<td>4.5 - 69</td>
<td>3.6 - 145 (367 outlier?)</td>
</tr>
<tr>
<td>rat, ventral/dorsal root</td>
<td>3.6 - 51.4</td>
<td>(2.1 - 8.1)</td>
<td>n.d.</td>
<td>7.6 - 17</td>
</tr>
<tr>
<td>rat, ventral root</td>
<td>~5 - ~58</td>
<td>(2.5 - 8.6)</td>
<td>110 - 750</td>
<td>~9 - ~36</td>
</tr>
<tr>
<td>mouse, ventral root</td>
<td>~1.5 - ~10</td>
<td>(1.4 - 3.6)</td>
<td>20 - 240</td>
<td>(13 - 25)</td>
</tr>
<tr>
<td>Xenopus, dorsal &amp; ventral root</td>
<td>(7.1 - 490)</td>
<td>~3 - ~25</td>
<td>n.d.</td>
<td>&gt;0 - 350</td>
</tr>
<tr>
<td>rat, peripheral sensory and motor nerves</td>
<td>3.7 - 35.6</td>
<td>(2.2 - 6.8)</td>
<td>n.d.</td>
<td>13.3 - 38.2</td>
</tr>
<tr>
<td>cat, monkey DRGs</td>
<td>4.9 - 380</td>
<td>2.5 - 22</td>
<td>1 - 1000</td>
<td>2 - ~35</td>
</tr>
<tr>
<td>lizard, dorsal root</td>
<td>&lt;0.1 - 4</td>
<td>(0.35 - 2.3)</td>
<td>~4.4 - ~50</td>
<td>~7 - ~59.9</td>
</tr>
<tr>
<td>lizard, dorsal root</td>
<td>&lt;0.1 - 4</td>
<td>(0.35 - 2.3)</td>
<td>~4.4 - ~57.1</td>
<td>~7.1 - ~99.7</td>
</tr>
<tr>
<td>rat, saphenous nerve (unmyel.)</td>
<td>0.24</td>
<td>(0.6)</td>
<td>~13.8</td>
<td>~63.5</td>
</tr>
<tr>
<td>rat, motor fibres</td>
<td>0.5 - 12.5 (1)</td>
<td>(0.8 - 4)</td>
<td>17 - ?</td>
<td>9.2 or 33 - ?</td>
</tr>
<tr>
<td>rat, sural nerve</td>
<td>1 - 18</td>
<td>(1.1 - 4.8)</td>
<td>20 - 350</td>
<td>4.5 - 40</td>
</tr>
<tr>
<td>mouse &amp; rat, sciatic nerve</td>
<td>&lt;0.5 - &gt;36</td>
<td>(0.08 - &gt;6.8)</td>
<td>6.7 - 206</td>
<td>n.d.</td>
</tr>
<tr>
<td>mouse, sciatic n. node</td>
<td>1 - 2.2</td>
<td>(1.1 - 1.7)</td>
<td>39 - 139</td>
<td>n.d.</td>
</tr>
<tr>
<td>mouse, sciatic n. node</td>
<td>1.4 - 11.8</td>
<td>(1.3 - 3.9)</td>
<td>38 - 120</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat, sciatic nerve</td>
<td>~1 - ~54</td>
<td>(1.1 - 8.3)</td>
<td>&gt;0 - ~300</td>
<td>n.d.</td>
</tr>
<tr>
<td>cat, sympath. postgangl. fibre</td>
<td>0.1 - 1.3</td>
<td>(0.35 - 1.3)</td>
<td>13 - 44.8</td>
<td>34.4 - 80.2</td>
</tr>
<tr>
<td>chicken, oculo-mot. nerve: motor axon</td>
<td>16.7-35.2</td>
<td>(4.6 - 6.7)</td>
<td>47.3 - 78.9</td>
<td>1.34 - 4.72</td>
</tr>
<tr>
<td>chicken, oculo-mot. nerve: motor axon</td>
<td>14.2 - 23.8</td>
<td>(4.3 - 5.5)</td>
<td>41.3 - 106.5</td>
<td>1.35 - 4.47</td>
</tr>
<tr>
<td>rat, optical nerve</td>
<td>~0.9</td>
<td>(1.1)</td>
<td>30.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>rat, olfactory nerve</td>
<td>(0.01 - 0.07; ≤7.1)</td>
<td>0.08-0.3; rarely ≤3</td>
<td>1 - 10</td>
<td>n.d.</td>
</tr>
<tr>
<td>cat, optical nerve &amp; tract</td>
<td>&gt;0 - 13</td>
<td>(&gt;0 - 4.1)</td>
<td>&gt;0 - ~500</td>
<td>(&gt;0 - ~38.4)</td>
</tr>
<tr>
<td>pike, olfactory nerve</td>
<td>(0.01 - 0.07)</td>
<td>0.1 - 0.3</td>
<td>4±1</td>
<td>n.d.</td>
</tr>
<tr>
<td>trout, Mauthner axon</td>
<td>3.8 - 587.8</td>
<td>2.2 - 27.4</td>
<td>(~250 - ~10,400)</td>
<td>17.7 - 96</td>
</tr>
<tr>
<td>mouse, cereb. granule cells</td>
<td>(0.002 - 0.02)</td>
<td>0.05 - 0.15</td>
<td>2 - 2.4</td>
<td>1.7 - 3.6</td>
</tr>
<tr>
<td>lamprey, central neurons</td>
<td>(9.9 - 1,590)</td>
<td>4 - 45</td>
<td>130 - 2530</td>
<td>(13 - 16)</td>
</tr>
</tbody>
</table>

**Arthropods**

|crayfish, ventral ganglion |350 - 5322 |(21 - 82) |11,588 - 264,056 |5.8 - 81.6 |absent |absent|Nadelhaft, 1974|
|crayfish, ventral ganglion |(~7500) |100 - 200 |590 - 184,000 |1.4 - 14.7 |absent |absent|Viancour et al., 1987|
|cockroach, ventral connectives |(36 - 907) |6.8 - 34 |693 - 11,934 |(~19 - 13.2) |absent |absent|Smith et al., 1977|