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Microtubular cytoskeleton-based cell outgrowths: from pseudopodia to axons and dendrites

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Summary

While the formation of actin-based pseudopodia is presumably an ancestral eukaryotic feature, microtubule-based pseudopodia represent later modifications that were acquired independently in different lineages. Mapping morphological information onto molecular phylogenetic trees led to different scenarios of the microtubule-based outgrowths evolution. The recent advance in the field of genomic and cell biology allows for tracking particular modifications in cytoskeletal and regulatory genes. At the same time, the picture is still dramatically incomplete, due to non-sufficient morphological data for many groups and the paucity or lack of molecular markers for robust phylogenetic reconstructions, where true eukaryotic diversity is still considerably undersampled. The obvious obstacle is also a lack of consistency and exactness in the definitions of many terms, some of which are discussed here. The importance of studying microtubule-based outgrowths function, morphology and evolution comes from the fact that they are also represented by medically relevant structures, e.g. axons, dendrites and tumor cells' microtentacles. Here we review the diversity, distribution and hypothetical evolutionary origin of microtubule-based cell outgrowths in eukaryotes with an emphasis on complex surveys, where the information on the cell structure and function is taken into account along with up-to-date phylogenomic reconstructions of the phylogenetic relationships.

Key words: amoebae, axopodia, filopodia, "heliozoans", "radiolaria", reticulopodia, haptonemata

Introduction

A microtubular cytoskeleton is the common feature of all eukaryotes (Koonin, 2010) and tubulin homologs are sometimes reported in prokaryotic cells (Yutin and Koonin, 2012). The role of microtubules in cell division, vesicular transport and ciliary movement is a subject of numerous reviews (e.g. Franker and Hoogenraad, 2013; Vicente and Wordeman, 2015; Stepanek and Pigino, 2016). However, the formation of both stable and very dynamic cell outgrowths gets significantly less attention. Primarily, microtubules provide the cytoskeletal basis for a variety of pseudopodia types. The formation of actin-based pseudopodia is widespread among eukaryotes and can most probably be traced to the last eukaryotic common ancestor (Richards and Cavalier-Smith, 2005; Yutin et al., 2009; Koumandou et al., 2013). Therefore pseudopodia, fully or partially supported

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and driven by a microtubular cytoskeleton, which are present in a broad variety of unrelated lineages, represent a derived character. It could be acquired by modification of LECA actin-based pseudopodia or after their loss (Pawlowski and Burki, 2009).

Pseudopodia are generally divided into four main types, namely lobopodia, filopodia, reticulopodia and axopodia (Karpov, 2001; Hausmann et al., 2003; Adl et al., 2019). Lobopodia are beyond the scope of this review because they virtually are never formed on the basis of a tubulin cytoskeleton, even though many lobose amoebae have interphase cytoplasmic microtubules-organizing centers (MTOCs) (Smirnov, 1996; Kudryavtsev, 2004) and microtubules might be involved in the process of amoeboid movement in lobose amoebae (Tekle and Williams, 2016). In almost all cases, the lobopodia are actin-driven, except for some unusual cases, such as major sperm protein-driven pseudopodia in the nematode spermatozoa (Roberts and Stewart, 1997). The rigid actin-based outgrowths - microvilli of holozoans (Sebé-Pedrós et al., 2013; Houdusse and Titus, 2021), haptopodia of *Aurigamonas* (Vickerman et al., 2005) – also are not reviewed here. Therefore, we will focus on reviewing a comparative structure and hypothesized evolutionary origin of filopodia, reticulopodia and axopodia.

Axopodia: different rays lead to different stars

Axopodia are non-anastomosing non-branching ray-like pseudopodia, supported by a bundle of microtubules (axoneme), that are connected by special linkers to form a three-dimensional lattice (Cachon et al., 1973; Yabuki et al., 2012). Pseudopodia meeting this definition are formed by Centroplasthelida (Fig. 1, A) in Haptista, by Microhelida in Cryptista, by Actinochrysia and Actinophryida in Heterokonta, by Ephelotidae in Alveolata, by Radiozoa in Rhizaria and also in Heliomonadida and Gymnosphaerida, that for now are *incertae sedis* taxa (Mikrjukov, 1997; Nikolaev et al., 2004; Cavalier-Smith et al., 2015).

The formation of complex and stable structures made of interconnected microtubules can be observed not only in axopodia, but also in a variety of other structures formed by protists (Grain, 1986; Chaaban and Brouhard, 2017). These include the ventral disc of *Giardia* (Nosala et al., 2017), cytopharyngeal baskets (Tucker, 1968) and a tentacle cytoskeleton (Bardele, 1972b) in ciliates, rods and vanes in the feeding apparatus of euglenids (Leander et al., 2007), and axostyles in oxymonads (Brugerolle and Joyon, 1973; McIntosh et al., 1973) and trichomonads (Hollande and Valentin, 1968; Tamm, 1978). The eukaryotic ciliary apparatus also represents such structure, especially taking into account its elaborate system of microtubular roots (Yubuki and Leander, 2013; Yubuki et al., 2016). Therefore, when discussing the evolutionary origin of axopodial structures, one should keep in mind that they both can emerge *de novo* or on the basis of preexisting structures, serving other functions.

The more or less easily explained cases are those where axopodia evolved relatively late, as in example of axopodia (prehensile tentacles) of Ephelotidae suctorians, that were a unique apomorphic addition to a typical system of the sucking tentacles (Mikrjukov, 1997; Dovgal, 2002). It is more difficult to find an evolutionary explanation for earlier cases, some of which gave rise to abundant and diverse lineages.

Interestingly, in two groups of "heliozoans", Actinophryida and Centroplasthelida, the presence of well-developed axopodia is accompanied by a complete loss of the cilium (Cavalier-Smith and Chao, 2003). This is not common, Eliáš and co-authors (2016) list only about 20 cases of cilium loss for all the eukaryotes. This suggests that axopodia in these two groups arose through some modifications of the ciliary apparatus components.

Cavalier-Smith and Scoble (2013) hypothesized that in case of Actinophryida, R2 microtubular roots (rhizoplasts) of their ciliated ancestors could be transformed into the axopodial axoneme. They show the sister relationships of Actinophryida and Raphidophyta flagellates, using 18S rDNA-based molecular phylogeny. This result needs further confirmation with an application of more genes, but the idea is apparently interesting, taking into account that the R2 roots of the raphidophyte *Haramonas* represent a double arcs of interconnected microtubules (Yamaguchi et al., 2008).

In the case of Centroplasthelida, it was shown that their axopodial MTOCs reacted with antibodies against centrioles of green algae and human centrosomes (Klewer et al., 1997). The behavior of the MTOC in the mitosis of these protists is also quite similar to that of centrioles in other organisms, as noted since the classic work of Schaudinn (1896). Thus, it is possible that Centroplasthelida converted their centrioles into the axopodial MTOC and lost the ability to assemble normal cilia.

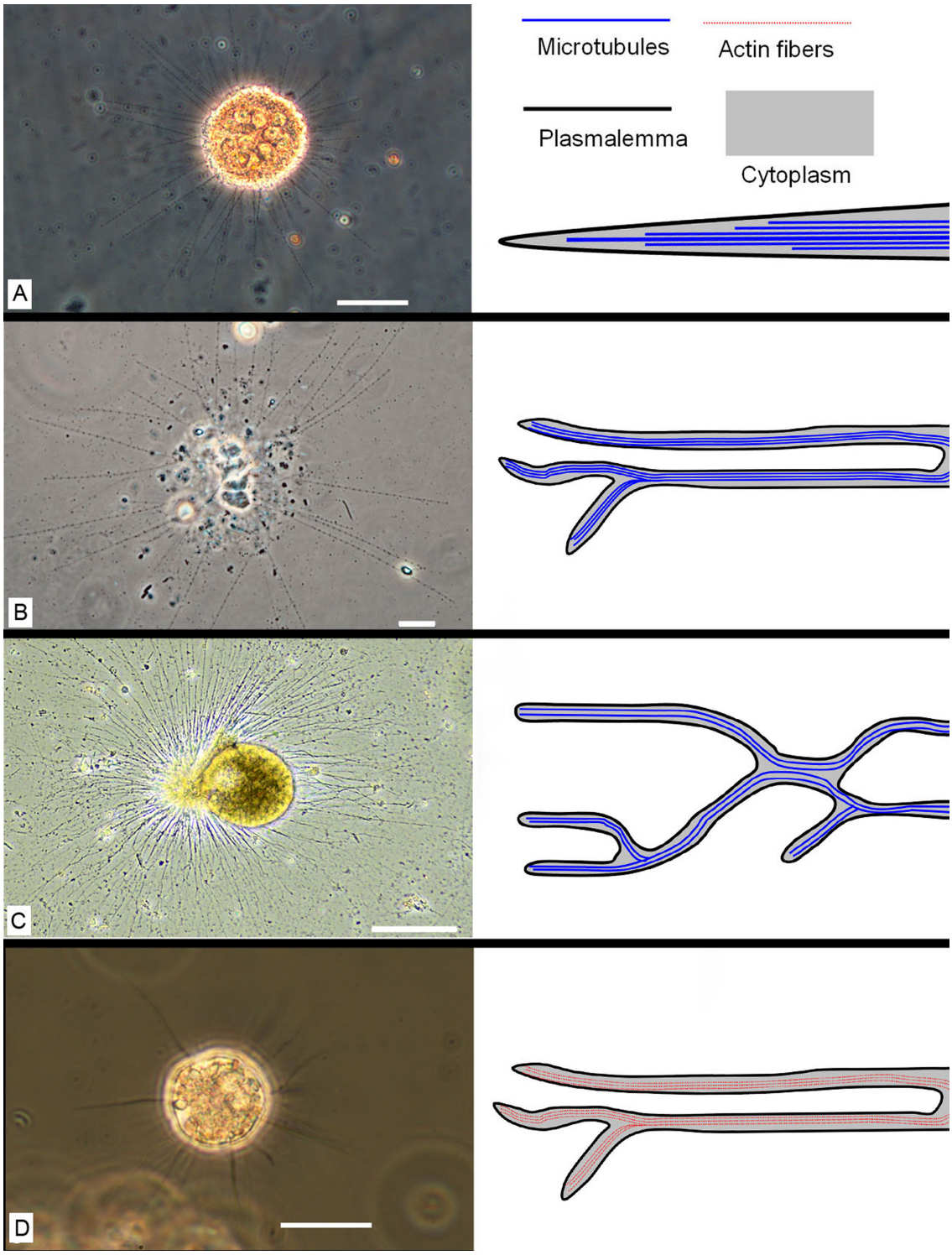

Fig. 1. Schematic representation of the pseudopodia types discussed in the text with micrographs examplifying representatives of protists with each pseudopodia type. A – Axopodia in *Yogsothoth cartheri* (Centroplasthelida); B – microtubule-driven filopodia in *Limnophila* sp.; C – reticulopodia in undetermined foraminifer; D – actin-driven filopodia in undetermined nucleariid amoeba. Scale bars: A – 50 μm, B, C – 10 μm; D – 20 μm. (B – Photo courtesy of Ferry Siemensma).

Cavalier-Smith and co-authors (2015) propose another scenario, where the Centroplasthelida MTOC is homologous to the fibrillar "haptonemal root" of the haptophytes, suggesting that haptonemata and centrohelid axopodia share a common origin. This is consistent with sister relationships between centrohelids and haptophytes confirmed with phylogenomics (Burki et al., 2016). Haptonemata are feeding organelles representing another example of microtubule-based cell outgrowths. In some species, it has been shown that in the basal part the haptonema microtubules are connected with cross-linkers (crossbanded structures) (Moestrup and Thomsen, 1986), which indeed resembles the axopodial microtubules. This hypothesis of the axopodial origin is interesting since it provides a morphological synapomorphy for Haptista (Haptophyta + Centroplasthelida), but unfortunately it does not explain a remarkable complete ciliary loss observed in Centroplasthelida. Cavalier-Smith and co-authors (2015) also postulate that the rhizostyle of cryptomonads, representing a single or double row of connected microtubules (Roberts, 1984; Kim and Archibald, 2013), is a homologous structure for a haptonemal microtubular bundle; it gave rise to the axonemes in axopodia of microhelids and heliomonad *Heliomorpha* (the latter is also included in Cryptista sensu Cavalier-Smith in Cavalier-Smith et al., 2015, despite the lack of molecular data).

In one of his last works, Cavalier-Smith (2018) provides a scrupulous analysis of the diversity and evolution of the kingdom Chromista – the assemblage of SAR, Haptista and Cryptista, which he believed to be holophyletic (contrary to the results of many other phylogenomic studies, e.g. Tice et al., 2021). In this paper, he recognizes a cytoskeletal synapomorphy of all Chromista – the bypassing microtubule band, which is not a part of a ciliary apparatus per se but bypasses centrioles extending from the cell apex into the cytoplasm. Cavalier-Smith argues that the presence of the bypassing band preadapts Chromista for evolving axopodia, since it can be easily transformed into the axopodial axoneme. Using this universal explanation, he connects the independent origin of axopodia (as well as many other tubulin-based structures, including the conoid of Apicomplexa) in a broad variety of protists with a bypassing band modification. Such a multiplied bypassing band is proposed to give axopodial axonemes in Radiozoa, Phaeodaria and Desmothoracida. The latter two are also axopodial in Cavalier-Smith's interpretation and contrary to the one in this paper (see below). Moreover, the similar assumption is made for all microtubule-based outgrowths of Granofilosea. The heliomonad *Tetradimorpha* has also been included in the Granofilosea (Yabuki et al., 2012), despite the lack of molecular evidence, separate from *Heliomorpha* – the other heliomonad genus, which is hypothesized to be a member of Cryptista. One of the reasons for this is that microtubules in the axopodia of *Tetradimorpha* are disordered (Brugerolle and Mignot, 1984). This fact requires further investigations since in their Fig. 4b the microtubules of *Tetradimorpha*, contrary to the authors' interpretation, seem to be organized in the pattern of hexavalent units-based sheet of equilateral triangles, similar to that in axostyles of the oxymonad *Pyrsonympha* (Bloodgood et al., 1974), and obviously have cross-links. Similarly, the microtubules in foraminiferal reticulopodia are postulated to evolve from bypassing bands. Microtubules in cryptomonad rhizostyles, haptonemata, and raphidophyte rhizoplasts that are important as probable axopodial precursors are also being reinterpreted as bypassing bands. The same origin is proposed for axonemes in the axopodia of Actinochrysia. This simple explanation for the origin of all types of axopodia is, of course, controversial but definitely worth further examination, which could prove to be true, at least in some proposed cases of axopodia origin.

Are there microtubule-based filopodia?

Filopodia are traditionally defined as threadlike or pointed pseudopodia (Hausmann et al., 2003; Cavalier-Smith et al., 2018; Adl et al., 2019). According to some definitions, filopodia never contain microtubules (Hausmann et al., 2003; Adl et al., 2019) or even always contain a bundle of microfilaments (Karpov, 2001). Adl et al. (2019) also emphasize that filopodia can be branching, but never form anastomoses. Thus, the thread shape demarcates filopodia from lobopodia, the absence of microtubules – from axopodia, and the absence of anastomoses – from reticulopodia.

Nevertheless, some authors (Bass et al., 2009; Margulis and Chapman, 2009) apply this term to microtubules-containing projections, particularly to the outgrowths of different Granofilosea (Rhizaria). For example, representatives of *Limnofila* spp. have branching thin pseudopodia with extremely

rare anastomoses (Fig. 1, B) that are supported by disordered microtubular bundles (Mikrjukov and Mylnikov, 1998). The same is true for the pseudopodia of other granofiloseans, the desmothoracid "heliozoans" (Bardele, 1972a; Brugerolle, 1985). Pseudopodia of such organization are hardly reticulopodia (contrary to the terminology of Mikrjukov and Mylnikov, 1998) due to the rarity of anastomosing. At the same time, the term axopodia (used by Brugerolle, 1985) is also problematic because the microtubules in the pseudopodia of desmothoracids are not ordered and these pseudopodia can branch. All these characters are atypical for axopodia. Overall, light microscopic appearance demonstrates the similarity of granofilosean pseudopodia with filopodia, and probably the latter is the right term that should be used, despite the presence of microtubules.

Similarly, the pseudopodia of the planktonic foraminifer *Globigerina* are also not reticulopodia, because they do not anastomose, thus not forming a net, and they are not axopodia because the microtubules are devoid of interconnecting linkers and disordered. The term filopodia is thus most applicable here as it is used by Febvre-Chevalier (1971). The *strahlen* (spiny rays) produced by the *strahlenkörper* (spiny-rayed stages) of the piroplasmid apicomplexan *Babesia* are also best described as filopodia, but not the axopodia as in Cavalier-Smith (1993). Their microtubules have a random distribution and are not linked (Weber and Friedhoff, 1977).

Thus, the term filopodia sometimes can be applied to microtubules-containing structures with no or rare anastomoses (to demarcate from reticulopodia) and no ordered and cross-linked axoneme (to demarcate from axopodia).

The hypothetical evolutionary origin of microtubule-based filopodia mentioned above is obviously different for all three cases. Granofilosean microtubules-containing filopodia probably originated from the typical actin-based filopodia (Fig. 1, D) of a common cercozoan ancestor with secondary incorporation of a microtubular cytoskeleton (Cavalier-Smith et al., 2018). The filopodia of *Globigerina* might be a specialization of the typical foraminifer reticulopodia that are also present, especially at early developmental stages (Adshead, 1966). Finally, the filopodia of *Babesia* are most probably a later innovation since no pseudopodial outgrowths are known among its apicomplexan relatives.

Reticulopodia - not only in foraminifers

Reticulopodia are best known as a feature that characterizes Foraminifera d'Orbigny 1826 (Bowser and Travis, 2002). In foraminifers, reticulopodia form a network of branching and anastomosing cytoplasmic threads, which is supported by disordered microtubules supplemented by actin bundles (Travis and Bowser, 1986) (Fig. 1, C). The ability of microtubules to transform reversibly into helical filaments, an alternative form of tubulin arrangement, which allows for faster cytoskeleton transformation (Welnhofer and Travis, 1998), was revealed in foraminifers with the use of electron microscopy. Later, the structurally different paralogs of canonical eukaryotic tubulins, α2-tubulin (Krabberød et al., 2017) and β2-tubulin (Habura et al., 2005), were recognized and suggested as a molecular basis of helical filament formation. Moreover, this tubulin duplication was shared with Radiozoa (the assemblage of all the classical "radiolaria" except Phaeodaria), as well as with Taxopodida (planktonic protists with aberrant oar-like axopodia) (Hou et al., 2013; Krabberød et al., 2017). The members of Radiozoa are mostly known as a prime example of axopodial protists. At the same time, the repertoire of pseudopodia they produce is much broader. Apart from axopodia, their ectoplasm produces branching and anastomosing pseudopodia, that are referred to as rhizopodia, filopodia or reticulopodia (Anderson, 1983; Suzuki and Aita, 2011; Ishitani et al., 2016). According to some very limited ultrastructural studies, these pseudopodia contain microtubules running across their axis, and in experiments, their movement is inhibited with Cytochalasin B, suggesting the involvement of actin (Anderson, 1983, p. 207). These pseudopodia can even be used for crawling along the substratum (*ibid.* p. 210). Therefore, it is possible that reticulopodia, containing both actin and helical-filament forming microtubules, were acquired from a common ancestor of Foraminifera and Radiozoa (Cavalier-Smith et al., 2018). The main discrepancy is Taxopodida that have not been observed to form reticulopodia, but possess α2 and β2 tubulins (Hollande et al., 1967; Krabberшd et al., 2017).

Reticulate morphotypes are not limited to Foraminifera and Radiozoa and are widely distributed across the eukaryotic tree (Berney et al., 2015). Nevertheless, it is usually not clear whether the reti-

culopodia of these organisms are homologous or just superficially similar structures, because many of them lack characterization at the ultrastructural or molecular levels, and often at both. Reticulopodia have emerged in haptophytes (*Reticulosphaera*), heterokonts (*Leukarachnion*), Amoebozoa (Variosea), Cercozoa (independently in Granofilosea (*Reticulamoeba*), Chlorarachnea, Thecofilosea (*Lecythium*), Imbricatea (*Kraken, Trivalvularis, Leptogromia*), and probably other independent cases) should be recognized (Cavalier-Smith et al., 2018). Phaeodaria that were shown not to be directly related to the other classical "radiolaria'' (Polet, 2004) and instead were classified in Cercozoa, have been poorly studied morphologically. One of the few ultrastructural studies has found no cross-linkers between the microtubules in the microtubular bundles supporting their pseudopodia (Cachon et al., 1973). Reshetnyak (1966) describes phaeodarian pseudopodia as thin, pointed, branching and anastomosing. This is exactly how pseudopodia look like in the classical drawings of Haeckel of the Challenger expedition (Haeckel, 1887, Fig. 10 on Plate 101, Fig. 1 on Plate 102). All of this suggests that Phaeodaria were only traditionally classified with axopodial protists and in fact have no true axopodia (Boltovskoy et al., 2017) and represent another rhizarian lineage, which acquired the reticulopodial morphotype.

Obviously, the mechanisms of reticulopodia formation in such distant organisms are expected to be different. For example, Mylnikov and Mylnikov (2011) did not find microtubules in the reticulopodial network of *Filoreta marina*, a representative of Endomyxa, a lineage sister to the Foraminifera + Radiozoa + Taxopodida clade. Krabberød and co-authors (2017) found duplications in two genes involved in the Arp2/3 complex, which facilitates actin filaments branching in Chlorarachnea and suggest that this allowed these protists to develop a reticulopodial morphotype. Morphological studies instead show that Chlorarachnea reticulopodia are microtubule-supported in *Chlorarachnion reptans* (Hibberd and Norris, 1984) and contain microtubular bundles with only short actin incorporations in *Cryptochlora perforans* (Dietz and Schnetter, 1996). Thus, it remains unclear whether these reticulopodia are actin filament-driven or microtubule-driven.

Therefore, reticulopodia have been acquired multiple times in the evolution of eukaryotes and are not restricted to the better-known foraminiferal pseudopodia. Most likely they have different inner structures driven by different mechanisms, but in general, their most probable evolutionary source is filopodia that have acquired the ability to form anastomoses through modification of their membrane characteristics. Such filopodia may be actin-driven or, as discussed in the previous section, already contain microtubular bundles (Cavalier-Smith et al., 2018).

Concluding remarks

Apart from a fundamental question about the origin and diversity of axopodia, filopodia and reticulopodia, there are examples where microtubule-based outgrowths are of outstanding practical importance. In animal neurons, the microtubules [here called neurotubules (Frixione, 2006)] pass inside the axons and dendrites, supporting their shape and taking part in the vesicular transport (Stephan et al., 2015). Microtubules are also crucial for migration, polarity and differentiation in neuronal development (Kapitein and Hoogenraad, 2015). Various factors causing the decrease of the microtubular cytoskeleton performance are responsible for multiple neurodevelopmental and neurodegenerative diseases (Hahn et al., 2019; Holzbaur and Scherer, 2011). In many aspects, neurotubules are different from canonical microtubules. In mature neurons, there is usually no single well-defined microtubules-organizing center, suggesting some unusual mechanisms of local microtubules nucleation (Yu and Baas, 1994). In axons, neurotubules have familiar plus end outward polarity, but it gets inverted, e. g., in dendrites of *Drosophila* and *Caenorhabditis*, and in mammalian dendrites the neurotubules of the opposite polarity are mixed (Tas et al., 2017; Tas and Kapitein, 2018). The regular arrangement is usually absent in neurotubules, but in axon initial segments, the part where action potential is initiated, they form the so-called fascicles – the bundles of 3-6 closely apposed microtubules united by electron-dense bridges. The fascicle organisation is proposed to play a role in polarized trafficking of organelles and vesicles (Leterrier et al., 2017). In more distal parts of the outgrowths, there is no bridges between microtubules detectable by electron microscopy but it is suggested, that they are still spaced by microtubules-associated proteins (Prokop, 2020) and sometimes regular cross-section patterns reminiscent of these in protist axopodia can be observed (see e. g. Fig. 2 A of Chen et al., 1992). According to the "orchestrated objective reduction"

theory, the tubulin dimers also play a substantial role in the maintenance of the cognitive processes and serve the physical basis of memory and consciousness (Hameroff and Penrose, 1996). Authors often appeal to complex behaviour, which some protists demonstrate in the absence of the nervous system (for the examples of such behaviour see Dexter et al., 2019; Gershman et al., 2021) suggesting that their microtubules provide the ability for cognitive processes. Remarkably, Hameroff (1998) discusses a high potential of consciousness presence in the heliozoan *Actinosphaerium* due to its microtubules-rich axopodia, but erroneously refers to it as "echinoderm" (p. 427). This theory is controversial and not broadly accepted (Khoshbin-e-Khoshnazar, 2007), but it emphasizes the remarkable similarity in the microtubular cytoskeleton of protists and neurons, which makes them advantageous model organisms for neuroscience (Brette, 2021).

Another example of the microtubules' considerable importance for the cell outgrowths formation are the so-called microtentacles formed by some types of tumor cells and having a considerable importance for metastasis (Matrone et al., 2010). Microtentacles are thin outgrowths that are supported by a loose bundle of several microtubules with plus end outward polarity suggesting the presence of a microtubules-organizing center (Killilea et al., 2017). Thus, by their organisation, microtentacles represent an example of microtubule-driven filopodia. Microtentacles are formed by free-floating tumor cells and facilitate their reattachment, thus allowing the cells' dissemination between tissues (Whipple et al., 2007). The formation of microtentacles can be provoked by chemostatic therapy, which blocks the functioning of the mitotic spindle, but at the same time facilitates the formation of microtubule-based cell protrusions (Balzer et al., 2010).

Thus, the understanding of the tubulin-based cell outgrowths formation is crucially important for answering fundamental questions such as reconstructing evolution of the major eukaryotic lineages, and at the same time, it is of great significance for medicine and neurosciences.

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