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Axon Formation, Extension, and Navigation: only a Neuroscience Phenomenon?

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Abstract

Understanding how neurons form, extend, and navigate their finger-like axonal and dendritic processes is crucial for developing therapeutics for the diseased and damaged brain. Although less well appreciated, many other types of cells also send out similar finger-like projections. Indeed, unlike neuronal specific phenomena such as synapse formation or synaptic transmission, an important issue for thought is that this critical long-standing question of how a cellular process like an axon or dendrite forms and extends is not primarily a neuroscience problem but a cell biological problem. In that case, the use of simple cellular processes – such as the bristle cell process of Drosophila – can aid in the fight to answer these critical questions. Specifically, determining how a model cellular process is generated can provide a framework for manipulations of all types of membranous process-containing cells, including different types of neurons.

Introduction

Our brains control such diverse abilities as movement, sensation, intelligence, speech, emotion, and memory only because our neurons communicate with one another and the rest of our body through highly organized networks. These neuronal networks or connections are assembled when neurons send out their stalk-like axon and dendrite appendages (Fig 1A). How do these appendages form? Extend? Change shape? What controls their length and directionality? How might we get them to regrow if they are damaged or diseased? These questions have captivated scientists for over a century [1–5] and are of the utmost importance to human health, yet they still remain largely unanswered. Compounding this problem is that researchers in these areas have also become interested in other related (but completely different) phenomena such as: What molecules are involved in setting-up particular neuronal connections and circuits? What are the neuronal circuits underlying specific behaviors? What differentiates one neuronal class from another? What range of morphological/biological events do specific axon guidance cues specify? While these are also questions of the utmost importance, they are not designed to uncover the mechanisms of neuronal process shape, extension, and navigation.

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The Difficulties of Deciphering the Mechanisms of Axon Formation, Extension, and Navigation

Over the years, a large number of diverse labs have made critical inroads into the mechanisms of neuronal process extension and pathfinding (e.g., reviewed in [1–21]). For example, ground-breaking work into the identification of extracellular guidance cues and their cell-surface receptors has provided a molecular basis for axon growth and guidance (reviewed in [3–5,7,12,18]). Likewise, elegant studies have begun to identify intracellular molecules required for transducing these extracellular signals into changes in the growth and orientation of neuronal extensions (reviewed in [9,11,13,16,20]). Adding to these critical molecular and biochemical studies is a long history of research that has sought to define the cell biology of neuronal extension and guidance, including the cytoskeletal and cell/substrate adhesive dynamics that accompany axon/dendrite elongation (e.g., reviewed in [6,8,14,15,17,19]). Collectively, this work has provided exciting insights into the mechanisms of axon extension and guidance, with important biomedical implications.

Despite these important advances, however, much remains to be learned about the mechanisms of neuronal growth and guidance [21]. Additionally, a sampling of recent results (e.g., [22–30]), including a revised view of the axon guidance cue Netrin [25,27,28], indicates that some of our long-held views on these mechanisms may be incomplete – further highlighting the importance of continuing to examine/reexamine these complex mechanisms and the fundamental functions of specific guidance cues (including by employing higher resolution approaches to study them). Moreover, similar to other cells, neuronal process extension and pathfinding require the dynamic assembly and disassembly of the structural elements – actin and tubulin cytoskeletal proteins – that control cellular shape (Fig 2A–C; [31,32]). Yet, while extracellular guidance signals have been identified that activate receptors on the surface of axons/dendrites (Fig 2D; [4,5]), how these cytoskeletal elements are precisely controlled by guidance cues/receptors remains enigmatic. Thus, it remains incompletely understood how neurons form a membranous process and how these membranous processes extend, orient in space, shape themselves, reach a certain diameter, modulate their extension velocity, form branches, know when to stop extending, and transition to a stable structure.

What has complicated our ability to clearly understand these events? While in many instances, employing neurons as a model can be seen as a “rosetta stone” for cell biology and is highly advantageous for deciphering fundamental biological mechanisms (e.g., the study of neuronal synaptic transmission provides great insight into membrane/vesicle fusion and is applicable interdisciplinarily to all cells/tissue systems [33]), the morphological complexity of neurons makes them more challenging when deciphering the mechanisms of axon formation, extension, and navigation. For example, neurons have the unusual ability to place parts of themselves (i.e., axons and dendrites, or collectively referred to as neurites) at locations far from their cell body. Consequently, far less is known of the organization and dynamics of F-actin and microtubules in these extending processes than in the cell body of a typical migrating cell. Furthermore, the movement (elongation) of axons and dendrites is much different than the movement (migration) of cells. Whereas migrating cells need to drag

along their soma and nucleus as they move from place to place, neurons do not. Instead, neurons send out long cylindrical extensions that stabilize and continue to elongate. Additionally, axons and dendrites often undergo branching and form complex arborizations that are much different than what is present in a typical cell (e.g., Fig 1A). Moreover, the *in vitro* and *in vivo* settings often utilized for studying these growth and guidance events are multicellular and complex, and therefore results are not always easy to interpret (e.g., as highlighted above for [22–30]). Likewise, although cell culture experiments are a crucial tool that have provided invaluable insights into the cytoskeletal dynamics of extending neurites and the mechanisms regulating them, they also have their limitations (e.g., see [8,14]), including that neurons are post-mitotic, for which there are a paucity of cell lines, and less work has been done to define the molecular and biochemical mechanisms of axonal cytoskeletal responses *in vivo.* Indeed, given the historical difficulties in labeling/following the extension of axons *in vivo* [34], significantly more is known about cell migration and the biology of the cytoskeleton and organelles in the soma than about their dynamic changes in neurites. Additionally, although progress is being made (e.g., [22,25]) and new technologies such as Super-resolution microscopy have been developed, the technical difficulties associated with seeing and defining what is happening inside of tiny axons and their extending growth cone tips *in vivo* (including the dynamics of their actin and microtubule cytoskeletons, the coordinated movement of proteins within them, the spatiotemporal activation of specific signaling cascades, etc.) have slowed our understanding of how neurons are specifically controlled to enable neurite formation, growth, and guidance.

Not just Neurons: other types of cells also extend membranous processes

So, are there additional strategies that might aid in the goal of further elucidating these mechanisms? Although less well appreciated, many other types of cells – from endothelial to immune to glial to bone to cancer cells – also send out similar finger-like processes that are crucial for their specialized functions (Fig 1B). Like neurons, these cells also depend on actin and microtubules to extend their processes. Indeed, it has long been observed that many different types of cells contain similarly shaped processes/extensions (Fig 1B; e.g., [35–38]) – and that motile cells have a common cell biological feature: the polarized formation of cytoplasmic protrusions that determine the direction of movement (e.g., Fig 3A–D; [38–44]). Likewise, not only do cellular extensions, including axons, form as the result of these protrusive events (e.g., Fig 3C–D), but the tips of elongating processes, including axons/growth cones, continually send out new protrusions (filopodia), which allow these cellular extensions to change directions (e.g., Fig 3E). Moreover, at least some of these non-neuronal cell types, such as endothelial cells (and their involvement in angiogenesis/vasculogenesis), use many of the same cues and signaling systems to alter their cellular morphology in ways similar to neurons [45–47]. Yet, as in the case of neurons and their processes, there is not a complete understanding of the mechanisms generating and shaping them. Indeed, despite the fact that some of these cells are much simpler in form than neurons or endothelial cells (see Fig 1B), for what appears to be a combination of reasons – e.g., a limited appreciation of the importance of studying these questions in a simple system, the absence of a unified research focus aimed at using a simple system to answer these questions, or the “right” simple system not being employed, etc. – there are no actionable

answers to these basic cell biological questions of how membranous processes form, extend, elongate, orient, and stop growing in any system.

A Complementary Strategy: other types of cellular process-containing cells as models/prototypes

Therefore, unlike neuronal specific phenomena such as synapse formation or synaptic transmission, cellular extensions are not unique to neurons. Thus, critical long-standing questions that the field is interested in answering – How do cellular processes such as axons form? Extend? Change shape? What controls their length and directionality? How might we get them to regrow if damaged? – may be thought of broadly as cell biological problems that can also be investigated in simpler non-neuronal membranous process-containing cells. Although these events in neurons are undoubtedly more complex and bring additional elements into the mix (e.g., the coordination of cytoskeletal dynamics with substrate/cell adhesion and the guidance of axons over long distances with multiple choice points, rather than the simple orientation/reorientation of membranous extensions), this reductionist approach of using simple cellular process-containing cells as a prototype to help address important long-standing questions takes a page from the long history of the elegant work using the simple eukaryote yeast as a prototype ("reference model") to answer important biological questions [48], so that one can then decipher more complex events than those occurring in yeast. Thus, a simple process-containing cell – such as the simple bristle cell of the fruit fly Drosophila (Figs 1B, 4A–B), as one example – is a similar powerful model that can be brought to bear and add to the research being done on these biomedically-critical questions.

A Simple Model: employing the bristle cell to decipher the molecular and cellular biology that builds a membranous process

Due to its simplicity, accessibility, large-size, and stereotypical actin and microtubule organization [49–52], the bristle process (Figs 1B, 4A–B) provides an attractive complementary tool for deciphering the mechanisms that generate and shape a cellular process. Specifically: **1)** The bristle cell is a simple isolated single cell with only one extending long process (Fig 4A–B; [49,52]), thereby making it morphologically less complex than a neuron or other membranous process-containing cells (e.g., compare the bristle cell in Fig 1B to the other membranous process-containing cells). **2)** The bristle cell's anatomy and development have been well-defined and are indistinguishable from cell-to-cell/animal-to-animal (Fig 4A–B; [49,52]). **3)** The bristle cell's prominent stereotypic organization (Fig 4A–B; [49,52]) promotes detailed cytoskeletal, membranous, and organelle resolution and characterization (including the ability to measure actin and microtubule dynamics in real-time). **4)** The bristle cell is an *in vivo* model (Fig 4A–B; [49,52]), and thus more emblematic of *in situ* (native) conditions than cell/tissue culturing. **5)** The bristle cell and its process are large in size (Fig 4A–B; [49,52]), which simplifies analysis and enables high-throughput visualization/delineation, including with a dissecting stereomicroscope. **6)** The bristle cell extends its process outside of the fly's body (Fig 4A–B; [49,52]), which allows a range of high-resolution and time-lapse imaging approaches

without requiring tissue processing/sectioning. **7)** The bristle cell's presence in Drosophila (Fig 4A–B; [49,52]) makes it amenable to efficient and extensive gene-editing/manipulation approaches (including tracking specific proteins over time and increasing or decreasing their levels in a bristle cell-specific/single cell-specific manner). **8)** The bristle cell's isolated nature (Fig 4A–B; [49,52]) allows for pharmacological manipulations and other selective alterations such as single-cell culturing. In the same way, available studies reveal that the molecules that shape the bristle process are similar to those used to shape axons and dendrites. In particular, mutations affecting Drosophila bristle shape, extension, and orientation have been known for over 100 years [53] – many of which have turned out to mutate specific actin and microtubule regulatory proteins that are also critical for axon growth and guidance such as bundlers, assemblers, and disassemblers [31]. Indeed, the bristle has long served as an *in vivo* model to characterize the molecules and mechanisms that regulate the cytoskeleton [49–52]. Thus, the bristle cell provides a precise high-resolution tractable tool for defining the cell and molecular biology that builds a membranous process.

Proof of Principle: gaining insights from simple cellular processes

This idea of using as a model the simplest of cellular processes – such as the bristle – has also arisen from our results using simple model systems to investigate the mechanisms of axon guidance. Specifically, multiple labs have been using the Drosophila model system to investigate how one of the largest families of axon guidance cues, the Semaphorins (with over 20 members conserved from invertebrates to humans; [54–56]), exert their effects (e.g., [29,57–64]). Semaphorins (Semas) are best known for their inhibitory/repulsive effects on extending axons [52,65] – but they also regulate the motility of cells throughout the body including those involved in immunity, angiogenesis, cardiovascular development, and cancer [66–69]. Semas elicit destabilizing effects on actin filaments (F-actin) that include a loss of F-actin, the decreased ability to polymerize new F-actin, a decrease in the number of F-actin bundles, and the regulation of F-actin-rich filopodia/spines/branches [52]. Yet, despite significant progress in the identification of Sema receptors (e.g., Plexins) and their signaling pathways [18,56,70,71], the molecules and mechanisms linking them to the control of the cytoskeleton have remained poorly understood.

To further understand these molecular and biochemical mechanisms, multiple groups have been searching for molecules that work together with Semas/Plexins. For example, we have been studying a large cytosolic protein, Mical, which has multiple protein interaction domains and is a member of a new family of Plexin-interacting proteins (the MICALs) [72]. MICALs are conserved from flies to humans and direct Sema-mediated repulsive axon guidance [72,73]. Yet, a major question that emerged early-on was: what is the specific role of MICAL proteins in axon guidance? MICALs showed similarity to oxidoreductase (Redox) enzymes [72], thereby suggesting the intriguing hypothesis that oxidation-reduction (Redox) signaling mechanisms might play a role in axon guidance [72]. Interestingly, as we tested this possibility in axons, we noticed that surviving *Mical* mutants exhibited defects in the shape, length, and orientation of their bristle cell processes (Fig 4C, compare middle and left; [74]). Likewise, elevating Mical levels dramatically rearranged bristle processes in a Redox-dependent manner – "transforming" unbranched bristles into branched structures (Fig

4C, right; Fig 4D, bottom [74]) with a similar degree of morphological complexity to that observed in navigating growth cones (Fig 4D, top). Harnessing this high-resolution, single cell model revealed that Mical does not alter bristle cell proliferation, differentiation, or survival – but specifically co-localizes with and controls the organization of F-actin during development [74]. Moreover, developing the bristle cell as a model has been instrumental in defining the effects of this Mical Redox enzyme on F-actin, revealing: 1) that Mical uses F-actin as a direct substrate – which it post-translationally oxidizes to simultaneously dismantle filaments and prevent polymerization [74,75], 2) that Mical is counteracted by a specific reductase enzyme called SelR/MsrB [76], 3) that MICALs do not function in an isolated manner but integrate with other well-known actin regulatory proteins and signaling pathways to drive cytoskeletal remodeling [29,77], and 4) that mammalian MICALs work in a similar manner biochemically and *in vivo* to Drosophila Mical [78]. Importantly, our findings using the bristle have now also been confirmed in neurons and their axonal and dendritic processes, as well as in *in vitro* systems and other cells including muscle, skin, and immune cells (e.g., [63,74,76,79–86]) – indicating that these important mechanisms have commonality in process extension among simple and complex cells and further validating the use of the bristle process as a model.

Thus, the bristle system has been an instrument for discovery of new molecular and biochemical mechanisms controlling the actin cytoskeleton. Likewise, the bristle system has provided a new understanding of the cellular mechanisms that generate and shape membranous processes. In particular, the repellent Sema is present in the dendrite that extends alongside the developing bristle (Fig 4E; [74]) and Sema activates Plexin/Mical signaling in the bristle to locally disassemble F-actin (Fig 4F; [74]). This F-actin disassembly leads to an increased complexity of the bristle process, with extending filopodia/branches (Fig 4F; [74]). The response of the bristle process to Sema/Plexin/Mical is therefore similar to the effect that cellular repellents including Semas, Slits, and Ephrins have on axons – triggering an initial disassembly of the actin cytoskeleton and collapse of the growth cone, but ultimately inducing the growth cone to reform in a more complex and branched organization (reviewed in [52]). Employing the cell biological resolution of the bristle cell process indicated that this branching results from a Mical-induced “transformation” of parallel-arranged bundles of F-actin, which are a hallmark of bristles and growth cone filopodia, into branched meshwork arrays of F-actin that are reminiscent of that seen in lamellipodia (Fig 4F; [74]). These and other observations have allowed the formulation of a model that repellents such as Semas disassemble (prune back) the actin network *in vivo*, and this pruning process initiates secondary events that serve to enhance cellular complexity/plasticity [52,74]. Strikingly, this Sema/Plexin/Mical-triggered bristle morphology is similar in appearance to what has been described when an elongating axon contacts a repellent *in vivo* (compare Fig 4G and 4H; **arrows**; [87]): the axonal growth cone “collapses” and then sends out a “back” branch that allows the axon to navigate away in a new direction (Fig 4G; [87]). Thus, a simple membranous process-containing cell, such as the bristle cell, is one such reductionist system that can provide new insights into the cellular, molecular, and biochemical mechanisms that generate membranous extensions.

Conclusions

Stimulating membranous process formation, extension, and navigation – in the diseased or damaged brain or spinal cord, for example – is crucial for curing many devastating pathologies. Yet, how can we hope to get cellular extensions such as axons to *regrow* if we do not know how they form, grow, and navigate? The observations highlighted in this review support the view that we can learn a great deal from simple systems, which can be applied back to more complex ones. Specifically, we propose that it will be advantageous to harness the attributes of simpler membranous process-containing cells and use them as a prototype for neurons. We suggest that simple model processes, such as the bristle process, provide a means to determine how a cellular process is built, thereby answering fundamental cell biological questions that are highly relevant to neuroscience, including defining 1) the membrane and organelle biology of a membranous process, 2) the cytoskeletal biology of a membranous process, 3) the cell-cell interactions specifying a process, 4) the membrane, organelle, and cytoskeletal biology of branch formation, 5) the gene expression of a process-containing cell, and 6) the genes that build a process. Of course, as new molecules and mechanisms are discovered using these simple but powerful model processes, they should continue to be tested in neurons using available approaches. Nevertheless, even if only basic mechanisms of formation and extension are translatable, understanding how one simple membranous process is built will provide a framework for manipulations of all types of membranous process-containing cells, including different types of neurons. In conclusion, it is our current opinion that this strategy provides a complementary approach to work being done in neurons, with the goal of significantly advancing the understanding, diagnosis, and treatment of diseases not only affecting the nervous system, but also many other tissues.

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HIGHLIGHTS

- Technical hurdles and complexity have hindered our knowledge of neuronal extension
- Other types of cells also extend similar axonal/dendritic-like membranous processes
- These other types of process-containing cells can serve as simple models for neurons
- These simple cells have uncovered mechanisms that form, extend, and navigate axons

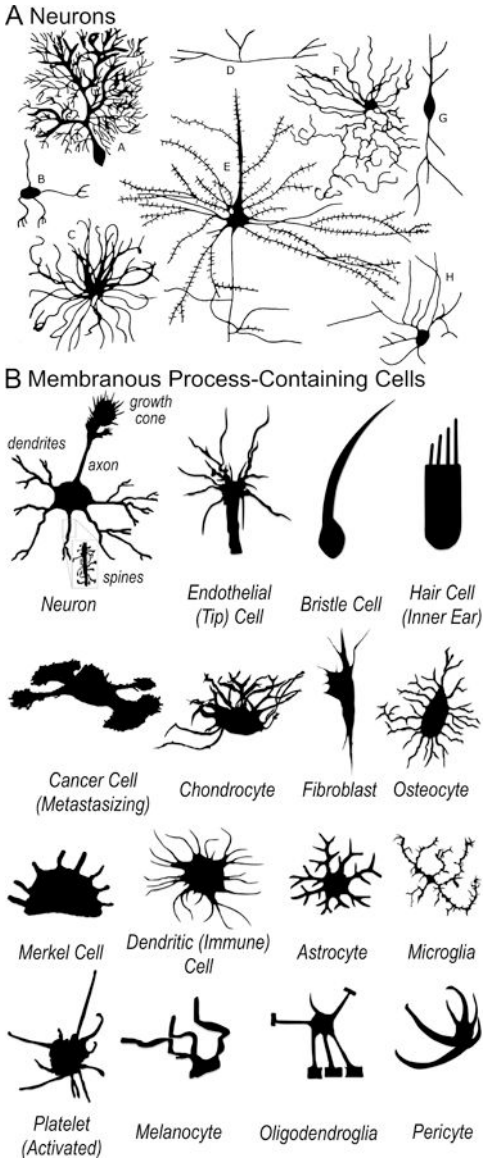

Figure 1. Neurons and other membranous process-containing cells.

(A) Neurons are the best-known of the membranous process-containing cells – but are also the most complex. Adapted from [88]. **(B)** Many different types of cells – including those within the nervous, cardiovascular, immune, and musculoskeletal systems – send out membranous extensions/processes. Many of these other process-containing cells are much simpler than neurons. The mechanisms of this process extension and the means to control it, so as to stimulate re-extension of neuronal processes or inhibit the extensions of metastasizing cancer cells, for example, are poorly understood. Adapted from [89–103].

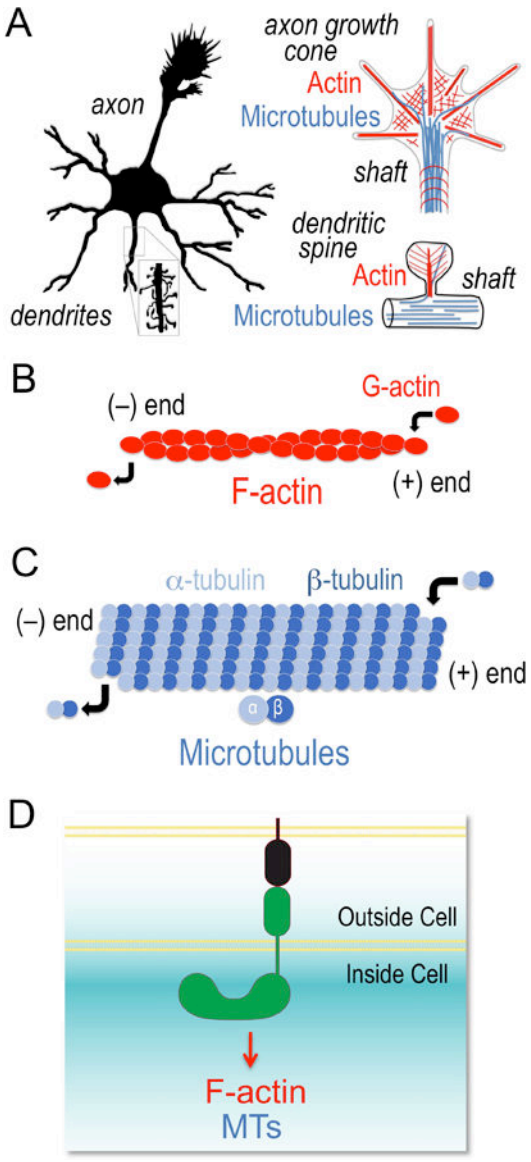

Figure 2. Control of F-actin and microtubule dynamics drive neuronal form and function.

(A–C) Neuronal extension, shape, connectivity, and function (A) is driven by regulating the ability of single actin proteins (G-actin) to form filaments (F-actin) (B) and single alpha (α) and beta (β) tubulin proteins to form microtubules (C). **(D)** Signals from the cell surface, such as from different guidance cues and their receptors control F-actin and microtubule (MT) dynamics through poorly understood means.

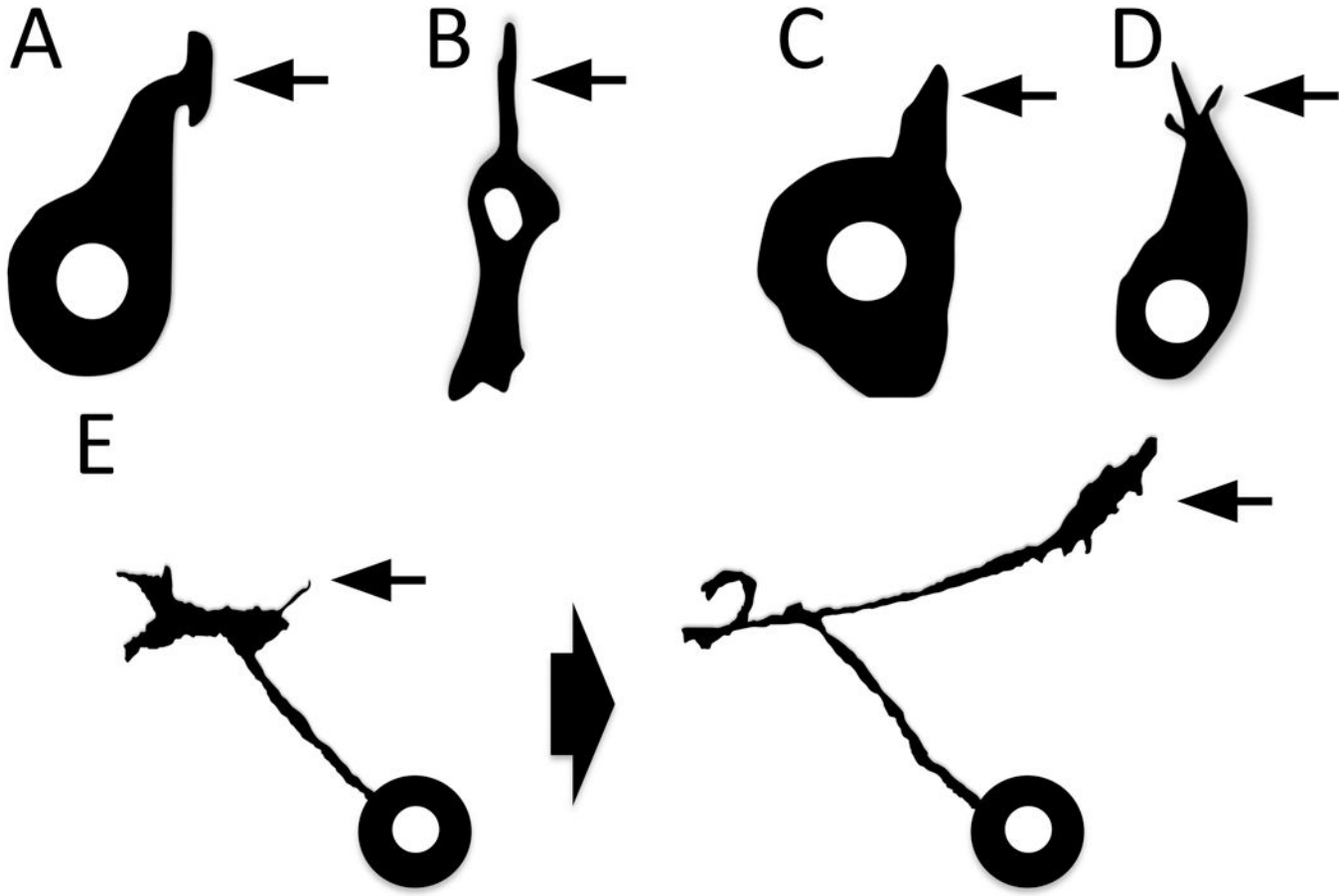

Figure 3. Development and morphology of membranous process extension and navigation.

(A–D) The extension of cellular processes (arrows) – from neutrophil (A) and mesenchymal (B) cells *in vitro* to bristle (C) and neuronal (D) cells *in vivo*. Adapted from [74,104–106].

(E) Note also the means by which axons extend "new" processes/change the direction of their process extension – via a new protrusion/filopodium (E, left arrow), which then forms the new growing cellular process/axon (E, right arrow). Adapted from [87].

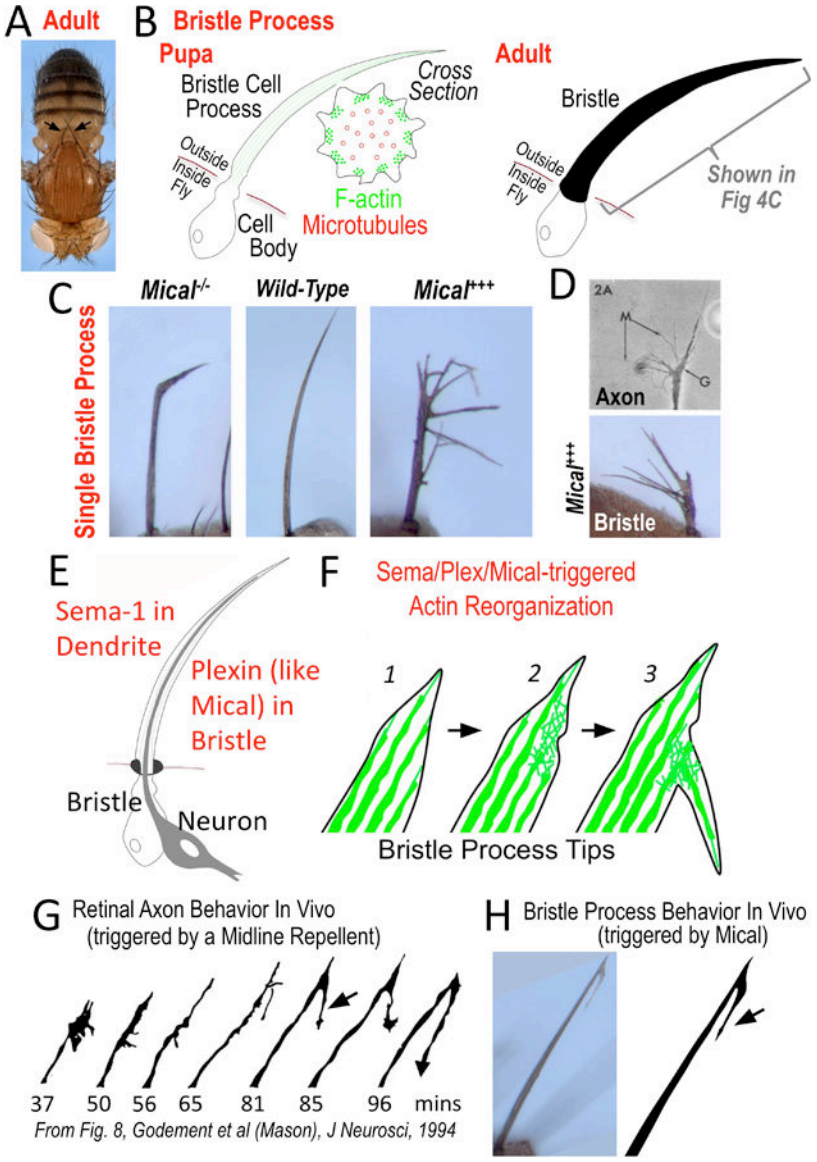

Figure 4. The bristle cell as a simple membranous process-containing cell.

(A–B) The bristle cell with its long process provides a model cellular process. (A) Note the long single cell bristle processes on the body of the adult fly (e.g., each arrow points to a single bristle cell with its process). Images reproduced with permission from [74]. (B, left) The stereotypical arrangement of F-actin and microtubules pushes out the bristle process during pupal development. (B, right) The bristle cell also secretes a chitin cuticle that wraps around its cellular process – preserving a record in the adult of its developmental history and allowing a rapid initial characterization (in different contexts and genetic backgrounds) without requiring tissue processing. **(C–D)** Changing Mical levels alters bristle process extension and morphology – including (D) inducing it to resemble an axon growth cone. Images reproduced with permission from [74,76,107]. (**E–F**) Sema (on dendrite) interacts with Plexin (on bristle), to activate Mical within the bristle to induce cellular remodeling and branching (F; [74]). (F) This remodeling and branching occurs through the disassembly of

F-actin (green) within bristle processes (2), which then allows currently unknown factors to form this actin-rich branch (3). **(G–H)** The Sema/Plexin/Mical-induced bristle branching (H, arrow) is remarkably similar to an axon's response *in vivo* to a repellent (G, arrow). In particular, the axon's (growth cone's) response is to decrease in size (G, compare 37mins to 50 and 56mins) and then form a new "back" branch (G, arrow) [87] – which is the same response seen in the bristle upon Mical activation (H; [74]). Image reproduced with permission from [87].