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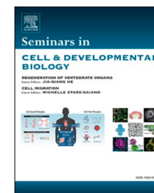

Review

Force: A messenger of axon outgrowth

Vittoria Raffa

Department of Biology, University of Pisa, SS12 Abetone e Brennero, 4, 56127 Pisa, Italy

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ABSTRACT

The axon is a sophisticated macromolecular machine composed of interrelated parts that transmit signals like spur gears transfer motion between parallel shafts. The growth cone is a fine sensor that integrates mechanical and chemical cues and transduces these signals through the generation of a traction force that pushes the tip and pulls the axon shaft forward. The axon shaft, in turn, senses this pulling force and transduces this signal in an orchestrated response, coordinating cytoskeleton remodeling and intercalated mass addition to sustain and support the advancing of the tip. Extensive research suggests that the direct application of active force is per se a powerful inducer of axon growth, potentially bypassing the contribution of the growth cone. This review provides a critical perspective on current knowledge of how the force is a messenger of axon growth and its mode of action for controlling navigation, including aspects that remain unclear. It also focuses on novel approaches and tools designed to mechanically manipulate axons, and discusses their implications in terms of potential novel therapies for re-wiring the nervous system.

1. Introduction

Axon outgrowth is a complex process through which the developing axon elongates and navigates the environment to reach the intended target. The developing axon can be regarded as a fine supramolecular machine consisting of interrelated parts with separate functions, and motion results from the cooperation of the various components. This motion is obviously caused by forces resulting from external stimuli. The axons possess molecular sensors for probing the extracellular environment and sensing stimuli. These sensors are mainly (but not exclusively) located at the leading edge (the growth cone, GC), and mostly consist of membrane receptors to bind various signaling molecules, guidance cues and molecular clutches, to probe the matrix and its stiffness [1]. An external stimulus causes the axon to change the relative positions of its components. The execution of coordinated movements is due to the many molecules that integrate this signal into a complex cytoskeletal remodeling. Elucidating all possible transduction pathways in response to extrinsic factors, the underlying axon navigation decisions and the effects on cytoskeletal changes remain a mystery to the scientific community. Identifying the biomechanics of the axon outgrowth presents another challenge, because the axon behaves like an active fluid consisting of interrelated elastic and viscous components [2]. The comprehension of axon outgrowth is beyond the bounds of conventional molecular biology and biophysics, as axons are a special type of machines that need not only energy but also new mass to operate. Thus, the main molecular strategies that enable the addition of new mass, i.e., axonal transport and local translation, appear to participate in the functioning of this machine somehow. Although our understanding of this issue has improved dramatically in recent years, its complex processes are far from being fully revealed. However, new methodological tools that can actively apply extremely low (below 10 nN) forces on axons have led to significant progress in recent years. Endogenously, force generation in response to extrinsic factors can be considered a downstream event of the signal transduction cascade in response to the stimulus, but also as an effector that regulates cytoskeleton remodeling and mass addition. To return to the analogy of supramolecular machines, the forces act as a gear or bridge between the sensing (stimulus) and the actuation (motion). The ability to finely tune this process through the generation of active forces has a dual advantage: the potential to perturbate the biological system and thus study the evoked response; and the ability to skip upstream events for direct and controlled navigation. In this review, I provide a summary of the current understanding of how exogenous force can be a tool for manipulating axonal outgrowth, focusing on the various biophysical, molecular and cellular effects.

E-mail address: vittoria.raffa@unipi.it.

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2. Which proteins generate the force required for motion?

2.1. Cytoskeleton structure

The kinetics of axon outgrowth obey Newton's first law of motion, which implies that a net force is required for axons to protrude or change direction. This poses the first question of how neurons generate forces. At the molecular level, many eukaryotic cells use the same biological building blocks to generate force. The cytoskeleton, along with its main components of actin, intermediate filaments and microtubules (MTs), is the central module. Actin and tubulin are monomeric proteins able to polymerize into long filaments and tubes, respectively. These polymers can be further arranged or cross-linked into networks and bundles. In a developing axon, cytoskeletal elements are organized in a very specific way. An actin filament-rich GC is connected to a microtubule-rich axon shaft (Fig. 1A). At the GC level, the central (C), transition (T) and peripheral (P) zones are characterized by different cytoskeletal architecture. Stable bundles of MTs are mainly located in the C zone, the meshwork of branched actin filaments can associate with dynamic MTs in the T zone, and in the P zone the cytoskeletal actin is organized in filopodia, and some exploratory tyrosinated MTs are able to extend to this region [3,4]. In the axon shaft, F-actin is organized in longitudinal trails while the cortical F-actin is organized by spectrin in periodically spaced rings underneath the axonal plasma membrane and MTs are organized in longitudinal bundles associated with intermediate filaments [5].

2.2. Unitary forces generated by cytoskeletal and motor proteins

The force generated by an actin filament under polymerization varies with the conditions of assembly, but it has been measured to be in the order of 1 pN [6], while the typical pushing force generated by a growing individual microtubule is in the range of 3–4 pN [7]. Interestingly, various molecules are typically associated with either actin or microtubules that stabilize or destabilize these biological polymers and their supramolecular structures, according to the cell requirements [8]. In addition, motor proteins such myosin, dynein and kinesin associate to these structures and convert chemical energy into mechanical activity. Myosins are a family of molecular motors that bind and slide actin filaments using ATP hydrolysis, generating a typical average unitary force of 3 pN [9]. Myosin motors in neurons, and particularly non-muscle myosin 2 (NMMII), associate and pull bundled actin filaments. Dynein proteins are MT walking motors that generate unitary stall forces of ~1 pN towards the minus end of microtubules and bind to MT with globular head domains and hydrolyze ATP during movement [10]. The kinesin superfamily of proteins are unipolar or bipolar molecular motors that move from the minus to the plus end of MT in a processive and ATP-dependent manner with unitary stall forces of ~5–6 pN [11]. Unipolar motors (e.g., kinesin-1) have a walking domain binding the MT and a domain binding the cargo or interacting with another filament. Bipolar motors (e.g., kinesin-5) have two walking domains connecting two MT parallel filaments [12]. Dynein and kinesin-1 generally promote MT bundle expansion, sliding or bulk translocation while kinesin-5 opposes it [12–14]. Motors generally exert higher forces in the axons, where they act as multiple-protein motors or in teams [15,16]. Section 4 explains how these unitary forces can combine and generate the force vectors required for motion in the GC and axon shaft.

3. Mechanosensitive (MS) proteins are required for signal mechanotransduction

The mechanotransduction process requires mechanosensitive (MS) proteins to convert mechanical forces into biochemical signals in cells. It is well known that the forces generated intracellularly by the cytoskeleton can be transmitted through adhesive clusters at the substrate interface and vice versa through a physical coupling between the F-actin filaments and the matrix [17,18]. The most remarkable examples of these signals in axons are the point contact (PC) adhesions at the GC, where MS proteins are concentrated into "molecular clutches" [19]. Of the various neuronal molecular clutches in PC adhesions, the complexes that link the actin to beta subunit of the integrin [20] or to cell adhesion molecule L1-CAM [21] have been extensively investigated. The molecular basis underlying the mechanosensitivity of MS proteins in neuronal integrin-based PC adhesions is better understood because they share many components found in non-neuronal focal adhesions, such as the recruitment of adhesion proteins talin, paxillin, vinculin, p130Cas, filamin and FAK in GCs [18,22]. In MS proteins, the application of mechanical forces often induces conformational changes. Molecule stretching can modulate mechanosensing pathways [23], and as protein functionality depends on sub-domain folding conformation, it is not surprising that mechanical tension can stretch proteins. This can expose cryptic domains or alter secondary, ternary or quaternary structures, even at the sub-domain level. Thus, molecular stretching can potentially increase or decrease protein-protein interactions [24], or interactions with membranes, and activate or inhibit enzymatic activity and modulate the association/dissociation lifetime of catch bonds [25]. Many MS proteins found in neuronal integrin-based PC adhesions show this behavior. Talin is a bridge protein that binds the integrin β-subunit with the FERM domain and actin with the C-terminal actin-binding domain. It also possesses 11 vinculin-binding sites (VBSs) in its rod domain, most of them unexposed to the water environment. Mechanical forces in the order of 20 pN have been reported to expose such cryptic VBSs, allowing vinculin to be recruited by talin [24,26]. Vinculin, in turn, anchors p130Cas, a protein presenting a central substrate domain (SD) that is intrinsically disordered. The stretching of this SD promotes p130Cas phosphorylation by Src kinases and the consequent activation of the small GTPase, Rap1 [27]. Similarly, filamin, another focal adhesion protein, has an amino-terminal actin binding the domain, Ig domains containing binding sites for integrins, and the von Willebrand receptor glycoprotein Ib (GPIbα) that arrange in pairs, thus autoinhibiting filamin interaction with these transmembrane proteins. A mechanical force of 2–4 pN has been found to be sufficient to overcome the autoinhibition, exposing the binding sites of integrins and GPIbα [28]. Tensile forces also activate FAK by unlocking its central phosphorylation site (Tyr576/577) from the autoinhibitory FERM domain [29]. Together, these mechanisms contribute to recruit proteins to the PC adhesion in a force-activatable fashion adaptor, which strengthen the connection between actin cytoskeleton and transmembrane complexes, which ultimately results in promoting the interaction of myosin II with actin filaments, actomyosin contraction and intracellular force generation. Interestingly, the force required for protein unfolding has been shown to be in the order of a few pN [30], and the force generated by PC adhesions is around 40 pN·μm^{-2} [31], making the mechanotransduction a regulated process.

Neurons also perceive force through another very important class of MS proteins, the Ca^{2+} channels, which transduce a mechanical stress into a biochemical signaling by the influx of Ca ions. A Ca influx can trigger various complex responses, depending on the local/global distribution and level of the Ca signal. MS channels can open in response to the direct application of force, in the range of 5–50 pN [32,33], or alteration to the curvature of the lipid-bilayer, which creates a tension that opens the channel, as reported for TRP channels [34]. The local activation of MS channels has been reported at the level of PC adhesions through direct binding to integrins or other components of the molecular clutches, as documented for DEG/ENaC and TRP ion channels [35,36], or through direct actomyosin contraction, as demonstrated for Piezo1 [37]. These results suggest an interesting scenario of spatially-restricted mechanical microdomains generation near the force-producing PC adhesions, thus eliciting discrete, local, and transient Ca2 + microdomains [38] that act as second messengers in the signal mechanotransduction.

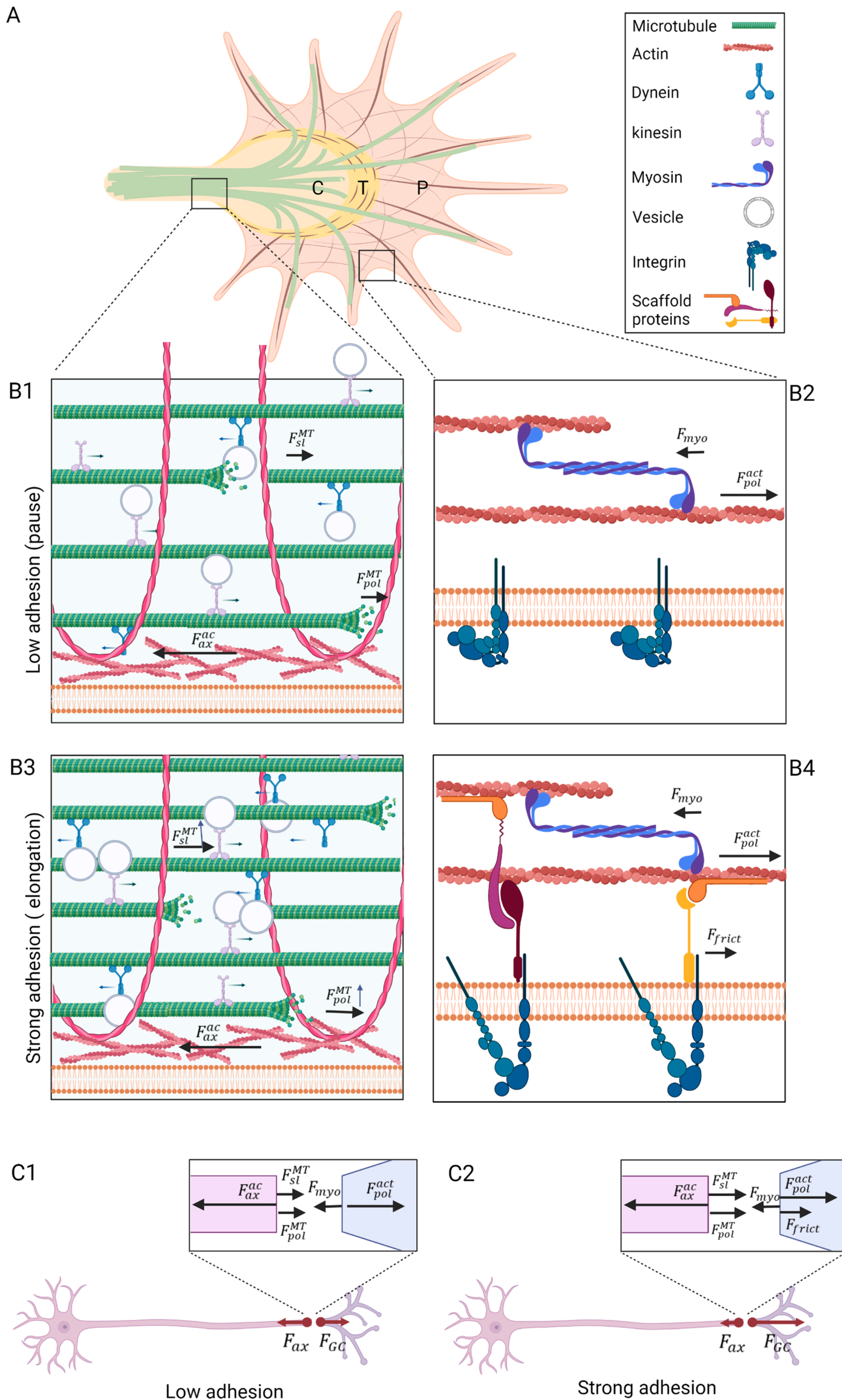

Fig. 1. A) The central (C), transition (T) and peripheral (P) zones of the GC. B) Forces generated in the axon under low (B1–2) or strong GC adhesion (B3–4). B1–3): F^{ac}_{ax} is the contractile force generated by the axonal actin cortex and the myosin-driven contraction; F^{MT}_{pol} generates from the MT assembly; F^{MT}_{sl} moves MTs anterogradely and is mainly powered by dyneins moving towards the minus-end and the kinesin-mediated MT sliding. B2–4) F^{ac}_{pol} generates from polymerization of actin filaments in the P domain; F_{myo} generates an actin RF powered by the myosin; F_{frict} opposes the adhesion force resulting from the linkage between the molecular clutches and the matrix at the adhesion points. C) The force vectors combine to give F_{GC}, the resulting contractile force generated by the GC that pulls the axon shaft, and F_{ax}, the resulting contractile force generated by the axon shaft that pulls the GC. Elongation is facilitated by the increase in F_{GC} and the decrease in F_{ax}. Created with BioRender.com.

4. Force vector generation in the axon

The GC generates a contractile force that pulls the axon shaft (F_{GC}) [39], which generates a contractile force that pulls the GC (F_{ax}) [40]. The balance between these two forces is responsible for tip protrusion or retraction. The strong contractile forces generated in the GC and the weaker contractile forces along the axon shaft account for the growth [2] (Fig. 1C2). For example, in the chick sensory neurons, the net contractile force generated by the axon ($F = F_{GC}$-F_{ax}) has been estimated to be about 0.6 nN [40]. Many mechanisms are responsible for the generation and modulation of these contractile forces, which result from different force vectors that add or counteract.

In the P domain, the network of actin filaments directly pushes the plasma membrane forward through polymerization (F^{ac}_{pol}). In a paused growth cone, membrane tension opposes this pushing force and a retrograde flow (RF) of actin is generated, powered by the actomyosin contraction (F_{myo}), which recycles actin filaments to the T domain where they de-polymerize [41,42] (Fig. 1B2). MTs explore filopodia by moving back and forward. Their forward movement is powered by dynein and stabilized through the tau-mediated interaction with actin filament bundles [43], while the back movements are coupled to the actin RF [44]. Under this condition, neither filopodia protrusion in the P domain nor MT translocation from the C to the T domains occur, and the contractile force generated at the GC is low (Fig. 1C1).

However, when the F-actin filaments are engaged in mature PC adhesions, the frictional traction forces resulting from the linkage with the matrix (F_{frict}) balance the force generated by myosin, slows down the actin RF [45,46], and generates a traction force onto the matrix at the adhesion point. Under this condition, a $F^{act}_{pol}$of several piconewtons pushes the filopodial (1–5 pN) and lamellipodial (up to 20 pN) edge forward [47,48] (Fig. 1B4). Aplysia growth cones can develop traction forces at the adhesion site of up to 100 nN [49]. As the traction force significantly increases at the adhesion site, the Rho-dependent actomyosin-contractility leads to massive MT translocation through association with actin bundles (C domain) and actin arcs (T zone), while in the P domain [50], MTs simply advance towards the adhesion site because of actin RF attenuation and actin clearance [51]. This results in a net contractile force that pulls the axon shaft (Fig. 1C2).

The main contribution to the contractile force generated by the axon shaft is from the axonal actin cortex and the myosin-driven contraction (F^{ac}_{ax}), which produces both circumferential and longitudinal contractile forces along the axon [52]. However, the contractile force is partially attenuated by other forces generated in the shaft. Specifically, the MT assembly generates a compressional force that pushes against the GC (F^{MT}_{pol}). Additionally, the relative sliding between MT and the F-actin cortex, which is powered by dynein, and between two different MTs, powered by kinesin-1, produce an extension of MT arrays that push against the GC (F^{MT}_{sl}) [53] (Fig. 1B1–3). The relatively low net contractile force pulling the GC results from the sum of these counteracting forces along the axon (Fig. 1C1–2).

A concise summary of the main force vectors endogenously generated in the axon is schematically depicted in Fig. 1. Importantly, these force vectors are finely modulated by both extrinsic and intrinsic factors, as the growth proceeds in a "stop and go" fashion, with periods of motion separated by pauses, even in an unchanged environment [54].

5. Tools for the generation of active mechanical stimuli

5.1. Types of stimulation

Recent advances in the fields of micro- and nano-technologies have provided many tools for the fine control of the generation of precise mechanical stimuli for neurites. Each type of technology possesses unique and specific features designed for examining particular biological questions. There are approaches that are primarily aimed at generating a strain or a stress, the latter could be tractional, compressional or shear stress. Stress and strain are directly coupled but, depending on the specific technique, one is applied in a controlled manner and the other is induced. The stimulation modality can be acute, chronic or cyclic. Acute stimuli generally consist of the generation of strong forces (1–100 nN order) for a short time (considered one day or less here) because their prolonged application could cause neuron death or axon breaking. In chronic stimulation, the forces are weaker (<1 nN) and a continuous loading can be applied for days or weeks. Cyclic stimulations usually involve the application of higher forces (nN order) at low frequencies (Hz or below) and can include a duty cycle. However, distinguishing these different schemes is biologically important. The application of high force is often associated with neurite retraction [55] while lower forces generally promote axonal outgrowth [56] and different pathways of signal mechanotransduction are activated in response to these different stimuli. For example, the response evoked by high force is particularly relevant to the study of some pathological conditions such as nerve injury or neurodegeneration [57]. Conversely, chronic stimuli are particularly relevant in developmental biology, as they mimic the endogenous generation of forces, e.g., during "towed growth", in which an increase of body mass puts the axons of integrated neurons into a condition of stretching [58]. The application of cyclic mechanical stimuli has profound significance in the context of aging, as throughout a lifespan nervous tissue is exposed to long-term mechanical stresses [59]. Technologies can also differ in terms of the target. Some methods stimulate a single cell while others stimulate a population of cells or of tissues and they have a higher translational potential. The location of the stimulus is extremely important when examining the evoked response. Force can be applied into a specific point of the axon or can be distributed. In some cases, the tension localizes at the GC, while in others the whole axon is stretched. Exogenous forces are generally external to the cell membrane but it is also possible to generate force intracellularly.

5.2. Methods and tools

Table 1 provides a brief summary of some popular technologies and their main features. The first tool proposed for stretching neurites was the force-calibrated microneedle (MN). A MN attached to the GC is moved perpendicular to the neurite's long axis through a hydraulic micromanipulator [60,61]. The restrained bead interaction (RBI) is a variant in which the MN restrains a microbead coated with antibodies to attach cell adhesion molecules in the GC [17]. Other popular methods are optical tweezers (OT) [62] and magnetic tweezers (MTw) [63] in which optical or magnetic microbeads, respectively, manipulate the GC through electromagnetic traps. The use of magnetic nanoparticles (MNP) has recently gained popularity, as these can be simply added to the cell growth medium and are spontaneously internalized by cells. MNP interaction with the elastic constrained components of the neurites (e.g., membranes and the cytoskeleton) can stretch these components under the effect of a magnetic field gradient [64]. Magnetic actuation is also applied in magnetic microposts (MM), which consist of an array of vertically aligned polymer pillars incorporating magnetic nanowires [65]. These have been used to grow cells on the top of the array and the adherent neurites can be stretched when external magnetic fields induce a torque in the nanowires. A micro-stepper motor (MSM) can be used to stretch axons by growing neurons on two overlapping membranes and displacing the top membrane across the lower stationary membrane [66]. Neurons have also been cyclically stretched by culturing them on the top surface of elastomer chambers (EC) mounted in a stretcher device. The frequency of the stretch application can be in the order of tens or hundreds of mHz and the duration can be of several days [67]. Atomic force microscopy (AFM), primarily developed for measuring forces, can also be modified for manipulating a cell adherent on a substrate, by pulling the neurite directly or indirectly (e.g., through a bead) when attached to the cantilever [55].

Table 1
Main methods for the application of exogenous forces and the most common features.

Method	Stimulus		Type			Force		Through-put		Cellular		Location	
	Strain	*Stress*	*Acute*	*Chronic*	*Cyclic*	*<1nN*	*>1nN*	*1 cell*	*High*	*Intra*	*Extra*	*Distributed*	*Point*
MN/RBI	■	■	■			■	■	■			■		■
AFM	■		■		■	■	■	■			■		■
OT		■	■			■		■			■		■
MTw		■	■			■		■			■		■
MNP		■		■		■			■	■		■	
MM		■		■	■		■		■		■	■	
MSM	■			■	■		■		■		■	■	
EC	■			■	■		■		■		■	■	

MN: microneedle; RBI: restrained bead interaction; AFM: atomic force microscopy; OT: optical tweezers; MTw: magnetic tweezers; MNP: magnetic nanoparticles; MM: magnetic microposts; MSM: micro-stepper motor; EC: elastomer chambers. These features refer to the standard set-up, variations are possible, but they are not considered here. In terms of the different types of stimulus (stress versus strain), the table indicates which of these is applied. In terms of force, the table indicates the typical unitary force generated.

6. Force and axonal outgrowth

Force induces axon growth at a rate of 0.1–1 $\mu m \cdot h^{-1} \cdot pN^{-1}$ for both central and peripheral nervous system neurons, independent of the magnitude of the force (from 10 pN to 100 nN), the technology used for stretching the neurites, the location of the force and the model [39,61, 68–71]. The process is generally referred to as “stretch growth” [56,66]. A continuous loading is required for inducing stretch growth and when the load is removed, neurites resume the elongation rate of tip growth [72].

6.1. Force and guidance

The direction of axon growth can be easily manipulated by applying

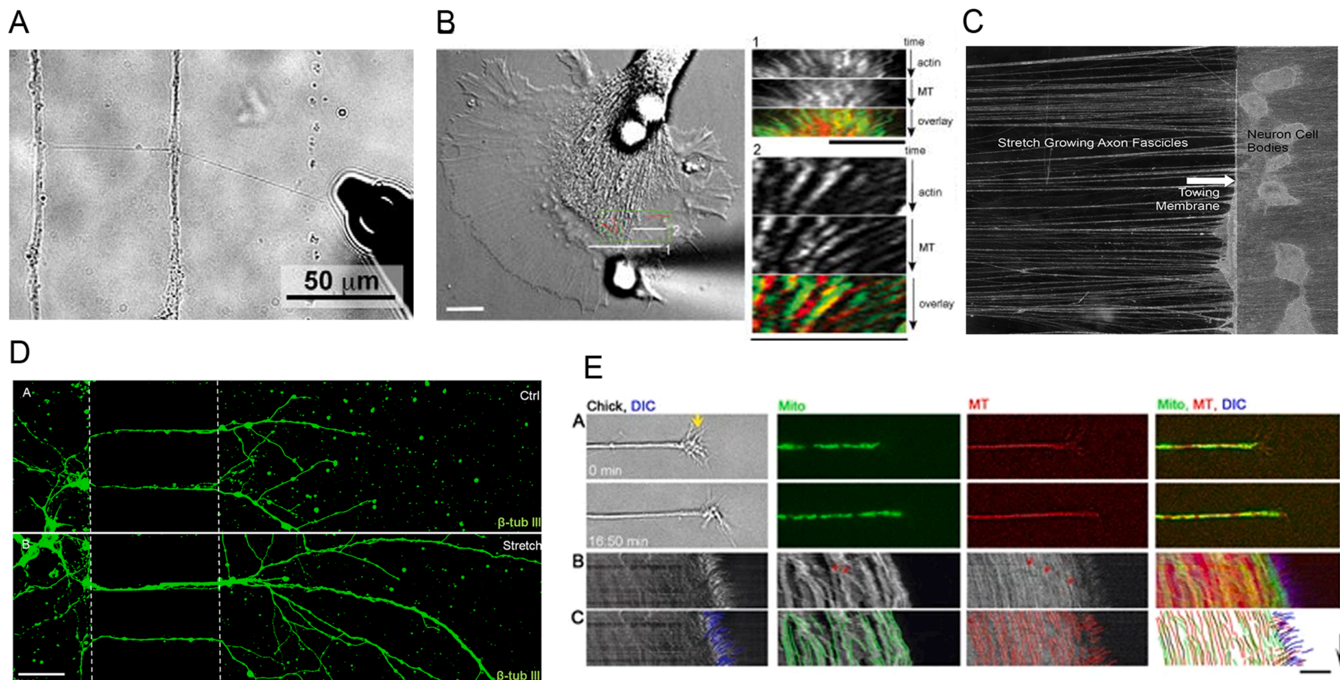

Fig. 2. Stretch growth occurs independently of the site of force application and mass addition takes place along the entire axon. A) Initiation, elongation, and connection of new neurites in primary rat hippocampal neurons pulled using PDL beads and AFM [73]. Reprinted with the permission of the Journal of Neuroscience. B) DIC image of a live Aplysia growth cone pulled via RBI. Microtubules (red) and actin fibers (green) in the T zone and C domain re-orient toward the bead during the traction period. Scale bar: 10 µm. [51] Reprinted with the permission of Wiley Online Library. C) Integrated axons of DRG neurons stretched via MSM. Axons respond to the strain imposed by growing in length and fasciculating [102]. Reprinted with the permission of Elsevier. D) Pulling the axon shaft of hippocampal neurons via MNPs induces axon outgrowth [71]. Scale bar: 50 µm. Reprinted with the permission of the Journal of Neuroscience. E) A chick DRG growth cone was monitored during elongation. The tension generated by the GC induces the anterograde translocation of axonal MTs (red) and docked mitochondria (green), as shown in kymographs. The yellow arrow marks the T zone. Red arrows indicate a mitochondrion and MT speckle undergoing fast transport. Arrow 10 min and bar 10 µm [79]. Reprinted with the permission of Springer Nature.

extremely low mechanical forces (Fig. 2A) [73]. The MNP-labelled neurites of PC12 cells were pulled with a 0.5 pN tangential force for 72 h, by inducing a net rotation of the main axis in the direction of the force vector, with a mean angle displacement of 30° [64]. This result is consistent with another study in which a shear stress of 0.15 pN was generated by trapping and spinning an optical bead via OT. The GC of a goldfish retinal ganglion cell adjacent to the bead turned 30° in 10 min in response to this force [74]. The increase in shear stress of up to 20pN was able to turn the GC about 90° in 1 h [75]. Another study identified an optimal force range of 4.5–70 pN for re-positioning the MNP-labelled neurites of cortical neurons via magnetic fields [76]. Although these forces are very low compared to the 0.1–0.5 nN force typically generated by the GCs of mammalian neurons [77,78], their effect should be understood in the context of dynamic molecular clutches. Integrin receptors and their ligands at the PC adhesions form transient clutches that bind and unbind with specific kinetics under mechanical loading (traction). When the adhesion site is also subjected to shear stress, the integrin detaching from its molecular partner can slip in the direction of the stress and forms a new bond with the adjacent ligand, behaving as a molecular "walker". This process has been mathematically modelled with the Bell theory and the model corroborates the experimental evidence [64]. The ability to control neurite navigation using pN tangential forces suggests that the shear stress generated in vivo by fluidic flows is crucial to shaping and wiring the nervous tissue connectome [75]. Indeed, the use of mechanical force may also be a potentially effective therapeutic strategy to re-shaping a damaged nervous system, particularly if mechanical and chemical stimulation are combined [63].

6.2. On-axis force at the tip

The application of on-axis force induces axon elongation. When Aplysia GCs are pulled via RBI, the adhesion forms soon after bead placement (latency) and MTs preferentially explore the nascent adhesion site [17]. The PC adhesions subsequently mature (interaction phase), the actin RF slows maximally to 15% of the unrestrained condition and the contractile force at the GC increases. This is accompanied by a dramatic cytoskeletal remodeling, consisting of the bulk translocation of the MT bundles from the C and T domains towards the adhesion site (together with actin arcs and bundles, respectively) (Fig. 1B) and protrusive growth of the leading edge [51,79]. This process is found to be generally spatially restricted to the on-axis bead interaction. The dynamic MTs invading the adhesion site carry signals involved in neurite outgrowth [80] such as the active Src kinase, whose tyrosine phosphorylation activity promotes filopodia motility [81]. For example, the PC adhesions have been found to recruit the α-tubulin acetyltransferase 1 (αTAT1) that promotes MT acetylation [82], which in turn causes the GEF-H1 to detach from the MTs and to activate RhoA, promoting actomyosin contractility. This and many other mechanisms contribute to a positive loop for regulating the generation of high traction forces. The application of pN forces exclusively to filopodia via MTw elicited filopodial protrusion but no advancing cone growth, highlighting that the generation of a traction force is essential for axon growth [83].

Stretched axons not only increase in length but also in volume, while maintaining a normal cytoskeletal ultrastructure, indicating that mechanical tension stimulates the addition of new mass [84]. Thus, there is a maximum threshold value of the elongation rate. Chick forebrain neurites are observed to thin and break in less than 10 min when elongated at 3 μm·min^{-1} via MTw [85]. Similarly, axons of dorsal root ganglion (DRG) neurons stretched by MSM were found to break when the strain exceeded about 2% of the initial axon length, but if a conditioning time is applied between each step (i.e., 2 μm displacements every 172 s), elongation proceeds without disconnection [66]. This is not surprising, as sustained axon growth is dependent on the supply of proteins, lipids, cytoskeletal elements and organelles [86]. A large amount of this mass is added to the GC through the bulk translocation of MTs transporting molecular cargoes and organelles into the protruding GC. Mass addition was previously thought to exclusively occur at the tip, while the axon shaft remained stationary [45]. However, subsequent studies using various axonal markers report that so-called "intercalated" mass addition occurs, meaning that new mass is added along the length of the axon to prevent thinning [56,61]. This evidence, together with the observation that the axon shaft is pulled by the traction force generated by the GC even when it pauses [87], suggests that the axon shaft lengthens in response to the F_{GC} and this stretching leads to the intercalated mass addition along the axon [56,79](Fig. 2E). Many subsequent studies have involved pulling the whole axon rather than the GC, which are aimed at assessing the contributions of the axon shaft to the process [61].

6.3. On-axis force at the axon shaft

The axon behaves as an active partner of the GC in axon outgrowth [88,89]. Stretched axons may possess an intrinsic capacity to elongate (Fig. 2C-D), above that imposed by the GC [73]. The GC may also limit the axon's intrinsic capacity for rapid growth [90]. A significant remodeling of the axon microtubule cytoskeleton in response to the force has been identified. An increase in the linear density of MTs in the axon shaft of the hippocampal neurons was found, and similar results have been reported for axons stretched intracellularly via MNP [71] or extracellularly via MM [65]. Similarly, an increase in tubulin content and MT formation in the stretch direction was found in the neurites of primary cortical neurons subjected to a cyclic strain [67]. An increase in MTs can cause an increase in the pulling forces F_{pol}^{MT} and F_{sl}^{MT} that counteract the contractile force F_{ax}, facilitating axon outgrowth (Fig. 1C2 vs Fig. 1C1). This mechanism can be regarded as a positive force generation loop: a pN force induces a substantial increase (20–40%) in the MT linear density in the axon shaft [65,71,91], each MT generates a pN pulling force, and the resulting net force (F_{pol}^{MT} plus F_{sl}^{MT}) substantially increases in magnitude (in the nN range) (Fig. 1B3 vs Fig. 1B1). The majority of MTs in the formed axon have a polarity with the plus end oriented towards the GC, which may explain why a force oriented from the axon hillock to the tip is productive for growth but not if it is directed from the tip to the soma [72]. Why MTs respond to force and how tension influences MT dynamics remain unclear. Nocodazole (MT-destabilizing activity), but not Paclitaxel (MT-stabilizing activity) have been found to block stretching and growth, highlighting that MT stabilization is an essential factor [71]. MTs have been found to be tension sensors per se and an increase in traction force can slow down MT depolymerization [92]. Tension can also promote net MT assembly [93] and stabilization [94]. MAPs are another possible target that modulate the stability/instability dynamics of MTs [95]. For example, tau re-positioning in response to force has been identified [76]. Finally, the theory that motor proteins can behave as "mechanical strain-dependent direction-switching force generators" has been proposed [96] and a switch in the walking direction in response to force could account for changes in MT sliding or expansion. Thus, the interaction between external force, the stabilization of axonal MTs, and axon growth deserves attention and requires further investigation.

The sustained axon growth of stretched axons is a process demanding high levels of mass and energy, and which closely relies on the axonal transport of vesicles, granules, and organelles. Accumulations of endoplasmic reticulum (ER) and ER cisternae were found in the stretched axon shaft [65,71,91], which are required in the control of many functions such as lipid biosynthesis, protein translation and calcium storage. Mechanical tension has been found to be directly involved in modulating vesicle dynamics [97]. The large dense core vesicles in Aplysia neurites spend more time undergoing active (molecule motor-driven) transport, and have increased mobility and motion, when the neuron is stretched via EC [98]. Vesicles were also found to cluster at the pre-synaptic terminal after the neurites were pulled for 30 min [99]. Synaptic vesicle

accumulation occurs in the absence of $Ca2+$ influx, suggesting that the observed enhancement is directly mediated by the force [97]. The modulation of the vesicle transport may be due to the stabilization of MTs or the direction-switching ability of molecular motors under tension, as discussed above. Stretch-driven actin polymerization [100] may create a structural scaffold that tethers vesicles at the synapse [99,101], which could explain why vesicle accumulation persists after the removal of the stimulus [97].

7. Force and calcium signals

DRG neurons subjected to a strain via MSM do not show global changes in terms of calcium influx [103]. However, a 0.1–1 nN stretch via MNPs triggers calcium influx into the cortical neurons, thus increasing the magnitude and frequency of intracellular $Ca2+$ waves. This discrepancy may be because these starch-coated MNPs were not internalized but instead associated with the cell membranes, where the MS channels are mainly located: the force stretches the lipid membrane, modulating the open probability of mechano-sensitive N-type $Ca2+$ channels [104]. Indeed, the treatment with an inhibitor of the mechano-sensitive N-type $Ca2+$ channels blocked the calcium influx in stretched cells [105]. However, when the axons of hippocampal neurons were stretched with a 10 pN force via MNPs, the axonal level of calcium was reduced by 75% but the elongation rate doubled relative to the control cultures [71], consistent with evidence that a rapid elongation rate is associated with low calcium transients [106]. Interestingly, the neurons returned to the control levels 30 min after the stimulus was removed [71]. These data together suggest that the location of the stimulus and the magnitude of the force influence the response. Neurons have evolved to establish distinct molecular pathways in response to the "traumatic" stress associated with calcium influx or the low forces associated with developmental stretch. When the force exceeds a critical value, a calcium influx occurs in the neurite through the MS channel, which leads to the breaking of the adhesion sites, possibly due to the activation of calpain, which induces the proteolytic cleavage of talin [107] or cofilin-mediated actin depolymerization [108], along with GC collapse and neurite retraction. A local tension exceeding a value of 274 $pN \cdot \mu m^{-2}$ at the GC of PC12 cells stretched via AFM causes a calcium influx through the MS channels [55]. The process is transient: the calcium levels propagated from the GC towards the soma first increase but disappear after tens of seconds. A new GC is then formed from the retracted axon and starts re-growing in the opposite direction to the traumatic stretch [55]. The generation of distinct force microdomains in the matrix is likely to be a well-conserved mechanism for finely shaping the morphology of the nervous system during development [109–111].

8. Cross-talk between chemical and mechanical signals

Mechanical and chemical signals together control axon growth, but our current understanding of their cross-talk is limited [112]. The axons of hippocampal neurons stretched via MNPs do not respond to brain-derived neurotrophic factor (BDNF), suggesting that force may interfere with BDNF signal transduction [71]. Force can control signal transduction downstream of chemical guidance cues. Many neurotrophic factors have been found to stimulate the GC-mediated elongation in a similar way to force. Guidance cues, such as the nerve growth factor (NGF) [113], netrin-1 [114], the BDNF [115] and semaphorins [116] transduce a chemical signal into traction forces and stimulate axon outgrowth by increasing the contractile force generated by the GC (F_{GC}). Repulsive cues, such as slit-2 [117] and EphrinA [118], destroy PC adhesions, and have an opposite action. Netrin-1 offers a remarkable example as it binds its receptor (DDC), which activates its downstream effectors (Cdc42, Rac1), inducing PAK-mediated shootin1 phosphorylation. Phosphorylated shootin1 mediates the linkage between RF and L1-CAM, strengthens the connection between actin cytoskeleton and transmembrane complexes, and promotes the reduction of actin RF, the generation of force, and neurite outgrowth [119]. The traction force generated by the GC before or after 1 h netrin-1 stimulation was found to be about 25 and 35 $pN\ \mu m^{-2}$, respectively. Interestingly, netrin-1 also modulates the MT dynamics in the filopodia where dynamic MTs are "captured" by a direct interaction with DCC [120]. The stimulus also induces a rapid movement of RNA granules from the C zone into the filopodia, via association with actin filaments [121] or DCC-stabilized MTs [120]. Additionally, DCC, which colocalizes with the translation machinery in neurites, was found to detach from these components (such as eIFs and ribosomal subunits) upon netrin-1 stimulation [122], which can promote the local translation of the mRNA translocated at the P zone, such as β-actin [123], DSCAM [124] and Par3 [125]. These mechanisms are essential for GC steering because an asymmetric netrin-1 signal elicits an asymmetric β-actin mRNA translation [126]. The coordination among the maturation of PC adhesions, the MT dynamics, local transport and local translation has been reported for many chemoattractant and chemorepulsive signals [127–129]. Testing this effect in direct mechanical stimulation would also be of benefit in future studies.

9. Conclusions

Axons are sensory-motor machines that have evolved to sense their local extracellular environment, interpret any mechanical and chemical signals, execute specific programs for navigating towards the target during development, and, after reaching it, accommodating the growth of the organism. It is well known that the axon shaft is under tension throughout its lifetime. During axon pathfinding, this force is powered by the growth cone that responds to matrix stiffness and guidance cues, thus directing the cytoskeletal changes that put the axon shaft under traction. Later, in integrated axons, stretching occurs as the body grows. Indeed, the force generation is finely regulated and can transduce multiple programs of growth, switching from promoting elongation to retraction depending on its intensity, in terms of a threshold value that is likely context-dependent. Forces exceeding this threshold can cause neurite thinning or breaking, or the opening of MS channels that consequently activate calcium-dependent proteases that cleave adhesion points. Conversely, when the force is below this threshold, axon outgrowth is promoted, but the discovery of the signal transduction still remains a puzzle for the scientific community. The general consensus is that tension induces an immediate response, which remodels the axonal cytoskeleton. However, the most interesting question concerns which molecular mechanisms orchestrate cytoskeleton changes and the supply of new building blocks. Recent data suggests that tension may directly or indirectly stimulate the stabilization of microtubules. This could generate a positive loop that decreases the axon contractile force and promotes tip advance, while also favoring the transport and biosynthesis of the molecules, vesicle and organelles required to sustain the intercalated mass addition [91]. However, this intriguing hypothesis awaits formal proof. Nevertheless, tension clearly has a central role, and thus the direct application of active forces to the axon can promote elongation, manipulate axon guidance, or counteract the effects of repellent molecules, thus enabling axon regeneration in inhibitory environments [63]. This could represent a plausible therapeutic strategy in the near future, and therefore adds a new and fascinating dimension to the study of axon mechanobiology.

Declaration of Interest

None.

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