Scuola Internazionale Superiore di Studi Avanzati - Trieste

Role of Myosin II and Arp 2/3 in the motility and force generation of Neuronal Growth Cones

Thesis submitted for the degree of

"**Doctor of Philosophy**"

Academic year 2014 – 2015

Candidate

Wasim Amin Sayyad

Supervisor

Prof. Vincent Torre

International School for Advance Studies - ISAS)

SISSA - Via Bonomea 265 - 34136 TRIESTE - ITALY

Scuola Internazionale Superiore di Studi Avanzati – Trieste

Role of Myosin II and Arp 2/3 in the motility and force generation of Neuronal Growth Cones

Thesis submitted for the degree of
"Doctor of Philosophy"

Academic year 2014 – 2015

Candidate
Wasim Amin Sayyad

Supervisor
Prof. Vincent Torre

Scuola Internazionale Superiore di Studi Avanzati (SISSA) (International School for Advance Studies - ISAS), Via Bonomea 265, 34136 Trieste, Italy

Dedicated to my Parents……..……..

Declaration

The work presented in this thesis was carried out at the International School for Advanced Studies, Trieste, between September 2010 and October 2014, under the supervision of Professor Vincent Torre. This thesis led to the following published and unpublished articles

1) "The role of myosin II in force generation of DRG filopodia and lamellipodia", **Wasim A. Sayyad***, Ladan Amin*, Paolo Fabris, Erika Ercolini, Vincent Torre. Scientific Reports 5, 7842; DOI: 10.1038/srep07842 (2015)

2) "Role of Arp2/3in force generation and motility of the Neuronal Growth cone", **Wasim A. Sayyad**, Paolo Fabris, Vincent Torre. **Submitted** to Scientific Reports

Table of contents

Abstract

Differentiating neurons have to find chemical cues to form the correct synaptic connections with the other neurons so that they can create a functional neuronal network. During their development differentiating neurons project neurites, at the distal part of which there is a growth cone (GCs). The growth cone has highly motile structures, referred as lamellipodia and filopodia. Lamellipodia and filopodia sense the environment and process the mechanical and chemical stimulus and also exert forces. During my work for the completion of my PhD thesis, I used Optical Tweezers, video imaging and immunocytochemistry to quantify the motility and the force exerted by lamellipodia and filopodia from Dorsal Ganglion (DRG) neurons. I have also precisely quantified the role of some proteins and signaling pathways which regulate the motility of the DRG GCs.

The first part of my results entitled, "The role of myosin-II in force generation of DRG filopodia and lamellipodia", characterizes the role of Myosin II in growth cone dynamics. Myosin II has been shown to control the retrograde flow of actin polymers, to be involved in the orchestration of actin and microtubules (MTs) dynamics and to possess contractile activity. GCs advance due to combined effects of the adhesion of lamellipodia and filopodia on the substrate and the contractile activity of Myosin II. Therefore, I probed the functional role of Myosin II on GCs dynamics by using its specific inhibitor, Blebbistatin. I show that the force exerted by lamellipodia decreased but surprisingly the force exerted by filopodia increased upon treatment with Blebbistatin. Moreover I show that the well organized and distributed structures of lamellipodia and filopodia of the GCs depend on the activity of Myosin II and confirmed the coupling between actin and microtubule dynamics.

The next chapter, "The role of Rac1 in force generation of DRG neurons", describes the function of Rac1 and its downstream effector Arp2/3 in lamellipodia and filopodia formation and dynamics. It is well known that Rac1 Rho-GTPase acts as a switch between GTP bound active state and GDP bound inactive state. I observed that GCs retract following partial inhibition of Arp2/3 but recover their usual motility within 5-10 minutes. I found that this recovery is caused by the activation of Rac1. This indicates that Rac1 acts as switch and activates upon Arp2/3 inhibition, possibly through integrin pathways. I also confirmed that the

activity of Arp2/3 not only regulates the formation of lamellipodia but also controls the dynamics and formation of filopodia.

Chapter 1

Introduction

Locomotion is an uncompromising part of life and essentially all living organisms on this planet exhibit some sort of movement starting from cellular level. For a specific movement an individual cell can convert the stored chemical energy into mechanical energy through organelles (Bray D 2001).

Neurons are among the most specialized cells in a living organism and are able to self-organize in precisely wired networks. Differentiating neurons during their development project neurites. At its tip each neurite contains a growth cone (GC) which crawls in search of chemical cues (Goodman 1996). In this way a neurite makes synapses with the other neurons or cells and connects the peripheral body part to the central nervous system (Ghashghaei et al. 2007; Trivedi & Solecki 2011). Through these contacts neurons receive and transmit information from and to other cells. This permits the processing of sensory information, organ function regulation, movement control, and higher functions like memory, thought, and self-awareness (Engle 2010). Correct wiring of the developing nervous system is essential for proper function during adulthood. Therefore, it is very important to understand the dynamics and directed movement of the GC before it makes synapses, which has been described with different theoretical models and experimental approaches (Mogilner 2009). The main objective of this thesis is to study the intrinsic interesting process behind the motility of GCs and to characterize the role of proteins and signaling pathways regulating the process in Dorsal Root Ganglion (DRG) neurons.

I will first describe the structure and function of GCs, then the role of important proteins and signaling pathways regulating the force and motility of GCs. At the end of this section I will briefly explain the theoretical models proposed in vitro force measurements for the force generation in biological systems.

1.1 Neuronal growth cone:

The neuronal growth cone (GC) is the highly motile structure of the differentiating neuron at the distal part of the neurite. In 1890 it is first observed and named by the Nobel Laureate Santiago Ramon y Cajal. He reported that growth cones navigate through developing tissues to

their targets and the contacts between the neurons are not continuous but are contiguous (López-Muñoz et al. 2006).

1.1.1 Structure and function of Growth cone

The structure of the growth cone is similar to a human hand. The neurite resembles a forearm where the lamellipodia are the palm, from which finger like filopodia emerge. The GCs can sense and process the chemical and mechanical stimulus through lamellipodia and filopodia. Structurally, a growth cone is divided into three regions namely Peripheral (P), Transitional (T) and Central (C) (Bridgman 1989; Forscher 1988)(Figure 1).

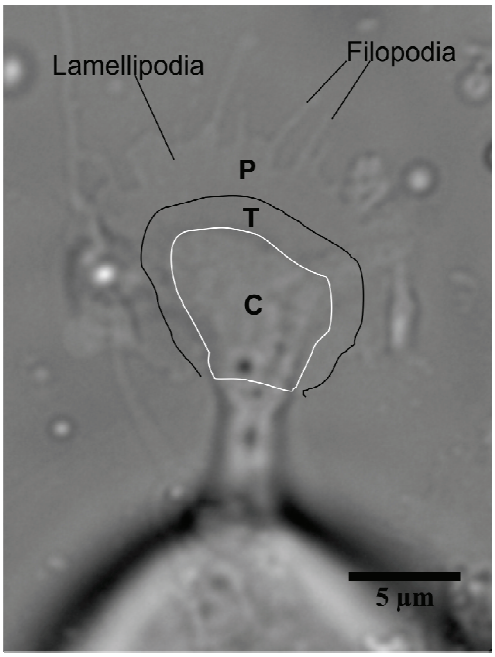

Figure 1*: Phase contrast image of DRG Growth Cone. The area bounded by the white line, between the white and black lines and the area outside the black line but inside the leading edge of lamellipodia shows the central region (C), transition (T) and peripheral (P) regions of the growth cone respectively. Note the presence of thin lamellipodial veil and spiky filopodia in the peripheral region.*

The peripheral region is a highly dynamic actin rich part of the growth cone. The actin monomers are polymerized into filaments making the dense sheet -like structure lamellipodia and small spike-like structure filopodia emerging from the lamellipodia. Depending upon the cell type and species, the size of lamellipodia varies (Mongiu et al. 2007). Lamellipodia and

filopodia attach on the substrate by the adhesion molecules present on their plasma lemma. With the help of Myosin II contractile property growth cones gain traction on the substrate and move forward (Ketschek et al. 2007; Heidemann 1990). Filopodia act as mechanical devices that penetrate the environment, sensing the guidance cues and steering the growth cone. Lamellipodia unable to make stable adhesions at the leading edge project upward and move as an rolling crest back along the dorsal cell surface toward the cell body forming a ruffle (Borm et al. 2005).

Compared to the peripheral region, the central region is thicker and consists of dense microtubule (MT) arrays. It is enriched with cellular organelles such as mitochondria and exocytotic vesicles. The depolymerised actin filaments and the microtubules also terminate in this region. Microtubule arrays extended from the axonal shaft support growth cone movement and serve as the track for transport of membranous organelles.

The transition zone plays an important role in regulating actin rich peripheral region and MT rich central region. The transition region limits the incursion of the MTs into the P region with the help of actin arc (Medeiros et al. 2006). Recent studies have shown that microtubules often penetrate into the peripheral region of the growth cone and even invade filopodia (Schaefer et al. 2002; Dent & Kalil 2001).

The guidance molecules and the adhesive substrate guide the growth cone to their synaptic target. (Lowery & Van Vactor 2009). The receptors present on the growth cone activate by the guidance molecules which further activates different signaling pathways. Through these signaling pathways GC can turn toward (attraction) or away from (repulsion) the guidance cue (Goodman 1996).

1.1.2 Actin dynamics during leading edge protrusion and Growth cone motility

The cytoskeleton is essential in every cell biological process which provides architectural shape, mechanical strength and at the same time the flexibility for the cellular motility (Bruce A, 2002). The cytoskeleton has three main structural components: microfilaments, intermediate filaments, and microtubules. Intermediate filaments are the most rigid filaments and are fundamental for structural rigidity of cells and the overall cell shape (Howard 2001). The actin filaments push the cell membrane at the periphery of the cell thereby cause protrusion and they also act as tracks for the movement of myosin molecules. Microtubules play a key role in

intracellular transportation of mitochondria or vesicles and they also act as a track for the dynein and kinesin motors (Wickstead & Gull 2011).

Actin is the most abundant protein in eukaryotes and can undergo polymerization into helical filament. The actin filament is polar in nature due to the orientation of all the subunits in one direction. Because of this, the polymerization is faster at one (plus or barbed) end than the other (minus or pointed) end of the filament. In addition to this, there is a large number of proteins which regulate the dynamic assembly and spatial organization of actin filaments. These proteins are divided by following their role in the actin polymerization process. These proteins i) promote the nucleation of actin such as the Arp2/3 complex or formins, ii) affect the depolymerizing factor (ADF/Cofilin) family, iii) associate to monomeric actin, such as Profilin and β-thymosin and iv) cap the ends of filaments (Small et al. 2002; Pollard & Borisy 2003).

Under physiological conditions, ATPbound G-actin is incorporated into growing filaments at the barbed end. ATP-actin is then converted into ADP-actin by slow hydrolysis as actin monomers are shifted along the filament toward the pointed ends. Proteins like ADF/cofilin and myosin II help to sever actin filaments and remove actin monomers from their pointed ends close to the C-domain (Marsick 2010; Meberg 2000; Medeiros et al. 2006). These ADP-actin monomers are then transferred to the growth cone leading edge and their cyclic nucleotides exchanged by profilin, so as to provide a steady ATP-actin monomer pool ready for polymerization (Pollard 2000). The cycle of addition of G-actin-ATP monomers to actin filament's barbed end and simultaneous disassembly of F-actin-ADP monomers at the pointed end where the ADP is subsequently changed into ATP, is known as "treadmilling".

The net protrusion of lamellipodia depends on the actin 'treadmilling' together with actin retrograde flow. Where retrograde flow refers to the backward flow of the F-actin network away from the growth cone leading edge into the C-domain. The retrograde flow occurs due to contractile activity of myosin II pulling on the pointed-end of actin filaments in the P-domain, and by the push exerted on the membrane by the incorporation of new actin monomers against the growth cone leading edge (Medeiros et al. 2006).

When the rate of actin polymerization overtakes the actin retrograde flow, the GC protrudes (Lowery & Van Vactor 2009). This allows the addition of actin monomers/oligomers to actin filaments in close contact with the membrane pushing the cellular membrane forward, leading to the protrusion. Growth cones use substrate adhesions to slow down the overlying retrograde flow of actin filaments and cause leading edge protrusion (Alexandrova et al. 2008).

Mitchison and Kirschner proposed that an intracellular molecular clutch, formed by interactions between growth cone transmembrane adhesive receptors and the extracellular environment, would couple to the overlying flow of actin filaments to slow down their retrograde rate (Mitchison & Kirschner 1988). Specifically, when integrins engage adhesive substrates, they recruit proteins like talin and vinculin to their intracellular domain. This engages the overlying retrograde flow of actin filaments to slow it down (Thievessen et al. 2013; Zhang et al. 2008). Similarly, catenins couple with N-cadherin to the actin cytoskeleton to slow down the actin retrograde flow (Bard et al. 2008). Formation of these 'clutches' together with Myosin II contractile activity, provide traction to pull and move the central region of the GC closer to the peripheral region , leading to axon lengthening. Therefore, substrate adhesion causes leading edge protrusion by decrease in retrograde flow and also Myosin II driven growth cone advance (Betz et al. 2011; Fass & Odde 2003).

1.2 Myosin II:

Myosin is a superfamily of motor proteins that plays an important role in growth cone motility, cellular locomotion, morphology and cell division (Conti et al. 2004; Vicente-Manzanares et al. 2009; Forscher 1988). Myosin molecules hydrolyze ATP to walk along, propel the sliding of or produce tension on actin filaments. Myosin has the general architecture of a globular head domain that contains the ATPase activity, a neck region contains actin binding domains, and a tail domain that interacts with other myosin molecules or cargo. Myosin can associate to actin filaments to form the actomyosin complex, which can generate force.

The non muscle myosin II (NMII) is a subfamily which can self assemble into bipolar filaments through tail-tail interactions. NM II molecules are composed of three pairs of peptides: two heavy chains of 230 kDa, two 20 kDa regulatory light chains (RLCs) that regulate NMII activity and two 17 kDa essential light chains (ELCs) that stabilize the heavy chain structure (Vicente-Manzanares et al. 2009). The three myosin II isoforms NMIIA, NMIIB and NMIIC have similar structural and dynamical properties but have slightly different localizations and functions. Depending upon the cellular specificity and developmental stage of the cell the localization of the NM II differs (Betapudi 2010; Conti & Adelstein 2008; Wylie & Chantler 2008). In neurons, NMIIA is an important regulator of retraction and promotes the adhesion with formation of focal contact sites (Yu et al. 2012; Conti & Adelstein 2008), NMIIB is required for

the outgrowth of neuritic processes (Wylie & Chantler 2008; Bridgman & Dave 2001) while NMIIC, is thought to regulate cell membrane extension and the formation of focal contacts shows separate but coupled activities with NMIIA and NMIIB (Wylie & Chantler 2008).

In neuronal GC, the balance between the rate of polymerization and myosin base retrograde flow of actin determines growth cone protrusion or retraction. Myosin II controls the retrograde flow of actin by severing the actin filaments at their pointed end (Medeiros et al. 2006). Recent studies have shown that actomyosin complex formed by binding Myosin II with actin filaments, exert a contractile force on actin filaments in the transition zone of the GC. This contracts the actin meshwork and breaks the filaments. Moreover, NMII generates small traction forces in lamellipodia that stabilize nascent adhesions and promotes their transition to focal complexes through actin meshwork (Shutova et al. 2012).

1.3 Arp2/3 :

Actin related protein 2/3 complex (Arp2/3) is widely studied actin binding protein for its involvement in lamellipodia formation and protrusion (Suraneni et al. 2012; Wu et al. 2012). The initial formation of an actin filament, known as nucleation, posses an energetic barrier for growth cones (Mullins et al. 1998; Cooper et al. 1983). During this period, growth cones rely on nucleators that bind and stabilize a trimeric complex of actin monomers, Stabilization of this intermediate complex by actin nucleators permits the stable and continuous self assembly of subsequent actin monomers into the growing filament. Arp2/3 consists of seven subunits, two of its subunits; the Actin-Related Proteins ARP2 and ARP3 closely resemble the structure of monomeric actin and serve as nucleation sites for new actin filaments. Arp2/3 has very little nucleation activity on its own and it can be increased by interaction with nucleation promoting factors (NPF), such as the members of the WASP/WAVE family of proteins (Campellone & Welch 2010; Millard et al. 2004). Activated Arp2/3 by WASP/WAVE family bind to the side of a pre-existing actin filament and nucleate a new daughter branch at a particular 70 ° angle (Fujiwara et al. 2002; Amann & Pollard 2001). Therefore, Arp2/3 complex simultaneously controls nucleation of actin polymerization and branching of filaments.

Two models were proposed for the branching of actin filament through Arp2/3. Inside a branching model it is assumed that the Arp2/3 complex binds to the side of a pre-existing filament at a point different from the nucleation site (Rouiller et al. 2008). In a barbed end branching model, Arp2/3 only associates at the barbed end of growing filaments, allowing for

the elongation of the original filament and the formation of a branched filament (Dayel & Mullins 2004). Recent results favor the former model(Smith et al. 2013).

The Arp2/3 complex is an important actin nucleator and lacks of its activity is lethal for unicellular to multicellular organisms (Fujiwara et al. 2002). In non neuronal cells Arp2/3 complex takes part in lamellipodial protrusion and directed migration(Suraneni et al. 2012) haptotaxis (Wu et al. 2012), filopodia formation (Svitkina et al. 2003), adhesions maturation and organization (Wu et al. 2012), membrane trafficking (Rozelle & Machesky 2000), and endocytosis (Merrifield & Qualmann 2004). However, in neuronal GCs, the role of Arp2/3 in actin dynamics and guidance studies often report ambiguous findings. Previously, it was reported that Arp2/3 did not take part in lamellipodia protrusion, or filopodia formation and P-domain of the growth cones had less branched actin. Moreover it had been also shown that the Arp2/3 subunits were enriched in the growth cone C-domain and that it acts as a negative regulator of axon elongation (Strasser et al. 2004). Soon after, it was reported that neurons did have a branched actin network close to the leading edge and that it was dependent on Arp2/3 function. Moreover, Arp2/3 inhibition reduced lamellipodial protrusion, filopodia formation and the rate of the actin retrograde flow (Korobova & Svitkina 2008). Yang et al. later confirmed that the actin nucleation activity of Arp2/3 was localized to the leading edge of growth cones and that its inhibition led to an increase in the rate of the actin retrograde flow (Yang et al. 2012).

Emerging evidence also indicate that Arp2/3 is recruited to nascent integrin adhesions through interaction with FAK and vinculin, which further required to strengthen the link between integrin and cytoskeleton (Ilić et al. 1995; Saunders et al. 2006). Furthermore, Beckham et al. reported that Arp2/3 inhibition weaken integrin, an extracellular membrane attachment resulting in either a translocation or treadmilling of mature adhesions (Beckham et al. 2014).

1.4 Rho GTPase Signalling:

Generally, attractive guidance cues can lead to an adhesion formation, leading edge protrusion and growth cone motility, while repulsive guidance cues can remove substrate adhesions, stop leading edge protrusion and slow down growth cone advancement (Gomez & Letourneau 2014). These cytoskeletal rearrangements provide the growth cone with motility, directionality, and the necessary traction to steer growth cones to their targets. The particular effect that a guidance cue has on a growth cone is dependent on membrane receptor complexes, the signaling pathways, and crosstalk between them.(Huber et al. 2003).

Rho family GTPase has distinct and specific roles in the regulation of growth, maintenance and retraction of GCs (Ridley 2006). The small GTPases of the Rho family act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state, a process that is regulated by GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins). GEFs catalyze the conversion to the GTP-bound state and GAPs accelerate the intrinsic rate of hydrolysis of bound GTP to GDP. Additionally, GDIs (GDP-Dissociation Inhibitors) have been described to capture Rho in both GTP and GDP-bound states and allow it to cycle between cytosol and membranes. In its active state, Rho GTPases interact with their specific downstream targets and perform their cellular function (Boureux et al. 2007; Ridley 2006).

RhoA, Rac1 and CDC42 are well-studied members of the Rho family GTPase controlling distinct cytoskeletal elements. Activation of Rac1 stimulates actin polymerization to form lamellipodia, CDC42 induces the polymerization of actin to form filopodia or microspikes which are parallel actin bundles within the lamellipodium and Rho regulates the bundling of actin filaments into stress fibers and the formation of focal adhesion complexes.

Rho GTPase is also involved in crosstalk, regulating different processes required in GC dynamics. The auto-inhibited NWASP is relieved by binding of activated Cdc42 and PIP2. Active N-WASP binds toArp2/3 and increases the actin nucleation rate of Arp2/3 by 70-fold (Zalevsky et al., 2001). This in turn increases the actin filament polymerization process. The ADF/Cofilin causes depolymerization at the minus end of the filaments. This provides a constant pool of ATP-actin monomers at the barbed end of actin filament to maintain polymerization for axonal growth. The activity of ADF/cofilin is negatively regulated through phosphorylation by LIM kinase (Bamburg 1999). Lim kinase is a downstream effector of PAK which activates by Rac and CDC42 pathways (Schwartz 2004). Moreover, LIM kinase is also activated by the Rho kinase (ROCK), the downstream effecter of RhoA (Torka et al. 2006). Therefore, activation of PAK by Rac and Cdc42, and of ROCK by RhoA leads to the inactivation of ADF/cofilin, controlling the actin polymerization process.

Myosin II activity can directly control the rate of the F-actin retrograde flow, which can have direct effects on the growth cone leading edge protrusion and retraction (LIN et al. 1996). The contractile activity of myosin II has shown to be up regulated by ROCK or myosin light

chain kinase (MLCK) phosphorylation (Bresnick, 1999). ROCK is a direct downstream target of RhoA, and MLCK activity can be downregulated by PAK. Therefore, the F-actin retrograde flow, increases due to RhoA activation while it decreases by Rac and Cdc42 activation (Huber et al. 2003). Moreover, in case of adhesion formation, it has been shown that the initial formation of integrin-based adhesions in growth cones is dependent on Rac activation and that stabilization of such adhesions requires the activation of RhoA, along with the concomitant dowregulation of Rac activity (Woo & Gomez 2006).

So far we have learnt about the structure and function of GCs, the Actin polymerization process - the main source of motility in GCs, some of their regulatory proteins -Myosin II and Arp2/3- and finally about the important functions of the Rho GTPase signaling pathways in the GC dynamics. Let us now see the force generation process in GC and some of the models used to explain it.

1.5 Force generation by growth cone:

During differentiation, growth cones explore the surrounding environment by exerting a force. The force is essential for protrusion, turning, branching and for the overall motility of the growth cone. The force exerted by the growth cone is the effect of different processes such as actin and microtubule dynamics coupled with myosin-based retrograde actin flow and also adhesion to extracellular substrate (Dent & Gertler 2003; Suter & Forschert 1998; Vitriol & Zheng 2012). Previous investigations in vivo using Atomic Force Microscopy (Prass et al. 2006) and opposing liquid flow (Bohnet et al. 2006) were limited to a temporal resolution in the 100 ms range and sensitivity of 50–100 pN; these experimental limitations can be overcome by using optical tweezers (Bustamante et al. 2000; Neuman & Block 2004) providing a ms resolution and pN sensitivity. Quantitative characterization of the force exerted by lamellipodia and filopodia during neuronal differentiation and migration enabled us to understand the dynamical properties of force generation.

1.5.1 Theoretical models for force generation:

The complex process of actin filaments polymerization is the main source of GCs leading edge protrusion (Pollard & Borisy 2003). Although the process is not fully understood, the theoretical models with the known molecular events can help in understanding the force generation process in the overall movement of the cell.

Basically, two theoretical models have been proposed to explain the force generation by actin polymerization: ratchet models (Mogilner & Oster 1996; Mogilner & Oster 2003), and autocatalytic models (Carlsson 2003; Carlsson 2001). The straightforward way to demonstrate how these models work is through the quantitative measurement of the force-velocity relation which explains how the force (F) exerted by the actin filament network is related to the velocity (v) of their growing ends (Carlsson 2003; Mogilner & Oster 2003).

1.5.1a Ratchet Model:

Earlier 'Brownian Ratchet model' (Peskin et al. 1993) considered that, an actin filament is a rigid rod that has hindered growing, once it has reached the membrane. However, later, when it was confirmed that the actin filament is an elastic filament that can bend in response to the load, the 'elastic ratchet model' was proposed (Mogilner & Oster 1996). This model suggested that thermal fluctuation of filaments create a gap between their tips. An actin monomer (which is 2.7 nm in size) can easily insert itself between these gaps if the bending of the filament away from the membrane is sufficiently large (angle > ~30°) and the filament is long enough (>~70 nm) (Mogilner & Oster 1996; Mogilner & Oster 2003). Therefore, an elongated filament consequently applies an elastic force on the membrane and moves it forward. This model explained the force generation by a single polymerizing actin filament but not the complex leading edge protrusion due to actin network. 'Tethered Ratchet model' is the extended model of the 'Brownian ratchet model' in which the transient attachment of the actin filament to the membrane is considered (Mogilner & Oster 2003). There are two filaments: the working filaments are the filaments that are not attached to the membrane and can exert a force on it, and attached filaments, which cannot exert a force on the membrane. The working filaments, supply the motile force by polymerization. Thus, the tethered elastic Brownian ratchet model presumes that new actin filaments/branches are generated independently of existing branches, and attempts to understand force generation from the group of actin filaments in the network.

1.5.1b Autocatalytic Model:

The main assumption of the Autocatalytic model is that the new actin branches are generated from the existing one which is different from the Brownian ratchet model (Carlsson 2003). This model starts with a single filament and considers that the branching occurs only near the obstacle. A network grew with a well-defined velocity and structure from this filament. The

most surprising result of these simulations was that the growth velocity is independent of the opposing force. This occurs when branching is limited to the region near the obstacle. It is because an increase in the opposing force causes more filament subunits to be in the region of the membrane. This causes increased branching, so that the number of filaments grows proportionally with opposing force. Therefore, force per filament (and thus growth velocity) becomes independent of the opposing force. A simplified mathematical treatment of branching and nucleation (Carlsson 2003) showed that when nucleation of filaments occurs by non-branching mechanisms, the velocity depends very strongly on the opposing force. The independence of the velocity from the opposing force in branching nucleation was confirmed by later stochastic-growth simulations based on autocatalytic branching (Schaus et al. 2007) .

Even if in the single filament Fv relationships are similar for both types of model, the predicted Fv relationships for a network growing against a load are very different from the tethered elastic Brownian ratchet and the autocatalytic models. Fluctuations of contact between the tips of actin filaments and the surrounding membrane is an essential feature of Brownian ratchet models leading to Fv relationships in which v decreases exponentially with increasing values of F. On the other hand, in autocatalytic models, when an obstacle is encountered, the actin network - due to the activity of the controlling proteins – originates a new branch, so that the velocity v remains constant for increasing values of F. Indeed, it has been recently shown that in both DRG and hippocampal neurons the Force-velocity relationships (Fv) is consistent with a common autocatalytic model of force generation, indicating that molecular mechanisms of force generation of GC from CNS and PNS neurons are similar (Amin et al. 2013).

Recently, the force generated due to contractile activity of Myosin II together with actin filament have also been simulated (Dasanayake et al. 2011). They estimate the effects of the network structure via simulation of myosin minifilament motion through a random two-dimensional actin network.

The simulation has shown that contraction is a very general feature of myosins that move along actin filaments, provided that myosins on one filament are coupled to those moving on other filaments.

Chapter 2

Materials, Methods and Results

2.1 The role of myosin-II in force generation of DRG filopodia and lamellipodia

Wasim A. Sayyad, Ladan Amin, Paolo Fabris, Erika Ercolini & Vincent Torre

Accepted in **Scientific reports**, December 2014.

Abstract:

Differentiating neurons process the mechanical stimulus by exerting the protrusive forces through lamellipodia and filopodia. We used optical tweezers, video imaging and immunocytochemistry to analyze the role of non-muscle myosin-II on the protrusive force exerted by lamellipodia and filopodia from developing growth cones (GCs) of isolated Dorsal Root Ganglia (DRG) neurons. When the activity of myosin-II was inhibited by 30 μM Blebbistatin protrusion/retraction cycles of lamellipodia slowed down and during retraction lamellipodia could not lift up axially as in control condition. Inhibition of actin polymerization with 25 nM Cytochalasin-D and of microtubule polymerization with 500 nM Nocodazole slowed down the protrusion/retraction cycles, but only Cytochalasin-D decreased lamellipodia axial motion. The force exerted by lamellipodia treated with Blebbistatin decreased by 50 %, but, surprisingly, the force exerted by filopodia increased by 20-50 %. The concomitant disruption of microtubules caused by Nocodazole abolished the increase of the force exerted by filopodia treated with Blebbistatin. These results suggest that; i- Myosin-II controls the force exerted by lamellipodia and filopodia,; ii- contractions of the actomyosin complex formed by filaments of actin and myosin have an active role in ruffle formation; iii- myosin-II is an essential component of the structural stability of GCs architecture.

Introduction

During development, neurons are able to self-organize in precisely wired networks and are able to establish the appropriate synaptic connections. Neuronal navigation requires the existence of highly motile structures able to probe the mechanical properties of the surrounding environment and to search for the chemical cues leading to the formation of correct synaptic connections(Ghashghaei et al. 2007; Solecki et al. 2006). Neuronal exploration is guided by growth cones (GCs) located at the neurite tips(Song & Poo 2001; C. Goodman 1996). GCs are composed of lamellipodia of different sizes, depending on the cell type and species from which thin filopodia with a submicron diameter emerge(Mongiu et al. 2007). The primary source of motility in GCs is the polymerization of actin filaments(Mogilner & Oster 1996; Pollard & Borisy 2003), controlled by a large set of regulatory proteins, such as Arp2/3, WASP, etc(Pak et al. 2008) and molecular motors seem to participate in the overall process by controlling several aspects of the process.

The addition of actin monomers/oligomers to actin filaments in close contact with the membrane pushes the cellular membrane forward exerting a protrusive force(Mogilner & Oster 1996; Raucher & Sheetz 2000). An important determinant of force generation is the turnover of actin filaments, during which actin monomers or small oligomers are added to the barbed end of actin filaments (polymerization) and are removed from the other end (depolymerization). In this process the non-muscle myosin-II plays an important role: indeed myosin-II controls the retrograde flow of actin polymers by severing the actin filaments at their pointed end, providing the necessary treadmilling mechanism(Medeiros et al. 2006). Myosins constitute a superfamily of motor proteins with major roles in several cellular processes such as cell adhesion, migration and division(Vicente-Manzanares et al. 2009). Myosin molecules, like all motor proteins, can walk along, propel and slide by other molecules and can produce tension on actin filaments. Generation of tension and force requires metabolic energy, usually provided by ATP hydrolysis and therefore myosins have appropriate catalytic sites in their amino-terminal (head) region. Myosin can associate to actin filaments to form the actomyosin complex, which can generate force. Like muscle myosin-II, non-muscle myosin-II (NMII) molecules are formed by three pairs of peptides with different molecular weight and function(Vicente-Manzanares et al. 2009). The three myosin-II isoforms NMIIA, NMIIB and NMIIC have similar structural and dynamical properties but have slightly different kinetics properties. Their major difference seems to reside in their regulation properties and different proteins control them through distinct phosphorylation sites(Vicente-Manzanares et al. 2009).

Myosin-II seems to be involved in the orchestration of actin polymerization /depolymerization but also of microtubules (MTs) dynamics. Indeed, it has been shown that actin oligomers driven by myosin-II interact with growing MTs and that myosin-II-dependent compressive force is necessary for MTs dynamics (Burnette et al. 2008) to form axons. The existence of a coupling between actin and MT dynamics is also supported by the observation that inhibition of myosin-II with Blebbistatin markedly accelerates axon growth and promotes the reorganization of both actin and MTs in GCs(Hur et al. 2011). In this study we used Blebbistatin, selective potent inhibitor of myosin-II to assess the effect of myosin-II on the motility of the DRG GCs. Blebbistatin blocks the myosin in an ADP bound state which precedes the force generating step and therefore inhibits the actomyosin contraction(Allingham et al. 2005).

We have used Optical Tweezers (OT), to analyze the role of myosin-II in the force generation of DRG GCs lamellipodia and filopodia. OT provide a quantitative characterization of the exerted force with millisecond time resolution and pN sensitivity(Bustamante et al. 2000). We have also used video imaging to characterize and quantify the 3D motion of lamellipodia, during which lamellipodia lift up vertically by some microns(Krotkov 1988). By combining these experimental methods with the use of inhibitors of cytoskeletal functions and of immunocytochemistry, we have explored the role of contractions of the actomyosin complex in the protrusion/retraction cycles, observed in lamellipodia of developing neurons. Here we confirm that myosin-II not only controls the retrograde flow of actin(Medeiros et al. 2006) but it is also an essential component of the structural stability of GCs architecture regulating the coupling of actin filaments and microtubules dynamics and plays a fundamental role in the force generation of lamellipodia and - to some extent - also in filopodia.

RESULTS

Large and highly motile lamellipodia emerge from dissociated neurons from DRG after 6-12 hours of culture(Amin et al. 2012; Amin et al. 2011; Shahapure et al. 2010). These lamellipodia can exert forces larger than 20 pN and their leading edge can move with a speed of 30-100 nm/s(Shahapure et al. 2010). In our preparation, motility is restricted to lamellipodia and filopodia of dissociated neurons from DRG, which do not migrate and their soma remains approximately in the same position on the dish for several hours. After 2-3 days of culture, dissociated neurons establish physical contacts and motility of lamellipodia and filopodia is reduced. Therefore, we analyzed the effect of inhibitors of, myosin-II (Blebbistatin), actin polymerization (Cytochalasin-D) and microtubule polymerization (Nocodazole) on lamellipodia and filopodia after 24-48 hours of culture, when their motility is more pronounced.

The effect of Blebbistatin, Cytochalasin-D and Nocodazole on lamellipodia protrusion/retraction cycles

Lamellipodia emerging from the soma of DRG neurons protrude and collapse continuously and their protrusion/retraction cycles were followed by video imaging (see Materials and Methods). By analyzing these image sequences with Algorithm I, described in the Materials and Methods section, the average distance of the lamellipodium leading edge was measured from a reference point (C) chosen at the base of the lamellipodium (see Materials and

Methods) and the periods of protrusion/retraction cycles were calculated (red bars in Fig. 1). When 30 μM Blebbistatin was added to the medium bathing of the neuronal culture, protrusion/retraction cycles of lamellipodia could be observed but with a period 30-50 % longer than in control condition and after 15 minutes lamellipodia shrank (Fig. 1a and 1b). When a higher concentration of Blebbistatin was used, such as 100 μM, lamellipodia shrank within 2-3 minutes and motility was completely suppressed.

In control condition, during protrusion/retraction cycles, lamellipodia also moved upwards by 2-5 μm: indeed, at a focal plane 3 or 4 μm above the coverslip their leading edge could be seen well in focus. By using Algorithm II described in the Materials and Methods section, the number of pixels of a lamellipodium in focus at different heights, i.e. at 2, 3 and 4 μm above the coverslip, was counted and followed in time (Fig. 1c). This algorithm allowed quantifying the extent of the axial motion and the effect of different inhibitors of cytoskeletal proteins on this axial motion. After the addition of 30 μM Blebbistatin to the bathing medium, the period of protrusion/retraction cycles increased from an average of 96.1±3.3 s in control condition to 136.7±5.9 s (Fig. 1b). Lamellipodia not only prolonged the duration of their protrusion/retraction cycles (Fig. 1a and b) but also reduced the average height reached during these cycles in the presence of 30 μM Blebbistatin (Fig. 1c). Indeed, the fraction of pixels in focus at 2 μm above the coverslip increased, while those in focus at 3 and 4 μm above the coverslip decreased (Fig. 1c).

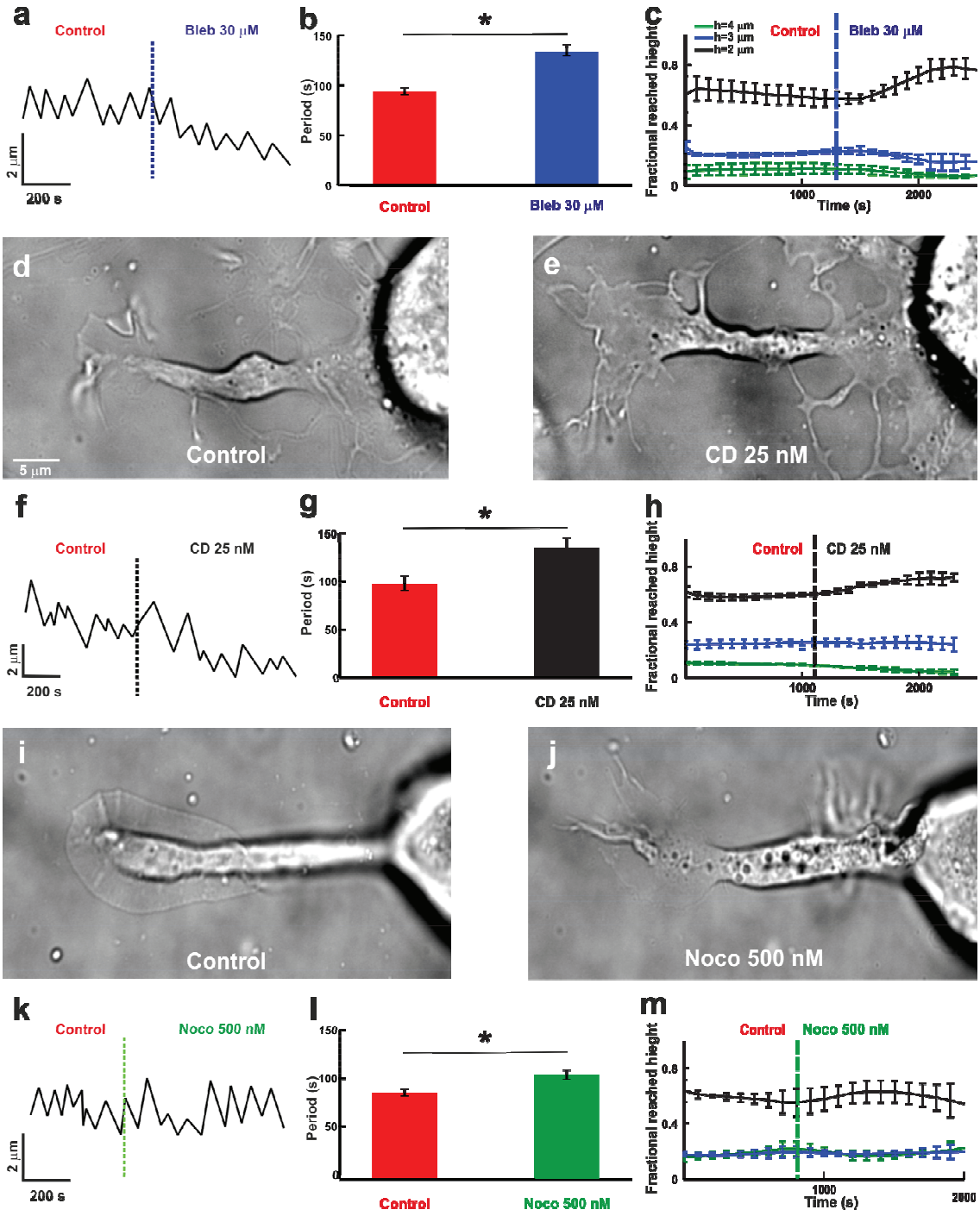

Figure 1. The effect of Blebbistatin, Cytochalasin-D and Nocodazole on protrusion/retraction cycles.

*(**a**) Cycles of protrusion/retraction of lamellipodia vs time. The dotted line represents the time of inhibitor addition. (**b**) Average periods of lamellipodia protrusion/retraction cycles*

*in control condition (red) and in the presence of 30 µM Blebbistatin (Bleb 30 µM, blue), from n= 8 neurons. Student t-test showed that the data significantly differs with respect to control, P<0.005. Data represents mean ± SEM. (**c**) The fraction of pixels in focus of lamellipodia in different focal planes (h=2, 3 and 4 µm) above the coverslip. Data averaged from 8 experiments. The vertical bar indicates the SEM and the vertical broken line indicates the time at which the drug was added. (**d-e**) Images of lamellipodia emerging from a DRG neuron in control condition (d) and after treatment with 25 nM Cytochalasin-D (CD 25 nM) (e), Scale bar, 5 µm. (**f-h**) As in (a-c) but in the presence of 25 nM Cytochalasin-D, from n=10 neurons. (h) as in (c) but for 10 experiments. (**i-j**) Images of lamellipodia emerging from a DRG neuron in control condition (i) and after treatment with 500 nM Nocodazole (Noco 500nM,). (**k-m**) As in (a-c) but in the presence of 500 nM Nocodazole, from n=8 neurons. (m) As in (c) but for 7 experiments. All the data were checked with chi-square test for Normal distribution before applying the student's t test.*

Treatment with a concentration of 100 µM Blebbistatin invariably led to the suppression of lamellipodia motility. We also investigated the effect of other inhibitors known to affect and abolish motility, but acting on different biochemical targets. Cytochalasin-D is a well-known and specific inhibitor of actin filament polymerization(Cooper 1987). Cytochalasin-D bound to the barbed end of actin filaments blocking the addition of new actin monomers or oligomers. Concentrations of Cytochalasin-D, such as 50 or 100 nM caused lamellipodia to shrink completely and abolished almost entirely the GCs motility, confirming the fundamental role of actin filament polymerization. Nocodazole inhibits the microtubules polymerization(Dent & Kalil 2001) and, in our experiments, lamellipodia motion was almost entirely abolished in the presence of 1 µM of Nocodazole. Blebbistatin, Cytochalasin-D and Nocodazole reduced lamellipodia motility but did not have the same effect on lamellipodia and filopodia morphology: lamellipodia treated with Cytochalasin-D shrank and showed the formation of small ruffles but did not acquire the 'filopodish' appearance (Fig. 1d and e) observed in lamellipodia treated with Blebbistatin (see Discussion). Lamellipodia treated with Nocodazole shrank showing neither small ruffles nor the filopodish appearance as seen in Cytochalasin-D and Blebbistatin respectively (Fig. 1i and j). Addition of 25 nM Cytochalasin-D and 500 nM Nocodazole did not abolish the protrusion/retraction cycles (Fig. 1f and k).

The period of protrusion/retraction cycles increased from an average of 95.2±7.3 s in control condition to 131.5±9.8 s in the presence of 25 nM Cytochalasin-D (Fig. 1g) and 110.9±5.0 s in the presence of 500nM Nocodazole (Fig. 1l). The same concentration of Cytochalasin-D also reduced the ability of treated lamellipodia to lift up along the vertical direction during these protrusion/retraction cycles: the fraction of edges seen in focus at focal planes higher than 3 μm significantly decreased and lamellipodia edges seen in focus at a plane 2 μm above the coverslip became much more frequent (Fig. 1h). The application of 500 nM Nocodazole caused a transient shrinkage of GCs, but, within a couple of minutes, lamellipodia were able to lift up in the vertical direction almost as in control condition (Fig. 1m). This differential effect of Blebbistatin and Cytochalasin-D versus Nocodazole indicates a major role of myosin-II and actin polymerization in lamellipodia axial motion and a minor role of microtubules.

The effect of Blebbistatin, Cytochalasin-D and Nocodazole on the force exerted by lamellipodia

Having analyzed the effect of Blebbistatin, Cytochalasin-D and Nocodazole on the kinetics of protrusion/retraction cycles, we used OT to analyze changes of the force exerted by lamellipodia and filopodia caused by these inhibitors. Untreated lamellipodia pushed trapped beads (Fig. 2a-c) exerting maximum forces up to 10-20 pN as previously described(Cojoc et al. 2007) and often a bead could be displaced out of the optical trap. Lamellipodia of DRG treated with 30 μM Blebbistatin could also pull and push a trapped bead (Fig. 2e-g) but with a lower force (Fig. 2h).

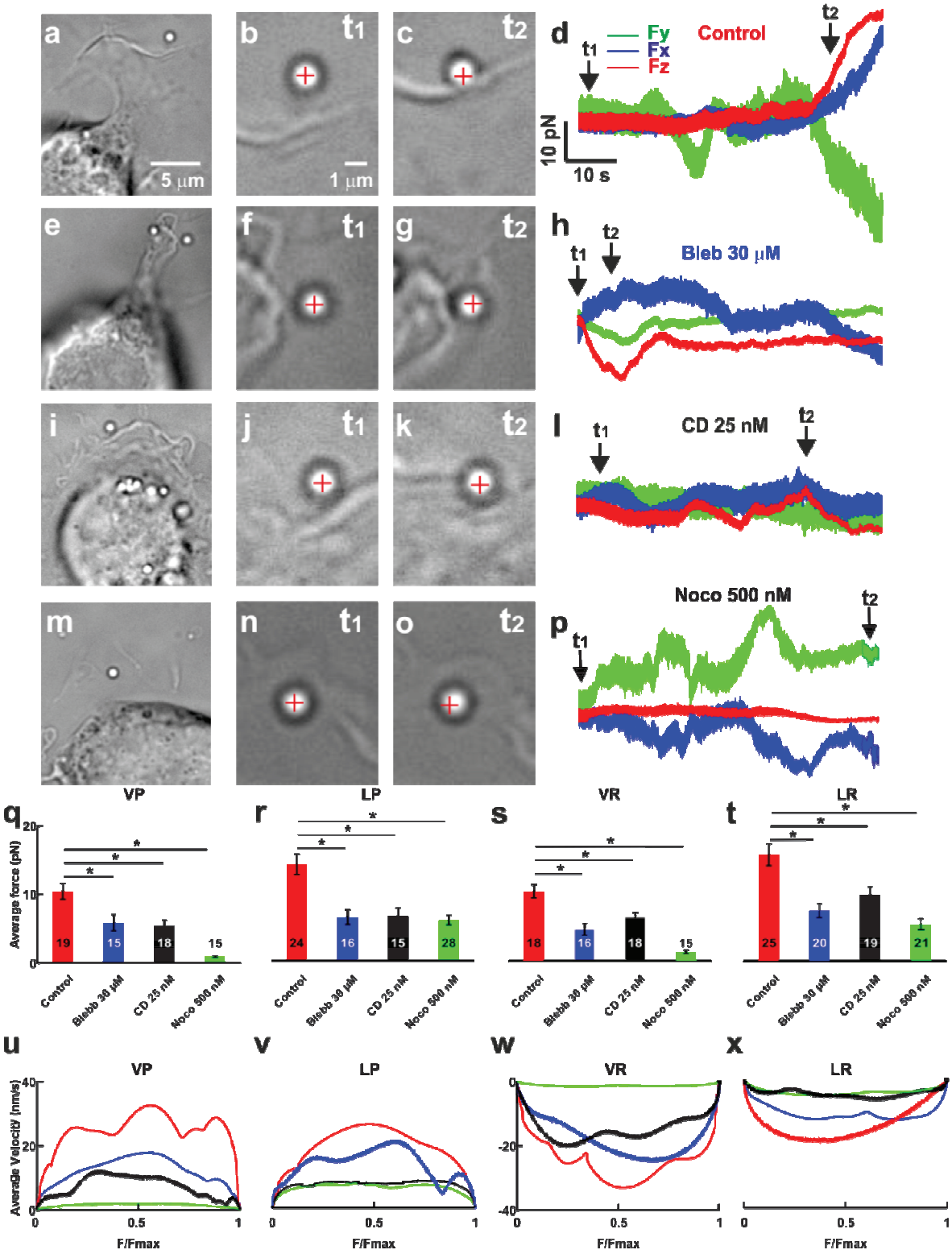

Figure 2. The effect of Blebbistatin, Cytochalasin-D and Nocodazole on the force generated by lamellipodia. *(a) Low-resolution image of a bead trapped in front of a*

lamellipodium emerging from the soma of a DRG neuron in control condition. Scale bar, 5μm ***(b-c)*** *High-resolution images during a push. At* t_1 *the bead is in the optical trap (b) and when the lamellipodium grows, at* t_2*, it pushes the bead (c). The cross indicates the center of the optical trap. Scale bar, 1μm.* ***(d)*** *The three components* F_x*,* F_y*, and* F_z *of the force exerted when the lamellipodium pushes the bead.* ***(e-h)*** *As in (a-d) but in the presence of Blebbistatin (Bleb 30μM).* ***(i-l)*** *As in (a-d) but in the presence of Cytochalasin-D (CD 25 nM).* ***(m-p)*** *As in (a-d) but in the presence of Nocodazole (Noco 500nM). The trap stiffness is* $k_{x,y} = 0.10$ *pN/nm.,* $k_z = 0.03$ *pN/nm.* ***(q-t)*** *Comparison of the force exerted by lamellipodia in control condition (red), 30μM Blebbistatin (blue), 25 nM Cytochalasin-D (black) and 500nM Nocodazole (green) and in all four different stereotyped behaviors: LP (lateral push), LR(lateral retraction), VP (vertical push) and VR (vertical retraction). In each case, by using the student t-test, the force measured in the presence of inhibitors was lower than the one measured in control condition with a significance *P<0.005. Data represent mean ± SEM.* ***(u-x)*** *Average Fv relationship,* $(FV)_{avg}$*, normalized to* F_{max} *for VP(u), LP(v), VR (w) and LR (x). All the data were checked with chi-square test for Normal distribution before applying the student's t test.*

The addition of 25 nM Cytochalasin-D and 500 nM of Nocodazole caused lamellipodia to shrink, reduced neuronal motility and the amplitude of generated forces (Fig.2i-p). In several experiments we were able to measure the maximum force exerted by the same lamellipodia in control condition and in the presence of inhibitors. These measurements were then divided into four different stereotyped behaviors: vertical push (VP), vertical retraction (VR), lateral push (LP) and lateral retraction (LR), where vertical refers to the push or pull of the bead in the axial direction (perpendicular to the plane of the coverslip) and lateral refers to the push or pull of the bead in the lateral direction (parallel to the plane of the coverslip) (Fig. 2q-t). In these experiments all tested inhibitors reduced the force by about 50 %, in case of LP. While in case of LR, Blebbistatin and Cytochalasin-D reduced the force by 40-50 %, but Nocodazole reduced the force by 75% compared to control condition. For VP and VR the force was decreased in Blebbistatin and Cytochalasin-D by about 50% and in Nocodazole by more than 80 % compared to control condition (Table 1). In the great majority of experiments, treatment with 25 nM Cytochalasin-D and 500 nM of Nocodazole for longer than 30-50 minutes completely abolished GCs motility.

Table 1

	Control	Blebbistatin 30 µM (n=4)	Cytochalasin-D 12.5 nM (n=3)	Cytochalasin-D 25 nM (n=4)	Nocodazole 500 nM (n=3)
j_+ (nm)	5.10	3.05*	3.60*	2.46*	4.9
j_- (nm)	4.90	2.96*	3.60	2.35*	5.4
A_+ events/s	157.3	135.10*	138.26	110.99*	56.04*
A_- events/s	155.5	125.68*	157.74	153.25	51.60*

Table 1. The effect of different inhibitors on the maximum force exerted by lamellipodia *Average maximum force exerted by lamellipodia in control condition (second column), in the presence of 30 µM Blebbistatin (third column), 25 nM Cytochalsin D (fourth column) and 500 nM Nocodazole (fifth column) for vertical push (first row), lateral push (second row), vertical retraction (third row) and lateral retraction (fourth row). Student t-test showed that the data significantly differs with respect to control, *P<0.05 and **P<0.005. Data represents mean ± SEM*

The average Fv relationships $(FV)_{avg}$ were computed from the measured displacements (see Materials and Methods). Fv relationships obtained from a single experiment were normalized to F_{max} and averaged to obtain average Fv relationships, $(FV)_{avg}$ (Shahapure et al. 2010). At the beginning, the bead was in the trap far from the lamellipodia and its velocity was zero. During push the lamellipodia leading edge moved toward the trapped bead with constant velocity. Before coming to a solid contact with the bead, the bead velocity increased but later - after complete contact – beads and lamellipodia moved with the same velocity. Therefore $(FV)_{avg}$ relationships after an initial rise of v exhibited a flat shape, during which the mean velocity remained constant while the force increased (Fig. 2u-x). The analysis of the Force-velocity (Fv) relationships (Fig. 2u-x) shows that both inhibitors did not modify the shape of the Fv relationships but reduced the maximal velocity v for both vertical and lateral pushes and retractions. Lamellipodia velocity was reduced more potently by 500 nM Nocodazole than 25 nM Cytochalasin-D and 30 µM Blebbistatin (compare green, black and blue traces in Fig. 2 u-x).

These results show that Nocodazole, Blebbistatin and Cytochalasin-D reduce the maximal force exerted by protruding lamellipodia and the maximal velocity of their leading edges.

Changes of noise during force generation with Blebbistatin and Cytochalasin-D

A remarkable feature of force generation during vertical and lateral push is the concomitant increase of noise when the lamellipodia push the bead(Amin et al. 2011). This increase of noise is not present when the lamellipodium retracts, pulling the bead away from the optical trap. We have previously shown(Amin et al. 2011) that in controlled GCs, the relation between the variance of the measured displacement σ^2 and the exerted force F is upward convex and σ^2 increases from about 50 nm^2 to 150 nm^2 as the force also increases (Fig. 3a, b and c, red traces) and that this increase of σ^2 is abolished by Jasplakinolide, inhibiting actin filament depolymerisation(Bubb 2000). In GCs treated with 12.5 and 25 nM Cytochalasin-D the relation between F and σ^2 was flat and almost no increase of σ^2 was observed even when the force exceeded 8 pN (grey and black traces in Fig. 3a). In the presence of 30 μM Blebbistatin, a small increase of σ^2 from about 40 to 60 nm^2 was observed (blue trace in Fig. 3b). In case of 500 nM Nocodazole σ^2 increased from 30 to 70 nm^2 (green trace in Fig. 3c)

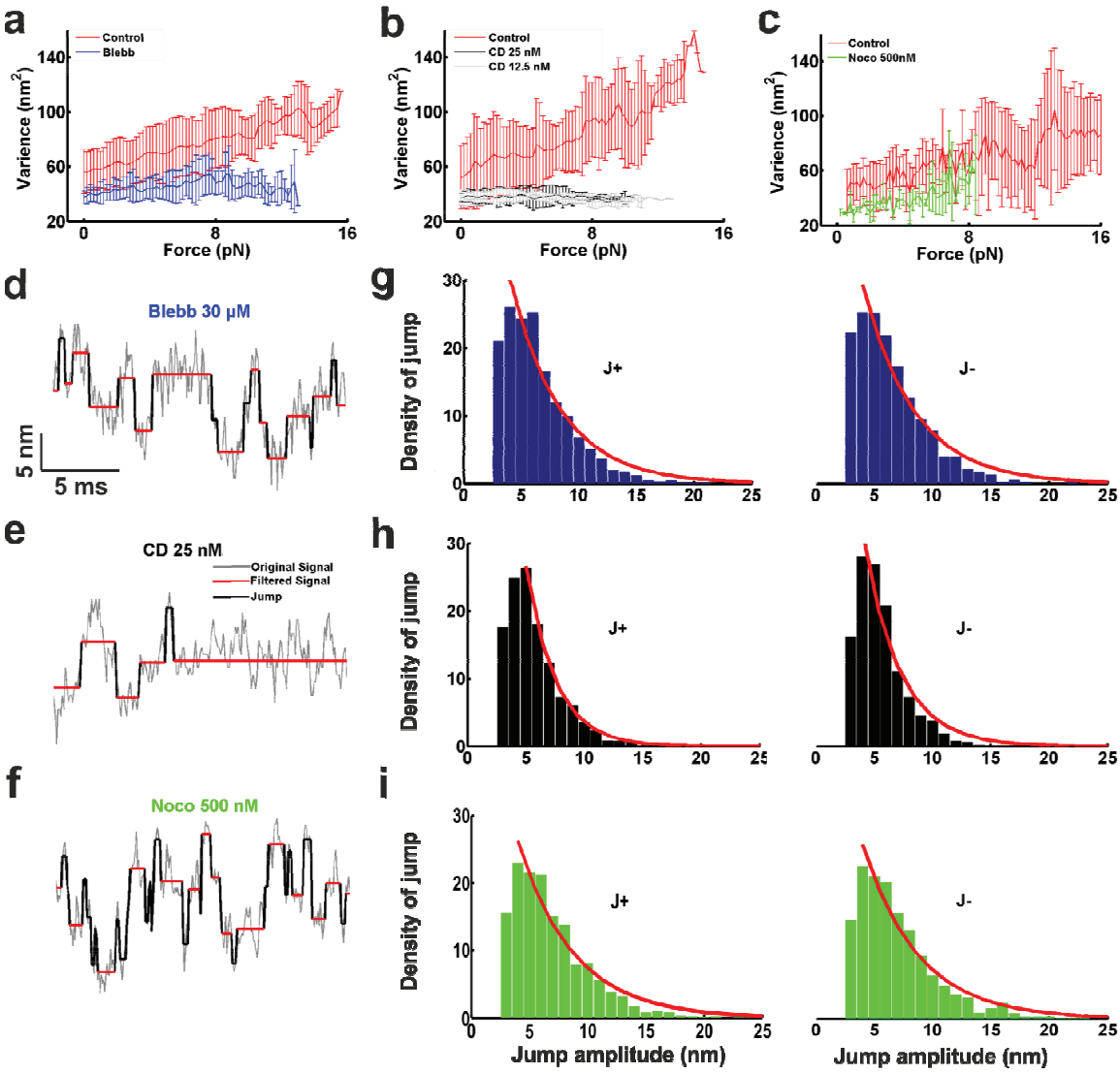

Figure 3. The effect of Blebbistatin and Cytochalasin-D on the elementary events underlying force generation.

*(**a**) Average force – variance relationship for lateral pushes in control condition (red curve) and in the presence of Cytochalasin-D (CD 25 nM, black and grey curves). (**b**) As in (a) but in the presence of Blebbistatin (Bleb 30µM, blue curve). (**c**) As in (**a**) but in the presence of Nocodazole (Noco 500nM, green curve) (**d-f**) Magnification of the z component during push in the presence of Cytochalasin-D (d), in the presence of Blebbistatin (e) and in the presence of Nocodazole (f). Original traces were filtered by the*

*nonlinear diffusion algorithm, resulting in a smooth component and jumps. Jumps were not detected frequently during a push in the presence of Cytochalasin-D and Nocodazole but more often during a push in the presence of Blebbistatin. (**g-i**) Density of forward j^+ and backward j^- jumps during pushes in the presence of Cytochalasin-D (g), in the presence of Blebbistatin (h) and in the presence of Nocodazole (i). Because of a residue noise, jumps with an amplitude lower than 2 nm could not be detected.*

Following bead adhesion to the lamellipodium membrane(Amin et al. 2011), σ^2 could decrease to less than 6 nm^2 and subsequently, when the lamellipodium pushed the bead, forward and backward jumps constituting the elementary events underlying force generation appeared. In the presence of 500nM Nocodazole, 25 nM Cytochalasin-D, forward and backward jumps could be observed but were less frequent than in control condition (Fig. 3d and f). Also in the presence of 30 μM Blebbistatin forward and backward jumps were observed and were more frequent (Fig. 3e and h) than those observed in the presence of Cytochalasin-D. The amplitude of forward j^+ and backward jumps j^- were exponentially distributed (Fig. 3g, h and i) and were fitted by the equations $A_+ e^{-j+/j+*}$ and $A_- e^{-j-/j-*}$ where A_+ and A_- are the frequency of forward and backward jumps, respectively and j^{+*} and j^{-*} are the mean amplitude of forward and backward jumps, respectively. Mean values of these parameters obtained in control condition and in the presence of Nocodazole, Cytochalasin-D and Blebbistatin are shown in Table 2. In control condition the mean values of j^{+*} and j^{-*} were 5.1±1.3 and 4.9±1.2 nm respectively with corresponding rates A_+ and A_- of 157.3±12.2 and 155.5±11.1 events/s respectively. In the presence of both Blebbistatin and Cytochalasin-D the mean values of forward and backward jumps j^{+*} and j^{-*} decreased by about 50 %, in agreement with the reduced or absence of noise which increased during force generation caused by the addition of the two inhibitors (Fig. 3a and b). These inhibitors, however, had a different action on the jump frequency: larger concentrations of Cytochalasin-D progressively reduced A_+ , i.e. the rate of the appearance of forward jumps but not of backward jumps, in agreement with the known effect of Cytochalasin-D that blocks actin filament polymerization (Cooper 1987). Blebbistatin reduced both the forward and backward rates A_+ and A_-. In the presence of Nocodazole the mean values of the forward and backward jumps j^{+*} and j^{-*} remained the same, but the jump frequency was reduced by more than 60% (Fig. 3c and Table. 2).

Table 2

	Control	Blebbistatin 30 μM (n=4)	Cytochalasin-D 12.5 nM (n=3)	Cytochalasin-D 25 nM (n=4)	Nocodazole 500 nM (n=3)
j_+ (nm)	5.10	3.05*	3.60*	2.46*	4.9
j_- (nm)	4.90	2.96*	3.60	2.35*	5.4
A_+ events/s	157.3	135.10*	138.26	110.99*	56.04*
A_- events/s	155.5	125.68*	157.74	153.25	51.60*

Table 2. Jump Frequency and amplitude.

*Amplitudes of positive jumps (j_+, second row) and negative jumps (j_-, third row), frequency of positive jumps(A_+, fourth row) and negative jumps (A_-, fifth row) of the control (second column), Blebbistatin (third column), Cytochalasin-D12.5 nM,(fourth column), Cytochalasin-D 25 nM (fifth column) and Nocodazole (sixth column) respectively. Power analysis is used to determine a required sample size. * indicates sufficient sample size for 80% power assuming a 5% significance level.*

Blebbistatin makes filopodia able to exert a larger force

Nocodazole, Blebbistatin and Cytochalasin-D reduced the amplitude of the force exerted by DRG lamellipodia, but, rather surprisingly, we observed that the force exerted by filopodia treated with Blebbistatin was larger than in untreated filopodia.

In control condition, when filopodia emerged from lamellipodia (Fig. 4a), they moved randomly in space searching for chemical cues before they retracted. These filopodia could exert forces very rarely exceeding 4 pN when a trapped bead was kept in their random motion (Fig. 4b). From the same neurons, the force exerted by filopodia after the addition of 30 μM Blebbistatin was measured (Fig. 4c-d). In these conditions, filopodia emerging from lamellipodia that had shrunk were still able to exert a force which was often larger (Fig. 4d) and were also able to exert a significant force along a vertical direction (compare red traces in Fig.

4b and d). Collected data from 12 neurons show that the average force exerted by filopodia was 2.7 pN in control condition and increased to 4.2 pN in the presence of 30 µM Blebbistatin. However, filopodia in the presence of 500 nM Nocodazole together with 30 µM Blebbistatin exerted forces of 2.6±0.2 pN (Fig. 4h) similar to those observed in control condition.

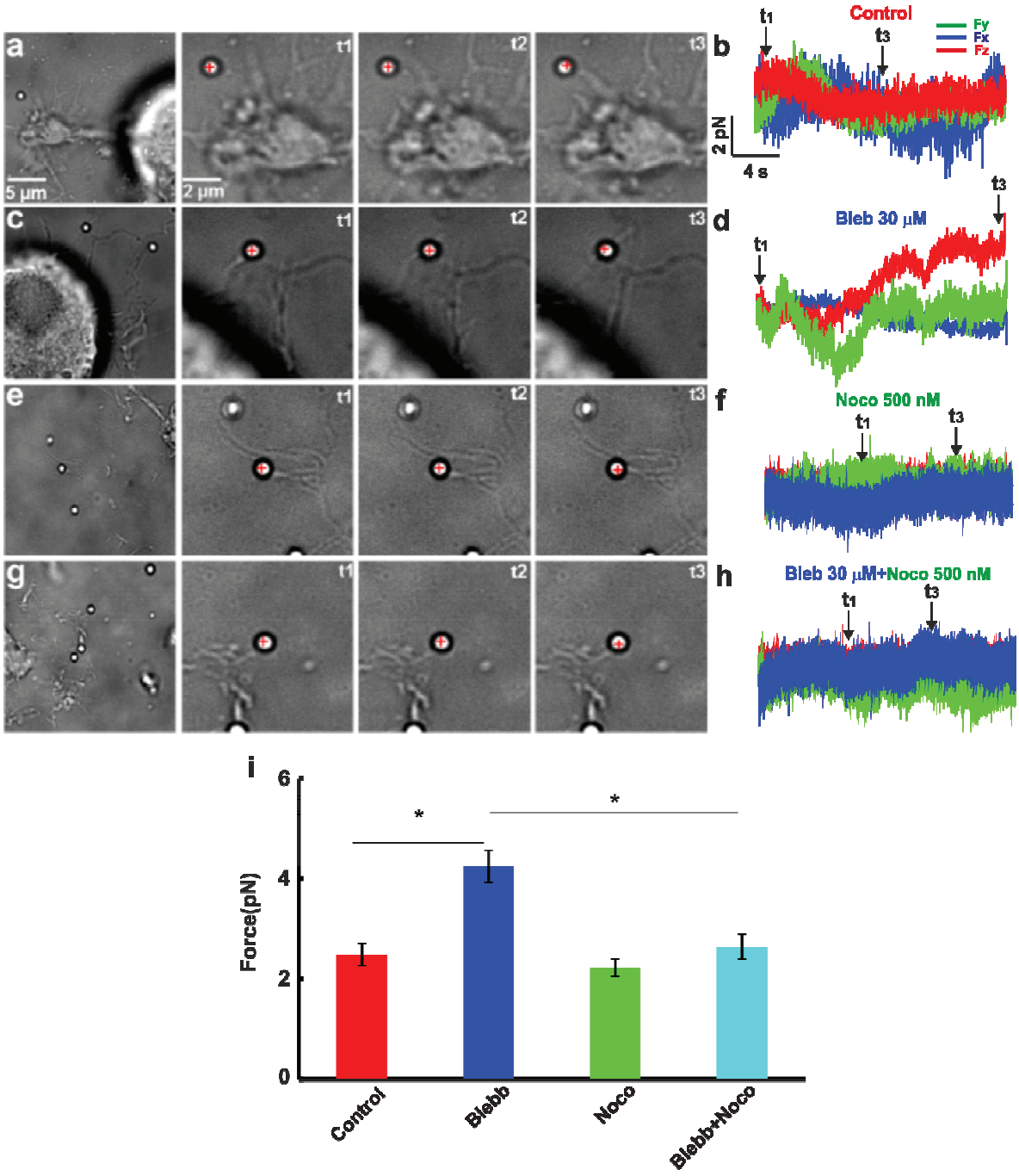

Figure 4. The effect of Blebbistatin on the force exerted by DRG filopodia. *(**a**) Images of a bead trapped in front of a filopodium emerging from a GCs of DRG neuron. At t_1 the bead is in the optical trap and at t_2-t_3 the filopodium pushes the bead. The cross indicates the centre of the optical trap. (**b**) The three components F_x, F_y and F_z of the force exerted by the filopodium. (**c-d**) As in (a-b) but in the presence of Blebbistatin (Bleb 30μM, blue). (**e-f**) As in (a-b) but in the presence of Nocodazole (Noco 500nM). (**g-h**) As in (a-b) but in the presence of Nocodazole+Blebbistatin (Blebb+Noco). (**i**) Filopodia force in Control, in presence of Blebbistatin, Nocodazole and Nocodazole+Blebbistatin. By using the student t-test, the force measured in the presence of inhibitors was lower than the one measured in control condition with a significance *P<0.005. Data represent mean ± SEM. The trap stiffness is $k_{x,y}$=0.10 pN/nm, k_z=0.03 pN/nm. All the data were checked with chi-square test for Normal distribution before applying the student's t test.*

These results show that Blebbistatin reduces the amplitude of the force exerted by lamellipodia but increases the force exerted by filopodia of DRG neurons (Fig. 4i); this increase of the force exerted by filopodia is abolished by the concomitant application of Nocodazole.

DISCUSSION

The present manuscript describes the effect of the inhibition of myosin-II on the morphology, kinetics and dynamics of lamellipodia and filopodia emerging from the soma and GCs of DRG neurons. Our results confirm that myosin-II not only controls the retrograde flow of actin(Medeiros et al. 2006) but also controls and regulates the structural stability of GCs architecture managing the coupling of actin filaments and microtubules dynamics. Our results also show that the contractions of the actomyosin complex formed by filaments of actin and myosin have an active role during lamellipodia retractions. Let us now discuss more in detail these issues.

There are three isoforms of myosin-II in GCs, which have often a different localization in GCs(Hur et al. 2011; Wylie & Chantler 2008) possibly underlying different functions(Betapudi 2010; Wylie & Chantler 2001). We examined the localization of NMIIA and NMIIB in DRG GCs by immunostaining. We determined simultaneously the cellular distribution of actin, tubulin and one of the two myosin isoforms, i.e. NMIIA and NMIIB (see Fig. SI1). The staining for NMIIB was preferentially localized in the central domain and transition zone of the GCs, in

agreement with previous observations(Hur et al. 2011; Medeiros et al. 2006) and very rarely we detected staining in the filopodia. In contrast, we observed a more diffuse staining of NMIIA, present in the central and transition zone of the GCs, but also in its periphery, near its leading edge, and occasionally also in some filopodia. We analyzed also the actin and tubulin distribution in lamellipodia emerging from the soma of differentiating DRG neurons. Lamellipodia sprouting from the soma had an extensive network of actin filaments interspersed with rare filaments of microtubules. Also in these lamellipodia staining of NMIIA was clearly present at their leading edge, while staining for NMIIB was more restricted near the soma and rarely extended to the leading edge of lamellipodia.

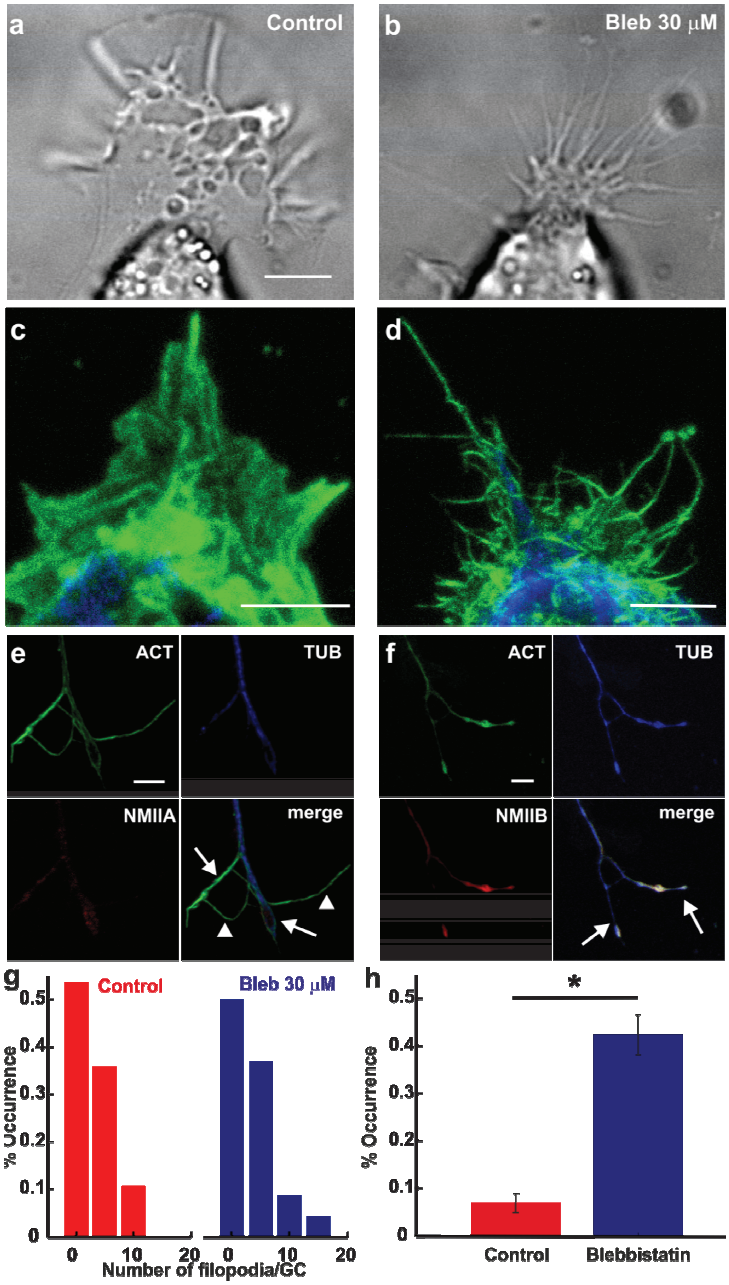

Figure 5. The effect of Blebbistatin on GCs morphology. *(**a-b**) Lamellipodium emerging from a DRG neuron in control condition and after treatment with 30 μM Blebbistatin, respectively. Note the 'filopodish' appearance of the lamellipodia after Blebbistatin treatment. (**c**) Immunostaining of DRG lamellipodium in control condition for actin (green) and tubulin (blue) staining. (**d**) As in (c) but in the presence of 30 μM Blebbistatin. (**e**) Immunostaining of a GC after Blebbistatin treatment for actin, NMIIA and tubulin and merge of the three staining. Arrows and arrowheads indicate filopodia with and without a*

clear staining for tubulin, respectively. ***(f)*** *Immunostaining of a GC after Blebbistatin treatment for actin, NMIIB and tubulin and merge of the three staining.* ***(g)*** *The average number of filopodia per GC before (red) and after treatment with Blebbistatin (blue).* ***(h)*** *The fraction of filopodia with a staining for microtubules in control condition (red bar) and after Blebbistatin treatment (blue bar). Student t-test showed that data significantly differ when compared to the control, P<0.05. All data were checked with chi-square test for Normal distribution before applying the student's t test. Scale bar, 5µm.*

After treatment with 20-50 µM Blebbistatin, a powerful inhibitor of both myosin-II isoforms (Kovács et al. 2004), lamellipodia emerging from the soma and from GCs distant from the soma, changed their morphology, lost their sheet-like structure and appeared 'filopodish' (Fig. 5a-d). After Blebbistatin treatment (Fig. 5d-f) sparse actin filaments were clearly visible and they did not appear to be joined by the usual actin network. Untreated GCs at the tip of long neurites had the core of microtubules surrounded by a mesh of actin filaments and very rarely microtubules entered the filopodia, which were primarily composed of actin filaments. After treatment with Blebbistatin, the terminal ends of neurites were not only composed of actin filaments but also of microtubules at the most distant GCs tips (Fig. 5e and f). The average number of filopodia per GCs in untreated DRG neurons was 7.5±1.2 and was 6.8±1.2 after treatment with 30 µM Blebbistatin (Fig. 5g).

If the mean number of filopodia per GC was not significantly affected by myosin-II inhibition, treatment with Blebbistatin had a profound effect on the distribution of microtubules inside the filopodia: in control condition the fraction of filopodia emerging from GCs exhibiting a staining for microtubules was 0.07± 0.02 (Fig. 5h, red bar) but after Blebbistatin treatment it increased to 0.42±0.04 (Fig. 5h, blue bar), showing that inhibition of NMII elevated the presence of microtubules inside filopodia. Filaments of NMII could cross-link actin filaments providing the network with a diffuse lateral connectivity gluing together the sparse actin filaments resulting in a sheet-like overall structure. Inhibition of NMII destroyed this connectivity leading to the observed 'filopodish' appearance.

Contractions of the actomyosin complex play a fundamental role in several cellular processes such as changes of the cellular shape(Roh-Johnson et al. 2012), cell migration(Solecki

et al. 2009; Betapudi 2010) cell-cell and cell-matrix adhesion(Pasapera et al. 2010), cell division and cell differentiation(Vicente-Manzanares et al. 2009). During the cycles of protrusion/retraction here analyzed (Fig.1) lamellipodia ruffle after their maximal protrusion. These events seem to precede the usual lamellipodium retraction and, given the localization of NMIIA at the lamellipodium periphery, they are most likely originated from contractions of the actomyosin. These observations suggest a dual and complementary role for the two myosin-II isoforms: NMIIA located also at the periphery of lamellipodia, undergoing ruffle formation, could mediate a contraction of the actomyosin complex initiating retraction and NMIIB located more centrally near the transition region of the lamellipodium could control actin turnover(Medeiros et al. 2006). Numerical simulations of the actomyosin complex have shown that generated stresses are overwhelmingly contractile and force chains play a major role(Kim 2014; Lenz et al. 2012; Dasanayake et al. 2011).

The ruffle formed during the retraction of the lamellipodia could be the artifact of the 2D substrate. Lamellipodia ruffle forms because of inefficient formation of focal adhesion(Borm et al. 2005), while in 3D matrices the motility of the cell switches between adhesion-dependent mesenchymal (elongated) and adhesion-independent amoeboid (rounded) cell motility(Ulrich et al. 2010).

When NMII was inhibited by Blebbistatin, we observed two significant morphological changes: lamellipodia lost their sheet-like appearance and became 'filopodish' (Fig. 5a and b) and filopodia emerging from GCs had a higher proportion of microtubules inside (Fig. 5g and h) in agreement with previous findings(Rösner et al. 2007). These morphological changes were mirrored by the observation that filopodia treated with Blebbistatin exert a larger force (Fig. 4). The mean flexural rigidity of microtubules is 2.2×10^{-23} Nm^2 which is almost 1000 times larger than that of actin filaments and equals to 7.3×10^{-26} Nm^2 (Gittes et al. 1993): therefore, filopodia from GCs treated with Blebbistatin are expected to have a larger stiffness and to exert a larger force. When microtubule polymerization was concomitantly inhibited by Nocodazole (Fig.4), filopodia exerted a force comparable to that observed in control condition.

These observations are consistent with the emerging view that inhibition of NMII promotes axon regeneration(Hur et al. 2011). Chondroitin sulfate proteoglycans (CSPGs), major components of the extracellular matrix in the CNS, inhibit axonal regeneration after injury,

through the activation of NMII by phosphorylation of regulatory myosin light chain (RLC) ultimately remodeling cytoskeletal dynamics(Yu et al. 2012). Inhibition of NMII by Blebbistatin promotes axon outgrowth irrespective of the presence of CSPGs in both CNS and PNS neurons(Hur et al. 2011; Yu et al. 2012) providing therefore a promising pharmacological/chemical treatment for neuronal regeneration.

The results reported in the present manuscript confirm the essential role of NMII in cytoskeletal dynamics and in the orchestration of both actin and MT dynamics in GCs(Burnette et al. 2008; Hur et al. 2011; Yu et al. 2012). As shown in Fig. 5, after Blebbistatin treatment, the proportion of filopodia with MTs inside them increases from 0.07 to 0.42 suggesting that Blebbistatin has facilitated the growth of MTs filaments. The biochemical pathway through which NMII affects MT dynamics is not known and it is probably not involving the Rho-kinase (ROCK)(Hur et al. 2011): indeed, inhibition of NMII promotes axon growth but not the inhibition of the Rho-ROCK pathway. On the other hand, repulsive guidance molecule (RGMa) induces neurite outgrowth inhibition through RhoA and Rho-kinase dependent phosphorylation of NMIIA RLC resulting in F-actin reduction(Kubo et al. 2008). These findings suggest, therefore, mechanistically distinct actin- and MT-based GC responses.

MATERIALS AND METHODS

Neuron preparation

Wistar rats at postnatal days 10 to 12 (P10-P12) were sacrificed by decapitation after anesthesia with CO_2 in accordance with the Italian Animal Welfare Act. The Ethics Committee of the International School for Advanced Studies (SISSA-ISAS) has approved the protocol (Prot.n. 2189-II/7). After dissection, Dorsal Root Ganglias (DRG) were incubated with trypsin (0.5 mg/ml; Sigma-Aldrich, Milan, Italy), collagenase (1 mg/ml; Sigma-Aldrich) and DNase (0.1 mg/ml; Sigma-Aldrich) in 5 ml Neurobasal medium (Gibco, Invitrogen, Milan, Italy) in a shaking bath (37°C, 35-40 min). After mechanical dissociation, they were centrifuged at 300 rpm, resuspended in culture medium, and plated on poly-L-lysine-coated (0.5 μg/ml; Sigma-Aldrich) coverslips. Neurons were incubated for 24 h to 48 h and nerve growth factor (50 ng/ml; Alomone Labs, Jerusalem, Israel) was added before performing the measurements.

Quantification of lamellipodia motility

Z-stack phase contrast imaging was performed to quantify the kinetics of protrusion/retraction cycles of lamellipodia in lateral and axial directions. Stacks of images at a frequency of 0.1-1 Hz were acquired. Every stack of images was composed of an image, focused at the plane containing the coverslip where neurons were cultured and images focused at 1,2,3,4,5 and 6 microns above the coverslip. Two algorithms were developed: Algorithm I was designed to quantify in a semi-automatic way the time course of protrusion/retraction cycles and Algorithm II was designed to quantify the vertical motion of lamellipodia during these cycles.

Algorithm I

Images focused on the coverslip plane at different times of the protrusion/retraction cycles (Fig. 6a, t_1-t_3) were analyzed: edges were extracted using standard procedures(Marthon, Ph.; Thiesse, B.;Bruel 1986) and the contour of the neuron was obtained (red line in Fig. 6b). A reference point at the base of the lamellipodium was selected (red cross in Fig. 6b) and an angle covering the lamellipodium was drawn (green shadow in Fig. 6b). The mean distance between the red cross and the points forming the detected contour inside the green shadow was computed and plotted (Fig. 6c). In this plot, representing the mean distance of the lamellipodium leading edge from the reference point, local maxima and minima were detected (green and red asterisks, respectively, in Fig. 6c). The interval between a successive green and red point was taken as the period of that protrusion/retraction cycle.

Algorithm II

Algorithm II was based on classical depth-from-focus algorithms introduced in Computer Vision(Krotkov 1988) to recover 3D information from stacks of images acquired at different focal planes. These algorithms were used to restore the lamellipodia motion in the vertical direction. Briefly, for each pixel (i,j) and for each image I(i,j,h) acquired at a focal plane h microns above the coverslip, the gradient ∇ I(i,j,h) was computed. The point at location (x,y) has the height h if the feature at point (x,y) is in focus on the plane h, determined as the plane for which ∇ I(i,j,h) has the maximum value. Images of the neuron taken from different focal planes separated by 1 μm are shown in Fig. 6d (h=1,…,6μm) from which ∇I(i,j,h) was computed.

In order to characterize the vertical motion ability of a lamellipodium for each value of h, we computed the fraction of pixels - in a given region of interest - in focus at the height h (Fig. 6e). In this way we could quantify the effect of Blebbistatin, Cytochalasin-D and Nocodazole on the ability of lamellipodia to lift up vertically.

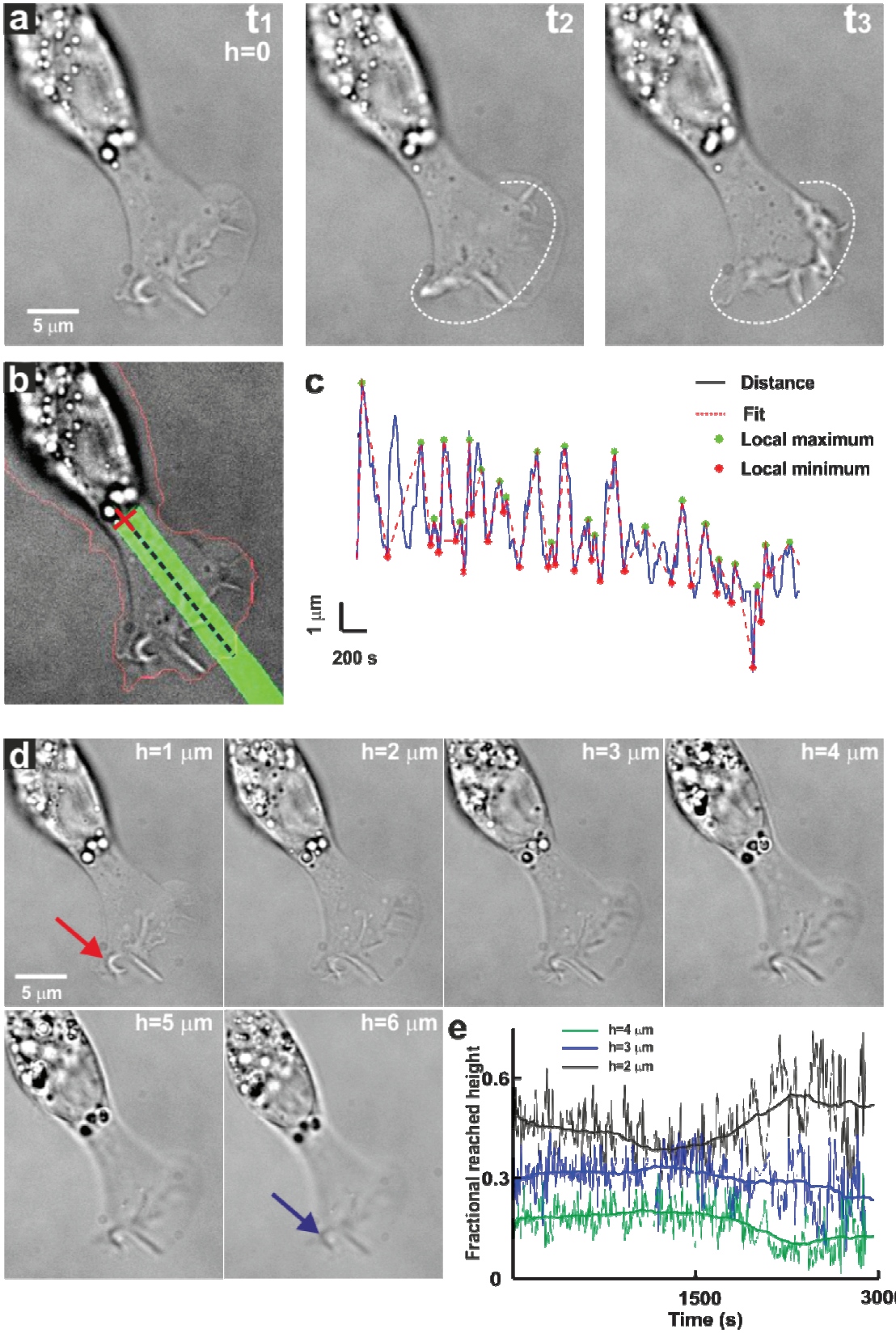
a
t1
h=0
5 μm
t2
t3
b
c
Distance
Fit
Local maximum
Local minimum
1 μm
200 s
d
h=1 μm
h=2 μm
h=3 μm
h=4 μm
5 μm
h=5 μm
h=6 μm
e
h=4 μm
h=3 μm
h=2 μm
Fractional reached height
0.6
0.3
0
1500
3000
Time (s)

Figure 6. Characterization of lamellipodial protrusion/retraction cycles and of vertical motion. *(**a**) From left to right: three images of the lamellipodium undergoing cyclic waves of protrusion (t_2) and retraction (t_1 and t_3) in control condition; the white dotted line represents the leading edge of the lamellipodia. Scale bar, 5 µm. (**b**) Diagram of the method used for the semi-automatic detection of protrusion/retraction cycles. See text for more details. (**c**) Time evolution of the distance of the lamellipodium leading edge from the reference point indicated by the red cross in (b). Local maxima and minima represent maximal protrusion and retraction, respectively. (**d**) Stack of 6 images acquired from 6 focal planes at distance h from the coverslip where neurons were cultured. Scale bar, 5 µm. Red and blue arrows indicate section of lamellipodia above and below the focused plane, respectively. The pixels above focus appear brighter and the pixels below appear darker. (**e**) Fractional pixels in focus of lamellipodia in different focal planes (h=2, 3 and 4 µm) above the coverslip indicating the fractional reached height by lamellipodia. The continuous solid lines are smoothing over a time window of 100 s.*

Force Measurements

The Optical Tweezers (OT) set-up used for force measurements, the procedures followed to compute the Force-Velocity (Fv) relations and the elementary events were as previously described (Amin et al. 2011; Shahapure et al. 2010). The optical tweezers set-up was built as described in Ref. 22. In brief, the dish containing the differentiating neurons and the beads (PSI-1.0 collagen; G.Kisker GbR, Steinfurt, Germany) was placed on a microscope stage. The temperature of the dish was maintained at 37^{o} C using a Peltier device. Bead position x,y and z was determined using back focal plane (BFP) detection which relies on the interference between forward scattered light from the bead and unscattered light. The BFP of the condenser was imaged onto a QPD, and the light was converted to differential outputs digitized at 10 kHz and low-pass filtered at 5 kHz.

Computation of Fv relationships

The velocity $\mathbf{v} = (v_x, v_y, v_z)$ of the bead was obtained by numerical differentiation of its sampled position $\mathbf{x} = (x(n), y(n), z(n))$ n = 1,…N. Numerical differentiation was computed either by convolution of the position components x(n), y(n) and z(n) with the derivative of a Gaussian

filter $1/[\sigma(2\pi)^{1/2}]$ $\exp(-t^2/\sigma^2)$ (Gaussian filtering) or by Linear regression. Gaussian filters corresponding to cut-off frequencies of 0.2, 1 and 10 Hz were used. Further details can be found in Ref. 19.

Jumps determination by non linear diffusion filtering

In order to detect jumps, we used an algorithm based on non linear diffusion[43,44]. The algorithm is based on the Toolbox of Frederico D'Almeida (see http://www.mathworks.com/matlabcentral/fileexchange/3710-nonlinear-diffusiontoolbox). Further details can be found in Ref. 18.

Immunostaining

Cells were fixed in 4% paraformaldehyde containing 0.15% picric acid in phosphate-buffered saline (PBS), saturated with 0.1 M glycine, permeabilized with 0.1% Triton X-100, saturated with 0.5% BSA in PBS (all from Sigma-Aldrich, St.Louis, MO) and then incubated for 1h with primary antibodies: mouse monoclonal antibody against neuronal class III β-tubulin-TUJ1 (Covance, Berkeley, CA) and rabbit polyclonal antibodies against myosin-IIA and IIB (both from Sigma-Aldrich, St.Louis, MO). The secondary antibodies were goat anti-rabbit 594 Alexa (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and anti-mouse IgG_{2a} biotynilated (Santa Cruz Biotechnology, Santa Cruz, CA) and the incubation time was 30 min. F-actin was marked with Alexa Fluor 488 phalloidin, whereas biotin was identified by Marina Blue-Streptavidin (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and incubated for 30 min. All the incubations were performed at room temperature (20-22°C). Cells were examined using a Leica DMIRE2 confocal microscope (Leica Microsystems GmbH, Germany) equipped with DIC and fluorescence optics, diode laser 405nm, Ar/ArKr 488nm and He/Ne 543/594nm lasers. The fluorescence images (1024x1024 pixels) were collected with a 63X magnification and 1.3 NA oil-immersion objective. Leica LCS Lite and Image J by W. Rasband (developed at the U.S. National Institutes of Health and available at http://rsbweb.nih.gov/ij/) were used for image processing.

References

Agero, U. et al., 2003. Cell surface fluctuations studied with defocusing microscopy. *Physical Review E*, 67(5), p.051904. Available at: http://link.aps.org/doi/10.1103/PhysRevE.67.051904 [Accessed November 8, 2014].

Alexandrova, A.Y. et al., 2008. Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. *PloS one*, 3(9), p.e3234. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2535565&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Allingham, J.S., Smith, R. & Rayment, I., 2005. The structural basis of blebbistatin inhibition and specificity for myosin II. *Nature structural & molecular biology*, 12(4), pp.378–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15750603 [Accessed June 6, 2014].

Amann, K. & Pollard, T., 2001. Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proceedings of the National ...*, 98(26), pp.15009–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=64974&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2014].

Amin, L. et al., 2013. Comparison of the force exerted by hippocampal and DRG growth cones. *PloS one*, 8(8), p.e73025. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3749134&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Amin, L. et al., 2011. The elementary events underlying force generation in neuronal lamellipodia. *Scientific reports*, 1, p.153.

Amin, L. et al., 2012. The role of membrane stiffness and actin turnover on the force exerted by DRG lamellipodia. *Biophysical journal*, 102(11), pp.2451–60.

Bamburg, J.R., 1999. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annual review of cell and developmental biology*, 15, pp.185–230. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10611961 [Accessed December 20, 2014].

Bard, L. et al., 2008. A molecular clutch between the actin flow and N-cadherin adhesions drives growth cone migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(23), pp.5879–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18524892 [Accessed November 18, 2014].

Beckham, Y. et al., 2014. Arp2 / 3 Inhibition Induces Amoeboid-Like Protrusions in MCF10A Epithelial Cells by Reduced Cytoskeletal- Membrane Coupling and Focal Adhesion Assembly. , 9(6).

Betapudi, V., 2010. Myosin II motor proteins with different functions determine the fate of lamellipodia extension during cell spreading. *PloS one*, 5(1), p.e8560.

Betz, T. et al., 2011. Growth cones as soft and weak force generators. *Proceedings of the National Academy of Sciences of the United States of America*, 108(33), pp.13420–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3158236&tool=pmcentrez&rendertype=abstract [Accessed December 6, 2014].

Bohnet, S. et al., 2006. Weak force stalls protrusion at the leading edge of the lamellipodium. *Biophysical journal*, 90(5), pp.1810–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1367330&tool=pmcentrez&rendertype=abstract [Accessed November 27, 2014].

Borm, B. et al., 2005. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Experimental cell research*, 302(1), pp.83–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15541728 [Accessed September 9, 2014].

Boureux, A. et al., 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Molecular biology and evolution*, 24(1), pp.203–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2665304&tool=pmcentrez&rendertype=abstract [Accessed November 20, 2014].

Bray D, 2001. No Title. In *Cell Movements: From Molecules to Motility*. New York: Garland, pp. 3–60.

Bridgman, P. & Dave, S., 2001. Myosin IIB is required for growth cone motility. *The Journal of ...*, 21(16), pp.6159–6169. Available at: http://www.jneurosci.org/content/21/16/6159.short [Accessed December 17, 2014].

Bridgman, P.C., 1989. The organization of myosin and actin in rapid frozen nerve growth cones. *The Journal of Cell Biology*, 108(1), pp.95–109. Available at: http://www.jcb.org/cgi/doi/10.1083/jcb.108.1.95 [Accessed December 10, 2014].

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and P.W., 2002. Molecular Biology of the Cell. In pp. 973–975.

Bubb, M.R., 2000. Effects of Jasplakinolide on the Kinetics of Actin Polymerization. AN EXPLANATION FOR CERTAIN IN VIVO OBSERVATIONS. *Journal of Biological Chemistry*, 275(7), pp.5163–5170. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed October 20, 2014].

Burnette, D.T. et al., 2008. Myosin II activity facilitates microtubule bundling in the neuronal growth cone neck. *Developmental cell*, 15(1), pp.163–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2548298&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Bustamante, C., Macosko, J.C. & Wuite, G.J., 2000. Grabbing the cat by the tail: manipulating molecules one by one. *Nature reviews. Molecular cell biology*, 1(2), pp.130–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11253365.

Campellone, K.G. & Welch, M.D., 2010. A nucleator arms race: cellular control of actin assembly. *Nature reviews. Molecular cell biology*, 11(4), pp.237–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2929822&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Carlsson, a E., 2003. Growth velocities of branched actin networks. *Biophysical journal*, 84(5), pp.2907–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302854&tool=pmcentrez&rendertype=abstract.

Carlsson, A., 2001. Growth of branched actin networks against obstacles. *Biophysical journal*, 81(October). Available at: http://www.sciencedirect.com/science/article/pii/S0006349501758420 [Accessed December 18, 2014].

Cojoc, D. et al., 2007. Properties of the force exerted by filopodia and lamellipodia and the involvement of cytoskeletal components. *PloS one*, 2(10), p.e1072.

Conti, M. a. & Adelstein, R.S., 2008. Nonmuscle myosin II moves in new directions. *Journal of Cell Science*, 121(3), pp.404–404. Available at: http://jcs.biologists.org/cgi/doi/10.1242/jcs.03496 [Accessed August 26, 2014].

Conti, M.A. et al., 2004. Defects in cell adhesion and the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *The Journal of biological chemistry*, 279(40), pp.41263–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15292239 [Accessed December 17, 2014].

Cooper, J. a, 1987. Effects of cytochalasin and phalloidin on actin. *The Journal of cell biology*, 105(4), pp.1473–8.

Cooper, J.A. et al., 1983. Kinetic evidence for a monomer activation step in actin polymerization. *Biochemistry*, 22(9), pp.2193–2202. Available at: http://pubs.acs.org/doi/abs/10.1021/bi00278a021 [Accessed December 20, 2014].

Dang, I. et al., 2013. Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature*, 503(7475), pp.281–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24132237 [Accessed October 17, 2014].

Dasanayake, N.L., Michalski, P.J. & Carlsson, A.E., 2011. General Mechanism of Actomyosin Contractility. *Physical Review Letters*, 107(11), p.118101. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3226764&tool=pmcentrez&rendertype=abstract [Accessed September 18, 2014].

Dayel, M.J. & Mullins, R.D., 2004. Activation of Arp2/3 complex: addition of the first subunit of the new filament by a WASP protein triggers rapid ATP hydrolysis on Arp2. *PLoS biology*, 2(4), p.E91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=387265&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Dent, E. & Kalil, K., 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. *The Journal of Neuroscience*, 21(24), pp.9757–9769. Available at: http://www.jneurosci.org/content/21/24/9757.short [Accessed November 26, 2014].

Dent, E.W. & Gertler, F.B., 2003. Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance. *Neuron*, 40(2), pp.209–227. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627303006330.

Dent, E.W., Gupton, S.L. & Gertler, F.B., 2011. The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harbor perspectives in biology*, 3(3). Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3039926&tool=pmcentrez&rendertype=abstract.

Egile, C. et al., 2005. Mechanism of filament nucleation and branch stability revealed by the structure of the Arp2/3 complex at actin branch junctions. *PLoS biology*, 3(11), p.e383. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1278936&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Engle, E., 2010. Human genetic disorders of axon guidance. *Cold Spring Harbor perspectives in biology*, 2(3), p.a001784. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2829956&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Etienne-manneville, S., 2002. Rho GTPases in cell biology. , 420(December), pp.629–635.

Fass, J.N. & Odde, D.J., 2003. Tensile force-dependent neurite elicitation via anti-beta1 integrin antibody-coated magnetic beads. *Biophysical journal*, 85(1), pp.623–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1303117&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Forscher, P.S.S.J., 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *The Journal of Cell Biology*, 107(4), pp.1505–1516.

Friesland, A. et al., 2012. Small molecule targeting Cdc42 – intersectin interaction disrupts Golgi organization and suppresses cell motility. , pp.2–7.

Fujiwara, I. et al., 2002. Visualization and force measurement of branching by Arp2/3 complex and N-WASP in actin filament. *Biochemical and biophysical research*

communications, 293(5), pp.1550–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12054693.

Gao, Y. et al., 2001. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. *The Journal of biological chemistry*, 276(50), pp.47530–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11595749 [Accessed December 3, 2014].

Ghashghaei, H.T., Lai, C. & Anton, E.S., 2007. Neuronal migration in the adult brain: are we there yet? *Nature reviews. Neuroscience*, 8(2), pp.141–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17237805 [Accessed June 4, 2014].

Gittes, F. et al., 1993. Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape. *The Journal of cell biology*, 120(4), pp.923–34.

Gomez, T.M. & Letourneau, P.C., 2014. Actin dynamics in growth cone motility and navigation. *Journal of neurochemistry*, 129(2), pp.221–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24164353 [Accessed November 10, 2014].

Goodman, C., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–377.

Goodman, C.S., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8833447 [Accessed December 10, 2014].

Hall, A., 2012. Rho family GTPases. *Biochemical Society transactions*, 40(6), pp.1378–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23176484 [Accessed October 10, 2014].

Heidemann, S.R., 1990. Growth cone behavior and production of traction force. *The Journal of Cell Biology*, 111(5), pp.1949–1957. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2116337/ [Accessed December 14, 2014].

Hinz, B. et al., 1999. Quantifying lamella dynamics of cultured cells by SACED, a new computer-assisted motion analysis. *Experimental cell research*, 251(1), pp.234–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10438589.

Howard, J., 2001. Mechanics of motor proteins and the cytoskeleton.

Huber, A.B. et al., 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual review of neuroscience*, 26, pp.509–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12677003 [Accessed December 17, 2014].

Hur, E.-M. et al., 2011. Engineering neuronal growth cones to promote axon regeneration over inhibitory molecules. *Proceedings of the National Academy of Sciences of the United States of America*, 108(12), pp.5057–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3064397&tool=pmcentrez&rendertype=abstract [Accessed May 28, 2014].

Ilić, D. et al., 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, 377(6549), pp.539–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7566154 [Accessed November 1, 2014].

Jacquemet, G. et al., 2013. Rac1 is deactivated at integrin activation sites through an IQGAP1-filamin-A-RacGAP1 pathway. *Journal of cell science*, 126(Pt 18), pp.4121–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3772387&tool=pmcentrez&rendertype=abstract [Accessed October 7, 2014].

Ketschek, A., Jones, S. & Gallo, G., 2007. Axon extension in the fast and slow lanes: Substratum□dependent engagement of myosin II functions. *Developmental neurobiology*, 67(10), pp.1305–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2674262&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Kim, T., 2014. Determinants of contractile forces generated in disorganized actomyosin bundles. *Biomechanics and modeling in mechanobiology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25103419 [Accessed November 26, 2014].

Korobova, F. & Svitkina, T., 2008. Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells. *Molecular biology of the cell*, 19(4), pp.1561–74. Available at: http://www.molbiolcell.org/content/19/4/1561.short [Accessed December 17, 2014].

Kovács, M. et al., 2004. Mechanism of blebbistatin inhibition of myosin II. *The Journal of biological chemistry*, 279(34), pp.35557–63.

Kozma, R., Ahmed, S. & Best, A., 1995. The Ras-Related Protein Cdc42Hs and Bradykinin Promote Formation of Peripheral Actin Microspikes and Filopodia in Swiss 3T3 Fibroblasts. , 15(4), pp.1942–1952.

Krotkov, E., 1988. Focusing. *International Journal of Computer Vision*, 1(3), pp.223–237. Available at: http://link.springer.com/10.1007/BF00127822 [Accessed November 26, 2014].

Kubo, T. et al., 2008. Myosin IIA is required for neurite outgrowth inhibition produced by repulsive guidance molecule. *Journal of neurochemistry*, 105(1), pp.113–26.

Lee, C.-S. et al., 2010. Myosin II directly binds and inhibits Dbl family guanine nucleotide exchange factors: a possible link to Rho family GTPases. *The Journal of cell biology*, 190(4), pp.663–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2928003&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Lenz, M., Gardel, M.L. & Dinner, A.R., 2012. Requirements for contractility in disordered cytoskeletal bundles. *New journal of physics*, 14. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3496381&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2014].

LIN, C. et al., 1996. Myosin Drives Retrograde F-Actin Flow in Neuronal Growth Cones. *Neuron*, 16(4), pp.769–782. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627300800975 [Accessed December 20, 2014].

López-Muñoz, F., Boya, J. & Alamo, C., 2006. Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain research bulletin*, 70(4-6), pp.391–405. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17027775 [Accessed October 1, 2014].

Lowery, L.A. & Van Vactor, D., 2009. The trip of the tip: understanding the growth cone machinery. *Nature reviews. Molecular cell biology*, 10(5), pp.332–43. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714171&tool=pmcentrez&rendertype=abstract [Accessed September 3, 2014].

Marsick, B., 2010. Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin□1. *Developmental ...*, 70(8), pp.565–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2908028&tool=pmcentrez&rendertype=abstract [Accessed December 15, 2014].

Marthon, P., Thiesse, B. & Bruel, A., 1986. No Title. In O. D. Faugeras & R. B. Kelley, eds. *Proceedings SPIE 0595*. pp. 318–327. Available at: http://proceedings.spiedigitallibrary.org/proceeding.aspx?articleid=1241265 [Accessed September 21, 2014].

Marthon, Ph.; Thiesse, B.;Bruel, A., 1986. Edge Detection By Differences Of Gaussians. In *Proceedings SPIE 0595*. pp. 318–327. Available at: http://dx.doi.org/10.1117/12.952277.

Meberg, P., 2000. Signal-regulated ADF/cofilin activity and growth cone motility. *Molecular neurobiology*, 21, pp.97–107. Available at: http://link.springer.com/article/10.1385/MN:21:1-2:097 [Accessed December 15, 2014].

Medeiros, N. a, Burnette, D.T. & Forscher, P., 2006. Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nature cell biology*, 8(3), pp.215–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16501565 [Accessed June 5, 2014].

Merrifield, C. & Qualmann, B., 2004. Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. *European journal of cell ...*, 83(1), pp.13–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15085951 [Accessed December 17, 2014].

Millard, T.H., Sharp, S.J. & Machesky, L.M., 2004. Signalling to actin assembly via the WASP (Wiskott – Aldrich syndrome protein) -family proteins and the Arp2 / 3 complex. , 17, pp.1–17.

Mitchison, T. & Kirschner, M., 1988. Cytoskeletal dynamics and nerve growth. *Neuron*, 1(9), pp.761–772. Available at: http://linkinghub.elsevier.com/retrieve/pii/0896627388901249 [Accessed December 11, 2014].

Mogilner, a & Oster, G., 1996. Cell motility driven by actin polymerization. *Biophysical journal*, 71(6), pp.3030–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1233792&tool=pmcentrez&rendertype=abstract [Accessed May 27, 2014].

Mogilner, A., 2009. Mathematics of cell motility: have we got its number? *Journal of mathematical biology*, 58(1-2), pp.105–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2862828&tool=pmcentrez&rendertype=abstract [Accessed December 19, 2014].

Mogilner, A. & Oster, G., 2003. Force generation by actin polymerization II: the elastic ratchet and tethered filaments. *Biophysical journal*, 84(3), pp.1591–605. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302730&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Mongiu, A.K. et al., 2007. Kinetic-structural analysis of neuronal growth cone veil motility. *Journal of cell science*, 120(Pt 6), pp.1113–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17327278 [Accessed May 29, 2014].

Montalvo-Ortiz, B.L. et al., 2012. Characterization of EHop-016, novel small molecule inhibitor of Rac GTPase. *The Journal of biological chemistry*, 287(16), pp.13228–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3339933&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Mullins, R.D., Heuser, J.A. & Pollard, T.D., 1998. The interaction of Arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), pp.6181–6186. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC27619/.

Neuman, K.C. & Block, S.M., 2004. Optical trapping. *The Review of scientific instruments*, 75(9), pp.2787–809.

Nimnual, A.S., Taylor, L.J. & Bar-Sagi, D., 2003. Redox-dependent downregulation of Rho by Rac. *Nature cell biology*, 5(3), pp.236–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12598902 [Accessed November 9, 2014].

Nolen, B.J. & Pollard, T.D., 2007. NIH Public Access. , 26(3), pp.449–457.

Pak, C.W., Flynn, K.C. & Bamburg, J.R., 2008. Actin-binding proteins take the reins in growth cones. *Nature reviews. Neuroscience*, 9(2), pp.136–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18209731 [Accessed June 3, 2014].

Pasapera, A.M. et al., 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *The Journal of cell biology*, 188(6), pp.877–90.

Peskin, C., Odell, G. & Oster, G., 1993. Cellular motions and thermal fluctuations: the Brownian ratchet. *Biophysical journal*, 65(1), pp.316–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1225726&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Pollard, T., 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annual review of ...*, 29, pp.545–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10940259 [Accessed December 15, 2014].

Pollard, T.D., 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annual review of biophysics and biomolecular structure*, 36, pp.451–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17477841 [Accessed July 10, 2014].

Pollard, T.D. & Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, 112(4), pp.453–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12600310.

Prass, M. et al., 2006. Direct measurement of the lamellipodial protrusive force in a migrating cell. *The Journal of cell biology*, 174(6), pp.767–72. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064331&tool=pmcentrez&rendertype=abstract [Accessed October 26, 2014].

Raucher, D. & Sheetz, M.P., 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *The Journal of cell biology*, 148(1), pp.127–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2156205&tool=pmcentrez&rendertype=abstract.

Ridley, A.J., 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology*, 16(10), pp.522–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16949823 [Accessed September 10, 2014].

Roh-Johnson, M. et al., 2012. Triggering a cell shape change by exploiting preexisting actomyosin contractions. *Science (New York, N.Y.)*, 335(6073), pp.1232–5.

Rosenfeldt, H. et al., 2006. Rac inhibits thrombin-induced Rho activation: evidence of a Pak-dependent GTPase crosstalk. *Journal of molecular signaling*, 1, p.8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1769496&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Rösner, H. et al., 2007. Attenuation of actinomyosinII contractile activity in growth cones accelerates filopodia-guided and microtubule-based neurite elongation. *Brain research*, 1176, pp.1–10.

Rouiller, I. et al., 2008. The structural basis of actin filament branching by the Arp2/3 complex. *The Journal of cell biology*, 180(5), pp.887–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2265399&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Rozelle, A. & Machesky, L., 2000. Phosphatidylinositol 4, 5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Current Biology*, 10(6), pp.311–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10744973 [Accessed December 17, 2014].

Saunders, R.M. et al., 2006. Role of vinculin in regulating focal adhesion turnover. *European journal of cell biology*, 85(6), pp.487–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16584805 [Accessed November 1, 2014].

Schaefer, A.W., Kabir, N. & Forscher, P., 2002. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *The Journal of cell biology*, 158(1), pp.139–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173029&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Schaus, T.E., Taylor, E.W. & Borisy, G.G., 2007. Self-organization of actin filament orientation in the dendritic-nucleation/array-treadmilling model. *Proceedings of the National Academy of Sciences of the United States of America*, 104(17), pp.7086–91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1855413&tool=pmcentrez&rendertype=abstract.

Schnelzer, A., Bokoch, G.M. & Jolla, L., 2004. Phosphorylation of RhoGDI by Pak1 Mediates Dissociation of Rac GTPase. , 15, pp.117–127.

Schwartz, M., 2004. Rho signalling at a glance. *Journal of cell science*, 117(Pt 23), pp.5457–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15509861 [Accessed November 10, 2014].

Shahapure, R. et al., 2010. Force generation in lamellipodia is a probabilistic process with fast growth and retraction events. *Biophysical journal*, 98(6), pp.979–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2849058&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Shutova, M. et al., 2012. Functions of nonmuscle myosin II in assembly of the cellular contractile system. *PloS one*, 7(7), p.e40814. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3396643&tool=pmcentrez&rendertype=abstract [Accessed June 2, 2014].

Small, J.V. et al., 2002. The lamellipodium: where motility begins. *Trends in Cell Biology*, 12(3), pp.112–120. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0962892401022371.

Solecki, D., Trivedi, N. & Govek, E., 2009. Myosin II motors and F-actin dynamics drive the coordinated movement of the centrosome and soma during CNS glial-guided neuronal migration. *Neuron*, 63(1), pp.63–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2737100&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Solecki, D.J., Govek, E.-E. & Hatten, M.E., 2006. mPar6 alpha controls neuronal migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(42), pp.10624–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17050699 [Accessed June 13, 2014].

Song, H. & Poo, M., 2001. The cell biology of neuronal navigation. *Nature cell biology*, 3(3), pp.E81–8. Available at: http://www.nature.com/ncb/journal/v3/n3/abs/ncb0301_e81.html [Accessed June 13, 2014].

Stavenger, R. a et al., 2007. Discovery of aminofurazan-azabenzimidazoles as inhibitors of Rho-kinase with high kinase selectivity and antihypertensive activity. *Journal of medicinal chemistry*, 50(1), pp.2–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17201404.

Strasser, G. a et al., 2004. Arp2/3 is a negative regulator of growth cone translocation. *Neuron*, 43(1), pp.81–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15233919.

Suraneni, P. et al., 2012. The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *The Journal of cell biology*, 197(2), pp.239–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3328382&tool=pmcentrez&rendertype=abstract [Accessed September 22, 2014].

Suter, D.M. & Forschert, P., 1998. An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. , 3.

Svitkina, T.M. et al., 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *The Journal of cell biology*, 160(3), pp.409–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172658&tool=pmcentrez&rendertype=abstract [Accessed November 19, 2014].

Thievessen, I. et al., 2013. Vinculin-actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth. *The Journal of cell biology*, 202(1), pp.163–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3704983&tool=pmcentrez&rendertype=abstract [Accessed September 16, 2014].

Torka, R. et al., 2006. ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. *Experimental cell research*, 312(19), pp.3857–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17010335 [Accessed November 26, 2014].

Trivedi, N. & Solecki, D.J., 2011. Neuronal migration illuminated: A look under the hood of the living neuron. *Cell Adhesion & Migration*, 5(1), pp.42–47. Available at: http://www.landesbioscience.com/journals/celladhesion/article/13609/ [Accessed June 13, 2014].

Ulrich, T. a et al., 2010. Probing cellular mechanobiology in three-dimensional culture with collagen-agarose matrices. *Biomaterials*, 31(7), pp.1875–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19926126 [Accessed September 9, 2014].

Vicente-Manzanares, M. et al., 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature reviews. Molecular cell biology*, 10(11), pp.778–90. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2834236&tool=pmcentrez&rendertype=abstract [Accessed May 26, 2014].

Vitriol, E. a & Zheng, J.Q., 2012. Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. *Neuron*, 73(6), pp.1068–81. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3319373&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Wickstead, B. & Gull, K., 2011. The evolution of the cytoskeleton. *The Journal of cell biology*, 194(4), pp.513–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3160578&tool=pmcentrez&rendertype=abstract [Accessed July 15, 2014].

Woo, S. & Gomez, T.M., 2006. Rac1 and RhoA promote neurite outgrowth through formation and stabilization of growth cone point contacts. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(5), pp.1418–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16452665 [Accessed December 9, 2014].

Wu, C. et al., 2012. Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell*, 148(5), pp.973–87. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3707508&tool=pmcentrez&rendertype=abstract [Accessed July 23, 2014].

Wylie, S.R. & Chantler, P.D., 2008. Myosin IIC: a third molecular motor driving neuronal dynamics. *Molecular biology of the cell*, 19(9), pp.3956–68. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed November 26, 2014].

Wylie, S.R. & Chantler, P.D., 2001. Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nature cell biology*, 3(1), pp.88–92.

Yang, Q. et al., 2012. Arp2/3 complex-dependent actin networks constrain myosin II function in driving retrograde actin flow. *The Journal of cell biology*, 197(7), pp.939–56. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3384413&tool=pmcentrez&rendertype=abstract [Accessed October 15, 2014].

Yu, P. et al., 2012. Myosin II activity regulates neurite outgrowth and guidance in response to chondroitin sulfate proteoglycans. *Journal of neurochemistry*, 120(6), pp.1117–28. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3296867&tool=pmcentrez&rendertype=abstract [Accessed May 30, 2014].

Zhang, W. et al., 2012. Self-Assembling Peptide Nanofiber Scaffold Enhanced with RhoA Inhibitor CT04 Improves Axonal Regrowth in the Transected Spinal Cord. *Journal of Nanomaterials*, 2012, pp.1–10. Available at: http://www.hindawi.com/journals/jnm/2012/724857/ [Accessed November 26, 2014].

Zhang, X. et al., 2008. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nature cell biology*, 10(9), pp.1062–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2746969&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Acknowledgments

We acknowledge the financial support of the following projects within the Seventh Framework Programme for Research of the European Commission: the FOCUS Project n. FP7-ICT-270483, the NEUROSCAFFOLDS Project n. 604263. We thank M. Lough for editing the paper.

Author Contributions Statement

WS and LA equally contributed to this work. VT conceived the project and designed the experiments. WS, LA and EE performed the experiments and WS, LA analyzed the data. WS, LA, EE and PF contributed to reagents/materials/analysis tools. VT, WS and LA wrote the paper.

2.2 The role of Rac1 in force generation of DRG neurons

Wasim A. Sayyad, Paolo Fabris & Vincent Torre

Submitted to **Scientific Reports**

Abstract:

We used optical tweezers, video imaging and immunocytochemistry to analyze the role of Rac1 in the motility and force generation of lamellipodia and filopodia from developing growth cones of isolated Dorsal Root Ganglia neurons. When the activity of Rac1 was inhibited by EHop-016, the period of lamellipodia protrusion/retraction cycles increased and the actin retrograde flow rate decreased; moreover, the axial force exerted by lamellipodia was reduced dramatically. Inhibition of Arp2/3 by a moderate amount of CK-548 caused a transient retraction of lamellipodia followed by a complete recovery of their usual motility. This recovery was abolished by the concomitant inhibition of Rac1. The filopodia length increased upon inhibition of both Rac1 and Arp2/3, but the speed of filopodia protrusion increased when Rac1 was inhibited and decreased instead when Arp2/3 was inhibited. These results suggest that Rac1 acts as a switch that activates upon inhibition of Arp2/3 and it also controls the filopodia dynamics necessary to explore the environment.

Introduction

Neurons are specialized cells responsible for exchanging information with other neurons or cells through synapses (López-Muñoz et al. 2006). During development, differentiating neurons explore the surrounding environment in order to form the correct contacts and they use highly motile structures called growth cones (GCs) located at the tip of their neurites (C. S. Goodman 1996; Song & Poo 2001). GCs consist of a flat extension, named 'lamellipodium' with varying width from which finger-like submicron diameter structures called filopodia emerge (Mongiu et al. 2007). The process of polymerization of actin filaments is the main source of GC protrusion, which is regulated and controlled by several proteins such as Arp2/3, cofilin, formin… and molecular motors, such as myosin,... controlling different features of cellular motility (Pak et al. 2008).

Actin related protein 2/3 complex (Arp2/3) is widely studied for its involvement in lamellipodia formation and protrusion (Suraneni et al. 2012; Wu et al. 2012). Arp2/3 consists of seven subunits and promotes the formation of branched actin filament networks (Pollard 2007; Pollard & Borisy 2003). Arp2/3 not only regulates the branching of actin filaments but it is also involved in the formation and dynamics of filopodia (Yang et al. 2012; Korobova & Svitkina 2008). Inhibition of Arp2/3 causes lamellipodia retraction and an increase of the retrograde flow rate (Yang et al. 2012). Arp2/3 is inactive in its native state and the members of the Wiskott-Aldrich syndrome protein (WASP) family, downstream of Rac1 and CDC42 pathways activate the Arp2/3 complex to nucleate new filaments (Campellone & Welch 2010; Millard et al. 2004). Rac1 binds the WAVE (WASP family Verprolin Homology Domain-containing protein) complex to release active WAVE, which promotes actin polymerization through activation of Arp2/3. WASP and WIP (WASP-interacting protein), downstream effectors of CDC42 interact directly with Arp 2/3 complex to promote filopodia formation. Recently a new protein called Arpin has been shown to be part of the Rac1-Arpin-Arp2/3 inhibitory circuit playing a major role in steering during cell migration (Dang et al. 2013).

Rho family GTPase has distinct and specific roles in the regulation of growth, maintenance and retraction of GCs (Ridley 2006). RhoA, Rac1 and CDC42 are well-studied members of Rho family GTPase controlling distinct cytoskeletal elements. Activation of Rac1 stimulates actin polymerization to form lamellipodia, CDC42 induces the polymerization of actin to form

filopodia or microspikes which are parallel actin bundles within the lamellipodium and Rho regulates the bundling of actin filaments into stress fibres and the formation of focal adhesion complexes. The Rho family of GTP-binding proteins are activated by a variety of Growth factors, Cytokines, Adhesion molecules, Hormones, Integrins, G-proteins and other biologically active substances (Hall 2012; Ridley 2006). Biochemical approaches or analyses of the morphology of fixed cells have shown that Rho GTPase also involves crosstalk. This may occur through the Rac1/Cdc42 effecter PAK, which can negatively regulate Rho GEFs (Rosenfeldt et al. 2006) or other mechanisms including, via reactive oxygen species (Nimnual et al. 2003), phosphorylation and competitive binding of RhoGDI (Schnelzer et al. 2004) or binding of GEFs to actomyosin(Lee et al. 2010). Depending upon the concentration and localization of these Rho GTPase, mammalian cells showed different morphology, movement and behaviour (Etienne-manneville 2002).

In this study we have investigated the role of Rac1 in GC motility by using Optical Tweezers and specific inhibitors of Arp2/3 (CK-548) and Rac1 (EHop-016). Motility of lamellipodia and of filopodia was also followed and characterized by video imaging. By combining these techniques together with immunofluorescence we have explored the interaction between Rac1 and Arp2/3 complex and their role in the formation of lamellipodia and filopodia of Dorsal root Ganglion (DRG) GCs. Here we show that Rac1 acts as a switch and activates upon inhibition of Arp2/3.

Results:

After 6-8 hours of culture, differentiating DRG neurons have neurites emerging from their soma. At the tip of the protruding neurites, GCs lamellipodia and filopodia explore the environment and their motion continues for 1-3 days. The motility of lamellipodia and filopodia slows down when appropriate connections are established and the neuronal network is formed; the leading edge of these lamellipodia can move with a speed 30-100 nm/s exerting a force exceeding 20 pN (Shahapure et al. 2010). The effect of the inhibitors of specific proteins involved in the regulation of GC motility was analyzed after 24-48 hours of culture, when the motility of filopodia and lamellipodia is more pronounced. We focused on the analysis of inhibitors of small GTPases and of the Arp2/3 complex.

We used the small molecules CK-636, CK-548, CK-666 and CK-869 as inhibitors of the Arp2/3 complex. All these compounds at a high concentration, i.e. above 100 μM, abolished GC motility completely and in the experiments here described we used extensively CK548 as Arp2/3 inhibitor since it decreases the affinity of rhodamine-N-WASP-VCA for BtArp2/3 complex approximately twofold (Nolen & Pollard 2007). Furthermore, we tested two inhibitors of Rac1namely, EHop-016(Montalvo-Ortiz et al. 2012) and F56(Gao et al. 2001) and CDC42 inhibitor ZCL278 (Friesland et al. 2012). In addition to these, CT04 (CT) (Zhang et al. 2012) and GSK 269962 (GSK) (Stavenger et al. 2007) were also used as inhibitors of RhoA and Rock pathways respectively.

The effect of partial inhibition of Rac1 and Arp2/3 in lamellipodia motility

The involvement of Rac1 and Arp2/3 in lamellipodia motility of DRG GCs was studied by analyzing the effect of their inhibitors EHop-016 (EH) and CK-548 (CK) respectively and by quantifying lamellipodia motility using the two algorithms as described in the Materials and Methods section, based on the analysis of Z-stack phase contrast video imaging. From the image sequences, kymographs were obtained by using algorithm I. The ability of lamellipodia to lift up vertically was quantified by computing the fraction of pixels in focus at 5 μm above the coverslip obtained by using algorithm II (Fig. 1a, b).

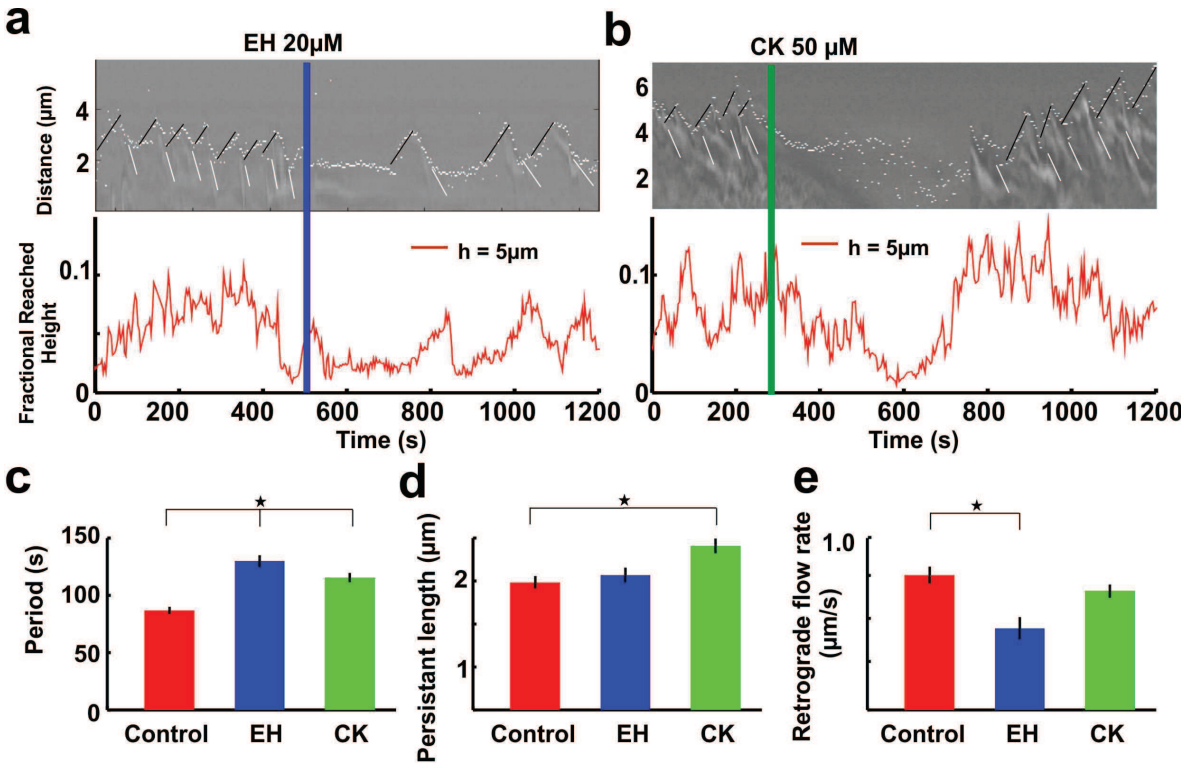

Figure 1. The effect of Rac 1 and Arp2/3 Inhibitor on the motility of lamellipodia

(a) *Kymograph (upper panel) showing the protrusion/retraction cycles of lamellipodia in control conditions and in 20 μM EH. White dots show the leading edge of lamellipodia and the white lines shows the retrograde flow rate of the lamellipodia. Fractional height (lower panel) reached by lamellipodia before and after 20 μM EH.* ***(b)*** *Same as in (a) but in the presence of 50μM CK. Descending white lines label retrograde flow of lamellipodia, and ascending black lines indicate lamellipodia protrusion.* ***(c)*** *Period of protrusion/Retraction cycles of lamellipodia in control conditions, with 20 μM EH and 50μM CK.* ***(d)*** *Persistence length of lamellipodia in control conditions, with 20 μM EH and 50μM CK.* ***(e)*** *Retrograde flow rate of lamellipodia in control conditions, with 20 μM EH and 50μM CK. Student t-test showed that the data significantly differ from the control conditions, *P<0.05. Data represents mean ± SEM.*

When Rac1 activity was inhibited by 20 μM EH lamellipodia still exhibited protrusion retraction cycles (Fig. 1a, upper panel) and could lift up in the axial direction (Fig. 1a, lower panel). Interestingly, lamellipodia of DRG GCs, treated with 50 μM CK showed a transient retraction and were not able to lift up vertically in a significant manner. However, treated

lamellipodia recovered their usual motility in 5-8 min (Fig. 1b, upper panel) and were able to lift up in the axial direction as in control conditions (Fig. 1b, lower panel). The average period of protrusion/retraction cycles of lamellipodia increased significantly, both in the presence of 20 μM EH (129.6±5.2 s) and 50 μM CK (115.1±4.2 s) respectively compared to control conditions (86.5±3.1 s) (Fig. 1c). The persistence length of lamellipodia i.e the maximum extension reached by the lamellipodia after which they start to retract, increased when Arp 2/3 was inhibited by 50 μM CK (1.90±0.09 μm) compared to control conditions (1.48±0.07 μm) (Fig. 1d). However there was no significant change in the persistence length of lamellipodia when Rac1 was inhibited (1.56±0.09 μm), but the retrograde flow rate decreased when Rac1 was inhibited (0.05±0.01 μm/s) compared to what observed in control conditions (0.08±0.01 μm/s) and in the presence of Arp2/3 inhibitors (0.07±0.00 μm/s) (Fig. 1e).

Rac1 activates when Arp2/3 is inhibited.

When the activity of Arp2/3 was inhibited by 100 μM of CK lamellipodia shrank and their motility was completely and permanently suppressed (Fig. 2a). Remarkably, when DRG neurons were treated with 50 μM CK, lamellipodia showed a transient retraction that continued for 5-8 minutes, but then lamellipodia recovered their usual motility restoring protrusion and retraction cycles and were able to lift up vertically almost as under control conditions (Fig. 1b). The results of these experiments suggest that following a partial inhibition of Arp2/3 another pathway is activated rescuing - to some extent - the usual GC motility. To test this possibility and to identify the origin of the recovery of motility in treated lamellipodia, we considered the Rho GTPase pathways, known to regulate many aspects of intracellular actin dynamics and GC metabolism (Boureux et al. 2007). The most extensively studied members of Rho GTPase family are Rho A, Rac1 and CDC42. Since Rac1 promotes the lamellipodia growth (Ridley 2006) we hypothesized that Rac1 could mediate the recovery of motility observed in Fig.3b. Lamellipodia were first treated with 20 μM EH, exhibited an increase in the period of protrusion/retraction cycles and could move up in the axial direction (Fig. 2b). Then the same lamellipodia were treated also with 50 μM CK: in this case, as expected, lamellipodia shrank but could not recover their motility even after 10-20 minutes of exposure to these inhibitors (Fig. 2b). We tested also the simultaneous application of 20 μM EH and of 50 μM CK, which were mixed and added to the medium bathing of the neuronal culture at the same time. Lamellipodia exposed simultaneously to the two inhibitors retracted and did not show any sign of motility even after

10-20 minutes (Fig. 2c).

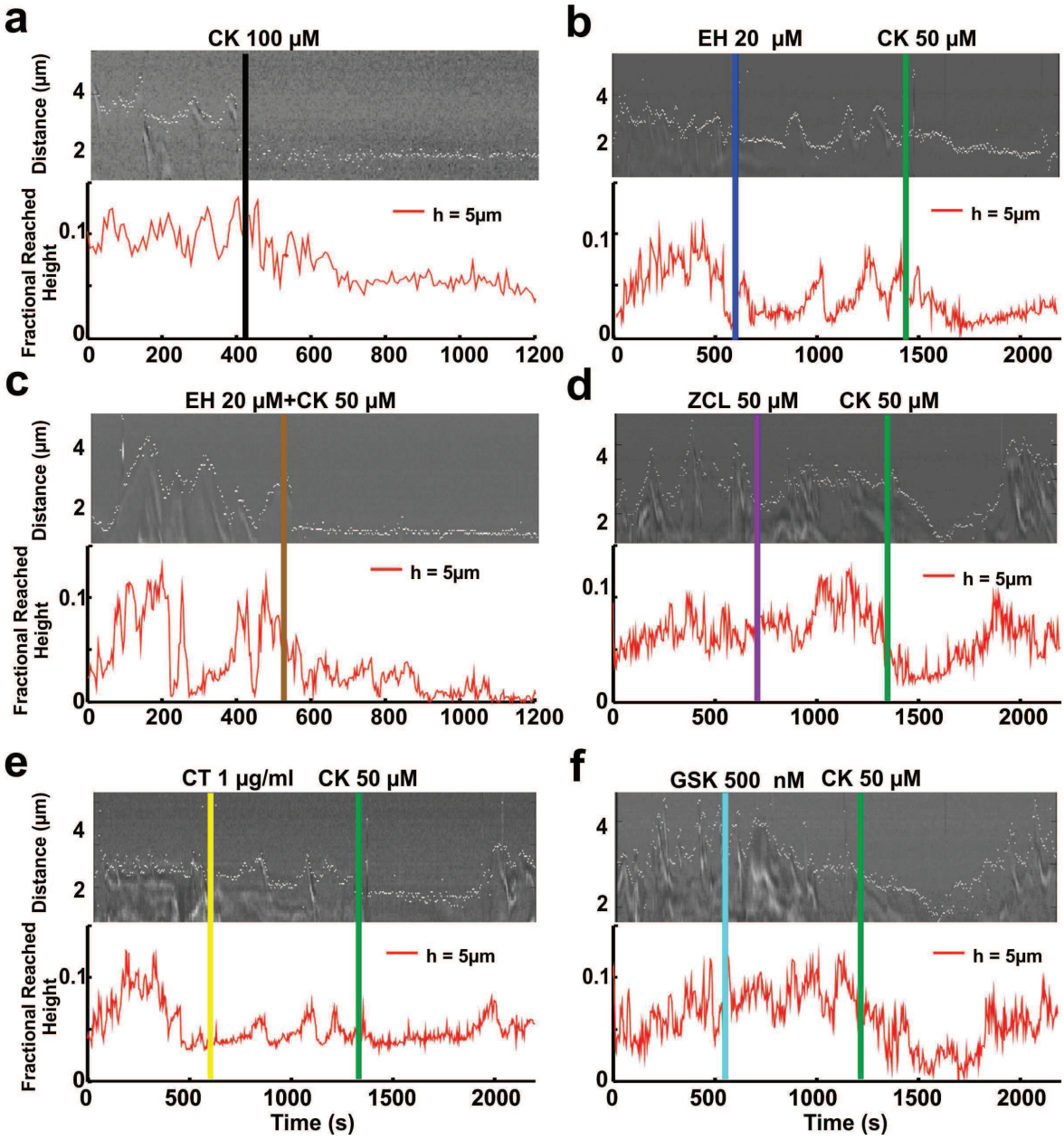

Figure 2: Rac1 restores lamellipodia's motion after transient retraction when Arp2/3 is inhibited

(a) *Kymograph (upper panel) and fractional height reached by lamellipodia (lower panel) in control conditions (before the black line) and in the presence of 100 µM CK (after the black line).* ***(b)*** *As in (a) but in the presence of 50 µM CK (green line) and of 20 µM EH (blue line).* ***(c)*** *As in (a) but in the presence of 50 µM CK and of 20 µM EH together (brown line).* ***(d)*** *As in (a) but in the presence of 50 µM ZCL (purple line) and of*

50 μM CK (green line). ***(e)*** *As in (a) but in the presence of 1μg/ml CT04 (yellow line) and 50 μM CK (green line)* ***(f)*** *As in (a) but in the presence of 500 nM GSK (cyne line) and 50 μM CK (green line). Lines show time at which the inhibitors were added. We observed the same behavior for all the above cases in n ≥ 8 experiments.*

In order to examine the possible role of the CDC42 pathway, we used ZCL-278 as a selective inhibitor which is known to target the binding site of the CDC42 guanine nucleotide exchange factor, intersectin (ITSN) and to hinder CDC42 activation(Friesland et al. 2012). When 50μM ZCL-278 was added lamellipodia did not show significant change in their motility. Subsequent exposure of 50 μM CK to the same lamellipodia shrank the lamellipodia as usual, but then lamellipodia did recover after approximately 8 minutes of exposure (Fig. 2d).

The Rho pathway activates the scaffolding proteins such as GDIA,WASP and IRSP53 and its downstream effector ROCK inactivates Cofilin, an actin depolymerization factor(Torka et al. 2006) and in this way Rho and Rock regulate actin cytoskeletal reorganization. In the Arp2/3 depleted situation, in order to see the role of RhoA and Rock in the lamellipodia recovery, lamellipodia were exposed to CT (Rho A inhibitor)(Zhang et al. 2012) and GSK (ROCK inhibitor) (Stavenger et al. 2007) independently, before the treatment with CK. In both situations lamellipodia recovered after 8 minutes of exposure and, at the end of their retraction, they were also able to reach the same height as in control conditions (Fig. 2e and f).

These results indicate that Rac1 is crucial for the recovery of the transient retraction of lamellipodia caused due to inhibition of Arp2/3.

Effect of Arp2/3 and Rac1 inhibitors on the force exerted by lamellipodia

Optical Tweezers was used to investigate the effect of the partial inhibition of Rac1 and Arp2/3 on the force exerted by lamellipodia. Lamellipodia in control condition pushed the trapped beads with force up to 10-20 pN as previously described (Cojoc et al. 2007) and often beads could be displaced out of the optical trap. The forces were measured from the same lamellipodia in control conditions and in the presence of the inhibitors. Exerted forces were analyzed according to four different stereotyped behaviors depending upon the direction in which lamellipodia exerting force on the bead: vertical push (VP), vertical retraction (VR), lateral push (LP) and lateral retraction (LR).

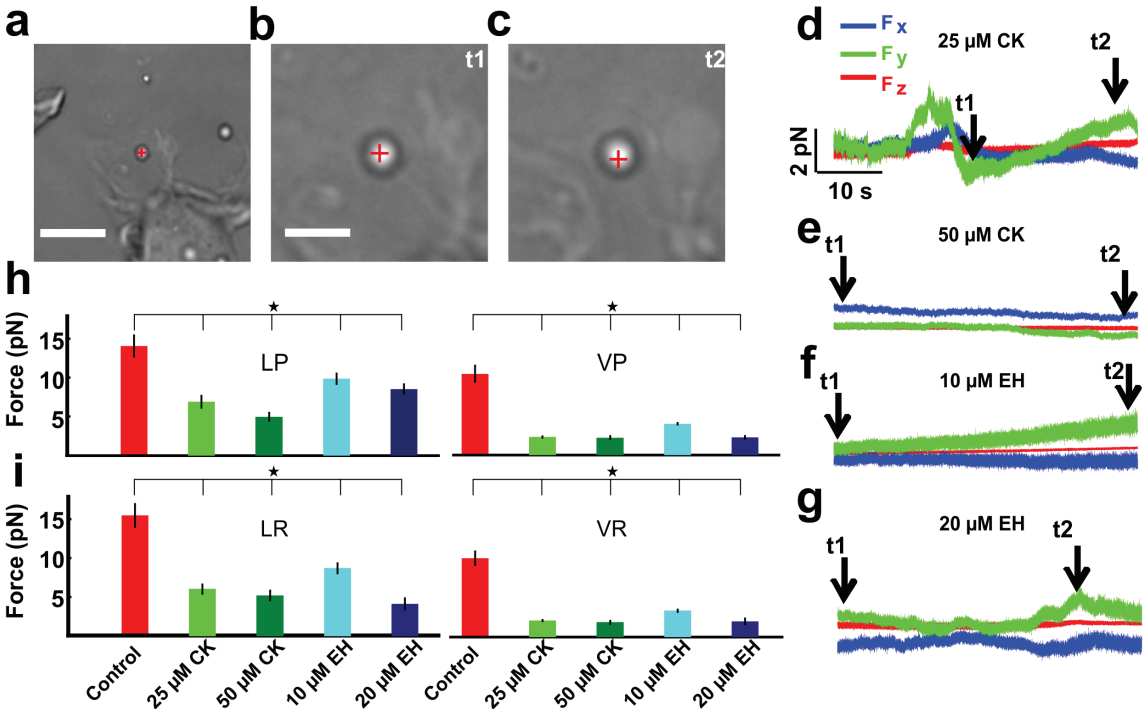

Figure 3: ***The effect of CK and EH on the force generated by lamellipodia.***

(a) *Low-resolution image of a bead trapped in front of a lamellipodium emerging from the soma of a DRG neuron in the presence of 25 µM CK (25 µM CK). Scale bar, 5µm.* ***(b-c)*** *High-resolution images during a push. At t_1 the bead is in the optical trap (b) and when the lamellipodium grows, at t_2, it pushes the bead (c). The red cross indicates the centre of the optical trap. Scale bar, 2µm.* ***(d)*** *The three components F_x, F_y, and F_z of the force exerted when the lamellipodium pushes the bead.* ***(e)*** *As in (d) but in the presence of 50 µM CK (CK 50 µM).* ***(f)*** *As in (d) but in the presence of 10 µM EH (EH 10 µM).* ***(g)*** *As in (d) but in the presence of 20 µM EH (EH 20 µM). The trap stiffness is $k_{x,y} = 0.10$, $k_z = 0.08$ pN/nm..* ***(h)*** *Comparison of the force exerted by lamellipodia in control conditions (red), with 25 µM CK (green), with 50 µM CK (dark green), with 10 µM EH (cyan) and with 20 µM EH (blue) and in all the four different stereotyped behaviours: LP, LR, VP and VR. In each case, by using the student t-test, the force measured in the presence of each inhibitor was lower than that measured in control conditions with a significance *P<0.005. Data represent mean ± SEM.*

Lamellipodia of DRG treated with a moderate concentration of Rac1 and Arp2/3 inhibitors were able to pull and push a trapped bead, but with a lower force compared to the force observed in control conditions (Table 1). In the lateral direction: in case of LP the lamellipodia force

decreased 30-40 % with increased in the inhibition of Rac1, however it decreased 50-65% when Arp2/3 was inhibited compared to control condition. The retractile force LR decreased by 40% when Rac1 was inhibited by 10μM EH, inhibition of Rac1 by 20 μM EHoP decreased the LR force more than 70 % probably due to a decrease in the retrograde flow rate. The retractile force LR decreased to 65% when Arp2/3 was inhibited. In the axial direction: when Rac1 was inhibited by 10 μM EHop, the lamellipodia force in VP and VR decreased more than 60 %. Besides, it decreased more than 75% in all the other VP and VR cases (Table 1).

Force (pN) N ≥ 15	**Control**	**EH 10 μM**	**EH 20 μM**	**CK 25 μM**	**CK 50 μM**
LP	14.0±1.5	9.9±0.8	8.5±0.7	6.9±0.9	5.0±0.6
VP	10.4±1.2	4.0±0.2	2.3±0.3	2.3±0.2	2.2±0.3
LR	15.5±1.6	8.7±0.8	4.1±0.8	6.0±0.7	5.2±0.7
VR	10.1±1.0	3.4±0.3	2.0±0.5	2.1±0.2	1.9±0.3

Table 1. The effect of different inhibitors on the force exerted by lamellipodia. *Average maximum force exerted by lamellipodia in control conditions (second column), in the presence of* 10 μM EH *(third column), of* 20 μM EH *(fourth column), of* 25 μM CK *(fifth column) and of* 50 μM CK *(sixth column) for lateral push (second row), vertical push (third row), lateral retraction (fourth row) and vertical retraction (fifth row) respectively.*

These results suggest that lamellipodia were not able to explore the surrounding environment with an equal force when Rac1 and Arp2/3 were inhibited when compared to control conditions. In addition, lamellipodia were not able to exert a larger force in the axial direction than in the lateral direction, when compared with the control conditions state.

The effect of Rac1 inhibitors on the rate of lamellipodia protrusion

Lamellipodia in the presence of 10-20 μM EH exerted a lower force but were still able to extend. In order to measure their rate of protrusion, we used the Nanopositioner feedback (see Materials and Methods section) which allows a precise and continuous measurement of the bead position by using Optical Tweezers. In control conditions, the speed of protrusion of lamellipodia could reach 100 nm/s (see black trace in Fig. 4) and was reduced to 30-50 nm/s in

the presence of 10 µM EH and to 10-20 nm/s in the presence of 20 µM EH.

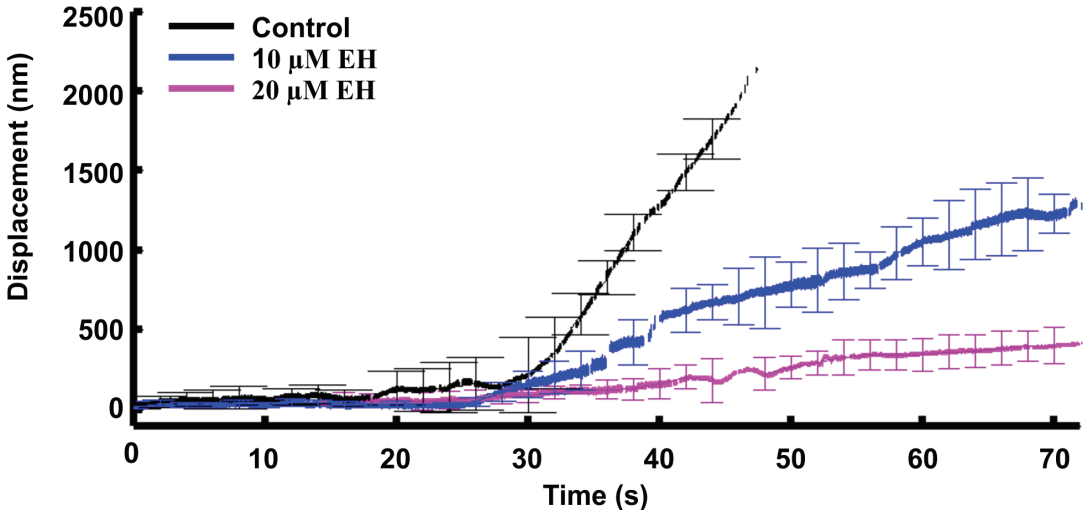

Figure 4. Total displacement of beads in control conditions, in the presence of 10µM EH and in the presence of 20 µM EH *Total displacement of the bead in control conditions (black), with 10µM EH (blue) and with 20µM EH (Magenta).*

These results indicate that inhibition of Rac1 has a similar effect on the amplitude of the force exerted by lamellipodia and on their protrusion rate.

Effect of Arp2/3 and Rac1 inhibitors on the force exerted by filopodia and their motility

The filopodia motility and the force exerted by them were quantified by video imaging, immunocytochemistry and Optical Tweezers (Table 2). The protruding filopodia tips were followed in different frames to calculate the filopodia protrusion rate and the maximum length of the filopodia was measured as described in the Materials and Methods section.

In DRG GC the length of the filopodia increased by 60 to 80 % when Arp2/3 was inhibited by 25 and 50 µM CK respectively. When Rac1 was inhibited by 10 µM EH the length of the filopodia increased by 20 %. Remarkably the filopodia length increased more than the double when the Rac1 was inhibited by 20 µM EH compared to control conditions (Fig 5a, b and e). The GCs were then fixed and stained with Alexa 488 phalloidin and imaged to observe the actin localization. The longer filopodia protruded from the GCs after the inhibition of Rac1 with 20 µM EH and showed an increase in the total F-actin compared to the controlled filopodia (Fig. 5 c and d).

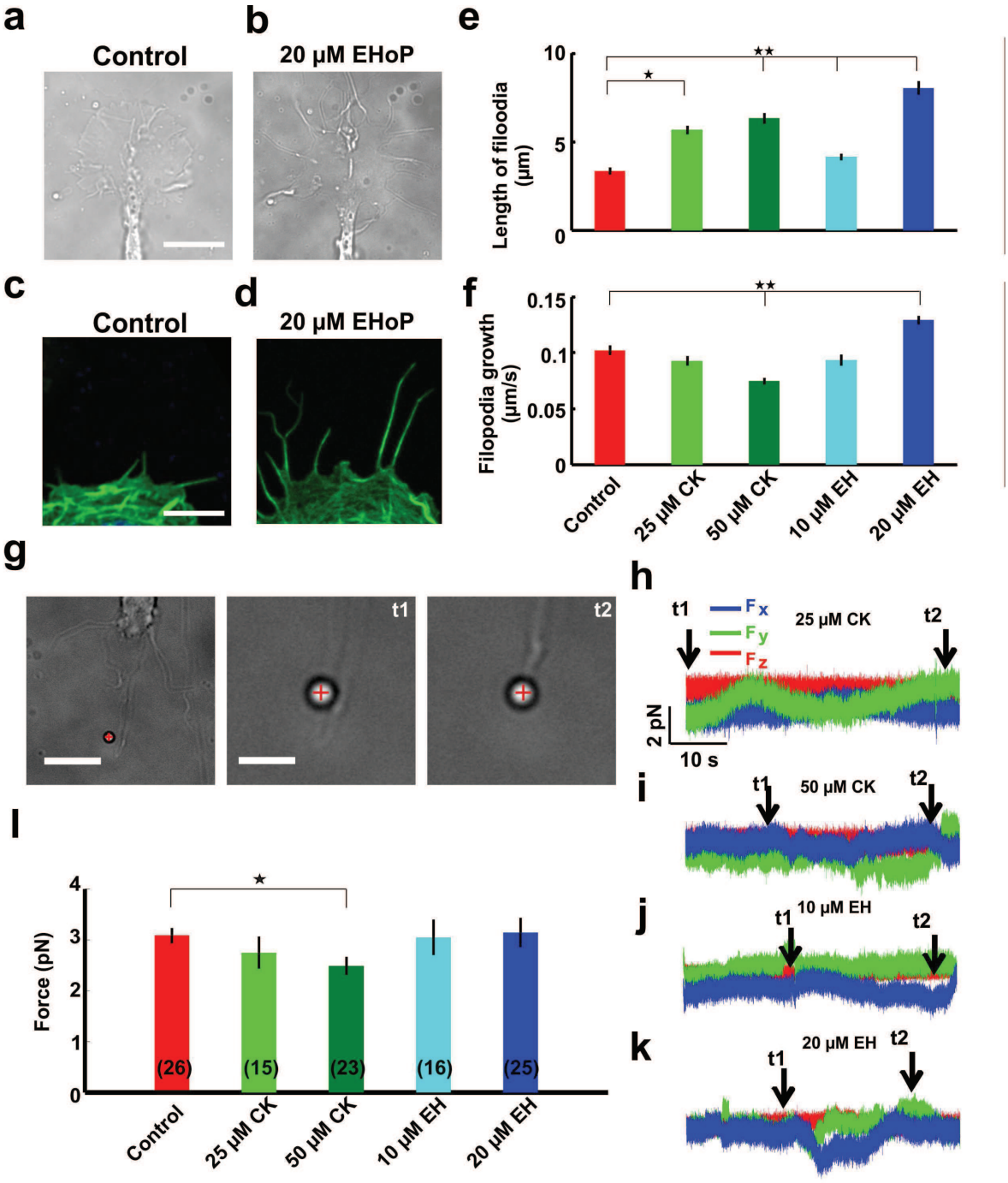

Figure 5: The effect of the CK and EH on the motility and force exerted by filopodia. ***(a-b)*** *Phase contrast images of GC before and after treatment with 20 µM EH. Note the length of filopodia in each case. Scale bar 5 µm.* ***(c-d)*** *Staining of F-actin by phalloidin in GC before and after treatment with 20 µM EH.* ***(e)*** *Rate of filopodia protrusion in control conditions (red), with 25 µM CK (green), with 50 µM CK (dark green), with 10 µM EH*

(cyan) and with 20 µM EH (blue). ***(f)*** *Maximum length of filopodia in control conditions (red) , with 25 µM CK(green), with 50 µM CK(dark green), with 10 µM EH (cyan) and with 20 µM EH (blue).* ***(g)*** *Images of a bead trapped in front of a filopodium emerging from a GC of DRG neuron in the presence of 25 µM CK. At t_1 the bead is in the optical trap and at t_2 the filopodium pushes the bead. The cross indicates the centre of the optical trap.* ***(h)*** *The three components F_x, F_y and F_z of the force exerted by the filopodium in the presence of 25 µM CK.* ***(i-k)*** *As in (h) but in the presence of 50 µM CK (i), in the presence of 10 µM EH (j) and in the presence of 20 µM EH (k) respectively.* ***(l)*** *Filopodia force in control conditions (red), in the presence of 25 µM CK (green), of 50 µM CK (dark green), of 10 µM EH (cyne) and of 20 µM EH (blue). The trap stiffness was $k_{x,y}$=0.10 pN/nm, k_z=0.08 pN/nm. By using the student t-test, the data differs with respect to the control conditions with a significance of *P<0.05 and **P<0.005. Data represent mean ± SEM. All the data were checked with chi-square test for Normal distribution before applying the student's t test.*

The protrusion rate of filopodia did not change when Rac1 and Arp2/3 were suppressed by their respective inhibitors with a lower concentration. However, it increased by 30 % when Rac1 was inhibited by 20 µM EH. In this case the extension of the filopodia length could be the effect of this increase in the filopodia protrusion rate together with the decrease of the retrograde flow rate. Surprisingly, the filopodia protrusion rate decreased by 30 % when Arp2/3 was inhibited by 50 µM CK (Fig. 5f).

Inhibition of Rac1 and Arp2/3 significantly decreased the force exerted by lamellipodia; however, the force exerted by filopodia did not change when Rac1 was inhibited and, with a lower concentration of its inhibitor, Arp2/3 was suppressed, if compared to control conditions. Very rarely filopodia emerged from lamellipodia exert a force that is larger than 4 pN in control conditions. The forces exerted by filopodia were measured in the same neuron before and after the addition of inhibitors of Rac1 or Arp2/3. In each case collected data from 10 neurons showed that the filopodia force did not changed when Rac1 was inhibited by 10-20 □M EH and when the Arp2/3 was inhibited by 25 µM CK. Inhibition of Arp2/3 with 50 µM CK decreased the filopodia force by 20 % when compared to control conditions. (Fig. 5l).

Filopodia	Control	CK 25 µM	CK 50 µM	EH 10 µM	EH 20 µM
Length (µm)	3.36±0.2	5.67±0.25*	6.33±0.3**	4.14±0.18**	8.04±0.39**
Growth rate (µm/s)	0.10±0.001	0.09±0.004	0.07±0.003**	0.09±0.005	0.13±0.004**
Force (pN)	3.08±0.15	2.74±0.31	2.48±0.18*	3.04±0.35	3.14±0.29

Table 2. Filopodia motility and force exerted by them

Maximum length (second row), protrusion rate (third row) and force exerted by filopodia (fourth row) in control conditions (second column), in the presence of 10 µM EH *(third column),of* 20 µM EH *(fourth column), of* 25 µM CK *(fifth column) and of* 50 µM CK *(sixth column). The student t-test has shown that data significantly differ with respect to control conditions *P<0.05 and **P<0.005. Data represent mean ± SEM.*

Discussion:

In this study we have characterized the role of Rac1 and Arp2/3 in the motility and force exerted by lamellipodia and filopodia of DRG GCs. Our results suggest that Rac1 acts as a switch that activates following the inhibition of Arp2/3. Moreover, Arp2/3 and Rac1 not only control the force exerted by lamellipodia but also the dynamics of filopodia.

The effect of the inhibition of Rac1 and Arp2/3 on lamellipodia motility

We followed and quantified the protrusion/retraction cycles of DRG lamellipodia by measuring their period, persistence length and retrograde flow rate using kymograph (see Fig. 6 of the Materials and Methods section).

Lamellipodia treated with a small amount of Rac1 and Arp2/3 inhibitors increased the period of their protrusion/retraction cycles (Fig. 1c). When Rac1 was inhibited, the retrograde flow rate decreased, leading to a longer retraction time and overall cycle period. However, when Arp2/3 was inhibited, the retrograde flow rate remained constant but the persistence length increased. The combination of these two effects increases the period of protrusion/retraction cycle (Fig. 1d and e).

The retrograde flow level decreased after the Arp2/3 complex was knocked down with siRNA in primary cultured hippocampal neurons and neuroblastoma cells (Korobova & Svitkina 2008) but increased when the Arp2/3 complex was inhibited by CK666 and CK869(Yang et al. 2012). We found that inhibition of Arp2/3 with 50 μM CK548 (Fig. 1b), after recovery of lamellipodium motility did not affect the retrograde flow rate. These differences are likely caused by specific cell interactions between the proteins controlling lamellipodia motility and slightly different actions of the used Arp2/3 inhibitors.

Recovery of motility following partial inhibition of Arp2/3

When Arp2/3 was partially inhibited by 50 μM CK548 lamellipodia transiently shrank for 5-8 minutes but then recovered their usual motility. The Rho family of GTPase signalling proteins plays a pivotal role in regulating actin cytoskeleton (Ridley 2006) and could be involved in the observed recovery of lamellipodia motility. The best characterized small GTPases of the Rho family are Rac1, Cdc42 and RhoA which act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state (Boureux et al. 2007). To determine the possible role of Rho GTPase signaling pathways, in the transient retraction and recovery of lamellipodia when Arp2/3 was inhibited, we used selective inhibitors of Rac1, Cdc42 and RhoA (Fig. 2).

Lamellipodia treated with an inhibitor of Rac1 showed increase in their period of protrusion/retraction cycle and could move in the axial direction. When the same lamellipodia were treated with 50 μM CK548, they showed the usual retraction but did not recover even after 10-20 minutes (Fig. 2b). Moreover, when treated with both Rac1 and Arp2/3 inhibitor together, lamellipodia shrank as usual but again they did not recover after 10-20 minutes of exposure (Fig. 2c). Both the above results suggest that Rac1 is activated when Arp2/3 is inhibited, through an unknown pathway.

A possible mechanism could be mediated by the Integrin pathways. Jacquemet, G. *et al.* suggested that the engagement of integrin followed by filamin-A, IQGAP1 and RacGAP1 enrollment, deactivates Rac1(Jacquemet et al. 2013). Ilić, D. *et* al. and Saunders, R. M. *et al.* reported that Arp2/3 is recruited to nascent integrin adhesions through interaction with FAK and vinculin, which further required to reinforce the link between integrin and cytoskeleton (Ilić et al. 1995; Saunders et al. 2006). Furthermore, Beckham et al. reported that Arp2/3 inhibition

impairs integrin, an extracellular membrane attachment resulting in either a translocation or treadmilling of mature adhesions (Beckham et al. 2014). Therefore, it is possible that inhibition of Arp2/3 could reduce the ligation and clustering of integrins and further suppress filamin-A, IQGAP1 and RacGAP1 recruitment, leading to an enhancement of Rac1 activity.

To study the role of the other Rho GTPase pathways in the transient retraction and recovery of lamellipodia upon Arp2/3 inactivation, inhibitors of the respective pathways were used. In all these cases lamellipodia showed recovery when treated with Cdc42, RhoA and ROCK inhibitors before the treatment with Arp 2/3 inhibitor (Fig. 2). Therefore, the involvement of these pathways in the recovery of lamellipodia motility can be discarded.

Arp2/3 controls the formation and dynamics of filopodia

In the active states Rac1, CDC42 and RhoA interact not only with their specific downstream targets but also cross talk (Ridley 2006). Specifically, activation of Cdc42 triggers a localized activation of Rac1, initiating the filopodia formation (Kozma et al. 1995). In our experiments the presence of actin was confirmed in the filopodia before and after the inhibition of Rac1 by using immunocytochemistry (Fig. 5). Inhibition of Rac1 remarkably increased the protrusion speed as well as the maximum length of the filopodia (Fig. 5e and f). Since Rac1 inhibition reduces the activation of Arp2/3, it is possible that Rac1 inhibition decreases the formation and protrusion of lamellipodia, leaving filopodia behind. Also Rac1 inhibition could also increase the availability of GTPs, possibly enhancing the Cdc42 activity and the formation of filopodia. In addition, abundance of actin filaments remains from lamellipodia formation and decreases in the retrograde flow rate (Fig. 1e), upon Rac1 inhibition could contribute to the formation of longer filopodia.

Korobova et al. found that inhibition of Arp2/3 reduced the lamellipodia protrusion as well as filopodia formation and dynamics (Korobova & Svitkina 2008). In our case we found that Arp 2/3 inhibition decreased the protrusion speed of filopodia but it increased the maximum length of filopodia (Fig. 5e and f). We also found that when Arp2/3 was inhibited the force exerted by filopodia decreased compared to control conditions. The above results indicate the direct involvement of the Arp2/3 in the formation and dynamics of filopodia. On the other hand, Rac1 inhibition increased the length of filopodia but it did not change the force they exert. This

indicates that, unlike Arp2/3, Rac1 may not directly take part in the formation and dynamics of filopodia (Fig. 5e-i).

In conclusion, here we have shown that Rac1 activates when Arp2/3 is inhibited possibly through the Integrin pathways acting as a feedback. Besides its role in lamellipodia formation Arp2/3 is directly involved in the formation and dynamics of filopodia while Rac1 does not involved in the activity of the force generation of filopodia.

MATERIALS AND METHODS

Neuron preparation

Wistar rats at postnatal days 10-12 (P10-P12) were sacrificed by decapitation after anaesthesia with CO_2 in accordance with the Italian Animal Welfare Act. The Ethics Committee of the International School for Advanced Studies (SISSA-ISAS) has approved the protocol (Prot.n. 289-II/7). After dissection, Dorsal Root Ganglia (DRG) were incubated with trypsin (0.5 mg/ml; Sigma-Aldrich, Milan, Italy), collagenase (1mg/ml; Sigma-Aldrich) and DNase (0.1 mg/ml; Sigma-Aldrich) in 5 ml Neurobasal medium (Gibco, Invitrogen, Milan, Italy) in a shaking bath (37°C, 35-40 min). After mechanical dissociation, they were centrifuged at 300 rpm, resuspended in the culture medium and plated on poly-L-lysine-coated (0.5 μg/ml; Sigma-Aldrich) coverslips. Neurons were incubated for 24 - 48 h and nerve growth factor (50 ng/ml; Alomone Labs, Jerusalem, Israel) was added before performing the measurements.

Quantification of lamellipodia and filopodia motility:

Neurons were maintained at 37 ^{o}C in the sample holder of the microscope stage capable of moving in X and Y directions with nanometer precision and imaged through 100 X oil immersed, 1.4 NA objective lens mounted on an inverted microscope (IX80, Olympus). Stacks of phase contrast images of neurons from DRG ganglia were obtained by Charge couple device (CCD) camera (Olympus Megaview) and by moving the objective lens vertically. Each stack contains images obtained in the focal plane of the objective, focussed on the coverslip where neurons were cultured i.e. at height 0 and at 1, 2, 3, 4, 5 and 6 micron above the coverslip. Stack of images were acquired with 0.1-1 Hz frequency to quantify the 3D motion of lamellipodia. Then for a further analysis, the time lapse image sequence for each height was extracted by using Xcellence software (Olympus) to create videos of different height. Two algorithms were

developed to quantify the dynamics of lamellipodia. Algorithm I was designed to quantify in a semi-automatic way the time course of protrusion/retraction cycles by using an improved version of the Kymograph (Hinz et al. 1999; Borm et al. 2005). Algorithm II was designed to quantify the vertical motion of lamellipodia during these cycles.

Algorithm I

Image sequences at height '0', i.e. the cover slip where neurons were cultured, was focused, was used to analyze the protrusion/retraction cycles of lamellipodia (Fig. 6). The lamellipodia edge were extracted from each image of the video by using the differences of Gaussian filter (Marthon et al. 1986). Lamellipodia edges were tracked and followed during the entire duration of the video (Fig. 6 a). A profile of the temporal movement of the lamellipodium edge was obtained. These profiles allowed to follow and quantify lamellipodia cycles of protrusion and retraction (Fig. 6d). Then the regions of interest of each line were cut and lined up with the time course, to obtain separate kymographs corresponding to each line (Fig. 6e).

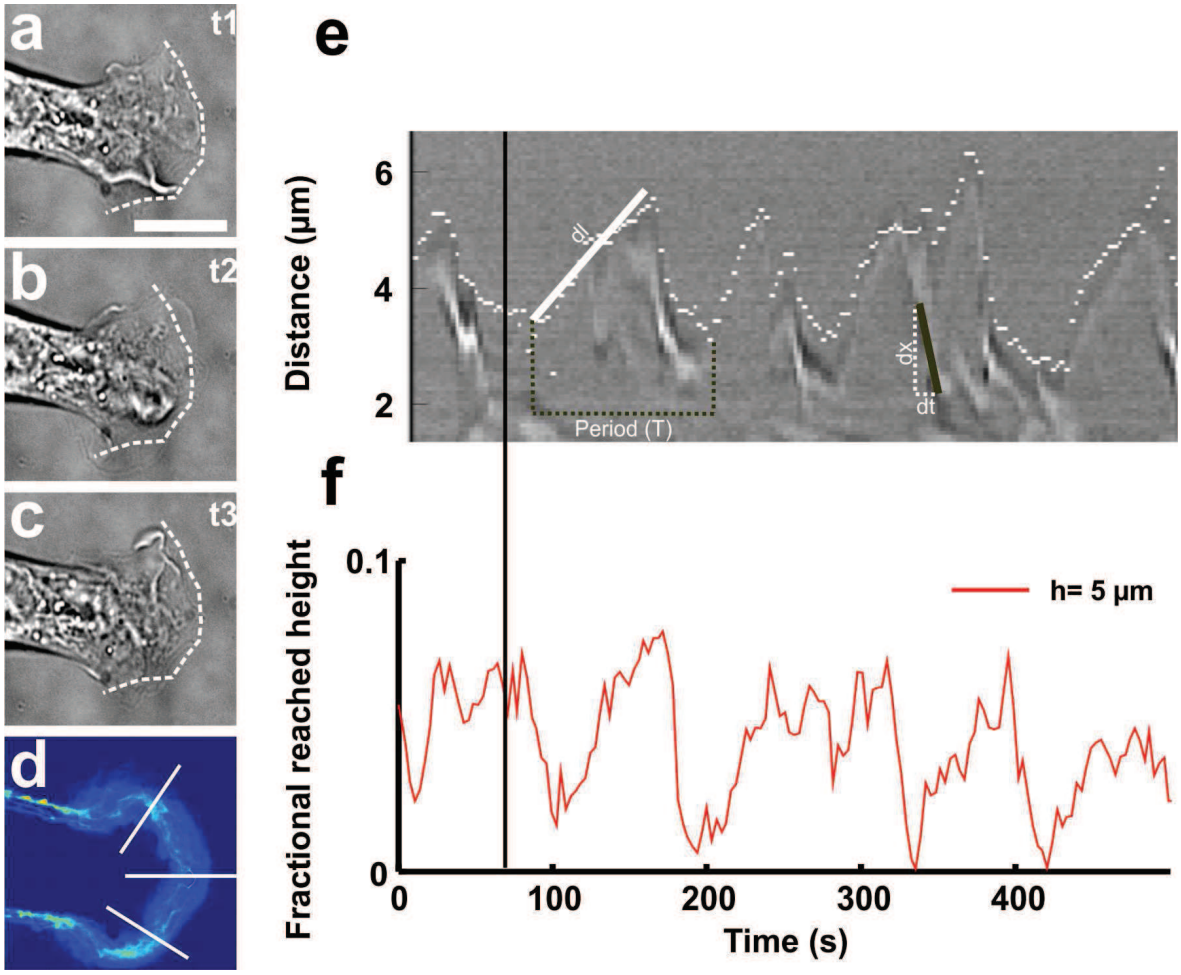

Figure 6 . Characterization of lamellipodial protrusion/retraction cycles and of vertical motion.

(a-c) *From top to bottom: images of the lamellipodium undergoing cyclic waves of protrusion (t_2) and retraction (t_1 and t_3) in control conditions; the white dotted line represents the leading edge of the lamellipodium. Scale bar, 5 µm.* ***(d)*** *The profile diagram of the positions of the lamellipodium edge during the time course. Increase in the colour intensity shows increase in the frequency of the lamellipodia edge to be present at particular space. White lines used to plot the kymographs.* ***(e)*** *Kymograph showing the protrusion/retraction cycles of lamellipodia. White dots show the leading edge of lamellipodia. The characteristic values of period of protrusion/retraction cycles of lamellipodia motion (black dotted line), the retrograde flow rate (black line) and persistence length of lamellipodia (white line) i.e. (T), (dx/dt) and (dl) respectively were*

*calculated along the label lines.**(f)** Fractional number of pixels in focus at 5µm height above the coverslip. The protrusion/retraction cycles of lamellipodia is also observed in terms of fractional reached height by lamellipodia. The black line shows the peak position of the fractional height where lamellipodia reaches the maximum in axial direction at the end of the retraction.*

The white dotted line in the kymograph shows the lamellipodia leading edge. The changes in the grey values show lamellipodia movements. Mainly the ascending white dotted parts of the dotted line show the protrusion of lamellipodia (white line showing single protrusion) while the descending white dotted parts of the line represent the retraction of lamellipodia. The time to complete one protrusion and retraction by the lamellipodia was considered as a period (T) of protrusion/retraction cycle of lamellipodia. The maximum protrusion length after which lamellipodia starts retracting (white line, dl; micrometers) was defined as the persistence length of lamellipodia. The dark appearances in the kymograph during each retraction of lamellipodia represent the retrogradely moving lamellipodia features (green line showing single retrograde flow, micrometer). The slope of the line drawn on these dark appearances was calculated to find out the retrograde flow rate (dx/dt ; micrometers per second) (Hinz et al. 1999; Borm et al. 2005) (Fig. 6 e). Each parameter, the period of the protrusion/retraction cycles of lamellipodia, the persistence length of lamellipodia and the retrograde flow rate, were calculated by extracting these features from many kymographs and averaged over for the statistical significance.

Algorithm II

Lamellipodia not only show periodic motion of protrusion and retraction (Fig. 6C) but, during retraction, they also lifts up and ruffle. To study the axial motion of GC lamellipodia, image sequences taken at different heights i.e. 0, 1, 2…6 were acquired and analysed. Algorithm II was based on the theory of defocusing, in which a pixel is assumed to be in focus at a specific height when its intensity equalises with the background intensity of the image of that height (Agero et al. 2003). The background intensity of the image for each height was computed as the median of pixel intensities of the image for that height. In this way, the fraction of pixels of the lamellipodium in focus at different heights, was extracted and plotted against time (Fig. 6f). In this manner it was possible to study the maximal height reached by the lamellipodia edge during retraction in different conditions. Usually lamellipodia lift up high

around the maximal retraction, so that their cyclic motility could be characterized both by the kymograph and by the fractional height that was reached (Fig. 4 and 6).

In order to quantify the motility of filopodia, phase contrast time lapse image sequences acquired at height '0' were analyzed. An Imagej (Image processing and analysis in Java) software was used to measure the maximum length of the filopodia and plug-in, 'manual tracking' were used to identify the protrusion rate of the filopodia.

Force Measurements

For force measurements we used the Optical Tweezers (OT) set-up previously described in Cojoc et al. (Cojoc et al. 2007). The trapping source was an ytterbium fiber laser operating at 1064 nm (IPG Laser GmbH, Burbach, Germany), which was coupled with an inverted microscope (IX81, Olympus, Milan, Italy) to the focusing objective (Olympus 100_ oil, NA 1.4). The dish containing the differentiating neurons and the beads (PSI- 1.0Collagen, G. Kisker GbR, Steinfurt, Germany) was placed on the microscope stage. The temperature of the dish was kept at 37^{o}C by a Peltier device. The bead position (x, y, z) was determined along all the axes with an accuracy of 2 nm using back focal plane detection, which relies on the interference between forward scattered light from the bead and unscattered light (Neuman & Block 2004). The back focal plane of the condenser was imaged onto a quadrant photodiode (QPD) (C5460SPL 6041, Hamamatsu, Milan, Italy), and the light intensity was converted to differential outputs digitized at 10 kHz and lowpass-filtered at 5 kHz. The z position of the bead was determined using the Gouy phase-shift effect (Neuman & Block 2004). The trap stiffness,(kx, ky, kz), and the detector sensitivity were calibrated using the power spectrum method (Neuman & Block 2004). The force exerted by the lamellipodium/filopodia, F, was taken as equal to -Ftrap. When the displacement of the bead from its equilibrium position inside the trap (dx, dy, dz) was <200 nm. Ftrap (Fx, Fy, Fz) was calculated as Fx = dxkx, Fy= dyky, and Fz = dzkz. All experiments of force recordings were monitored by video imaging with a CCD camera (Olympus Megaview) at a frame rate of 5 Hz. Visual inspection of recorded images made it possible to discard from the analysis all force recordings during which visible debris interfered with the optical determination of the bead position.

Nanopositioner feedback

In the OT setup, the detection of the position of the bead was based on the interference signal in the back focal plane monitored with Quadrant Photo Detector (QPD) (Neuman & Block 2004). Often lamellipodia were able to push the bead out of the linear range – typically 200 nm - in which the QPD could provide a reliable measurement. To overcome this situation, we used a feedback mechanism, based on a nanopositioner stage-Nanodrive (Mad City Labs, USA) was used (Fig. 7).

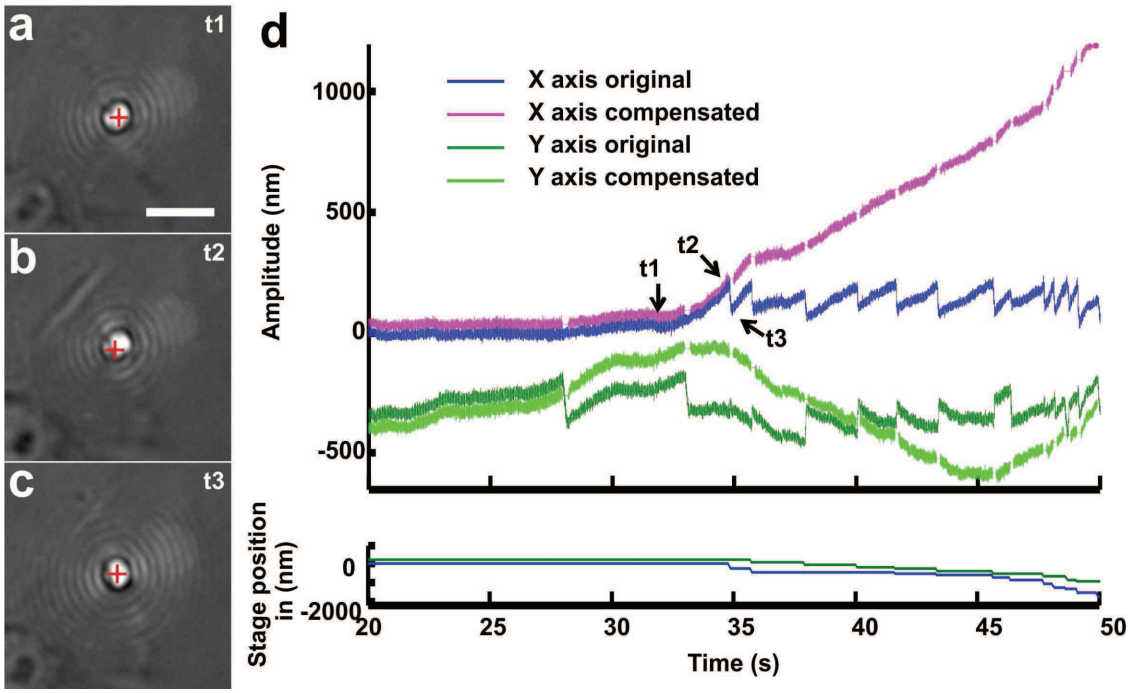

Figure7. The feedback and nanopositioner system

(a-c) High-resolution images of a bead trapped in front of a lamellipodium emerging from the soma of a DRG neuron in control conditions and during a push. At t_1 the bead is in the optical trap (a) Scale bar, 2µm.The lamellipodium grows, at t_2, tries to push the bead out of the trap (b). At t_3 the feedback mechanism of the Nano-drive redirects the bead back into the centre of the trap (c). The red cross indicates the center of the optical trap. (d) The X, Y components of the trace. The position of the bead (blue and green curve respectively, upper panel), compensated X, Y position of the bead (magenta and light green curve,

upper panel), corresponding X, Y position of the Nanodrive (Blue and green respectively, lower panel.

To summarize, from the detected x and y coordinates of the bead the displacement 'r' of the bead position from the centre of the trap was computed as sqrt (x^2+y^2). The nanodrive stage brings back the bead into the centre of the optical trap when r is larger than the threshold (which is usually set to be equal to 200 nm). By using the information of the displacement of the nanodrive stage (Fig. 7d, lower panel) and the bead position determined by the QPD (X,Y axis original in Fig. 7d) we recovered the x-y axis of the compensated displacement.

Immunostaining

Cells were fixed in 4% paraformaldehyde containing 0.15% picric acid in phosphate-buffered saline (PBS), saturated with 0.1 M glycine, permeabilized with 0.1% Triton X-100, saturated with 0.5% BSA in PBS (all from Sigma-Aldrich, St.Louis, MO) and then incubated for 1h with primary antibodies. The secondary antibodies were goat anti-rabbit 594 Alexa (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and anti-mouse IgG_{2a} biotynilated (Santa Cruz Biotechnology, Santa Cruz, CA) and the incubation time was 30 min. F-actin was marked with Alexa Fluor 488 phalloidin, whereas biotin was identified by Marina Blue-Streptavidin (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and incubated for 30 min. All the incubations were performed at room temperature (20-22°C). Cells were examined using a Leica DMIRE2 confocal microscope (Leica Microsystems GmbH, Germany) equipped with DIC and fluorescence optics, diode laser 405nm, Ar/ArKr 488nm and He/Ne 543/594nm lasers. The fluorescence images (1024x1024 pixels) were collected with a 63X magnification and 1.3 NA oil-immersion objective. Leica LCS Lite and Image J by W. Rasband (developed at the U.S. National Institutes of Health and available at http://rsbweb.nih.gov/ij/) were used for image processing.

Acknowledgments

We acknowledge the financial support of the following projects within the Seventh Framework Programme for Research of the European Commission: the FOCUS Project n. FP7-ICT-270483, the NEUROSCAFFOLDS Project n. 604263. We thank M. Lough for editing the

paper and Andrea Raffin for developing the LabViwe based algorithm for the working of Nanopositioner in feedback manner. The authors declare no competing financial interests.

References:

Agero, U. et al., 2003. Cell surface fluctuations studied with defocusing microscopy. *Physical Review E*, 67(5), p.051904. Available at: http://link.aps.org/doi/10.1103/PhysRevE.67.051904 [Accessed November 8, 2014].

Alexandrova, A.Y. et al., 2008. Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. *PloS one*, 3(9), p.e3234. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2535565&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Allingham, J.S., Smith, R. & Rayment, I., 2005. The structural basis of blebbistatin inhibition and specificity for myosin II. *Nature structural & molecular biology*, 12(4), pp.378–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15750603 [Accessed June 6, 2014].

Amann, K. & Pollard, T., 2001. Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proceedings of the National ...*, 98(26), pp.15009–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=64974&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2014].

Amin, L. et al., 2013. Comparison of the force exerted by hippocampal and DRG growth cones. *PloS one*, 8(8), p.e73025. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3749134&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Amin, L. et al., 2011. The elementary events underlying force generation in neuronal lamellipodia. *Scientific reports*, 1, p.153.

Amin, L. et al., 2012. The role of membrane stiffness and actin turnover on the force exerted by DRG lamellipodia. *Biophysical journal*, 102(11), pp.2451–60.

Bamburg, J.R., 1999. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annual review of cell and developmental biology*, 15, pp.185–230. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10611961 [Accessed December 20, 2014].

Bard, L. et al., 2008. A molecular clutch between the actin flow and N-cadherin adhesions drives growth cone migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(23), pp.5879–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18524892 [Accessed November 18, 2014].

Beckham, Y. et al., 2014. Arp2 / 3 Inhibition Induces Amoeboid-Like Protrusions in MCF10A Epithelial Cells by Reduced Cytoskeletal- Membrane Coupling and Focal Adhesion Assembly. , 9(6).

Betapudi, V., 2010. Myosin II motor proteins with different functions determine the fate of lamellipodia extension during cell spreading. *PloS one*, 5(1), p.e8560.

Betz, T. et al., 2011. Growth cones as soft and weak force generators. *Proceedings of the National Academy of Sciences of the United States of America*, 108(33), pp.13420–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3158236&tool=pmcentrez&rendertype=abstract [Accessed December 6, 2014].

Bohnet, S. et al., 2006. Weak force stalls protrusion at the leading edge of the lamellipodium. *Biophysical journal*, 90(5), pp.1810–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1367330&tool=pmcentrez&rendertype=abstract [Accessed November 27, 2014].

Borm, B. et al., 2005. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Experimental cell research*, 302(1), pp.83–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15541728 [Accessed September 9, 2014].

Boureux, A. et al., 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Molecular biology and evolution*, 24(1), pp.203–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2665304&tool=pmcentrez&rendertype=abstract [Accessed November 20, 2014].

Bray D, 2001. No Title. In *Cell Movements: From Molecules to Motility*. New York: Garland, pp. 3–60.

Bridgman, P. & Dave, S., 2001. Myosin IIB is required for growth cone motility. *The Journal of ...*, 21(16), pp.6159–6169. Available at: http://www.jneurosci.org/content/21/16/6159.short [Accessed December 17, 2014].

Bridgman, P.C., 1989. The organization of myosin and actin in rapid frozen nerve growth cones. *The Journal of Cell Biology*, 108(1), pp.95–109. Available at: http://www.jcb.org/cgi/doi/10.1083/jcb.108.1.95 [Accessed December 10, 2014].

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and P.W., 2002. Molecular Biology of the Cell. In pp. 973–975.

Bubb, M.R., 2000. Effects of Jasplakinolide on the Kinetics of Actin Polymerization. AN EXPLANATION FOR CERTAIN IN VIVO OBSERVATIONS. *Journal of Biological Chemistry*, 275(7), pp.5163–5170. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed October 20, 2014].

Burnette, D.T. et al., 2008. Myosin II activity facilitates microtubule bundling in the neuronal growth cone neck. *Developmental cell*, 15(1), pp.163–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2548298&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Bustamante, C., Macosko, J.C. & Wuite, G.J., 2000. Grabbing the cat by the tail: manipulating molecules one by one. *Nature reviews. Molecular cell biology*, 1(2), pp.130–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11253365.

Campellone, K.G. & Welch, M.D., 2010. A nucleator arms race: cellular control of actin assembly. *Nature reviews. Molecular cell biology*, 11(4), pp.237–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2929822&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Carlsson, a E., 2003. Growth velocities of branched actin networks. *Biophysical journal*, 84(5), pp.2907–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302854&tool=pmcentrez&rendertype=abstract.

Carlsson, A., 2001. Growth of branched actin networks against obstacles. *Biophysical journal*, 81(October). Available at: http://www.sciencedirect.com/science/article/pii/S0006349501758420 [Accessed December 18, 2014].

Cojoc, D. et al., 2007. Properties of the force exerted by filopodia and lamellipodia and the involvement of cytoskeletal components. *PloS one*, 2(10), p.e1072.

Conti, M. a. & Adelstein, R.S., 2008. Nonmuscle myosin II moves in new directions. *Journal of Cell Science*, 121(3), pp.404–404. Available at: http://jcs.biologists.org/cgi/doi/10.1242/jcs.03496 [Accessed August 26, 2014].

Conti, M.A. et al., 2004. Defects in cell adhesion and the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *The Journal of biological chemistry*, 279(40), pp.41263–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15292239 [Accessed December 17, 2014].

Cooper, J. a, 1987. Effects of cytochalasin and phalloidin on actin. *The Journal of cell biology*, 105(4), pp.1473–8.

Cooper, J.A. et al., 1983. Kinetic evidence for a monomer activation step in actin polymerization. *Biochemistry*, 22(9), pp.2193–2202. Available at: http://pubs.acs.org/doi/abs/10.1021/bi00278a021 [Accessed December 20, 2014].

Dang, I. et al., 2013. Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature*, 503(7475), pp.281–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24132237 [Accessed October 17, 2014].

Dasanayake, N.L., Michalski, P.J. & Carlsson, A.E., 2011. General Mechanism of Actomyosin Contractility. *Physical Review Letters*, 107(11), p.118101. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3226764&tool=pmcentrez&rendertype=abstract [Accessed September 18, 2014].

Dayel, M.J. & Mullins, R.D., 2004. Activation of Arp2/3 complex: addition of the first subunit of the new filament by a WASP protein triggers rapid ATP hydrolysis on Arp2. *PLoS biology*, 2(4), p.E91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=387265&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Dent, E. & Kalil, K., 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. *The Journal of Neuroscience*, 21(24), pp.9757–9769. Available at: http://www.jneurosci.org/content/21/24/9757.short [Accessed November 26, 2014].

Dent, E.W. & Gertler, F.B., 2003. Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance. *Neuron*, 40(2), pp.209–227. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627303006330.

Dent, E.W., Gupton, S.L. & Gertler, F.B., 2011. The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harbor perspectives in biology*, 3(3). Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3039926&tool=pmcentrez&rendertype=abstract.

Egile, C. et al., 2005. Mechanism of filament nucleation and branch stability revealed by the structure of the Arp2/3 complex at actin branch junctions. *PLoS biology*, 3(11), p.e383. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1278936&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Engle, E., 2010. Human genetic disorders of axon guidance. *Cold Spring Harbor perspectives in biology*, 2(3), p.a001784. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2829956&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Etienne-manneville, S., 2002. Rho GTPases in cell biology. , 420(December), pp.629–635.

Fass, J.N. & Odde, D.J., 2003. Tensile force-dependent neurite elicitation via anti-beta1 integrin antibody-coated magnetic beads. *Biophysical journal*, 85(1), pp.623–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1303117&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Forscher, P.S.S.J., 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *The Journal of Cell Biology*, 107(4), pp.1505–1516.

Friesland, A. et al., 2012. Small molecule targeting Cdc42 – intersectin interaction disrupts Golgi organization and suppresses cell motility. , pp.2–7.

Fujiwara, I. et al., 2002. Visualization and force measurement of branching by Arp2/3 complex and N-WASP in actin filament. *Biochemical and biophysical research communications*, 293(5), pp.1550–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12054693.

Gao, Y. et al., 2001. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. *The Journal of biological chemistry*, 276(50), pp.47530–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11595749 [Accessed December 3, 2014].

Ghashghaei, H.T., Lai, C. & Anton, E.S., 2007. Neuronal migration in the adult brain: are we there yet? *Nature reviews. Neuroscience*, 8(2), pp.141–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17237805 [Accessed June 4, 2014].

Gittes, F. et al., 1993. Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape. *The Journal of cell biology*, 120(4), pp.923–34.

Gomez, T.M. & Letourneau, P.C., 2014. Actin dynamics in growth cone motility and navigation. *Journal of neurochemistry*, 129(2), pp.221–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24164353 [Accessed November 10, 2014].

Goodman, C., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–377.

Goodman, C.S., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8833447 [Accessed December 10, 2014].

Hall, A., 2012. Rho family GTPases. *Biochemical Society transactions*, 40(6), pp.1378–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23176484 [Accessed October 10, 2014].

Heidemann, S.R., 1990. Growth cone behavior and production of traction force. *The Journal of Cell Biology*, 111(5), pp.1949–1957. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2116337/ [Accessed December 14, 2014].

Hinz, B. et al., 1999. Quantifying lamella dynamics of cultured cells by SACED, a new computer-assisted motion analysis. *Experimental cell research*, 251(1), pp.234–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10438589.

Howard, J., 2001. Mechanics of motor proteins and the cytoskeleton.

Huber, A.B. et al., 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual review of neuroscience*, 26, pp.509–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12677003 [Accessed December 17, 2014].

Hur, E.-M. et al., 2011. Engineering neuronal growth cones to promote axon regeneration over inhibitory molecules. *Proceedings of the National Academy of Sciences of the United States of America*, 108(12), pp.5057–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3064397&tool=pmcentrez&rendertype=abstract [Accessed May 28, 2014].

Ilić, D. et al., 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, 377(6549), pp.539–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7566154 [Accessed November 1, 2014].

Jacquemet, G. et al., 2013. Rac1 is deactivated at integrin activation sites through an IQGAP1-filamin-A-RacGAP1 pathway. *Journal of cell science*, 126(Pt 18), pp.4121–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3772387&tool=pmcentrez&rendertype=abstract [Accessed October 7, 2014].

Ketschek, A., Jones, S. & Gallo, G., 2007. Axon extension in the fast and slow lanes: Substratum□dependent engagement of myosin II functions. *Developmental neurobiology*, 67(10), pp.1305–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2674262&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Kim, T., 2014. Determinants of contractile forces generated in disorganized actomyosin bundles. *Biomechanics and modeling in mechanobiology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25103419 [Accessed November 26, 2014].

Korobova, F. & Svitkina, T., 2008. Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells. *Molecular biology of the cell*, 19(4), pp.1561–74. Available at: http://www.molbiolcell.org/content/19/4/1561.short [Accessed December 17, 2014].

Kovács, M. et al., 2004. Mechanism of blebbistatin inhibition of myosin II. *The Journal of biological chemistry*, 279(34), pp.35557–63.

Kozma, R., Ahmed, S. & Best, A., 1995. The Ras-Related Protein Cdc42Hs and Bradykinin Promote Formation of Peripheral Actin Microspikes and Filopodia in Swiss 3T3 Fibroblasts. , 15(4), pp.1942–1952.

Krotkov, E., 1988. Focusing. *International Journal of Computer Vision*, 1(3), pp.223–237. Available at: http://link.springer.com/10.1007/BF00127822 [Accessed November 26, 2014].

Kubo, T. et al., 2008. Myosin IIA is required for neurite outgrowth inhibition produced by repulsive guidance molecule. *Journal of neurochemistry*, 105(1), pp.113–26.

Lee, C.-S. et al., 2010. Myosin II directly binds and inhibits Dbl family guanine nucleotide exchange factors: a possible link to Rho family GTPases. *The Journal of cell biology*, 190(4), pp.663–74. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2928003&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Lenz, M., Gardel, M.L. & Dinner, A.R., 2012. Requirements for contractility in disordered cytoskeletal bundles. *New journal of physics*, 14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3496381&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2014].

LIN, C. et al., 1996. Myosin Drives Retrograde F-Actin Flow in Neuronal Growth Cones. *Neuron*, 16(4), pp.769–782. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627300800975 [Accessed December 20, 2014].

López-Muñoz, F., Boya, J. & Alamo, C., 2006. Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain research bulletin*, 70(4-6), pp.391–405. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17027775 [Accessed October 1, 2014].

Lowery, L.A. & Van Vactor, D., 2009. The trip of the tip: understanding the growth cone machinery. *Nature reviews. Molecular cell biology*, 10(5), pp.332–43. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714171&tool=pmcentrez&rendertype=abstract [Accessed September 3, 2014].

Marsick, B., 2010. Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin□1. *Developmental …*, 70(8), pp.565–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2908028&tool=pmcentrez&rendertype=abstract [Accessed December 15, 2014].

Marthon, P., Thiesse, B. & Bruel, A., 1986. No Title. In O. D. Faugeras & R. B. Kelley, eds. *Proceedings SPIE 0595*. pp. 318–327. Available at: http://proceedings.spiedigitallibrary.org/proceeding.aspx?articleid=1241265 [Accessed September 21, 2014].

Marthon, Ph.; Thiesse, B.;Bruel, A., 1986. Edge Detection By Differences Of Gaussians. In *Proceedings SPIE 0595*. pp. 318–327. Available at: http://dx.doi.org/10.1117/12.952277.

Meberg, P., 2000. Signal-regulated ADF/cofilin activity and growth cone motility. *Molecular neurobiology*, 21, pp.97–107. Available at: http://link.springer.com/article/10.1385/MN:21:1-2:097 [Accessed December 15, 2014].

Medeiros, N. a, Burnette, D.T. & Forscher, P., 2006. Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nature cell biology*, 8(3), pp.215–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16501565 [Accessed June 5, 2014].

Merrifield, C. & Qualmann, B., 2004. Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. *European journal of cell …*, 83(1), pp.13–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15085951 [Accessed December 17, 2014].

Millard, T.H., Sharp, S.J. & Machesky, L.M., 2004. Signalling to actin assembly via the WASP (Wiskott – Aldrich syndrome protein) -family proteins and the Arp2 / 3 complex. , 17, pp.1–17.

Mitchison, T. & Kirschner, M., 1988. Cytoskeletal dynamics and nerve growth. *Neuron*, 1(9), pp.761–772. Available at: http://linkinghub.elsevier.com/retrieve/pii/0896627388901249 [Accessed December 11, 2014].

Mogilner, a & Oster, G., 1996. Cell motility driven by actin polymerization. *Biophysical journal*, 71(6), pp.3030–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1233792&tool=pmcentrez&rendertype=abstract [Accessed May 27, 2014].

Mogilner, A., 2009. Mathematics of cell motility: have we got its number? *Journal of mathematical biology*, 58(1-2), pp.105–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2862828&tool=pmcentrez&rendertype=abstract [Accessed December 19, 2014].

Mogilner, A. & Oster, G., 2003. Force generation by actin polymerization II: the elastic ratchet and tethered filaments. *Biophysical journal*, 84(3), pp.1591–605. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302730&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Mongiu, A.K. et al., 2007. Kinetic-structural analysis of neuronal growth cone veil motility. *Journal of cell science*, 120(Pt 6), pp.1113–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17327278 [Accessed May 29, 2014].

Montalvo-Ortiz, B.L. et al., 2012. Characterization of EHop-016, novel small molecule inhibitor of Rac GTPase. *The Journal of biological chemistry*, 287(16), pp.13228–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3339933&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Mullins, R.D., Heuser, J.A. & Pollard, T.D., 1998. The interaction of Arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), pp.6181–6186. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC27619/.

Neuman, K.C. & Block, S.M., 2004. Optical trapping. *The Review of scientific instruments*, 75(9), pp.2787–809.

Nimnual, A.S., Taylor, L.J. & Bar-Sagi, D., 2003. Redox-dependent downregulation of Rho by Rac. *Nature cell biology*, 5(3), pp.236–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12598902 [Accessed November 9, 2014].

Nolen, B.J. & Pollard, T.D., 2007. NIH Public Access. , 26(3), pp.449–457.

Pak, C.W., Flynn, K.C. & Bamburg, J.R., 2008. Actin-binding proteins take the reins in growth cones. *Nature reviews. Neuroscience*, 9(2), pp.136–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18209731 [Accessed June 3, 2014].

Pasapera, A.M. et al., 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *The Journal of cell biology*, 188(6), pp.877–90.

Peskin, C., Odell, G. & Oster, G., 1993. Cellular motions and thermal fluctuations: the Brownian ratchet. *Biophysical journal*, 65(1), pp.316–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1225726&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Pollard, T., 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annual review of ...*, 29, pp.545–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10940259 [Accessed December 15, 2014].

Pollard, T.D., 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annual review of biophysics and biomolecular structure*, 36, pp.451–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17477841 [Accessed July 10, 2014].

Pollard, T.D. & Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, 112(4), pp.453–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12600310.

Prass, M. et al., 2006. Direct measurement of the lamellipodial protrusive force in a migrating cell. *The Journal of cell biology*, 174(6), pp.767–72. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064331&tool=pmcentrez&rendertype=abstract [Accessed October 26, 2014].

Raucher, D. & Sheetz, M.P., 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *The Journal of cell biology*, 148(1), pp.127–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2156205&tool=pmcentrez&rendertype=abstract.

Ridley, A.J., 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology*, 16(10), pp.522–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16949823 [Accessed September 10, 2014].

Roh-Johnson, M. et al., 2012. Triggering a cell shape change by exploiting preexisting actomyosin contractions. *Science (New York, N.Y.)*, 335(6073), pp.1232–5.

Rosenfeldt, H. et al., 2006. Rac inhibits thrombin-induced Rho activation: evidence of a Pak-dependent GTPase crosstalk. *Journal of molecular signaling*, 1, p.8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1769496&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Rösner, H. et al., 2007. Attenuation of actinomyosinII contractile activity in growth cones accelerates filopodia-guided and microtubule-based neurite elongation. *Brain research*, 1176, pp.1–10.

Rouiller, I. et al., 2008. The structural basis of actin filament branching by the Arp2/3 complex. *The Journal of cell biology*, 180(5), pp.887–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2265399&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Rozelle, A. & Machesky, L., 2000. Phosphatidylinositol 4, 5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Current Biology*, 10(6), pp.311–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10744973 [Accessed December 17, 2014].

Saunders, R.M. et al., 2006. Role of vinculin in regulating focal adhesion turnover. *European journal of cell biology*, 85(6), pp.487–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16584805 [Accessed November 1, 2014].

Schaefer, A.W., Kabir, N. & Forscher, P., 2002. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *The Journal of cell biology*, 158(1), pp.139–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173029&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Schaus, T.E., Taylor, E.W. & Borisy, G.G., 2007. Self-organization of actin filament orientation in the dendritic-nucleation/array-treadmilling model. *Proceedings of the National Academy of Sciences of the United States of America*, 104(17), pp.7086–91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1855413&tool=pmcentrez&rendertype=abstract.

Schnelzer, A., Bokoch, G.M. & Jolla, L., 2004. Phosphorylation of RhoGDI by Pak1 Mediates Dissociation of Rac GTPase. , 15, pp.117–127.

Schwartz, M., 2004. Rho signalling at a glance. *Journal of cell science*, 117(Pt 23), pp.5457–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15509861 [Accessed November 10, 2014].

Shahapure, R. et al., 2010. Force generation in lamellipodia is a probabilistic process with fast growth and retraction events. *Biophysical journal*, 98(6), pp.979–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2849058&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Shutova, M. et al., 2012. Functions of nonmuscle myosin II in assembly of the cellular contractile system. *PloS one*, 7(7), p.e40814. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3396643&tool=pmcentrez&rendertype=abstract [Accessed June 2, 2014].

Small, J.V. et al., 2002. The lamellipodium: where motility begins. *Trends in Cell Biology*, 12(3), pp.112–120. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0962892401022371.

Solecki, D., Trivedi, N. & Govek, E., 2009. Myosin II motors and F-actin dynamics drive the coordinated movement of the centrosome and soma during CNS glial-guided neuronal migration. *Neuron*, 63(1), pp.63–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2737100&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Solecki, D.J., Govek, E.-E. & Hatten, M.E., 2006. mPar6 alpha controls neuronal migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(42), pp.10624–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17050699 [Accessed June 13, 2014].

Song, H. & Poo, M., 2001. The cell biology of neuronal navigation. *Nature cell biology*, 3(3), pp.E81–8. Available at: http://www.nature.com/ncb/journal/v3/n3/abs/ncb0301_e81.html [Accessed June 13, 2014].

Stavenger, R. a et al., 2007. Discovery of aminofurazan-azabenzimidazoles as inhibitors of Rho-kinase with high kinase selectivity and antihypertensive activity. *Journal of medicinal chemistry*, 50(1), pp.2–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17201404.

Strasser, G. a et al., 2004. Arp2/3 is a negative regulator of growth cone translocation. *Neuron*, 43(1), pp.81–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15233919.

Suraneni, P. et al., 2012. The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *The Journal of cell biology*, 197(2), pp.239–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3328382&tool=pmcentrez&rendertype=abstract [Accessed September 22, 2014].

Suter, D.M. & Forschert, P., 1998. An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. , 3.

Svitkina, T.M. et al., 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *The Journal of cell biology*, 160(3), pp.409–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172658&tool=pmcentrez&rendertype=abstract [Accessed November 19, 2014].

Thievessen, I. et al., 2013. Vinculin-actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth. *The Journal of cell biology*, 202(1), pp.163–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3704983&tool=pmcentrez&rendertype=abstract [Accessed September 16, 2014].

Torka, R. et al., 2006. ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. *Experimental cell research*, 312(19), pp.3857–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17010335 [Accessed November 26, 2014].

Trivedi, N. & Solecki, D.J., 2011. Neuronal migration illuminated: A look under the hood of the living neuron. *Cell Adhesion & Migration*, 5(1), pp.42–47. Available at: http://www.landesbioscience.com/journals/celladhesion/article/13609/ [Accessed June 13, 2014].

Ulrich, T. a et al., 2010. Probing cellular mechanobiology in three-dimensional culture with collagen-agarose matrices. *Biomaterials*, 31(7), pp.1875–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19926126 [Accessed September 9, 2014].

Vicente-Manzanares, M. et al., 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature reviews. Molecular cell biology*, 10(11), pp.778–90. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2834236&tool=pmcentrez&rendertype=abstract [Accessed May 26, 2014].

Vitriol, E. a & Zheng, J.Q., 2012. Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. *Neuron*, 73(6), pp.1068–81. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3319373&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Wickstead, B. & Gull, K., 2011. The evolution of the cytoskeleton. *The Journal of cell biology*, 194(4), pp.513–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3160578&tool=pmcentrez&rendertype=abstract [Accessed July 15, 2014].

Woo, S. & Gomez, T.M., 2006. Rac1 and RhoA promote neurite outgrowth through formation and stabilization of growth cone point contacts. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(5), pp.1418–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16452665 [Accessed December 9, 2014].

Wu, C. et al., 2012. Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell*, 148(5), pp.973–87. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3707508&tool=pmcentrez&rendertype=abstract [Accessed July 23, 2014].

Wylie, S.R. & Chantler, P.D., 2008. Myosin IIC: a third molecular motor driving neuronal dynamics. *Molecular biology of the cell*, 19(9), pp.3956–68. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed November 26, 2014].

Wylie, S.R. & Chantler, P.D., 2001. Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nature cell biology*, 3(1), pp.88–92.

Yang, Q. et al., 2012. Arp2/3 complex-dependent actin networks constrain myosin II function in driving retrograde actin flow. *The Journal of cell biology*, 197(7), pp.939–56. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3384413&tool=pmcentrez&rendertype=abstract [Accessed October 15, 2014].

Yu, P. et al., 2012. Myosin II activity regulates neurite outgrowth and guidance in response to chondroitin sulfate proteoglycans. *Journal of neurochemistry*, 120(6), pp.1117–28. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3296867&tool=pmcentrez&rendertype=abstract [Accessed May 30, 2014].

Zhang, W. et al., 2012. Self-Assembling Peptide Nanofiber Scaffold Enhanced with RhoA Inhibitor CT04 Improves Axonal Regrowth in the Transected Spinal Cord. *Journal of Nanomaterials*, 2012, pp.1–10. Available at: http://www.hindawi.com/journals/jnm/2012/724857/ [Accessed November 26, 2014].

Zhang, X. et al., 2008. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nature cell biology*, 10(9), pp.1062–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2746969&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Chapter 3

Discussion

The elaborate wiring of the human nervous system is generated during fetal development by the motile behavior of nerve cells, through GCs. The main source of motility in the GCs is the process of actin polymerization which is regulated by several proteins. Therefore to study the dynamic of GC it is very essential to know the specific roles of these proteins in functioning of GCs. The development of optical and force microscopy techniques has significantly advanced our quantitative understanding of cell motility at the level of single molecules, collections of molecules, and whole cells. Along with it, time-lapse video microscopy produced early measurements of GCs movement and internal dynamics. This dissertation provides data on the role of Myosin II and Arp2/3 involved in force generation and motility of DRG growth cone. Using optical tweezers, we precisely measured the force generated by DRG lamellipodia and filopodia with high temporal resolution and picoNewton force sensitivity without causing any photo damage. With the help of Z stack phase contrast video imaging we could followed the complex 3D motion of lamellipodia.

The first chapter addresses the role of myosin-II in force generation of DRG filopodia and lamellipodia. Immunostaining experiments have shown that Myosin II isoform NMIIB is primarily localized in the central domain of GC and NMII A was present at the GCs leading edge. We found that, when Myosin II was inhibited by Blebbistatin lamellipodia lost their sheet-like structure and became ‘filopodish’ and they were not able to lift up during retraction. Moreover, higher proportion of microtubules inside filopodia was also found upon Bebbistatin treatment possibly due to removal of the crosslinkage of actin and NMIIB filaments at the transition region of the GCs. In addition to these results, Myosin II inhibition decreased the force exerted by lamellipodia by 30-50% compared to control conditions. However, an equal force exerted by the filopodia was found to be increased when Myosin II was inhibited. Morphological changes occurred due to inhibition of myosin could explain the force exertion by lamellipodia and filopodia. In particular, the intrusion of microtubules increased the stiffness of filopodia and thereby increased the force exerted by them. This conclusion was supported by the experiment that the force exerted by the filopodia did not change when Myosin II and microtubules

polymerization were inhibited simultaneously. These results suggest important role of myosin II in force generation of lamellipodia and filopodia and confirmed a coupling between actin and MT dynamics in the transition region of GCs.

The next chapter presents the data illustrating that Rac1 activates upon inhibition of Arp2/3 possibly through integrin pathways and that Arp2/3 not only controls the formation but also the dynamics of filopodia. We found that lamellipodia show transient retraction and recovery after inhibition of Arp2/3 and this recovery was abolished by simultaneous inhibition of Rac1 indicating that Rac1 activates upon Arp2/3 inhibition. Moreover, we also discovered that the length of filopodia increased in both Arp2/3 and Rac1 inhibition states but only those filopodia in the Arp2/3 inhibited condition exerted less force compared to the control conditions. By contrast, filopodia in Rac1 inhibition condition did not show any change in the force. These results point out that Arp2/3 directly controls the dynamics of filopodia while Rac1 may be involved indirectly through cross talk with CDC42 pathways.

In summary, this dissertation reports that, inhibition of Myosin II disrupts the structure and function of GCs - especially the microtubules generally constrained in the C region – and invade the P region of the GCs. Moreover, inhibition of Arp2/3 shows the transient retraction and recovery of lamellipodia due to the activation of Rac1 and decreases the force exerted by filopodia. Recently, It was reported that the outcome of Arp/2/3 inhibition strongly depends on preexisting levels of NM II contractile activity (Yang et al. 2012). Therefore, in the future it will be very interesting to study the functional interactions between them by localized activation or inhibition of one and probing the other.

In addition, there is a large number of regulatory proteins and signaling pathways controlling the dynamics of GCs and they have the potential to affect axonal regeneration and neurites outgrowth (Dent et al. 2011). Manipulating and altering the activities of these proteins under physiological conditions for proper functioning of GC may help improve therapeutic approaches for neurodegenerative diseases.

References:

Agero, U. et al., 2003. Cell surface fluctuations studied with defocusing microscopy. *Physical Review E*, 67(5), p.051904. Available at: http://link.aps.org/doi/10.1103/PhysRevE.67.051904 [Accessed November 8, 2014].

Alexandrova, A.Y. et al., 2008. Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. *PloS one*, 3(9), p.e3234. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2535565&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Allingham, J.S., Smith, R. & Rayment, I., 2005. The structural basis of blebbistatin inhibition and specificity for myosin II. *Nature structural & molecular biology*, 12(4), pp.378–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15750603 [Accessed June 6, 2014].

Amann, K. & Pollard, T., 2001. Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. *Proceedings of the National …*, 98(26), pp.15009–13. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=64974&tool=pmcentrez&rendertype=abstract [Accessed December 17, 2014].

Amin, L. et al., 2013. Comparison of the force exerted by hippocampal and DRG growth cones. *PloS one*, 8(8), p.e73025. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3749134&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Amin, L. et al., 2011. The elementary events underlying force generation in neuronal lamellipodia. *Scientific reports*, 1, p.153.

Amin, L. et al., 2012. The role of membrane stiffness and actin turnover on the force exerted by DRG lamellipodia. *Biophysical journal*, 102(11), pp.2451–60.

Bamburg, J.R., 1999. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annual review of cell and developmental biology*, 15, pp.185–230. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10611961 [Accessed December 20, 2014].

Bard, L. et al., 2008. A molecular clutch between the actin flow and N-cadherin adhesions drives growth cone migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(23), pp.5879–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18524892 [Accessed November 18, 2014].

Beckham, Y. et al., 2014. Arp2 / 3 Inhibition Induces Amoeboid-Like Protrusions in MCF10A Epithelial Cells by Reduced Cytoskeletal- Membrane Coupling and Focal Adhesion Assembly. , 9(6).

Betapudi, V., 2010. Myosin II motor proteins with different functions determine the fate of lamellipodia extension during cell spreading. *PloS one*, 5(1), p.e8560.

Betz, T. et al., 2011. Growth cones as soft and weak force generators. *Proceedings of the National Academy of Sciences of the United States of America*, 108(33), pp.13420–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3158236&tool=pmcentrez&rendertype=abstract [Accessed December 6, 2014].

Bohnet, S. et al., 2006. Weak force stalls protrusion at the leading edge of the lamellipodium. *Biophysical journal*, 90(5), pp.1810–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1367330&tool=pmcentrez&rendertype=abstract [Accessed November 27, 2014].

Borm, B. et al., 2005. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Experimental cell research*, 302(1), pp.83–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15541728 [Accessed September 9, 2014].

Boureux, A. et al., 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Molecular biology and evolution*, 24(1), pp.203–16. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2665304&tool=pmcentrez&rendertype=abstract [Accessed November 20, 2014].

Bray D, 2001. No Title. In *Cell Movements: From Molecules to Motility*. New York: Garland, pp. 3–60.

Bridgman, P. & Dave, S., 2001. Myosin IIB is required for growth cone motility. *The Journal of ...*, 21(16), pp.6159–6169. Available at: http://www.jneurosci.org/content/21/16/6159.short [Accessed December 17, 2014].

Bridgman, P.C., 1989. The organization of myosin and actin in rapid frozen nerve growth cones. *The Journal of Cell Biology*, 108(1), pp.95–109. Available at: http://www.jcb.org/cgi/doi/10.1083/jcb.108.1.95 [Accessed December 10, 2014].

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and P.W., 2002. Molecular Biology of the Cell. In pp. 973–975.

Bubb, M.R., 2000. Effects of Jasplakinolide on the Kinetics of Actin Polymerization. AN EXPLANATION FOR CERTAIN IN VIVO OBSERVATIONS. *Journal of Biological Chemistry*, 275(7), pp.5163–5170. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed October 20, 2014].

Burnette, D.T. et al., 2008. Myosin II activity facilitates microtubule bundling in the neuronal growth cone neck. *Developmental cell*, 15(1), pp.163–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2548298&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Bustamante, C., Macosko, J.C. & Wuite, G.J., 2000. Grabbing the cat by the tail: manipulating molecules one by one. *Nature reviews. Molecular cell biology*, 1(2), pp.130–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11253365.

Campellone, K.G. & Welch, M.D., 2010. A nucleator arms race: cellular control of actin assembly. *Nature reviews. Molecular cell biology*, 11(4), pp.237–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2929822&tool=pmcentrez&rendertype=abstract [Accessed July 9, 2014].

Carlsson, a E., 2003. Growth velocities of branched actin networks. *Biophysical journal*, 84(5), pp.2907–18. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302854&tool=pmcentrez&rendertype=abstract.

Carlsson, A., 2001. Growth of branched actin networks against obstacles. *Biophysical journal*, 81(October). Available at: http://www.sciencedirect.com/science/article/pii/S0006349501758420 [Accessed December 18, 2014].

Cojoc, D. et al., 2007. Properties of the force exerted by filopodia and lamellipodia and the involvement of cytoskeletal components. *PloS one*, 2(10), p.e1072.

Conti, M. a. & Adelstein, R.S., 2008. Nonmuscle myosin II moves in new directions. *Journal of Cell Science*, 121(3), pp.404–404. Available at: http://jcs.biologists.org/cgi/doi/10.1242/jcs.03496 [Accessed August 26, 2014].

Conti, M.A. et al., 2004. Defects in cell adhesion and the visceral endoderm following ablation of nonmuscle myosin heavy chain II-A in mice. *The Journal of biological chemistry*, 279(40), pp.41263–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15292239 [Accessed December 17, 2014].

Cooper, J. a, 1987. Effects of cytochalasin and phalloidin on actin. *The Journal of cell biology*, 105(4), pp.1473–8.

Cooper, J.A. et al., 1983. Kinetic evidence for a monomer activation step in actin polymerization. *Biochemistry*, 22(9), pp.2193–2202. Available at: http://pubs.acs.org/doi/abs/10.1021/bi00278a021 [Accessed December 20, 2014].

Dang, I. et al., 2013. Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature*, 503(7475), pp.281–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24132237 [Accessed October 17, 2014].

Dasanayake, N.L., Michalski, P.J. & Carlsson, A.E., 2011. General Mechanism of Actomyosin Contractility. *Physical Review Letters*, 107(11), p.118101. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3226764&tool=pmcentrez&rendertype=abstract [Accessed September 18, 2014].

Dayel, M.J. & Mullins, R.D., 2004. Activation of Arp2/3 complex: addition of the first subunit of the new filament by a WASP protein triggers rapid ATP hydrolysis on Arp2. *PLoS biology*, 2(4), p.E91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=387265&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Dent, E. & Kalil, K., 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. *The Journal of Neuroscience*, 21(24), pp.9757–9769. Available at: http://www.jneurosci.org/content/21/24/9757.short [Accessed November 26, 2014].

Dent, E.W. & Gertler, F.B., 2003. Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance. *Neuron*, 40(2), pp.209–227. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627303006330.

Dent, E.W., Gupton, S.L. & Gertler, F.B., 2011. The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harbor perspectives in biology*, 3(3). Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3039926&tool=pmcentrez&rendertype=abstract.

Egile, C. et al., 2005. Mechanism of filament nucleation and branch stability revealed by the structure of the Arp2/3 complex at actin branch junctions. *PLoS biology*, 3(11), p.e383. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1278936&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Engle, E., 2010. Human genetic disorders of axon guidance. *Cold Spring Harbor perspectives in biology*, 2(3), p.a001784. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2829956&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Etienne-manneville, S., 2002. Rho GTPases in cell biology. , 420(December), pp.629–635.

Fass, J.N. & Odde, D.J., 2003. Tensile force-dependent neurite elicitation via anti-beta1 integrin antibody-coated magnetic beads. *Biophysical journal*, 85(1), pp.623–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1303117&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Forscher, P.S.S.J., 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *The Journal of Cell Biology*, 107(4), pp.1505–1516.

Friesland, A. et al., 2012. Small molecule targeting Cdc42 – intersectin interaction disrupts Golgi organization and suppresses cell motility. , pp.2–7.

Fujiwara, I. et al., 2002. Visualization and force measurement of branching by Arp2/3 complex and N-WASP in actin filament. *Biochemical and biophysical research*

communications, 293(5), pp.1550–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12054693.

Gao, Y. et al., 2001. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. *The Journal of biological chemistry*, 276(50), pp.47530–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11595749 [Accessed December 3, 2014].

Ghashghaei, H.T., Lai, C. & Anton, E.S., 2007. Neuronal migration in the adult brain: are we there yet? *Nature reviews. Neuroscience*, 8(2), pp.141–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17237805 [Accessed June 4, 2014].

Gittes, F. et al., 1993. Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape. *The Journal of cell biology*, 120(4), pp.923–34.

Gomez, T.M. & Letourneau, P.C., 2014. Actin dynamics in growth cone motility and navigation. *Journal of neurochemistry*, 129(2), pp.221–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24164353 [Accessed November 10, 2014].

Goodman, C., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–377.

Goodman, C.S., 1996. Mechanisms and molecules that control growth cone guidance. *Annual review of neuroscience*, 19, pp.341–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8833447 [Accessed December 10, 2014].

Hall, A., 2012. Rho family GTPases. *Biochemical Society transactions*, 40(6), pp.1378–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23176484 [Accessed October 10, 2014].

Heidemann, S.R., 1990. Growth cone behavior and production of traction force. *The Journal of Cell Biology*, 111(5), pp.1949–1957. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2116337/ [Accessed December 14, 2014].

Hinz, B. et al., 1999. Quantifying lamella dynamics of cultured cells by SACED, a new computer-assisted motion analysis. *Experimental cell research*, 251(1), pp.234–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10438589.

Howard, J., 2001. Mechanics of motor proteins and the cytoskeleton.

Huber, A.B. et al., 2003. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual review of neuroscience*, 26, pp.509–63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12677003 [Accessed December 17, 2014].

Hur, E.-M. et al., 2011. Engineering neuronal growth cones to promote axon regeneration over inhibitory molecules. *Proceedings of the National Academy of Sciences of the United States of America*, 108(12), pp.5057–62. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3064397&tool=pmcentrez&rendertype=abstract [Accessed May 28, 2014].

Ilić, D. et al., 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, 377(6549), pp.539–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7566154 [Accessed November 1, 2014].

Jacquemet, G. et al., 2013. Rac1 is deactivated at integrin activation sites through an IQGAP1-filamin-A-RacGAP1 pathway. *Journal of cell science*, 126(Pt 18), pp.4121–35. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3772387&tool=pmcentrez&rendertype=abstract [Accessed October 7, 2014].

Ketschek, A., Jones, S. & Gallo, G., 2007. Axon extension in the fast and slow lanes: Substratum-dependent engagement of myosin II functions. *Developmental neurobiology*, 67(10), pp.1305–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2674262&tool=pmcentrez&rendertype=abstract [Accessed December 14, 2014].

Kim, T., 2014. Determinants of contractile forces generated in disorganized actomyosin bundles. *Biomechanics and modeling in mechanobiology*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25103419 [Accessed November 26, 2014].

Korobova, F. & Svitkina, T., 2008. Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells. *Molecular biology of the cell*, 19(4), pp.1561–74. Available at: http://www.molbiolcell.org/content/19/4/1561.short [Accessed December 17, 2014].

Kovács, M. et al., 2004. Mechanism of blebbistatin inhibition of myosin II. *The Journal of biological chemistry*, 279(34), pp.35557–63.

Kozma, R., Ahmed, S. & Best, A., 1995. The Ras-Related Protein Cdc42Hs and Bradykinin Promote Formation of Peripheral Actin Microspikes and Filopodia in Swiss 3T3 Fibroblasts. , 15(4), pp.1942–1952.

Krotkov, E., 1988. Focusing. *International Journal of Computer Vision*, 1(3), pp.223–237. Available at: http://link.springer.com/10.1007/BF00127822 [Accessed November 26, 2014].

Kubo, T. et al., 2008. Myosin IIA is required for neurite outgrowth inhibition produced by repulsive guidance molecule. *Journal of neurochemistry*, 105(1), pp.113–26.

Lee, C.-S. et al., 2010. Myosin II directly binds and inhibits Dbl family guanine nucleotide exchange factors: a possible link to Rho family GTPases. *The Journal of cell biology*, 190(4), pp.663–74. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2928003&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Lenz, M., Gardel, M.L. & Dinner, A.R., 2012. Requirements for contractility in disordered cytoskeletal bundles. *New journal of physics*, 14. Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3496381&tool=pmcentrez&rendertype=abstract [Accessed August 28, 2014].

LIN, C. et al., 1996. Myosin Drives Retrograde F-Actin Flow in Neuronal Growth Cones. *Neuron*, 16(4), pp.769–782. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0896627300800975 [Accessed December 20, 2014].

López-Muñoz, F., Boya, J. & Alamo, C., 2006. Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain research bulletin*, 70(4-6), pp.391–405. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17027775 [Accessed October 1, 2014].

Lowery, L.A. & Van Vactor, D., 2009. The trip of the tip: understanding the growth cone machinery. *Nature reviews. Molecular cell biology*, 10(5), pp.332–43. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714171&tool=pmcentrez&rendertype=abstract [Accessed September 3, 2014].

Marsick, B., 2010. Activation of ADF/cofilin mediates attractive growth cone turning toward nerve growth factor and netrin-1. *Developmental ...*, 70(8), pp.565–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2908028&tool=pmcentrez&rendertype=abstract [Accessed December 15, 2014].

Marthon, P., Thiesse, B. & Bruel, A., 1986. No Title. In O. D. Faugeras & R. B. Kelley, eds. *Proceedings SPIE 0595*. pp. 318–327. Available at: http://proceedings.spiedigitallibrary.org/proceeding.aspx?articleid=1241265 [Accessed September 21, 2014].

Marthon, Ph.; Thiesse, B.;Bruel, A., 1986. Edge Detection By Differences Of Gaussians. In *Proceedings SPIE 0595*. pp. 318–327. Available at: http://dx.doi.org/10.1117/12.952277.

Meberg, P., 2000. Signal-regulated ADF/cofilin activity and growth cone motility. *Molecular neurobiology*, 21, pp.97–107. Available at: http://link.springer.com/article/10.1385/MN:21:1-2:097 [Accessed December 15, 2014].

Medeiros, N. a, Burnette, D.T. & Forscher, P., 2006. Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nature cell biology*, 8(3), pp.215–26. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16501565 [Accessed June 5, 2014].

Merrifield, C. & Qualmann, B., 2004. Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 complex are recruited to sites of clathrin-mediated endocytosis in cultured fibroblasts. *European journal of cell ...*, 83(1), pp.13–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15085951 [Accessed December 17, 2014].

Millard, T.H., Sharp, S.J. & Machesky, L.M., 2004. Signalling to actin assembly via the WASP (Wiskott – Aldrich syndrome protein) -family proteins and the Arp2 / 3 complex. , 17, pp.1–17.

Mitchison, T. & Kirschner, M., 1988. Cytoskeletal dynamics and nerve growth. *Neuron*, 1(9), pp.761–772. Available at: http://linkinghub.elsevier.com/retrieve/pii/0896627388901249 [Accessed December 11, 2014].

Mogilner, a & Oster, G., 1996. Cell motility driven by actin polymerization. *Biophysical journal*, 71(6), pp.3030–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1233792&tool=pmcentrez&rendertype=abstract [Accessed May 27, 2014].

Mogilner, A., 2009. Mathematics of cell motility: have we got its number? *Journal of mathematical biology*, 58(1-2), pp.105–34. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2862828&tool=pmcentrez&rendertype=abstract [Accessed December 19, 2014].

Mogilner, A. & Oster, G., 2003. Force generation by actin polymerization II: the elastic ratchet and tethered filaments. *Biophysical journal*, 84(3), pp.1591–605. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1302730&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Mongiu, A.K. et al., 2007. Kinetic-structural analysis of neuronal growth cone veil motility. *Journal of cell science*, 120(Pt 6), pp.1113–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17327278 [Accessed May 29, 2014].

Montalvo-Ortiz, B.L. et al., 2012. Characterization of EHop-016, novel small molecule inhibitor of Rac GTPase. *The Journal of biological chemistry*, 287(16), pp.13228–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3339933&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Mullins, R.D., Heuser, J.A. & Pollard, T.D., 1998. The interaction of Arp2/3 complex with actin: Nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), pp.6181–6186. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC27619/.

Neuman, K.C. & Block, S.M., 2004. Optical trapping. *The Review of scientific instruments*, 75(9), pp.2787–809.

Nimnual, A.S., Taylor, L.J. & Bar-Sagi, D., 2003. Redox-dependent downregulation of Rho by Rac. *Nature cell biology*, 5(3), pp.236–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12598902 [Accessed November 9, 2014].

Nolen, B.J. & Pollard, T.D., 2007. NIH Public Access. , 26(3), pp.449–457.

Pak, C.W., Flynn, K.C. & Bamburg, J.R., 2008. Actin-binding proteins take the reins in growth cones. *Nature reviews. Neuroscience*, 9(2), pp.136–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18209731 [Accessed June 3, 2014].

Pasapera, A.M. et al., 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *The Journal of cell biology*, 188(6), pp.877–90.

Peskin, C., Odell, G. & Oster, G., 1993. Cellular motions and thermal fluctuations: the Brownian ratchet. *Biophysical journal*, 65(1), pp.316–24. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1225726&tool=pmcentrez&rendertype=abstract [Accessed December 18, 2014].

Pollard, T., 2000. Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annual review of ...*, 29, pp.545–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10940259 [Accessed December 15, 2014].

Pollard, T.D., 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. *Annual review of biophysics and biomolecular structure*, 36, pp.451–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17477841 [Accessed July 10, 2014].

Pollard, T.D. & Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, 112(4), pp.453–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12600310.

Prass, M. et al., 2006. Direct measurement of the lamellipodial protrusive force in a migrating cell. *The Journal of cell biology*, 174(6), pp.767–72. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064331&tool=pmcentrez&rendertype=abstract [Accessed October 26, 2014].

Raucher, D. & Sheetz, M.P., 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *The Journal of cell biology*, 148(1), pp.127–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2156205&tool=pmcentrez&rendertype=abstract.

Ridley, A.J., 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology*, 16(10), pp.522–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16949823 [Accessed September 10, 2014].

Roh-Johnson, M. et al., 2012. Triggering a cell shape change by exploiting preexisting actomyosin contractions. *Science (New York, N.Y.)*, 335(6073), pp.1232–5.

Rosenfeldt, H. et al., 2006. Rac inhibits thrombin-induced Rho activation: evidence of a Pak-dependent GTPase crosstalk. *Journal of molecular signaling*, 1, p.8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1769496&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Rösner, H. et al., 2007. Attenuation of actinomyosinII contractile activity in growth cones accelerates filopodia-guided and microtubule-based neurite elongation. *Brain research*, 1176, pp.1–10.

Rouiller, I. et al., 2008. The structural basis of actin filament branching by the Arp2/3 complex. *The Journal of cell biology*, 180(5), pp.887–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2265399&tool=pmcentrez&rendertype=abstract [Accessed November 18, 2014].

Rozelle, A. & Machesky, L., 2000. Phosphatidylinositol 4, 5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Current Biology*, 10(6), pp.311–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10744973 [Accessed December 17, 2014].

Saunders, R.M. et al., 2006. Role of vinculin in regulating focal adhesion turnover. *European journal of cell biology*, 85(6), pp.487–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16584805 [Accessed November 1, 2014].

Schaefer, A.W., Kabir, N. & Forscher, P., 2002. Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *The Journal of cell biology*, 158(1), pp.139–52. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2173029&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Schaus, T.E., Taylor, E.W. & Borisy, G.G., 2007. Self-organization of actin filament orientation in the dendritic-nucleation/array-treadmilling model. *Proceedings of the National Academy of Sciences of the United States of America*, 104(17), pp.7086–91. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1855413&tool=pmcentrez&rendertype=abstract.

Schnelzer, A., Bokoch, G.M. & Jolla, L., 2004. Phosphorylation of RhoGDI by Pak1 Mediates Dissociation of Rac GTPase. , 15, pp.117–127.

Schwartz, M., 2004. Rho signalling at a glance. *Journal of cell science*, 117(Pt 23), pp.5457–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15509861 [Accessed November 10, 2014].

Shahapure, R. et al., 2010. Force generation in lamellipodia is a probabilistic process with fast growth and retraction events. *Biophysical journal*, 98(6), pp.979–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2849058&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Shutova, M. et al., 2012. Functions of nonmuscle myosin II in assembly of the cellular contractile system. *PloS one*, 7(7), p.e40814. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3396643&tool=pmcentrez&rendertype=abstract [Accessed June 2, 2014].

Small, J.V. et al., 2002. The lamellipodium: where motility begins. *Trends in Cell Biology*, 12(3), pp.112–120. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0962892401022371.

Solecki, D., Trivedi, N. & Govek, E., 2009. Myosin II motors and F-actin dynamics drive the coordinated movement of the centrosome and soma during CNS glial-guided neuronal migration. *Neuron*, 63(1), pp.63–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2737100&tool=pmcentrez&rendertype=abstract [Accessed November 26, 2014].

Solecki, D.J., Govek, E.-E. & Hatten, M.E., 2006. mPar6 alpha controls neuronal migration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(42), pp.10624–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17050699 [Accessed June 13, 2014].

Song, H. & Poo, M., 2001. The cell biology of neuronal navigation. *Nature cell biology*, 3(3), pp.E81–8. Available at: http://www.nature.com/ncb/journal/v3/n3/abs/ncb0301_e81.html [Accessed June 13, 2014].

Stavenger, R. a et al., 2007. Discovery of aminofurazan-azabenzimidazoles as inhibitors of Rho-kinase with high kinase selectivity and antihypertensive activity. *Journal of medicinal chemistry*, 50(1), pp.2–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17201404.

Strasser, G. a et al., 2004. Arp2/3 is a negative regulator of growth cone translocation. *Neuron*, 43(1), pp.81–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15233919.

Suraneni, P. et al., 2012. The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *The Journal of cell biology*, 197(2), pp.239–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3328382&tool=pmcentrez&rendertype=abstract [Accessed September 22, 2014].

Suter, D.M. & Forschert, P., 1998. An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. , 3.

Svitkina, T.M. et al., 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *The Journal of cell biology*, 160(3), pp.409–21. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172658&tool=pmcentrez&rendertype=abstract [Accessed November 19, 2014].

Thievessen, I. et al., 2013. Vinculin-actin interaction couples actin retrograde flow to focal adhesions, but is dispensable for focal adhesion growth. *The Journal of cell biology*, 202(1), pp.163–77. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3704983&tool=pmcentrez&rendertype=abstract [Accessed September 16, 2014].

Torka, R. et al., 2006. ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. *Experimental cell research*, 312(19), pp.3857–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17010335 [Accessed November 26, 2014].

Trivedi, N. & Solecki, D.J., 2011. Neuronal migration illuminated: A look under the hood of the living neuron. *Cell Adhesion & Migration*, 5(1), pp.42–47. Available at: http://www.landesbioscience.com/journals/celladhesion/article/13609/ [Accessed June 13, 2014].

Ulrich, T. a et al., 2010. Probing cellular mechanobiology in three-dimensional culture with collagen-agarose matrices. *Biomaterials*, 31(7), pp.1875–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19926126 [Accessed September 9, 2014].

Vicente-Manzanares, M. et al., 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature reviews. Molecular cell biology*, 10(11), pp.778–90. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2834236&tool=pmcentrez&rendertype=abstract [Accessed May 26, 2014].

Vitriol, E. a & Zheng, J.Q., 2012. Growth cone travel in space and time: the cellular ensemble of cytoskeleton, adhesion, and membrane. *Neuron*, 73(6), pp.1068–81. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3319373&tool=pmcentrez&rendertype=abstract [Accessed December 2, 2014].

Wickstead, B. & Gull, K., 2011. The evolution of the cytoskeleton. *The Journal of cell biology*, 194(4), pp.513–25. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3160578&tool=pmcentrez&rendertype=abstract [Accessed July 15, 2014].

Woo, S. & Gomez, T.M., 2006. Rac1 and RhoA promote neurite outgrowth through formation and stabilization of growth cone point contacts. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(5), pp.1418–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16452665 [Accessed December 9, 2014].

Wu, C. et al., 2012. Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell*, 148(5), pp.973–87. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3707508&tool=pmcentrez&rendertype=abstract [Accessed July 23, 2014].

Wylie, S.R. & Chantler, P.D., 2008. Myosin IIC: a third molecular motor driving neuronal dynamics. *Molecular biology of the cell*, 19(9), pp.3956–68. Available at: http://www.jbc.org/cgi/doi/10.1074/jbc.275.7.5163 [Accessed November 26, 2014].

Wylie, S.R. & Chantler, P.D., 2001. Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nature cell biology*, 3(1), pp.88–92.

Yang, Q. et al., 2012. Arp2/3 complex-dependent actin networks constrain myosin II function in driving retrograde actin flow. *The Journal of cell biology*, 197(7), pp.939–56. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3384413&tool=pmcentrez&rendertype=abstract [Accessed October 15, 2014].

Yu, P. et al., 2012. Myosin II activity regulates neurite outgrowth and guidance in response to chondroitin sulfate proteoglycans. *Journal of neurochemistry*, 120(6), pp.1117–28. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3296867&tool=pmcentrez&rendertype=abstract [Accessed May 30, 2014].

Zhang, W. et al., 2012. Self-Assembling Peptide Nanofiber Scaffold Enhanced with RhoA Inhibitor CT04 Improves Axonal Regrowth in the Transected Spinal Cord. *Journal of Nanomaterials*, 2012, pp.1–10. Available at: http://www.hindawi.com/journals/jnm/2012/724857/ [Accessed November 26, 2014].

Zhang, X. et al., 2008. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nature cell biology*, 10(9), pp.1062–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2746969&tool=pmcentrez&rendertype=abstract [Accessed December 16, 2014].

Acknowledgement

I would like to thank my supervisor, Prof Vincent Torre for providing me the opportunity to work in his lab and supporting me throughout my PhD. I thank him for his constructive criticism towards my work which helped me to develop my right attitude towards Science. His support and mentoring has encouraged my professional and personal development.

I wish to express my sincere thanks to Manuela Lough for her comments and suggestions on my manuscripts and thesis to improve them. I would like to thank to my former colleagues Erika Ercolini and Ladan Amin. Specially, I would like to acknowledge Erika for helping in my experiments, to arrange data and to be professional. I thank to Jelena Ban for introducing me to Immunocytochemistry technique.

I am deeply grateful to my friend and fellow Paolo Fabris without whose computational knowledge it would have difficult for me to accomplish this work. He has been an excellent companion and made my time in Trieste very enjoyable with his jolly nature.

I would like to thank all my lab mates and friends Sourav, Manuel, Andrea, Francesco, Federico, Arin, Hiba and Monica for their support and encouragement. My special thanks to Arletta and Gosia for sharing pleasant Coffee time with me. I would also like to thank Aswini, Swathi, Priya, Elena, Ayisha and Indrajeet for being good friends.

I would like to thank all the lab technicians and student secretariat for their help. I also thank my dear friends from India Waseem, Amol, Omkar and Nitin

My special gratitude to all the rats, who had to sacrifice their life for my experiments.

I am deeply indebted to my family for their indefinite love and understanding. I thank God for blessing me with such a big-hearted parents. I also thank to my lovely sisters and cousins. Last but not the least, I am very grateful to my wife Shital who has made my life easy and comfortable during my PhD.