Neurite Branch Retraction Is Caused by a Threshold-Dependent Mechanical Impact

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ABSTRACT Recent results indicate that, in addition to chemical cues, mechanical stimuli may also impact neuronal growth. For instance, unlike most other cell types, neurons prefer soft substrates. However, the mechanisms responsible for the neuronal affinity for soft substrates have not yet been identified. In this study, we show that, in vitro, neurons continuously probe their mechanical environment. Growth cones visibly deform substrates with a compliance commensurate with their own. To understand the sensing of stiff substrates by growth cones, we investigated their precise temporal response to well-defined mechanical stress. When the applied stress exceeded a threshold of 274 $\pm$ 41 pN/μm^2, neurons retracted and re-extended their processes, thereby enabling exploration of alternative directions. A calcium influx through stretch-activated ion channels and the detachment of adhesion sites were prerequisites for this retraction. Our data illustrate how growing neurons may detect and avoid stiff substrates—as a mechanism involved in axonal branch pruning—and provide what we believe is novel support of the idea that mechanics may act as guidance cue for neuronal growth.

INTRODUCTION

The selective elimination of neuronal cell processes, or neurites, is an essential step during normal development and under certain pathological circumstances. Exuberant and/or erroneous neuronal connections need to be pruned to achieve precise connectivity; pruning occurs through retraction, degeneration, or a combination of both (1). Neurons for instance retract their processes from previously established connections to ''fine-tune'' their wiring (2,3). Furthermore, growing neurites elongate discontinuously; phases of extension alternate with phases of rest and withdrawal (4,5). Moreover, migrating neurons in the developing cortex may spontaneously withdraw their leading processes (6). However, compared to axon growth and guidance, axon retraction remains poorly understood.

Axon retraction is potentially very similar to repulsive axon guidance (1). It is known that negative biochemical guidance cues, such as ephrins (3,7) and semaphorins (8), may cause the retraction of neuronal processes. Neuronal growth cones, which are highly motile structures at the distal ends of neurites, detect these guidance cues and respond to them (9).

In addition to biochemical cues, neurons are susceptible to mechanical stimuli. Enlarged filopodia tips of Aplysia growth cones are for example very sensitive to force (10), and mechanical tension is an important regulator of axonal elongation and branching (11,12). Neurite growth can be accelerated by the application of tension above a critical threshold, with the elongation rate being proportional to the tension magnitude (13–16). Even more, axons can be generated de novo by solely applying mechanical tension to the neuron and thus breaking the symmetry of the cell (17,18). The tension along neurite branches in in vitro networks, which is maintained through the mechanical attachment of neurons to their substrate, controls the diameter of the neurites, and the junction geometry is determined by the equilibrium of tension forces (19,20). It has been suggested that tension—similar to a second messenger—may serve as a signal for axonal branch survival (11,12); however, an increase in tension along one branch may not only lead to its stabilization but also cause the retraction or elimination of axon collaterals (11). The application of low tensions to neurites may also lead to their retraction (12,13,21). Apart from mechanical tension, substrate compliance may influence neuronal growth as well. In vitro, neurons prefer to grow on soft substrates (22,23). In vivo, neurons grow along glial cells (24,25), which in the central nervous system (CNS) are significantly softer than their neighboring neurons (26), suggesting an involvement of mechanics in axonal branch pruning and/or neuronal guidance. However, the origin of neuronal mechano-responsiveness is not yet understood.

The importance of mechanics for individual cells is becoming increasingly clear through the results of several recent studies: The fate of stem cells could be directed by exclusively changing the mechanical properties of their environment (27), cell growth and development may depend on substrate compliance (23,28), and fibroblasts could be

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guided using substrate mechanics (29). Neurons grow in a mechanically very inhomogeneous environment (26). Moving toward an understanding of their response to different mechanical stimuli is crucial for understanding neuronal functioning. Ultimately, this knowledge may be exploited in the treatment of certain pathologies of the nervous system, particularly for the promotion of axonal growth after traumatic injury (23).

In this study, we assessed how neurons explore their mechanical environment, and we investigated possible active responses of their growth cones to mechanical stimuli. These stimuli were externally applied with full temporal control, which enabled the observation of correlated initiated events. Local mechanical stress application above a threshold of ~274 pN/μm^2 to growth cones caused a calcium influx through mechanosensitive, stretch-activated ion channels (SACs) with subsequent neurite retraction. Our results show how mechanical inputs are translated into biochemical signaling, explain neuronal mechano-responsiveness, provide an insight into a formerly unknown branch pruning mechanism, and clearly strengthen the idea that mechanical cues are involved in neuronal guidance.

MATERIALS AND METHODS

Sample preparation

All experiments involving primary cells were carried out in accordance with applicable German laws of animal protection. NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (PAA Laboratories GmbH, Pasching, Germany) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH), 100 U/mL penicillin/streptomycin, and 10 mM HEPES (both Sigma-Aldrich, Steinheim, Germany). PC12 cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing glutamin (85%; PAA Laboratories GmbH), horse serum (10%; ATCC, Manassas, VA), fetal calf serum (5%; PAA Laboratories GmbH), and 50 μg/mL gentamicin (PAA Laboratories GmbH). Differentiation was initiated by addition of nerve growth factor 2.5S from mouse submaxillary glands (Sigma-Aldrich) to the medium 3 days before measurement. Rat cortex E16 primary neurons were cultured in Neurobasal medium (Gibco, Paisley, UK) containing B-27 supplement 1:50 (Gibco), 200 μM l-glutamine (Biochrom AG, Berlin, Germany), and 50 μg/mL gentamicin (PAA Laboratories GmbH). Cells were plated on coverslip-bottomed petri dishes coated with 20 μg/mL laminin (Sigma-Aldrich) and incubated at 37°C and 5% CO_2. For the experiments, the medium was exchanged by CO_2 independent L-15 Leibovitz medium (Biochrom AG) containing B-27 supplement 1:50, and 200 μM l-glutamine. In a series of experiments, 10 mg/mL fluorescein isothiocyanate-dextran (4 kDa, Sigma-Aldrich) was added to L-15 medium modified as described above 10 min before mechanical stimulation (30). Fluorescence within NG108-15 and PC12 growth cones after stimulation was recorded with a Leica DM IRB microscope using a 100× oil immersion lens (NA = 1.35). In a further series of experiments, PC12 cells were treated with 25 μM $GdCl_3$ (Sigma-Aldrich). In these experiments, the medium was exchanged by an extracellular solution containing 136 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM $CaCl_2$, and 11 mM glucose.

Recording of substrate deformation

NG108-15 cells, which are used frequently as a model system to study in vitro neurite outgrowth (31) and that are the only cells with know internal mechanics (T. Betz, D. Koch, Y. B. Lu, K. Franze, T. Fuhs, and J. Kas, unpublished), have been cultured on polyacrylamide gels prepared as described previously (23) and coated with poly-L-lysine and laminin (Sigma-Aldrich). A scanning force microscope (SFM) (NanoWizard, JPK Instruments, Berlin, Germany) was used to determine the elasticity of the gels. To detect the deformation of the gels, fluorescent beads (FluoSphere, 100 nm Ø, Molecular Probes, Eugene, OR) were embedded into the gel; 24 h after plating the cells, the position of the beads was recoded using a confocal microscope (TCS2 AOBS, Leica, Bensheim, Germany), and a reference image of the relaxed gel was taken after the cells were removed by applying a 0.5% solution of trypsin for 30 min. The deformation was calculated by comparing the bead positions using a cross correlation algorithm (33).

SFM studies

SFM measurements were taken with a NanoWizard SFM (JPK Instruments) and an Autoprobe CP SFM (PSI, Sunnyvale, CA) at 37°C. The former was placed on an inverted microscope DM IRB (Leica Microsystems, Wetzlar, Germany). A bead with diameter of ~6 μm was glued to the cantilever, which is a soft leaf spring with known spring constant, and used as probe to create a well-defined contact area and to avoid cell damage (34). NG108-15 and PC12 cells were indented with the cantilever and indentation and cantilever deflection were recorded. The change in neurite length was measured using self-written software based in LabView. In the experiments investigating the mechanical properties of the growth cone, an oscillatory drive signal was fed to the SFM scanner signal through a lock-in amplifier (SR850, Stanford Research Systems, Sunnyvale, CA) (34,35). Phase as well as amplitude differences between the applied modulation and the cantilever response were recorded and could be used to calculate the complex Young's modulus of the cell using self-written software (34,35).

$[Ca^{2+}]_i$ measurements

PC12 cells and rat cortex E16 primary neurons were loaded with 1 μM Fura-2/AM (Invitrogen, Karlsruhe, Germany) 0.1% dimethyl sulfoxide (DMSO containing 2% Pluronic F-127, both Sigma-Aldrich). Cells were incubated for 30 min at 25°C. Subsequently, cells were washed with Hanks' balanced salt solution. A cell poker was set up on an IMic microscope (TillPhotonics; Zeiss C-Apochromat 40×, NA = 1.2, water immersion). Bright field and fluorescence images were taken for at least 5 min. TillVision software (TillPhotonics) was used for data analysis.

Interference reflection microscopy

PC12 cells were observed with epi-illumination on an inverted microscope (Leica DM IRB) using a 63× oil immersion objective (NA = 1.25). The light of a mercury lamp was partly reflected by a glass coverslip and coupled into the objective. Light reflected from the different interfaces in the sample was then imaged on a charge-coupled device. Regions that are in contact with the substrate or close to it appear darker than regions that are not adhered to the substrate (36).

RESULTS

Substrate deformation assays

To test if growth cones deform their environment to determine its mechanical properties, neurons were cultured on polyacrylamide gels of different compliance. The compliance of a material can be characterized by its Young's modulus E, which relates the stress σ exerted on it (in N/m^2 = Pa) to its strain (or deformation) γ (dimensionless). A larger E in a material corresponds to a higher resistance to deformation, i.e., to a higher stiffness. Fluorescent particles

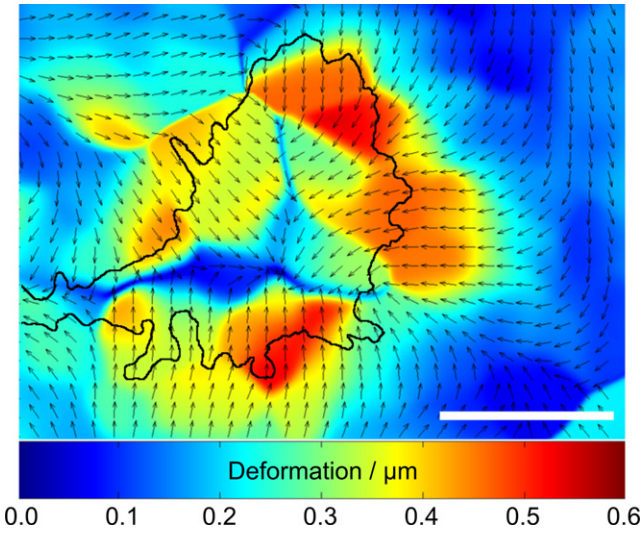

FIGURE 1 Deformation map of a growth cone plated on a 200 Pa polyacrylamide gel. The color coding gives the deformation of the gel; the arrows indicate the direction of deformation. The growth cone, whose edge is indicated by the black line, was able to deform the gel up to a maximum of 600 nm, and at substrate stiffness exceeding 400 Pa the deformations drop below optical resolution. Scale bar = 10 μm.

dispersed within the gels enabled the tracking of substrate deformations by observing the particle displacement (37). Extending growth cones exerted traction forces on their substrate; they significantly deformed soft substrates with a stiffness of $E \sim 200$ Pa (Fig. 1). Because the substrate deformation is proportional to the forces exerted, growth cone-induced substrate deformations are below optical resolution when the neurons are cultured on stiffer gels of $E \sim 400$ Pa (38). Thus, the ability of a growth cone to detectably deform its environment seems to be limited to substrates softer than ~300 Pa.

Mechanical stress application to growth cones

Application of large mechanical stress

To investigate the neuronal response to mechanical cues in a precise temporal order, NG108-15 growth cones were exposed to instant mechanical stress. Controlled mechanical stress was applied for 2 s to the leading edges of the cells' growth cones by an SFM cantilever modified as described (Fig. 2) (34). The stress $\sigma = F/A$, where F is the force (in pN) and A is the contact area (in μm^2), was on the order of 2000 pN/μm^2 (or Pa). In response to the mechanical deformation, active growth cones collapsed (Fig. 2, *A* and *B*). In 12 of 13 cases, the collapse was accompanied by the neuronal process' retraction, which started between 1 s and 3 min after stimulation, over a considerable distance (Fig. 2, *C–F*). This distance differed between individual cells and depended on the space between the growth cone's original position and the next adhesion site of the neurite on the substrate (cf. Figs. 3, 5, and 6).

The same set of experiments was repeated with PC12 cells, because their mechanics is well studied and their response to mechanical tension resembles that of primary neurons of the peripheral nervous system (PNS) (Fig. 3, and Movie S1 and Movie S2 in the Supporting Material) (13,21,39,40). Active growth cones collapsed within 30 s after mechanical stimulation (Fig. 3, *A* and *B*). In 19 of 24 cases, the collapse was accompanied by the retraction of the neuronal process over a considerable distance (Fig. 3, *C–G*). In 4 of 11 cases, neurites or neurite branches were even completely withdrawn (Movie S1). During the retraction, the neurites initially assumed a sinusoidal shape, similar to that of a relaxing coil spring (Fig. 3 *E*). When the retraction stopped and the neurite was not completely withdrawn, new growth cones formed. Simultaneously, the neurites tautened again (Fig. 3 *F*). In 2 of 11 cases, a further retraction occurred after the tautening of the neurite (Movie S2). Ultimately, 6 of 11 neurites started regrowing into a new direction (Fig. 3 *G*). In 5 of these 6 cases, in which the neurites were not attached to the substrate, the angle between the original path of the neurite and its new direction subtended up to 27° with an average angle of 9°; in the one case when the neurite was attached to the substrate, it kinked at the adhesion site, and the new growth direction differed by 106° from the original path. In 2 of 11 cases, neurites started branching subsequent to the retraction. The facts that the growth cones grew into a new direction after the application of a mechanical stimulus indicated that no irreversible damage was caused to the cells, and it further implied

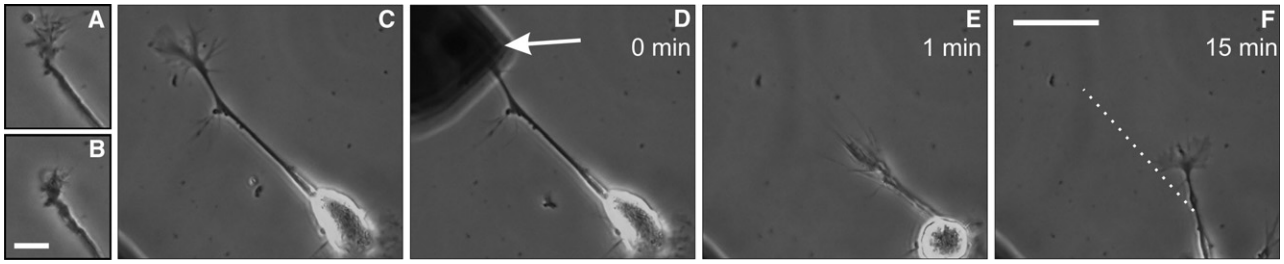

FIGURE 2 Response of NG108-15 processes to mechanical stimulation of their growth cone. (*A*) Phase contrast image of a neuronal growth cone. (*B*) After mechanical stress application, the growth cone collapsed. Scale bar = 10 μm, also applies for *A*. (*C–F*) Neurite retraction after mechanical stimulation. (*C*) NG108-15 cell, neurite is growing toward the left upper corner of the image. (*D*) When mechanical stress was applied to the leading edge of its growth cone (*arrow*: SFM cantilever), the growth cone collapsed and the neurite retracted (*E*). (*F*) Eventually, the neuronal process grew in a new direction (*dashed line*: initial growth direction). Scale bar = 30 μm, also applies for *C–E*.

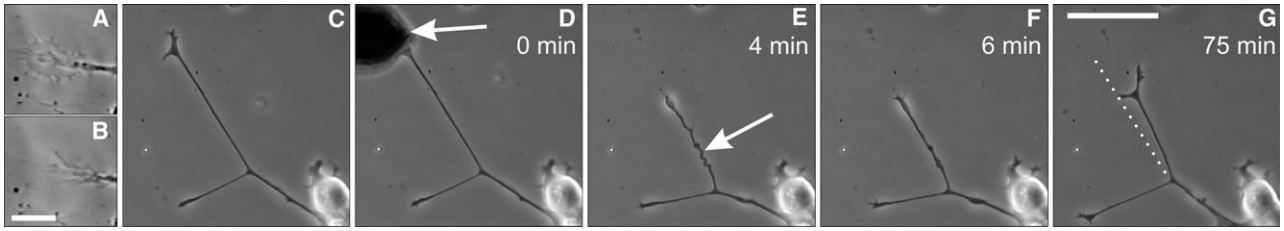

FIGURE 3 Response of PC12 processes to mechanical stimulation of their growth cone (cf. Movie S2). (*A*) Phase contrast image of a neuronal growth cone. (*B*) After application of mechanical stress, the growth cone collapsed. Scale bar = 10 μm, also applies for *A*. (*C–G*) Neurite retraction after mechanical stimulation. (*C*) A neurite is growing toward the left upper corner of the image. (*D*) When a mechanical suprathreshold stimulus (>274 pN/μm^2) was applied to the leading edge of its growth cone (*arrow*: SFM cantilever), the growth cone collapsed and the neurite retracted and assumed a coil-like shape (*arrow* in *E*). (*F*) Subsequently, a new growth cone established, tension recovered, and the neurite straightened again. (*G*) Finally, the neuronal process grew in a new direction; the dashed line indicates the initial direction of neurite growth. Scale bar = 50 μm, also applies for *C–F*.

that motile nerve cells actively avoid rigid contacts in their path.

Threshold stress required for neurite retraction

Such mechanoguidance would require that the cells only respond to mechanical stimuli above a well-defined threshold. Thus, we varied the stress applied by the SFM from 2000 pN/μm^2 down to 10 pN/μm^2. PC12 growth cone protrusion and motion was not disturbed at low stress levels. However, when a critical stress of 274 $\pm$ 41 pN/μm^2 (mean $\pm$ SE, $n = 13$) was applied, growth cone collapse and neurite retraction with consequential growth in a different direction were observed (Fig. 3, *C–G*). A further increase of mechanical stress above this threshold caused the same reactions, which thus seem to constitute an all-or-none response.

In addition to growth cones, all other morphologically distinct elements of neurons, including the processes and somata, were probed for their sensitivity to mechanical stress. In none of these experiments active changes in cellular morphology were observed. Process retraction could only be triggered if i), the stress exceeded the critical threshold; and ii), it was applied to the leading edge of the growth cone.

Cellular calcium response to mechanical stress

Calcium regulates numerous proteins that interact with the cytoskeleton as well as with the adhesion sites of a cell, and both are important for migration and growth control (41,42). Therefore, we monitored free intracellular calcium levels, $[Ca^{2+}]_i$, using the calcium sensitive fluorescent dye Fura-2 with time lapse microscopy (Fig. 4, Movie S3). $[Ca^{2+}]_i$ increased immediately after mechanical stress application (Fig. 4 *B*) from a resting level of 52 $\pm$ 8 nM and reached its maximum of 999 $\pm$ 168 nM (mean $\pm$ SE, $n = 6$) 3–5 s thereafter (Fig. 4 *C*). The elevated calcium level spread from the growth cone into the neurite and, depending on its length, eventually also in other neurite branches and in the neuron's soma (Fig. 4, *D* and *E*). Calcium propagation reached its maximum extension after 7 $\pm$ 3 s, and the calcium rise disappeared after 29 $\pm$ 14 s ($n = 6$).

Neurons of the PNS respond similarly to mechanical tension as those of the CNS (12–15,43). When primary CNS neurons were mechanically stimulated, a nearly identical temporal behavior of the calcium dynamics was observed (data not shown).

The application of mechanical stress at other locations along the neurites also caused a rise in $[Ca^{2+}]_i$, which spread toward the soma and the growth cone. Stress application to the somata caused only a very small, local increase in $[Ca^{2+}]_i$ that merely lasted for the duration of indentation.

To discriminate between calcium entry through unspecific, transient plasma membrane passages, as for example seen in fibroblasts contracting collagen matrices (30), and through specific channels, 4 kDa fluorescein isothiocyanate-dextran was added to the medium. No fluorescence was detected within growth cones and neurites after mechanical stimulation, indicating that transient membrane passages did not exist (data not sown). Gadolinium, which blocks

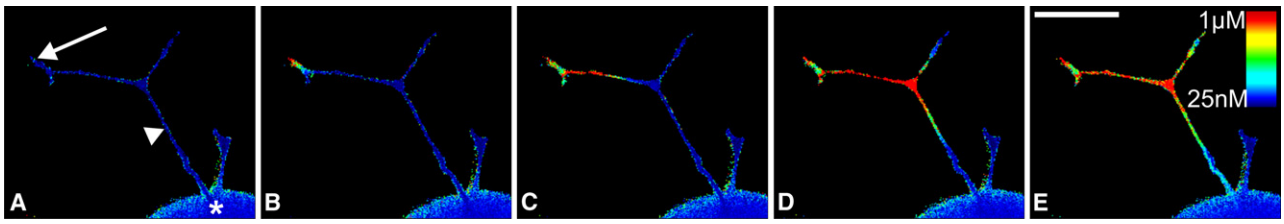

FIGURE 4 $[Ca^{2+}]_i$ within a mechanically stimulated neuron. (*A*) $[Ca^{2+}]_i$ before, (*B*) during, and (*C*) 5 s, (*D*) 15 s, and (*E*) 30 s after mechanical stimulation of the growth cone (*arrow* in *A*; *arrow head*: neurite; *asterisk*: soma). The increase in $[Ca^{2+}]_i$ spread from the area of mechanical contact into the neurite and in neighboring structures such as side branches if applicable (*D*,*E*). The color represents $[Ca^{2+}]_i$. Color coding and scale bar (50 μm) in *E* also apply for *A–D*.

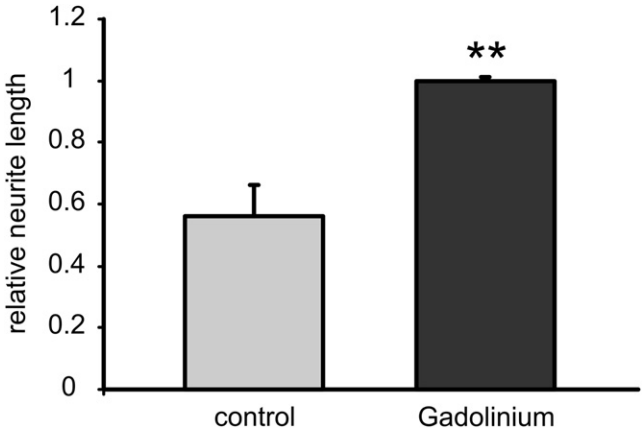

FIGURE 5 Relative change in neurite length 300 s after mechanical suprathreshold stimulation. In normal medium, neurites retracted to 56 ± 10% of their original length ($n = 12$, mean ± SE). When 25 μM gadolinium chloride, which is a blocker of SACs (44), was applied to the solution, no significant retraction of the neurites could be triggered ($n = 9$, mean ± SE, $p < 0.01$).

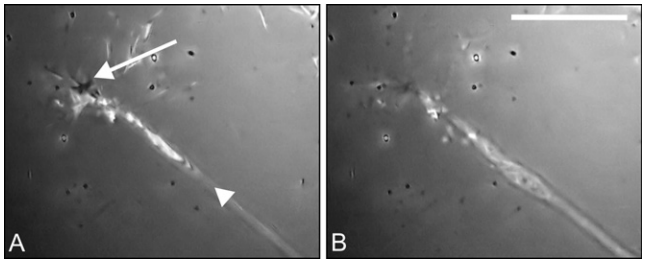

FIGURE 6 Interference reflection microscopy images of a neuronal growth cone. Structures that are in close contact to the substrate appear dark. (*A*) Growth cone before stress application. Adhesion sites, which are the dark structures visible, were densely packed at the growth cone's leading edge (*arrow*). The arrowhead points toward the direction of neurite extension. (*B*) Neuronal process 3 s after mechanical stimulation. The adhesion sites of the growth cone with the substrate disappeared concomitantly with increased $[Ca^{2+}]_i$. Scale bar = 25 μm, also applies for *A*.

stretch-activated cation-selective channels (44), suppressed neurite retraction (Fig. 5). When calcium was removed from the medium, growth cones also no longer responded to mechanical stimuli, i.e., they did not collapse and neurites were not retracted (data not shown), indicating a crucial role of calcium in neurite retraction.

These data suggest that a calcium influx via mechanosensitive ion channels in the membrane of growth cones is an essential step in their retractive response to the application of mechanical forces.

Cellular adhesion during mechanical stress application

It was already mentioned that calcium is involved in the regulation of cellular adhesions (41,42,45). Thus, we monitored neuronal adhesion sites during mechanical stress application using interference reflection microscopy (36) (Fig. 6). Adhesion sites were concentrated in PC12 growth cones (Fig. 6 *A*). When suprathreshold mechanical stress was applied to the leading edge of a growth cone, most of these adhesions disappeared and the neurite subsequently retracted (Fig. 6 *B*).

Mechanical properties of growth cones

The limitation of growth cones to deform only comparatively soft environments with a Young's modulus of up to ~300 Pa may either result from their maximal possible force exerted or from their own compliance. We determined the growth cones' mechanical properties using SFM cell rheology (34,35). The neurons' frequency dependent response to indentation revealed their complex Young's modulus $E^* = E' + iE''$, where $i = \sqrt{-1}$. The storage modulus E' characterizes the cells' elastic stiffness, and the loss modulus E'' reflects its viscous response to deformation. The governing elastic storage modulus $E' = 295 \pm 48$ Pa (mean ± SE, $n = 10$) found for PC12 growth cones (probed at a frequency $f = 100$ Hz) as well as that of NG108-15 growth cones (T. Betz, D. Koch, Y. B. Lu, K. Franze, T. Fuhs, and J. Kas, unpublished) is in the same range as the maximum substrate stiffness growth cones could visibly deform.

DISCUSSION

The external application of mechanical stress to growth cones allowed us to study neuronal responses to mechanical cues on a well-defined timescale, and thus helped to understand mechanisms underlying neuronal mechanosensitivity, which might be involved in axonal branch competition and pruning. On this timescale, however, growth cones can be considered passive, which is not representative for in vivo conditions. It can be reasonably assumed that, in vivo, the actively propagating growth cones come into contact with rather static, passive environmental compartments, which may differ in their mechanical properties. It has been shown that neurons in vitro grow better and extend more side branches on soft substrates (22). We show that neuronal growth cones constantly probe and pull on their mechanical environment, which may explain why neurons in vitro prefer soft substrates. Growth cones were able to detectably deform gels up to a stiffness of ~300 Pa (Fig. 1), which corresponds to their own compliance. This value is furthermore in excellent agreement with the observation that a transition from neurons with many branches to those with significantly less branches occurs at substrate stiffness of ~300 Pa (22). Thus, active mechanosensitive probing of the surrounding may provide positive stimuli for neuronal growth in a soft environment (below 300 Pa), whereas more rigid contacts deliver a negative feedback.

The application of external mechanical stimuli to neuronal growth cones sheds light on this negative feedback mechanism. We found a series of retraction and direction-changing events that was triggered when mechanical stress exceeding

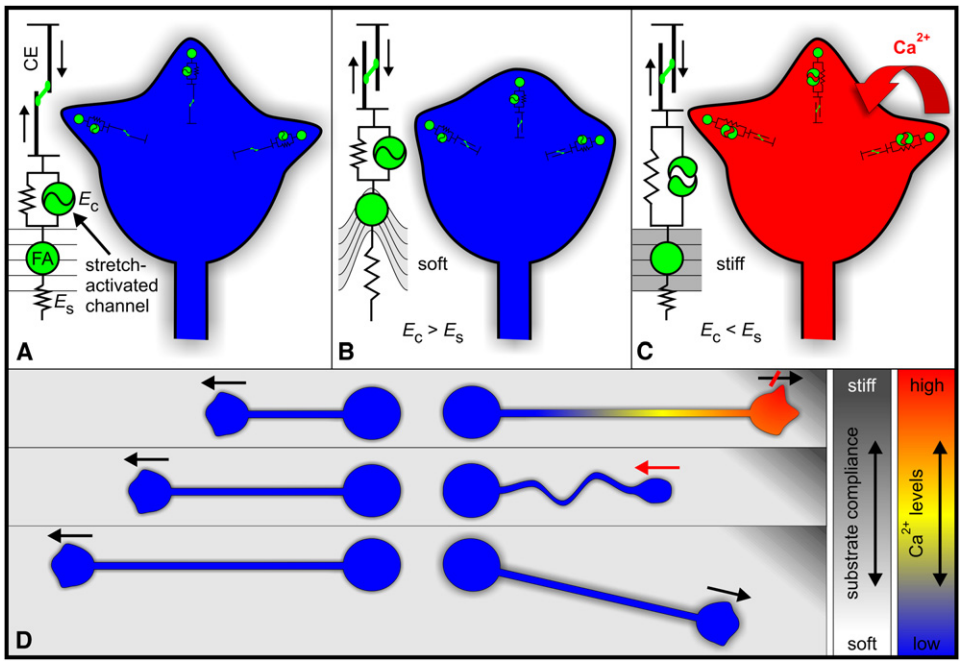

FIGURE 7 Scheme summarizing the effects of mechanical cues on neurite growth. (*A*) Growth cones are attached to the substrate by focal adhesions (*FAs*), which are connected to contractile elements (*CE*), and that may be linked to SACs. Neurons exert contractile forces (*arrows*) on their environment via FAs. These forces act on both focal adhesions and SACs. (*B*) If the substrate stiffness (E_s) is below the critical threshold, which corresponds to the growth cone's own compliance (E_c), the substrate is deformed and the channels remain closed. (*C*) When the substrate stiffness exceeds that of the growth cone, the force exerted on the SACs may be sufficient to trigger their opening, leading to a calcium influx from the extracellular space. (*D*) Illustration of neuronal growth in dependence on substrate compliance. *Left*: If the neuron grows on a soft substrate, the growth cone advances unhindered. *Right*: If the neuron approaches a hard substrate, more and more force generated by the contractile elements is transmitted to the SACs, until they open and calcium enters the interior of the cell, triggering growth cone collapse and neurite retraction by increasing its contractility and/or by destabilizing its focal adhesions. Subsequently, the growth cone adheres again to the substrate and grows into a new direction. This mechanism may slow down the effective neuronal growth rate in regions that are stiffer than the threshold, and it might ultimately be used as guidance cue during neuronal pathfinding.

a threshold of 274 pN/μm^2 was applied to the leading edges of growth cones. The following four steps were identified (Fig. 7):

1. Immediately after mechanical stress application, $[Ca^{2+}]_i$ increases locally and the elevated $[Ca^{2+}]_i$ spreads then into neighboring structures (Fig. 4). This calcium rise can be taken as the signature of a mechanosensitive event as similarly observed in fish keratocytes and fibroblasts (41,46). In our experiments, the dramatic increase in $[Ca^{2+}]_i$, the absence of transient plasma membrane passages, and the failure of neurite retraction when either a blocker of stretch-activated ion channels was applied or extracellular calcium was removed strongly indicate the presence of mechanosensitive ion channels in the growth cone membrane. Thus, the mechanically triggered opening of SACs may mediate a calcium influx from the extracellular space into the growth cone.
2. Subsequently, growth cones collapse and their adhesion sites lose contact with the substrate (Figs. 2, *A* and *B*, 3, *A* and *B*, and 6). The increase in $[Ca^{2+}]_i$ may directly trigger cytoskeletal changes that lead to the collapse of the growth cone and to an imbalance in the interplay between actin, microtubules and the associated motor proteins (7,39,47). The observed growth cone detachment may also be a direct consequence of the loss of adhesion sites after the increased $[Ca^{2+}]_i$ (42).
3. Thereafter, the neurites retract and assume a shape resembling a relaxing elastic coil spring (Fig. 3 *E*). In early development, many neuronal processes with wavy profiles were observed in situ (48). The sinusoidal shape of retracted neurites has furthermore been observed in in vitro experiments after neurite transection (19,39) and after the application of biochemical repulsive cues (49), suggesting similar pathways for mechanically and chemically activated retractions. The observed retraction is likely a consequence of an imbalance between the growth cone's adhesion and the tension of the neurite. As soon as the force required for growth cone detachment is below the tension associated with the neurite (19,50), the neurite may retract. This imbalance might arise either due to decreased growth cone adhesion, increased neurite tension, or a combination of both, and it is likely caused by the calcium increase (41,42). Our data thus suggest a central role of a calcium influx in axonal branch pruning. It seems unlikely that the detachment of growth cones was directly caused by their interaction with the SFM probe, because no strong adhesion between probe and growth cones has been observed (data not shown). Furthermore, forces were applied perpendicular to the growth cone surface, thus avoiding shear forces that might lead to their detachment. Whereas $[Ca^{2+}]_i$ increased immediately after mechanical stimulation and went back to normal levels only after ~30 s, neurite retraction started up to 3 min after mechanical stress application and lasted for up to 12 min. This retardation is another indication that the retraction is a secondary, delayed

response to the mechanical stimulation of the growth cone; it furthermore indicates that the calcium rise within the growth cones is the initial step required for neurite retraction.

4. Finally, if the process is not completely withdrawn, a new growth cone is established, adhering to the substrate (Fig. 3 *F*). Subsequently, the tension recovers and the neurite straightens again (Fig. 3 *G*). In some cases, a second retraction may occur, probably because the new adhesion site is weaker than the newly built-up tension of the neurite. Generally, however, the neurite starts regrowing into a new direction—away from the suprathreshold mechanical contact (Figs. 2 *F*, 3 *G*, and 7).

The presence of mechanosensitive ion channels in the growth cone membrane may thus explain how mechanics affects neuronal growth (Fig. 7) and axonal branch selection. Growth cones continuously exert forces on their environment. As long as the substrate is soft enough, they will deform it and continue growing. However, the stiffer the substrate, the more the exerted force is transmitted to cellular structures (Fig. 7, *A–C*). When the substrate stiffness exceeds a critical threshold value, which likely corresponds to the growth cones' own compliance, the force exerted on the SACs may exceed a threshold required to trigger their opening. As a consequence, calcium may enter the growth cone and trigger the observed response of the neurite (Fig. 7 *D*). Our data imply that the calcium influx is or induces the signal for axonal branch competition and pruning. In agreement with this hypothesis, in the developing *Xenopus* spinal cord axon retraction in vivo is associated with high frequencies of Ca^{2+} transients (5). Hence, substrates with suprathreshold compliance are less permissive for neuronal growth and may constitute a repellent cue by facilitating increased calcium influx into the growth cone. We determined the threshold value in vitro to be ~300 Pa.

In vivo, neurite retraction is assumed to occur as an effect of local guidance cues, which in neurons are detected by their growth cones (9). In agreement with previous studies, in which biochemical cues have been used to locally trigger neurite retraction (7), also in our experiments neurons only withdrew their processes when the signal was applied to their growth cones. Both the restriction of neuronal mechanoresponsiveness to the leading edges of their growth cones and the presence of a threshold value for the mechanical stimulation highly suggest an involvement of mechanics in axonal branch pruning and ultimately in neuronal guidance.

It is intriguing to speculate whether in vivo a similar critical mechanical threshold value exists as the 300 Pa found in our in vitro experiments. In this context, there are appealing data on the preferred pathways of neurons and their growth cones in vivo. Immature neurons in the developing cortex migrate along radial glial cells (24), and axons, which are growing in situ along superficial tissue layers, may turn right-angled and send their growth cones into deeper layers, again following radial glial fibers (25). Radial glial cells in the adult retina are the softest cells in this tissue. Their stiffness barely reaches 200 Pa, whereas that of all other cellular structures in the retina exceeds 300 Pa (26). Neurons might thus use (glial cell) mechanics—in a complex interplay with other signals—as guidance cue during growth and migration.

SUPPORTING MATERIAL

Three movies are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01294-6.

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