Delayed Retraction of Filopodia in Gelsolin Null Mice

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Abstract. Growth cones extend dynamic protrusions called filopodia and lamellipodia as exploratory probes that signal the direction of neurite growth. Gelsolin, as an actin filament-severing protein, may serve an important role in the rapid shape changes associated with growth cone structures. In wild-type (wt) hippocampal neurons, antibodies against gelsolin labeled the neurite shaft and growth cone. The behavior of filopodia in cultured hippocampal neurons from embryonic day 17 wt and gelsolin null (*Gsn*$^-$) mice (Witke, W., A.H. Sharpe, J.H. Hartwig, T. Azuma, T.P. Stossel, and D.J. Kwiatkowski. 1995. *Cell.* 81:41–51.) was recorded with time-lapse video microscopy. The number of filopodia along the neurites was significantly greater in *Gsn*$^-$ mice and gave the neurites a studded appearance. Dynamic studies suggested that most of these filopodia were formed from the region of the growth cone and remained as protrusions from the newly consolidated shaft after the growth cone advanced. Histories of individual filopodia in *Gsn*$^-$ mice revealed elongation rates that did not differ from controls but an impaired retraction phase that probably accounted for the increased number of filopodia long the neutrite shaft. Gelsolin appears to function in the initiation of filopodial retraction and in its smooth progression.

NEURONAL growth cones are highly motile structures that contain the mechanical elements necessary to implement a repertoire of behaviors that determine the rate and direction of advance, as well as the receptor elements that link environmental cues to the implementation of specific behaviors. (Trinkaus, 1984; Bray and Hollenbeck, 1988; Goodman and Shatz, 1993). The lamellipodia and filopodia, which constantly protrude and retract at the tips of incipient axons and dendrites (Goldberg and Burmeister, 1986; Rivas et al., 1992), are the most active processes, and the detailed molecular assemblies that mediate this activity are mostly unknown. Clearly, actin filaments are the major cytoskeletal component in these structures and must be involved in lamellipodial and filopodial motility (Forscher and Smith, 1988; Bridgman and Dailey, 1989; Lewis and Bridgman, 1992). Disruption of actin filaments by the depolymerizing agent cytochalasin abolished protrusive activity (Forscher and Smith, 1988) and even retraction of both lamellipodia and filopodia (Marsh and Letourneau, 1984; Bentley and Toroian, 1986; Forscher and Smith, 1988; Chien et al., 1993). One signaling molecule for actin polymerization is calcium ion (Ca^{2+}), and many studies have demonstrated that increased intracellular Ca^{2+} due to depolarization, as well as treatments with neurotransmitter or Ca^{2+} ionophore, alter the morphology and motility of lamellipodia and filopodia (Cohan and Kater, 1986; Lankford and Letourneau, 1989; Cohan, 1992; Grumbacher-Reinert and Nicholls, 1992; Rehder and Kater, 1992; Neely, 1993; Neely and Gesemann, 1994; Zheng et al.,1996). Also within growth cones are actin-binding proteins such as certain myosin isoforms, tropomyosin, α-actinin, actin depolymerizing factor (ADF),[1] filamin, and gelsolin (Bamburg and Bray, 1987; Bridgman and Dailey, 1989; Letourneau and Shattuck, 1989; Sobue and Kanda, 1989; Miller et al., 1992; Tanaka et al., 1993; Tanaka and Sobue, 1994). Although much is known about the in vitro effects of these proteins on actin organization and polymerization, how these actin-associated proteins act in vivo to affect the motility of lamellipodia and filopodia is largely unknown.

Gelsolin is a good candidate for a Ca^{2+}-sensitive factor capable of regulating the dynamics of actin filaments in growth cones. Gelsolin severs actin filaments in a Ca^{2+}-dependent manner and caps the plus ends of the severed filaments, preventing the addition of actin monomers (Yin and Stossel, 1979; Yin et al., 1981; Lamb et al., 1993). Binding of gelsolin to phosphoinositides (PPIs) causes the release of gelsolin from the filament end, providing a site for rapid actin monomer addition (Janmey et al., 1987; Janmey and Stossel, 1987, 1989). Micromolar Ca^{2+} also ac-

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1. *Abbreviations used in this paper*: ADF, actin depolymerizing factor; DIC, differential interference contrast; PPI, phosphoinositides; VEC, video enhanced contrast; wt, wild type.

tivates gelsolin to nucleate actin filament growth from monomers, resulting in the formation of short actin filaments (Yin et al., 1981; Janmey et al., 1985). Thus, during repetitive waves of high Ca^{2+}/low PPIs alternating with low Ca^{2+}/high PPIs, gelsolin can disassemble an existing actin filament architecture through its severing activity and then lead to site-directed reformation of an alternative structure, contributing to the motile changes occurring in cells.

This model of the function of gelsolin during motility is supported by studies of non-neuronal cells. Overexpression of gelsolin in cultured NIH 3T3 fibroblasts results in increased motility as assessed by tissue culture wound healing and filter transmigration assays (Cunningham et al., 1991). Moreover, gelsolin-null (*Gsn*$^-$) mice, generated by gene targetting methods (Witke et al., 1995), have grossly normal embryonic development and longevity but defects in hemostasis, inflammation, and tissue remodeling. Cultured dermal fibroblasts from these *Gsn*$^-$ mice migrated more slowly in comparison to wild-type (wt) mice and had increased amounts of actin stress fibers.

To determine the possible role of gelsolin during neurite elongation, we compared the morphology and motility of neuronal growth cones in cultures from *Gsn*$^-$ and wt mice. In high resolution time-lapse video microscopy images, considerably more filopodia were present along neurites of *Gsn*$^-$ mice. A comparison of the behaviors of filopodia in these mice leads to the observation that gelsolin has a critical role in filopodial retraction.

Materials and Methods

Cell Culture

Hippocampal neuronal cultures were obtained from either wt (C57LB/6 or BALB/c) or *Gsn*$^-$ mice (C57LB/6 or BALB/c; Witke et al., 1995) at embryonic day 17. Culture procedures generally followed those described (Goslin and Banker, 1991) with some minor modifications. The brief procedures are as follows. Brains were sterily removed from CO_2-killed mice and placed in the sterile cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+}. Hippocampi were carefully dissected, and meninges were removed. Hippocampal cells were dissociated enzymatically with 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for 15 min and mechanically by triturating with a fire-polished Pasteur pipette. The dissociated cells were plated onto 24.5-mm circular glass coverslips (No. 1.5; German) coated with laminin (20 μg/ml; Sigma Chemical Co.). The cells were cultured in a humidified 5% CO_2 incubator at 37°C for up to 3 d.

Video Microscopy

Dissociated hippocampal neurons were observed with a 60× planapochromat oil immersion objective (1.4 NA), 100 W mercury light source under an inverted microscope (Diaphot 300; Nikon, Inc., Melville, NY). For video-enhanced contrast differential interference contrast (VEC-DIC) microscopy, a matching high resolution (1.4 NA) oil immersion condenser was used. For time-lapse video microscopy, hippocampal cells cultured for 1 to 3 d were mounted in a Dvorak-Stotler chamber and kept at 37°C by means of an air stream stage incubator (Nicholson Precision Instruments, Gaithersburg, MD). A fiberoptic light scrambler (Knudsen Technical Video, Woods Hole, MA), heat filters, and a monochromatic green filter (546 nm) were used to achieve even illumination and reduce damage to cells. DIC images were amplified with 4× relay lens and detected with a Newvicon camera (C2400; Hamamatsu). Images were taken every 5 s, summed by using Image-1 (Universal Imaging, West Chester, PA), and stored on laser discs with an optical memory disc recorder (OMDR; LVR-5000A, Sony). For matching fluorescence and DIC images on fixed cells, images were magnified with a 2× relay lens, summed for 256 frames, and the background subtracted. DIC images were captured with a Newvicon camera, and fluorescence images were captured under regular fluorescence microscopy with a silicon-intensified target camera (C2400; Hamamatsu). These images were stored in the computer discs directly. Photographs were printed using Adobe Photoshop.

Immunocytochemistry

Dissociated hippocampal cultures were fixed with 4% paraformaldehyde and 3% sucrose in PHEM buffer solution (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) at 37°C for 30 min and permeabilized with 0.1% Triton for 2 min. After blocking with 5% goat serum in PBS buffer for 30 min, cells were incubated with primary antibodies in the blocking solution overnight at 4°C. Cells were then washed three times at 5-min intervals and incubated with secondary antibodies diluted at 1:100 (Jackson ImmunoResearch Laboratory, West Grove, PA) for 1 h. Cells were mounted on slides in Slow Fade™ mounting medium (Molecular Probes, Inc., Eugene, OR) after three washes. The primary antibodies used were affinity purified polyclonal antibody against recombinant murine gelsolin (kindly provided by Toshifumi Azuma, Brigham and Women's Hopital, Boston, MA) at 1:250 dilution and monoclonal antibody against neuronal specific βIII tubulin at 1:200 (Sigma Chemical Co.), with secondary antibodies fluorescein-conjugated donkey anti–rabbit and donkey anti–mouse, respectively. To label actin filaments, cells were incubated overnight at 4°C with 20 U of rhodamine phalloidin in the blocking solution along with the primary antibody.

Quantification

Video sequences of cultured hippocampal neurons from either wt or *Gsn*$^-$ mice were obtained using a high resolution 60× lens and an additional 4× relay lens. The images were quantified using the "measure curve length" function in Image-1 software. The average time recorded for individual sequences was ~30 min. The interval between frames was 5 s, to ensure capturing the detailed dynamic behaviors of growth cones and neurites. 7–10 evenly spaced frames at 3-min intervals were measured for each sequence. The length and number of filopodia along the neurite were measured within 10 μm of the neurite from the neck of the growth cone. Using the stated magnification, the width across the monitor screen was 45 μm, making it possible to visualize both the growth cone and neurite for every image quantified. To ensure the healthy status of sampled cells, only those sequences that lasted for at least 20 min were quantified. The computation and statistical analysis of the data were performed with Microsoft Excel 5.0.

The duration of filopodia were measured for each sequence. When >10 filopodia were present within the 10-μm segment of the neurite, only the first 10 filopodia were measured. In a few cases, filopodia did not fully retract after 30 min, the time over which most of the sequences were recorded; the duration of these filopodia were entered as 30 min. Life histories for individual filopodia were obtained by measuring the length of filopodia frame by frame and graphed as length versus time. The measurements for the elongation rate and retraction rate of filopodia were made by using the KaleidaGraph program. Lamellipodial activity was measured by determining the change in lamellipodial area at 5-s intervals.

Results

Gelsolin Is Present in Neuronal Growth Cones of Wild-Type Mice

To explore a possible role for gelsolin in neurite elongation, we first investigated the expression pattern of gelsolin in cultured embryonic hippocampal neurons from wt mice. Hippocampal cells were cultured overnight on a laminin substrate, which is found in the extracellular space of the embryonic rat hippocampus (Gordon-Weeks et al., 1989). The distribution of gelsolin was studied by immunostaining fixed, cultured hippocampal cells. In this study, an affinity-purified polyclonal antibody raised against recombinant gelsolin was used. 1-d-old cultures from wt and *Gsn*$^-$ mice were fixed and immunostained. Both neurons and non-neuronal cells stained with the affinity-purified polyclonal antibody against gelsolin. In neurons, gelsolin

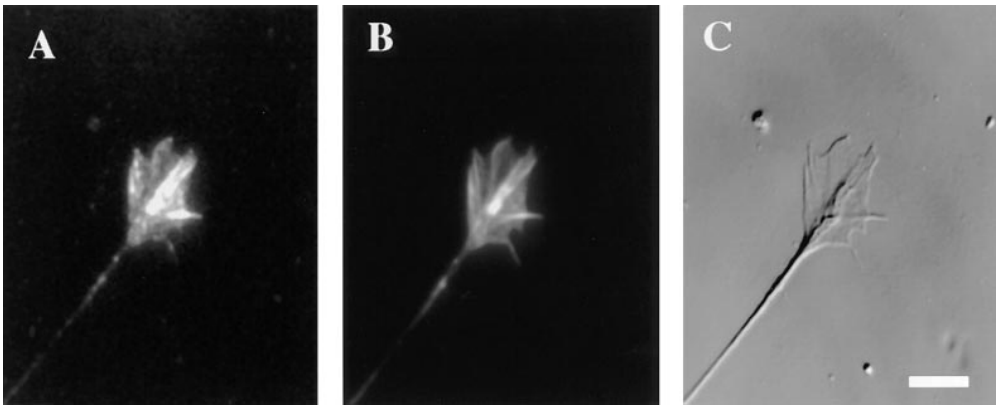

Figure 1. Fluorescence images of hippocampal neurons stained with affinity-purified polyclonal antibody to gelsolin from E17 wt mice (*A*) and rhodamine phalloidin (*B*). (*C*) Matching DIC image. Note in wt mice (*A*), not only the neurite but also the growth cone stained and colocalized with actin (*B*). Bar, 5 μm.

immunoreactivity was present in the neurites and appeared concentrated in growth cones where it was expressed in the central domain as well as in the leading edge of the lamellipodia and filopodia (Fig. 1). It co-localized with actin filaments as labeled by rhodamine phalloidin (Fig. 1).

Growth Cones and Neurites of Gsn⁻ Hippocampal Neurons

Embryonic day 17 hippocampal cells from wt and *Gsn*⁻ mice were dissociated and cultured on laminin-coated coverslips for 1 to 3 days. Hippocampal neurons from wt mice cultured on laminin substrate typically had a small round or oval cell body averaging 6–8 μm diam and usually had a long neurite and several short processes. Growth cones at the tips of neurites were ~7–10 μm across. Typically, the region proximal to the growth cone of wt neurons was fairly smooth with few filopodia, as shown in Fig. 2. Neuronal growth cones and neurites from *Gsn*⁻ mice were similar in size and morphology to those of wt mice. However, there were considerably more filopodia along the extending neurite of *Gsn*⁻ mice than along those of wt mice, as shown in Fig. 3. To quantitate this observation, a region of the neurite 10 μm proximal to the neck of the growth cone was analyzed.

Filopodia from 17 time-lapsed sequences of wt growth cones and 18 sequences of *Gsn*⁻ growth cones were quantitated. Filopodia along the neurite were quite dynamic, making it important to sample data not only from different neurons but also from a single neuron at different time points. For each sequence, the length and number of filopodia along the neurite were measured in 7–10 frames, taken in 3-min intervals. Results from these sequences showed that the average number of filodopia in each frame was significantly more ($P < 0.001$) in *Gsn*⁻ mice 2.64 ± 0.15, $n = 180$) compared to wt mice (1.20 ± 0.11, $n = 164$; Fig. 4 *A*); however, the average length for a single filopodia was not significantly longer in *Gsn*⁻ mice than in wt mice ($P > 0.05$; Fig. 4 *B*).

To investigate the cytoskeletal organization of filopodia along neurites in *Gsn*⁻ mice, neuronal cultures from *Gsn*⁻ mice were fixed and double stained with an antibody to neuron-specific βIII tubulin to assess microtubules and rhodamine phalloidin to assess actin filaments. Cells identified as neurons by DIC optics (Fig. 5 *C*) contained the βIII isoform of tubulin in the neurite shaft (Fig. 5 *B*), similar to wt neurons (data not shown). Filopodia from growth cones and neurites of *Gsn*⁻ mice were labeled with rhodamine phalloidin (Fig. 5 *A*) but did not generally contain microtubules. Therefore, filopodia along neurites in *Gsn*⁻ mice, like control mice, were mainly actin based.

Delayed Retraction Accounts for the Increased Numbers of Filopodia in Gsn⁻ Mice

Because we hypothesized that neurons lacking gelsolin may have motility defects, the initial observations concentrated on rapid shape changes of lamellipodia and filopodia using time-lapse video enhanced contrast DIC microscopy. Measurements of neurite elongation did not reveal significant differences in growth rates. The average rate of neurite elongation in wt neurons was 24.00 ± 5.09 μm/h ($n = 17$), and in *Gsn*⁻ mice was 29.85 + 3.91 μm/h ($n = 18$; $P > 0.05$). In time-lapse sequences, most of the protrusive activity arose from the growth cone, with far less activity in the neurite shaft. Protrusive activity dropped off steeply proximal to the neck of the growth cone. The increased numbers of filopodia in *Gsn*⁻ mice may be due to enhanced de novo elaboration of filopodia in the region of the neurite just behind the growth cone or the retention of filopodia, which failed to retract, after growth cone advance. Based upon time-lapse video data from wt and *Gsn*⁻ mice, only ~15–25% of the filopodia along the region of the neurite just proximal to the growth cone arose de novo from the consolidated neurite. There was no significant difference in number of filopodia formed de novo from the neurite shaft between wt and *Gsn*⁻ mice. In contrast, the majority of neurite filopodia (75–85%) were elaborated originally in the region of the growth cone and were retained on the neurite after the growth cone advanced. It was this population of filopodia, originally formed from growth cones that contributed most to the increased number of filopodia along neurites in *Gsn*⁻ mice. These results suggested that the retraction of filopodia in *Gsn*⁻ mice might be delayed relative to wt mice.

To test this hypothesis, 17 sequences from wt mice and 18 sequences from *Gsn*⁻ mice were analyzed for the duration of filopodial persistence along neurites. The average duration was 4.80 ± 0.55 min ($n = 151$) for *Gsn*⁻ mice and 1.31 ± 0.15 min ($n = 124$) for wt mice ($P < 0.001$; Fig. 6 *A*). In wt mice, 62% of filopodia lasted 1 min or less. The curve of filopodial persistence declined steeply: 91% lasted <3 min and none lasted >10 min (Fig. 6 *B*). In con-

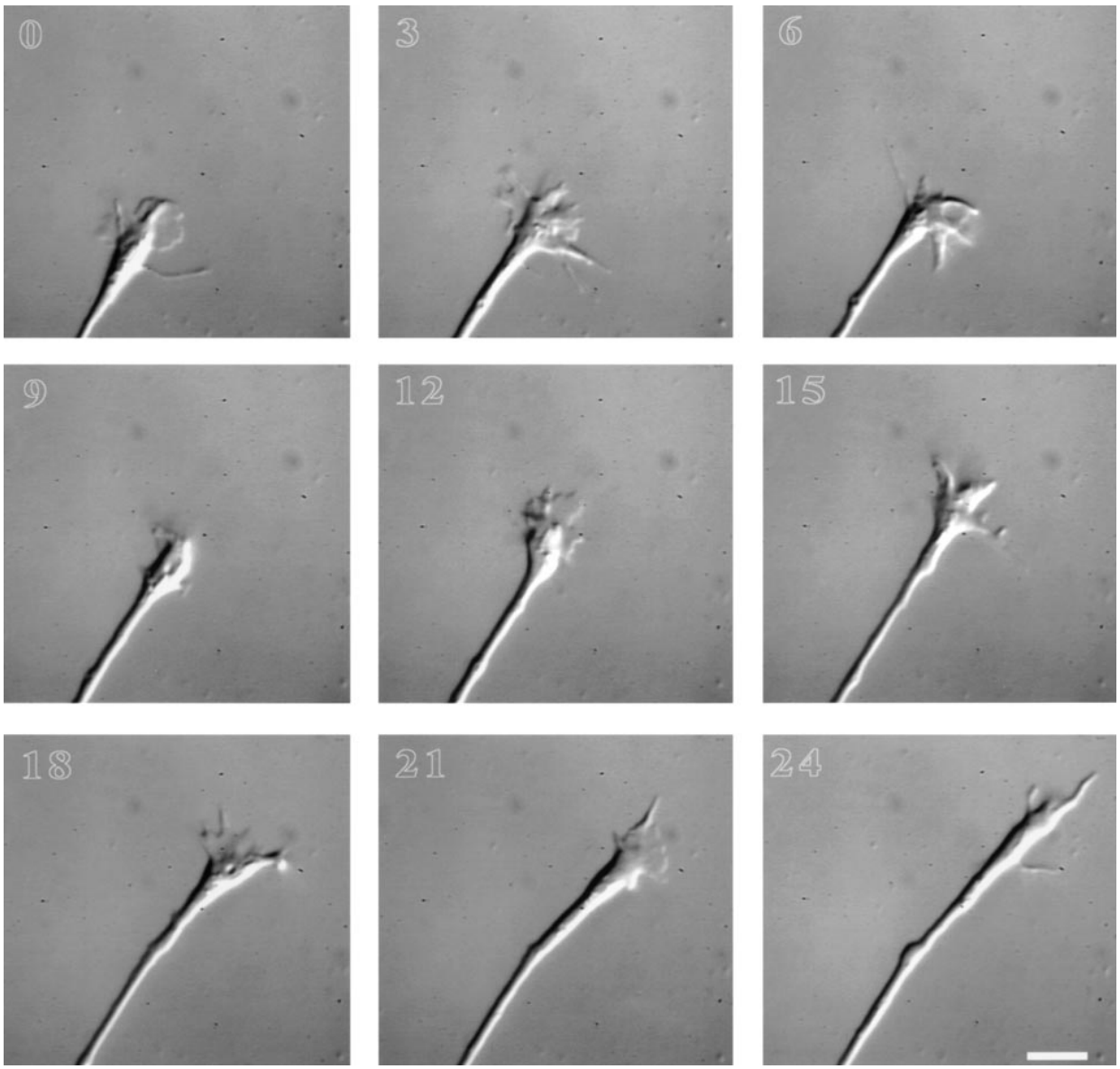

Figure 2. A sequence of VEC-DIC video images of a hippocampal growth cone from E17 wt mice growing on laminin-coated substrate. Images were recorded at 1 d in culture. The total length of the sequence shown is 24 min. Note growth cone extending smoothly with few filopodia, especially few filopodia from the shaft of the neurite. Bar, 5 μm.

trast, only 23% of filopodia along neurites in *Gsn*$^-$ mice retracted within 1 min, and the distribution curve exhibited a greater spread, with 12% of the filopodia persisting for >10 min. Filopodia that persisted for >30 min ($n = 5$) were assigned a 30-min duration, a strategy that would underestimate the number of long duration filopodia in *Gsn*$^-$ mice.

The life histories of 37 wt and 36 *Gsn*$^-$ individual filopodia were studied frame by frame. These histories demonstrated that filopodia went through elongation/pause phases and/or rapid linear retraction phases in both wt (Fig. 7 *A*) and *Gsn*$^-$ (Fig. 7 *B*) mice. During the elongation phase, the rate of elongation in *Gsn*$^-$ mice (0.048 ± 0.005 μm/s) did not significantly differ ($P > 0.05$) from wt mice (0.051 ± 0.004 μm/s; Fig. 7 *C*). But during the linear retraction phase, the rate of retraction was twofold slower ($P < 0.01$) in the *Gsn*$^-$ mice (0.09 ± 0.01 μm/s) compared to wt mice (0.16 ± 0.22 μm/s; Fig. 7 *D*). Furthermore, the dynamic pattern of filopodial retraction differed in *Gsn*$^-$ mice. In wt mice, most of the filopodia retracted in an "all or none" fashion (29/37, 78%); that is, once retraction began it continued until retraction was complete. Only 22% (8/37) of the filopodia retracted in a "staircase" fashion, e.g., cycles of elongation/pause phase followed by a rapid retraction phase. In contrast, the majority of the *Gsn*$^-$ filopodia retracted in a "staircase" fashion (24/36, 67%); the "all or none" behavior accounted for only 33% (12/36) of the events. Even though some filopodia in *Gsn*$^-$ neurites were capable of retracting as fast as those of wt mice, the retraction of the majority of filopodia in *Gsn*$^-$ mice was delayed. A statistical analysis showed that the filopodia from the *Gsn*$^-$ mice tended to pause for significantly

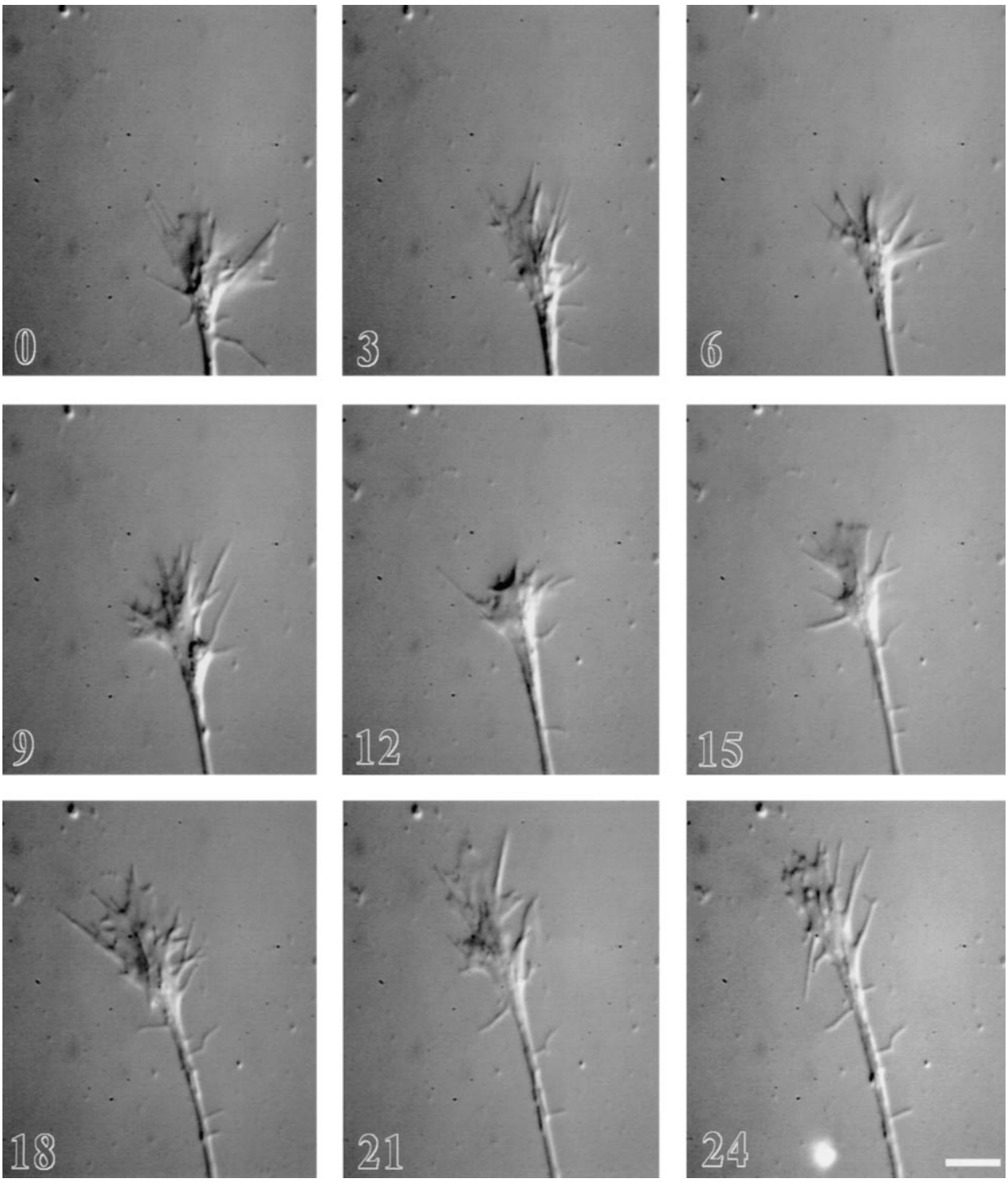

Figure 3. A sequence of VEC-DIC video images of a hippocampal growth cone from E17 *Gsn*$^-$ mice growing on laminin-coated substrate. The images were recorded at 1 d in culture. The total length of the sequence shown is 24 min. Note there were more filopodia in *Gsn*$^-$ mice, especially filopodia on the shaft of the neurite. Bar, 5 μm.

longer time periods than the wt mice (193.19 ± 43.87 s versus 49.32 ± 15.89 s; $P < 0.01$; Fig. 7 *E*).

Analysis of Lamellipodia

As described in invertebrate *Aplysia* neurons (Goldberg and Burmeister, 1986), neurites gradually elongated by cycles of filopodial and lamellipodial protrusions, engorgement of the central domain of the growth cone, and consolidation of neurite. Both the engorgement of the central domain of growth cones and the consolidation of neurites did not appear to differ between wt and experimental mice. The protrusive and retractive activity of growth cone lamellipodia from both wt and *Gsn*$^-$ mice was also ana-

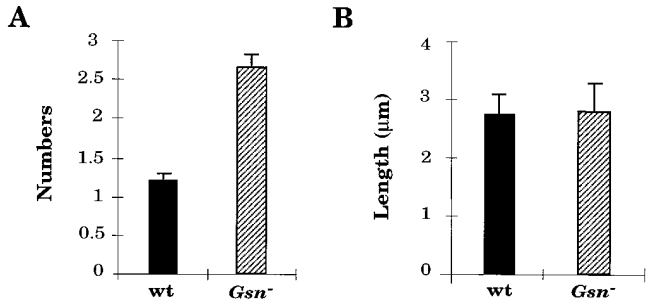

Figure 4. Graphs of length and number of filopodia along neurites from wt mice (*black bars*) and *Gsn*$^-$ mice (*hatched bars*). In *A*, Student *t*-test showed average numbers of filopodia along neurites were more in *Gsn*$^-$ mice ($P < 0.001$). *B* showed that the average length of individual filopodia along neurites in *Gsn*$^-$ mice did not significantly differ from wt mice. Error bar represents standard error.

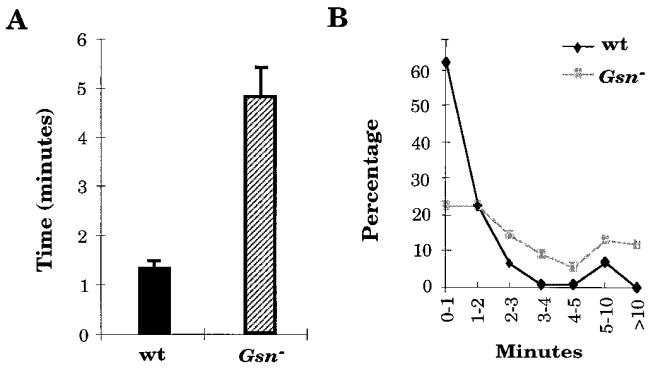

Figure 6. Duration of the extended phase of filopodia along neurites compared between wt mice (*A*, *black*; *B*, *diamond*) and *Gsn*$^-$ mice (*A*, *hatched*; *B*, *square*). Note the average duration from *Gsn*$^-$ mice was significantly longer than that from wt mice ($P < 0.001$ by Student *t*-test) in *A*. Error bar represents standard error. In *B*, x axis represents the duration in minutes, y axis represents the percentage of neurite filopodia in wt mice (*diamond*), and *Gsn*$^-$ mice (*square*) that remained extended for the corresponding time period. Note the marked difference in average, median, and extreme values of filopodial duration in *Gsn*$^-$ versus wt neurons.

lyzed. The changes in the area of lamellipodia at 5 s intervals in wt mice did not significantly differ ($P > 0.05$) from that in *Gsn*$^-$ mice. However, the peripheral domain of hippocampal growth cones grown on a laminin substrate are relatively loosely attached to the substrate compared to the central domain and the shaft.

The analysis of area does not reflect the frequent protrusions and retractions in the vertical plane, because filopodia that extend on the dorsal surface move out of focus over the time periods required to obtain their histories. Therefore an accurate quantitation of filopodial extensions and retractions from the growth cone itself was not technically possible. The difficulty in obtaining dynamic motility measurements in three dimensions may obscure some quantitative differences between wt and *Gsn*$^-$ mice. Nevertheless, there were so few filopodial extensions from the neurite shaft, that markedly increased number of filopodia along the region of the shaft under study had to be a direct reflection of a filopodial retraction defect in the growth cone.

Discussion

The molecular basis for the complexities of neuronal growth cone behavior is beginning to emerge. Highly complex motile phenomena such as turning clearly require ensemble behaviors in which signaling cascades implement the engagement of actin filaments with microtubules as they invade the distal region of the growth cone (Tanaka and Sabry, 1995). Filopodia exhibit a considerably more limited behavioral repertoire consisting of elongation and retraction. These behaviors are mediated by a single cytoskeletal system: actin filaments. Actin-associated proteins are leading candidates to regulate length changes of filopodia. Myosin is involved in the centripetal transport of actin filaments from the leading edge of the growth cone (Lin et al., 1996). In addition, myosin-V, a two-headed unconventional myosin present in growth cones that possesses Ca^{2+}–calmodulin-sensitive mechanochemical activities (Espreafico et al., 1992; Cheney et al., 1993) appears to be involved in filopodial extension (Wang et al., 1996). The local obliteration of this molecule by chromophore-assisted laser inactivation in neuronal growth cones from chick dorsal root ganglia decreased the rate of filopodial extension but did not alter the rate of filopodial retraction, leading us to conclude that extension and re-

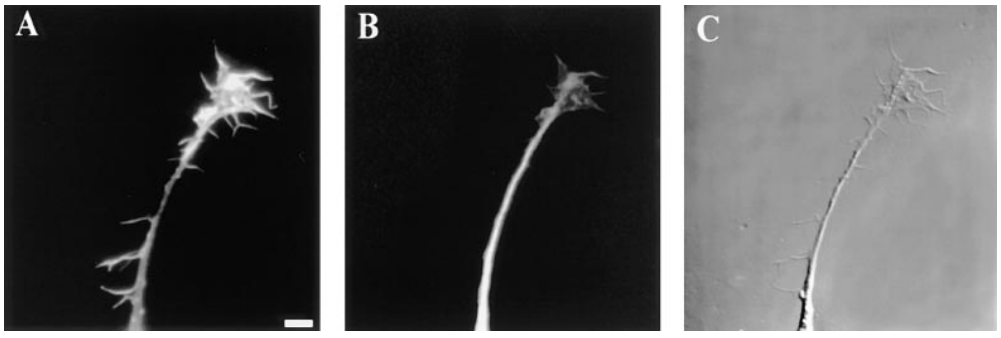

Figure 5. Matching double labeled fluorescence (*A* and *B*) and DIC (*C*) images of a hippocampal neurite and its growth cone from *Gsn*$^-$ mice cultured on laminin substrate. The neuron was fixed at 1 d in culture. *A* shows rhodamine phalloidin fluorescence indicating actin filaments. *B* shows fluorescein fluorescence derived from immunostaining with monoclonal antibody to neuron-specific βIII tubulin. Note that the filopodia along the neurite seen in the matching DIC image contained actin filaments (*A*) but not microtubules (*B*). Also, the cell was positive for neuron-specific βIII tubulin antibody (*B*), confirming the cell's neuronal identity. Bar, 5 μm.

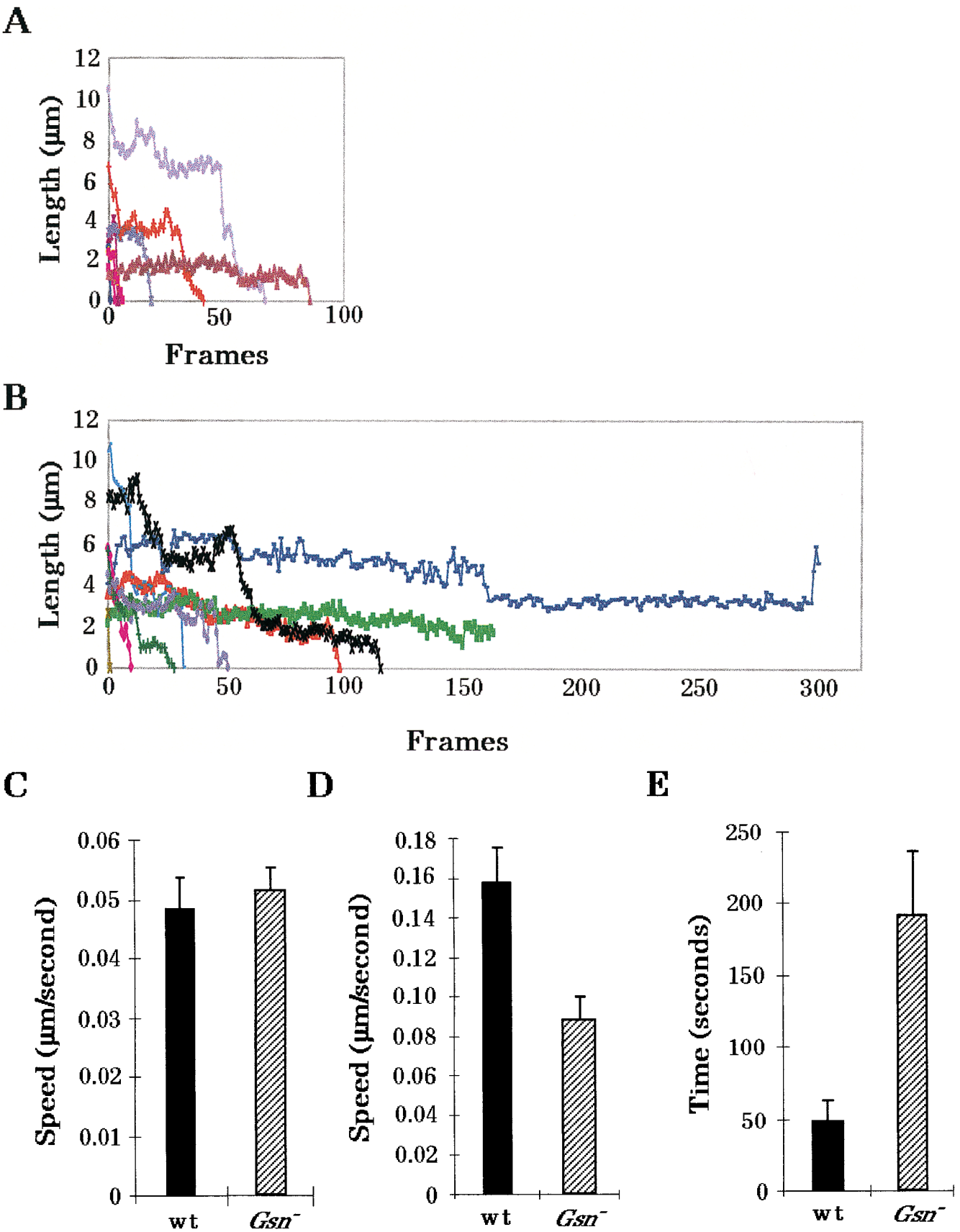

Figure 7. Graphs of life histories of several filopodia along neurites from both wt (*A*) and *Gsn*$^{-}$ mice (*B*). In the graphs, the x axis represents frames, which were 5 s apart, and the y axis represents the length of filopodia at any given time point. (*C*) The rate of elongation during the linear elongation phase was similar in both wt (*black bar*) and *Gsn*$^{-}$ mice (*hatched bar*). (*D*) The rate of retraction during the linear retraction phase was significantly slower ($P < 0.01$, Student *t*-test) in *Gsn*$^{-}$mice (*hatched bar*) compared to wt mice (*black bar*). (*E*) Further, the elongation/pause phase during retraction of filopodia in *Gsn*$^{-}$ mice (*hatched bar*) were significantly longer ($P < 0.01$, Student *t*-test) than those in wt mice (*black bar).* Error bar represents standard error.

traction are independently regulated. Here we have demonstrated that the retraction phase is dependent on gelsolin.

The observation that neurites from *Gsn*$^-$ mice contain many more filopodia than those from wt mice could be explained not only by impaired retraction but also by increased filopodial extrusion. However, an alteration in filopodia extrusion in the *Gsn*$^-$ is unlikely for several reasons. In general, filopodia tend to arise from the leading edge of the growth cone and retract from the base (Lewis and Bridgman, 1992). As was apparent from the time-lapse video images, most of the filopodia on the consolidated segment under study here arose within the growth cone, and as the growth cone advanced, the filopodia remained along the shaft of the newly consolidated neurite. Only rarely did filopodia arise from the shaft. Thus, even if increased filopodial extrusion did occur within the growth cone, these *Gsn*$^-$ filopodia failed to coordinate their retraction with the forward advance of the growth cone. Filopodia from *Gsn*$^-$ mice clearly remained extended for longer times than wt controls. The tendency of *Gsn*$^-$ mice to undergo a stuttering rather than a smooth retraction as well as a less steep slope of retraction (but normal rates of extension) also indicated that a retraction deficit existed. Although *Gsn*$^-$ mice had many more filopodia within the segment under study, the mean lengths of the individual filopodia did not differ from wt mice. Therefore, even though retraction was impaired, after achieving a certain length, filopodia did not continue to elongate. Therefore, filopodial growth is an independent process that is limited by factors other than the onset of retraction.

Gelsolin is the founding member of a family of six mammalian genes/proteins (Schafer and Cooper, 1995) with similar domain structure that are expressed in diverse cell types. All have the ability to bind to actin, and several have actin filament severing activity. Adseverin has the most structural and functional similarity to gelsolin, with Ca^{2+}-regulated actin filament severing activity, and its actin filament binding is inhibited by PPI, as well as phosphatidylinositol and phosphatidylserine (Maekawa and Sakai, 1990). Moreover, adseverin has been reported to be expressed in mammalian brain tissues, though its precise cellular localization is unknown (Tchakarov et al., 1990). Those facets of growth cone motility that are normal in the *Gsn*$^-$ mice may be due to the presence of adseverin in these regions or the actin regulatory protein ADF (Bamberg and Bray, 1987). ADF has complex effects on actin filaments that are dependent upon conditions of pH and ionic strength (Moon and Drubin, 1995), so that it may function to sever actin filaments and regulate actin dynamics in neuronal growth cones.

Given the filopodial retraction rates observed here and the known rates of actin depolymerization, it is unlikely that the retraction defect observed in the *Gsn*$^-$ mice is due to a dysfunction involving an effect of gelsolin on the depolymerization of actin filaments. Filopodial retraction occurs at rates from 0.09 to 0.16 μm/s, which corresponds to 32–59 monomers/s, based on the fact that 1 μm of polymerized actin contains 370 actin monomers (Hanson and Lowy, 1963). Since the most rapid off rate of actin monomers from an actin filament end is 0.18 monomers/s (Pollard, 1986), simple monomer dissociation cannot explain filopodia retraction. More likely, the nature of the dysfunction resulting in impaired filopodial retraction is due to a loss of the actin filament-severing activity of gelsolin. One potentially critical functional site may be the base of the filopodia where gelsolin may sever active filaments and thereby allow a coordinately engaged myosin motor to slide the filaments through the proximal actin mesh. In this model, filopodia remain extended as long as actin filaments at their base remain intact and prevent sliding of filaments into the shaft or growth cone. Few studies directly visualize well-preserved actin filaments in neuronal filopodia. In one study, negatively stained and freeze-etched EM of permeabilized growth cone from rat superior cervical ganglia explants contained tightly packed actin bundles in the core of their filopodia (Lewis and Bridgman, 1992). In quiescent filopodia, the filament bundles ended at the base of the filopodia; in contrast, in putatively active regions the bundles extended into the lamellar region. Although our experiments do not directly address filopodia protrusions from the growth cone itself, our model would predict that gelsolin severs actin filaments at the point where they extend into the lamella. Gelsolin may also release actin from sites of tension along the filopodia, where actin is linked to molecules such as vinculin and talin, which indirectly interact with the substrate. These explanations are consistent with the stuttering retraction pattern seen in the *Gsn*$^-$ mice and with the variation in retraction times, which may arise due to the quite different detailed organizational patterns of actin filaments around individual filopodia.

The tendency of the filopodia to remain along the consolidated neurite once the growth cone has advanced suggests a forward flow within the central cytoplasm while the filopodia hold on to their attachment sites to create the torque for the advancing growth cone. Because substrate interactions dominate neuronal cell growth under tissue culture conditions, neurons from *Gsn*$^-$ mice, placed in tissue culture, may enhance a phenotype that is less apparent *in vivo*. The *Gsn*$^-$ mice do not display any obvious neurologic deficits, but they have not been subjected to detailed anatomic and behavioral examination. The approach taken here of knocking out specific genes encoding growth cone proteins, may allow the systematic assignment of function to the many components of the growth cone.

We would like to thank Adriana Ferreira for assistance with some of the early observations and Philip G. Allen for helpful discussions.

M. Lu was supported by National Institutes of Health training grant (NS07009-21) through Dr. G. Strichartz. This work was supported by National Institutes of Health grants NS29031 (K.S. Kosik) and HL54188 and HL48743 (D.J. Kwiatkowski).

Received for publication 12 March 1997 and in revised form 8 July 1997.

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