5-22-2003

Filopodial Calcium Transients Regulate Growth Cone Motility and Guidance through Local Activation of Calpain

Estuardo Robles
University of Wisconsin-Madison, erobles@smith.edu

Anna Huttenlocher
University of Wisconsin-Madison

Timothy M. Gomez
University of Wisconsin-Madison

Neuron, Vol. 38, 597–609, May 22, 2003, Copyright ©2003 by Cell Press

Filopodial Calcium Transients Regulate Growth Cone Motility and Guidance through Local Activation of Calpain

Estuardo Robles,[1,2] Anna Huttenlocher,[3] and Timothy M. Gomez[1,2,*]
[1]Department of Anatomy
[2]Neuroscience Training Program
[3]Department of Pharmacology
University of Wisconsin
Madison, Wisconsin 53713

Summary

Spontaneous intracellular calcium ($[Ca^{2+}]_i$) transients in growth cone filopodia reduce filopodial motility, slow neurite outgrowth, and promote turning when generated asymmetrically; however, the downstream effectors of these Ca^{2+}-dependent behaviors are unknown. We report that Ca^{2+} transients in filopodia activate the intracellular protease calpain, which slows neurite outgrowth and promotes repulsive growth cone turning upon local activation. Active calpain alters the balance between tyrosine kinase and phosphatase activities in filopodia, resulting in a net decrease in tyrosine phosphorylation, which mediates both filopodial stabilization and reduced lamellipodial protrusion. Our findings indicate that locally generated Ca^{2+} signals repel axon outgrowth through calpain-dependent regulation of phosphotyrosine signaling at integrin-mediated adhesion sites.

Introduction

The formation of precise neural networks during development requires growth cones at the tips of elongating neurites to accurately respond to extracellular guidance cues (Tessier-Lavigne and Goodman, 1996). Filopodia are the antenna-like protrusions that extend from the growth cone and sample the extracellular environment for instructive cues (Kater and Rehder, 1995). In vivo studies have demonstrated that intact filopodia are necessary for proper axonal pathfinding and accurate synaptic targeting (Bentley, 1987; Chien et al., 1993). Filopodia also function during growth cone chemotropism, as turning responses are preceded by an increase in the number of filopodia extending toward the source of a chemoattractant (Zheng et al., 1996). The role of individual filopodia as sensory structures has been established by examining neurotransmitter-evoked Ca^{2+} elevations in isolated filopodia (Davenport et al., 1993). These findings indicate that individual filopodia express the molecular components necessary to transmit signals to the central region of the growth cone where they likely integrate to regulate growth cone behavior. These lines of evidence support an instructive role for filopodia as the initial source of intracellular signals following activation of surface-bound receptors by guidance cues.

Intracellular Ca^{2+} dynamics regulate many aspects of neuronal development, including differentiation, axon guidance, and synaptic plasticity (Gomez and Spitzer, 2000; Spitzer et al., 2000; Zucker and Regehr, 2002). For example, spontaneous Ca^{2+} transients in growth cones slow the rate of axon growth in vitro and in vivo (Gu et al., 1994; Gomez et al., 1995; Gomez and Spitzer, 1999). Importantly, many extracellular molecules affect neurite outgrowth by altering intracellular Ca^{2+} dynamics, including extracellular matrix (ECM) proteins (Snow et al., 1994; Kuhn et al., 1998), cell adhesion molecules (Bixby et al., 1994; Al-Mohanna et al., 1996), and myelin-associated glycoproteins (Bandtlow et al., 1993), as well as diffusible factors like netrin-1 (Hong et al., 2000), semaphorins (Behar et al., 1999), neurotrophins (Wang and Zheng, 1998), and neurotransmitters (Zheng et al., 1994). In addition, a gradient of $[Ca^{2+}]_i$ across the growth cone was shown to be necessary for chemotropic turning in an extracellular gradient of guidance molecules (Hong et al., 2000). Laser-induced photolysis of caged Ca^{2+} confirmed that a localized gradient of Ca^{2+} is sufficient to stimulate growth cone turning independent of extracellular guidance cues (Zheng, 2000). Despite many studies linking $[Ca^{2+}]_i$ changes to various growth cone behaviors, the specific downstream effectors that transduce Ca^{2+} signals into corresponding guidance decisions remain largely unidentified.

Our previous work demonstrated that growth cone filopodia exhibit brief, locally generated Ca^{2+} transients at sites of integrin receptor clusters (Gomez et al., 2001). The frequency of filopodial transients is substratum dependent and correlates with the strength of adhesion. Local Ca^{2+} signals function to reduce filopodial motility, which slows neurite outgrowth on substrata that stimulate high-frequency transients. In addition, transients generated asymmetrically on one side of the growth cone are sufficient to promote turning away from the site of Ca^{2+} activity. These findings suggest that filopodial interaction with repulsive guidance molecules in vivo may stimulate Ca^{2+} transients at their tips, leading to stabilization and growth cone turning. Due to the large surface to volume ratio in filopodia, this structure may represent a site where Ca^{2+} influx locally modulates adhesion and cytoskeletal dynamics, thereby regulating growth cone motility and guidance. The cellular targets of these frequency-encoded Ca^{2+} signals are unknown, and it remains unclear how this local activity stabilizes filopodia and promotes repulsive turning.

Neurons express many Ca^{2+}-regulated proteins that could mediate the observed effects of filopodial Ca^{2+} transients (Janmey, 1994). A potential candidate is the Ca^{2+}-activated protease calpain, which is known to have profound effects on cell motility. Calpains are neutral cysteine proteases activated by elevated intracellular Ca^{2+} (Sato and Kawashima, 2001). Upon activation, calpain cleaves key structural and signaling proteins in a limited manner, serving to modulate protein function. Inhibition of calpain in certain motile nonneuronal cells leads to impaired retraction of the trailing edge and reduced migration, suggesting that calpain normally functions to promote adhesion complex disassembly (Huttenlocher et al., 1997). In support of such a model,

*Correspondence: tmgomez@facstaff.wisc.edu

several known calpain substrates have been implicated in the regulation of cell adhesion and motility; these include protein kinases (PKC, FAK, and Src), phosphatases (calcineurin, PTP-1B), cytoskeleton-associated proteins (paxillin, α-actinin, and talin), and adhesion molecules (β-integrins). Although previous studies have shown that calpain functions in nerve growth cones (Song et al., 1994; Shea et al., 1995; Gitler and Spira, 1998), the role of calpain in growth cone motility and guidance downstream of naturally occurring Ca^{2+} signals has not been described.

We report that filopodial Ca^{2+} signals mediate changes in growth cone adhesion and motility through local calpain activation. Contrary to the previously described function of calpain in cell motility, we find that calpain activity reduces filopodial motility and slows process outgrowth. If activated locally on one side of a growth cone by high-frequency Ca^{2+} transients, calpain stabilizes filopodia and reduces veil protrusion, leading to repulsive turning away from this site. This local regulation of adhesion is mediated by a calpain-dependent shift in the balance between tyrosine kinase and phosphatase activities.

Results

Xenopus Spinal Neurons Express μ-Calpain

Of the 14 known members of the calpain family of proteases, μ-calpain is the predominant form expressed in neurons (Sato and Kawashima, 2001). The two principal calpain isoforms, μ- and M-calpain, are heterodimers consisting of a common regulatory domain and a catalytic domain that confers substrate selectivity (Sato and Kawashima, 2001). Using a μ-calpain-specific antibody, we first tested whether *Xenopus* spinal neurons express this isoform of the calpain catalytic subunit using immunocytochemistry and Western blotting. We find that all *Xenopus* spinal neurons examined in vitro express μ-calpain in their axons, growth cones, and individual filopodia (Figure 1A) regardless of culture substratum (data not shown). The expression pattern of μ-calpain is unchanged after chronic inhibition with calpastatin peptide (CPI, 1 μM; data not shown). This peptide inhibitor is analogous to the inhibitory domain of rat calpastatin, an endogenous protein inhibitor of both μ- and M-calpains. The uniform expression of μ-calpain within growth cones and filopodia suggests its activity is locally regulated within microdomains of elevated Ca^{2+}.

Calpain Inhibition Increases Filopodial Motility and Promotes Neurite Outgrowth

To assess the role of calpain in regulating filopodial adhesion and motility, we examined cultured spinal neurons under conditions that promote different frequencies of Ca^{2+} transients. We previously identified the ECM molecule tenascin (TN) as a protein that stimulates high-frequency filopodial Ca^{2+} transients (4.5 ± 0.5/min) (Gomez et al., 2001). In consequence, neurons on TN extend characteristically short neurites with stable, highly adherent growth cones and filopodia. By contrast, laminin (LN), another integrin binding ECM protein, stimulates a low frequency of filopodial Ca^{2+} transients (1.1 ± 0.2/min) and promotes extensive axon outgrowth. Poly-d-lysine (PDL), though known to stimulate high-frequency Ca^{2+} transients (5.1 ± 0.6/min), is not an integrin-dependent substratum. To explore the relationship between calpain activity and growth cone motility, we examined the effects of calpain inhibition on substratum-dependent neurite outgrowth. If calpain stabilizes filopodia downstream of Ca^{2+} transients, then inhibition of calpain should selectively promote neurite outgrowth on substrata that produce high-frequency Ca^{2+} transients. We find that inhibition of calpain with CPI selectively stimulates neurite outgrowth on TN in a dose-dependent manner (Figure 1B). Similar results were obtained using a second peptide inhibitor of calpain (ALLM; Figure 1B). In contrast, neurons cultured on LN exhibited no significant change in neurite lengths at any concentration of calpain inhibitor. Interestingly, neurite lengths on PDL, a potent filopodial transient promoting substratum, were not significantly affected by calpain inhibition, suggesting that Ca^{2+}-dependent reduction of growth may require downstream modulation of an integrin-dependent process.

To determine whether calpain inhibition promotes outgrowth on TN by increasing filopodial motility, we imaged live growth cones on TN treated with CPI. Filopodial stability was assayed by measuring the lifetime of filopodia, which corresponds to relative levels of adhesion (Bray and Chapman, 1985). We find that chronic inhibition of calpain activity with 1 μM CPI over 18–24 hr in culture results in a significant reduction in the average filopodial lifetime (Figure 1C). To confirm that this reflects a change in the motility of individual filopodia, neurons on TN were examined with time lapse imaging before and after acute application of 1 μM CPI for 30 min. Acute calpain inhibition resulted in a similar decrease in average filopodial lifetime within 30–60 min (Figure 1C). A large component of the effect of calpain inhibition on lifetime is due to a reduction in the number of stable filopodia (lifetime > 30 min). Both chronic and acute calpain inhibition resulted in significant decreases in the percentage of stable filopodia (69% versus 29% and 60% versus 44%, respectively). Together, these results confirm that calpain proteolysis functions to increase the stability of growth cone filopodia and reduce neurite outgrowth on TN.

Calpain Activity Is Both Ca^{2+} and Substratum Dependent

In order to directly assess calpain activity under conditions that regulate the frequency of filopodial Ca^{2+} transients, we used the fluorogenic calpain substrate CMAC, t-BOC-Leu-Met (t-BOC), as an in vitro bioassay for calpain proteolysis. The uncleaved calpain substrate is nonfluorescent and passes freely into cells. Once internalized, active calpain specifically cleaves the amino acid linker, unquenching the fluorescence of the coumarin fluorophore. Consistent with previous studies in myotubes (Alderton and Steinhardt, 2000), *Xenopus* neurons exhibit a linear rise in fluorescence during the first 15–20 min following introduction of 10 μM t-BOC into the culture medium. However, we find that the rate of fluorescence change is dependent on the culture substrata and greatest for neurons grown on TN (Figures 2A and 2B). This suggests that increased calpain activity on TN

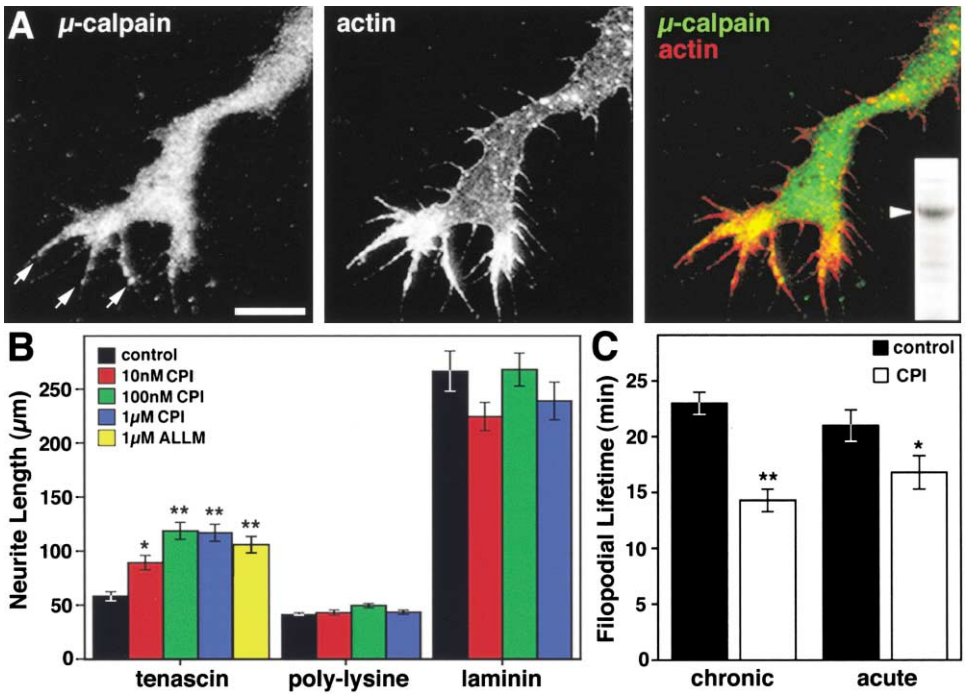

Figure 1. Calpain Expressed in *Xenopus* Neurons Decreases Growth Cone Motility

(A) Immunofluorescent labeling for μ-calpain (green) and actin filaments with Alexa 546-phalloidin (red) in growth cones reveal that μ-calpain is expressed homogeneously throughout the cytoplasm and in filopodia (arrows), whereas actin filaments are localized primarily to the leading edge. Inset: Western blot analysis of stage 22 *Xenopus* neural tubes. A band at approximately 80 kDa (arrowhead) corresponds to the predicted size of μ-calpain.

(B) Inhibition of calpain with CPI leads to a dose-dependent increase in neurite outgrowth on TN without affecting growth on LN or PDL ($n > 100$ neurites for each condition). ALLM, a second inhibitor of μ- and M-calpain, also promotes growth on TN.

(C) Inhibition of calpain leads to increased filopodial motility, as determined by decreased filopodial lifetime during chronic and acute CPI treatment of neurons on TN ($n > 65$ filopodia for each condition). $^{**}p < 0.001$; $^{*}p < 0.05$. Scale, 5 μm.

may be due to higher frequencies of filopodial Ca^{2+} transients. To verify that intracellular Ca^{2+} is sufficient to induce calpain proteolysis, neurons on LN were depolarized with 40 mM KCl during t-BOC imaging. Within 2 min of KCl stimulation, the rate of calpain proteolysis transiently increases and then subsides to a more stable rate. Although KCL depolarization activates calpain, it likely stimulates other Ca^{2+}-dependent targets, as this treatment induces the temporary collapse of filopodia and veil, which is not blocked by calpain inhibition (data not shown). These results indicate that calpain proteolysis in *Xenopus* growth cones is stimulated by elevated $[Ca^{2+}]_i$, suggesting that calpain may be locally activated by filopodial Ca^{2+} transients.

To determine the specificity of t-BOC as a calpain substrate, we measured its fluorescence in neurons on TN in the presence of 1 μM CPI. Inhibition of calpain reduced t-BOC fluorescence by 44% ± 5% as compared to control over the same incubation period ($p < 0.05$; Figure 2A). Incomplete inhibition of t-BOC fluorogenesis by CPI suggests either nonspecific substrate cleavage or partial calpain inhibition. Our data support the former possibility, as the percent reduction in t-BOC fluorogenesis upon pharmacological inhibition of calpain is similar to that observed upon abolition of Ca^{2+} transients. Perfusion of Ca^{2+}-free media (CFM) or chelation of intracellular Ca^{2+} with 200 nM BAPTA-AM both decreased t-BOC fluorescence to levels observed in the presence of CPI (45% ± 3% and 49% ± 5% reduction, respectively; $p < 0.05$; Figure 2B). These data are consistent with the observation that filopodia on TN become more motile after calpain inhibition (Figure 1C) and Ca^{2+} chelation (Gomez, et al., 2001). However, calpain in growth cones on TN is not maximally active, as depolarization with KCL further increased t-BOC fluorogenesis. Neurons on LN exhibit t-BOC fluorogenesis comparable to levels observed in the presence of CPI, suggesting calpain is not active in growth cones on LN (Figures 2A and 2B). Together these findings indicate that substratum-dependent Ca^{2+} signaling in filopodia may increase adhesion by activating calpain. Interestingly, neurons on PDL, an adhesive substratum that stimulates a high frequency of filopodial Ca^{2+} transients (Gomez et al., 2001), exhibit high levels of calpain proteolysis (Figure 2B), suggesting that Ca^{2+} influx independent of integrin activation is sufficient to activate calpain in the absence of downstream targets.

Ca^{2+} Transients in Growth Cones Are Sufficient to Activate Calpain Proteolysis

To determine if $[Ca^{2+}]_i$ transients are sufficient to stimulate calpain proteolysis, we used caged Ca^{2+} (NP-EGTA) to impose Ca^{2+} elevations of defined frequency in growth cones while measuring t-BOC fluorogenesis. Growth cones on LN were loaded with NP-EGTA and exposed to 300 ms pulses of UV light (360 ± 25 nm,

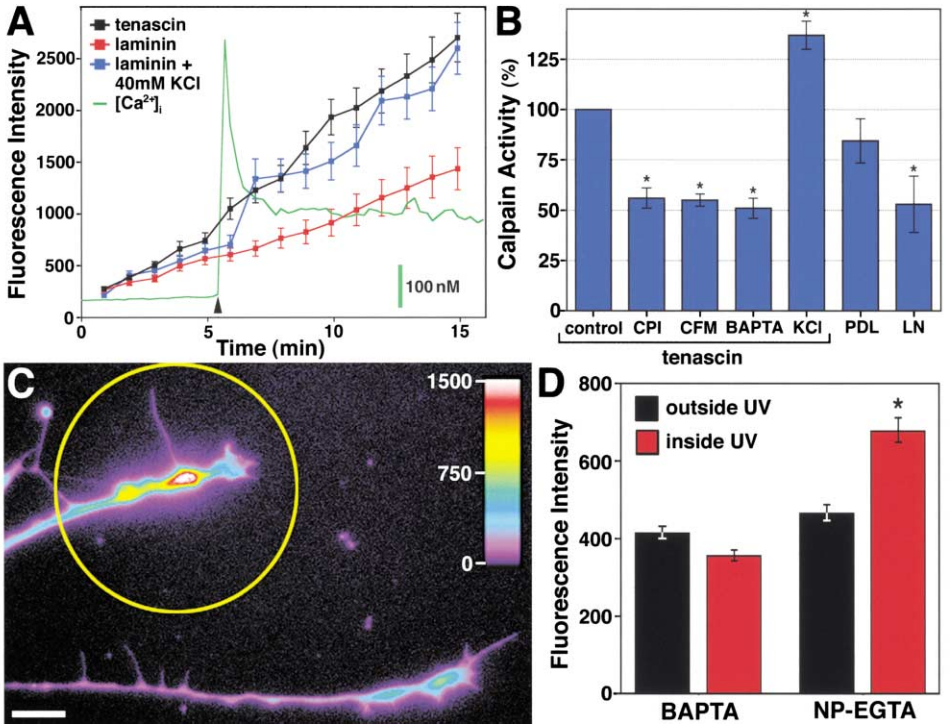

Figure 2. Ca^{2+} Transients and Culture Substratum Regulate Calpain Activity in Growth Cones

(A) Measurement of calpain proteolysis using t-BOC, a fluorogenic calpain substrate, reveals that calpain activity in growth cones is higher on TN as compared to LN. Fluorescent images were captured at 1 min intervals following addition of 10 μM t-BOC to the culture medium. Depolarization with 40 mM KCl accelerates calpain proteolysis in growth cones on LN, confirming that calpain activity is regulated by $[Ca^{2+}]_i$.

(B) Fluorescence intensity values, measured 15–20 min after t-BOC addition, expressed as percent induction of calpain activity relative to values obtained from growth cones on TN in control media (2 mM Ca^{2+}). $n > 50$ growth cones for each condition. t-BOC fluorogenesis is reduced on TN by direct inhibition of calpain with CPI and by blocking Ca^{2+} transients, but further increased by depolarization with 40 mM KCl. Growth cones on PDL exhibited calpain activity near that on TN; however, on LN, calpain activity is similar to that observed during calpain inhibition.

(C) Pseudocolor coumarin fluorescence image obtained following 15 min of imposed Ca^{2+} transients generated in a single growth cone by photorelease of caged Ca^{2+}. Yellow circle shows the area exposed to pulsed UV light (300 ms duration, 6/min). Pseudocolor scale represents relative fluorescence intensity.

(D) Summary of t-BOC proteolysis in experimental and control growth cones ($n \geq 15$ growth cones for each condition). Growth cones loaded with NP-EGTA and exposed to pulsed UV light exhibit significantly greater coumarin fluorescence compared to controls (*$p < 0.05$). In contrast, control neurons loaded with BAPTA-AM and exposed to pulsed UV light exhibit reduced t-BOC fluorescence, possibly due to photo-bleaching of the coumarin fluorophore. Scale, 10 μm.

6/min) for 15 min and compared with NP-EGTA-loaded, unphotolyzed control growth cones. To control for nonspecific effects of UV light and Ca^{2+} chelation, control cultures were loaded with an equal concentration of the photo-stable Ca^{2+} chelator, BAPTA-AM. UV exposure in all experiments was confined to growth cones, filopodia, and proximal neurites and was begun immediately following t-BOC application. After 15 min of imposed Ca^{2+} transients, t-BOC fluorescence in NP-EGTA-loaded growth cones was significantly greater than controls (Figures 2C and 2D). Although Ca^{2+} transients were not restricted to filopodia, these results confirm that imposed Ca^{2+} elevations with similar kinetics to spontaneous transients in filopodia are sufficient to stimulate increased levels of calpain proteolysis.

Local Filopodial Ca^{2+} Transients Promote Growth Cone Turning by Regulating Filopodial Adhesion and Cytoskeletal Remodeling

We have previously reported that Ca^{2+} transients generated in filopodia on one side of a growth cone promote repulsive turning (Gomez et al., 2001); however, the detailed behavior of growth cones during this turning response has not been examined. Local release of caged Ca^{2+} was used to impose high-frequency Ca^{2+} transients in specific filopodia of growth cones on LN. Growth cones loaded with NP-EGTA were positioned so their forward-projecting filopodia extend into a 12.5 μm region of pulsed UV light. As filopodia enter this region, Ca^{2+} transients are generated at a frequency of 6/min, which stimulates turning away from the site of local release. Close examination of turning behaviors reveals that a subset of filopodia exposed to Ca^{2+} transients become stabilized within the UV region, followed by a rapid loss of veil surrounding adherent filopodia and asymmetric protrusion of lamella opposite the site of Ca^{2+} transients (Figure 3). During morphologic remodeling, filopodia often remain stabilized within the region of UV photolysis, potentially providing an instructive signal guiding the growth cone away from and beyond the region of Ca^{2+} signaling. Inhibition of veil advance along adherent filopodia is similar to observations of chick dorsal root ganglion growth cones turning away from posterior sclerotome cells (Steketee and Tosney, 1999).

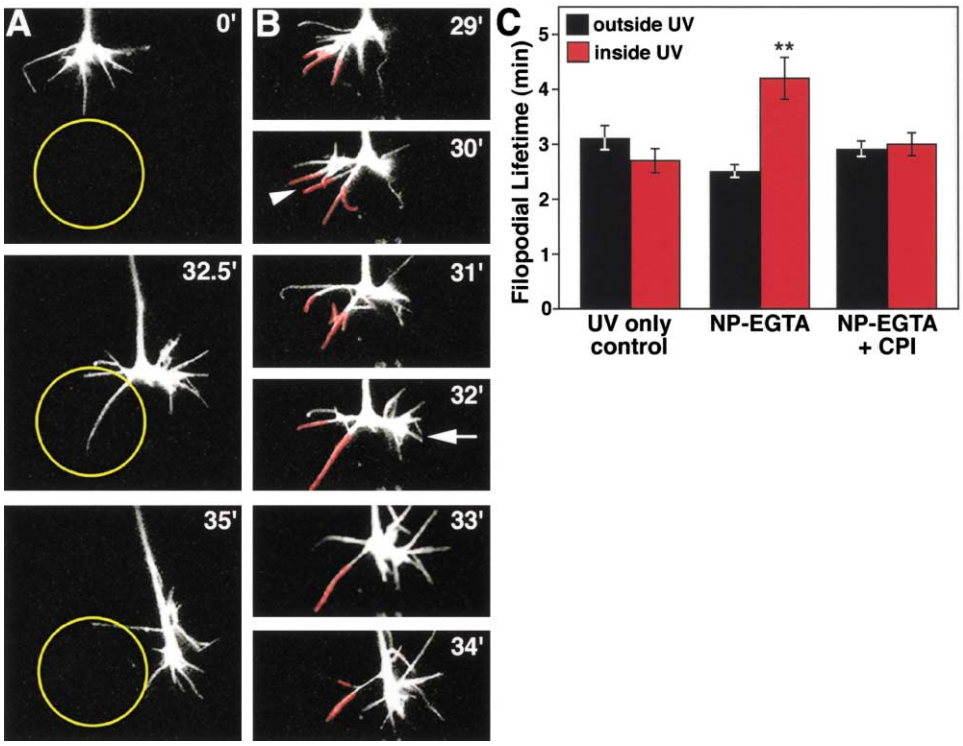

Figure 3. Imposed Ca^{2+} Transients Stabilize Filopodia and Promote Growth Cone Turning by Inhibiting Veil Protrusion

(A) Fluorescent images of a GFP-actin expressing growth cone turning away from the site of imposed filopodial Ca^{2+} transients. Yellow circle (12.5 μm diameter) denotes area exposed to UV light pulses (100 ms duration, 6/min). This growth cone is deflected 34° from its original trajectory over the 35 min sequence.

(B) Growth cone turning occurs by veil retraction (arrowhead) at the site of imposed Ca^{2+} transients and veil protrusion (arrow) on the side opposite imposed Ca^{2+} transients. Regions pseudocolored red indicate areas exposed to UV photolysis. Note that a single filopodium is stabilized within the UV region throughout the turning response.

(C) Locally generated Ca^{2+} transients selectively stabilize filopodia within the region exposed to UV light. Filopodial lifetime is increased for filopodia exposed to Ca^{2+} transients at a frequency of 6/min as compared to those outside the uncaging region (**$p < 0.001$). Unloaded control growth cones exhibit no significant difference in filopodial lifetimes inside and outside of the pulsed UV spot. Inhibition of calpain with 1 μM CPI abolishes the stabilizing effects of imposed Ca^{2+} transients on filopodial motility.

To confirm that imposed Ca^{2+} transients stabilize filopodia, we compared average lifetimes of filopodia inside and outside the region of UV exposure. Unloaded growth cones examined to control for potential effects of UV light on filopodial stability exhibited no significant difference in average lifetime inside (2.7 ± 0.2 min, $n = 74$) versus those outside (3.1 ± 0.2 min, $n = 96$; Figure 3C) the UV region. In contrast, filopodia loaded with NP-EGTA exhibit a 1.7-fold increase in the average lifetime within the UV region (4.1 ± 0.4 min inside versus 2.5 ± 0.1 min outside, $n = 58$ and 145, respectively, $p < 0.001$). Note that filopodia exhibit a modest but significant decrease in average lifetime when loaded with NP-EGTA as compared to unloaded control filopodia, possibly due to the Ca^{2+} buffering effects of unphotolysed NP-EGTA. These results confirm that Ca^{2+} transients are sufficient to increase filopodial adhesion and suggest they may locally activate calpain to regulate the stability of adhesion complexes as well as globally to stimulate contralateral veil protrusion.

Calpain Function Is Required for Ca^{2+}-Dependent Growth Cone Turning

To determine whether calpain activity is required for growth cone turning downstream of filopodial Ca^{2+} transients, we tested the response of growth cones to local release of caged Ca^{2+} in the presence of calpain inhibitor. These experiments were performed both on individual growth cones before and after calpain inhibition (Figure 4A; $n = 4$) and in separate control and experimental conditions (Figure 4B). Prior to CPI treatment, individual growth cones turn away from the site of imposed Ca^{2+} release, as previously demonstrated (Gomez et al., 2001). However, after 30 min incubation with calpain inhibitor (CPI), growth cones that had previously turned extend into the UV region with no significant deflection (Figure 4A). Results from 55 growth cones show that the average turning angles of unloaded growth cones (13° ± 1.5°; $n = 20$) was significantly less than NP-EGTA-loaded growth cones (24.3° ± 2.8°; $n = 19$; $p < 0.05$) but not significantly different from NP-EGTA-loaded and calpain-inhibited growth cones (15° ± 1.7°; $n = 16$; $p = 0.383$). Filopodial lifetime analysis confirmed that in addition to blocking repulsive growth cone turning, calpain inhibition abolished the selective stabilization of filopodia exposed to local Ca^{2+} transients (3.0 ± 0.2 min inside versus 2.9 ± 0.2 min outside, $n = 53$ and 85, respectively, $p = 0.51$; Figure 3C). These results confirm that local activation of calpain is specifically required for Ca^{2+}-dependent growth cone turning and suggest

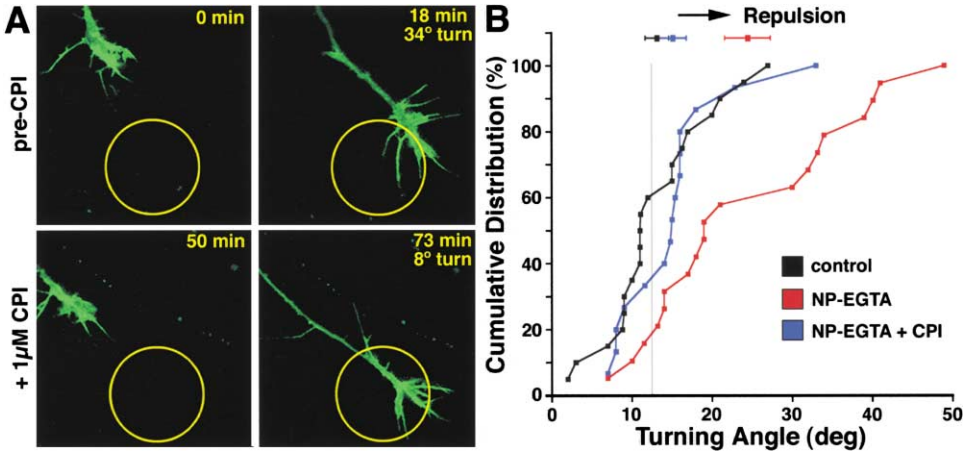

Figure 4. Calpain Activity Is Necessary for Ca^{2+} Transient-Mediated Growth Cone Turning

(A) Time lapse images of an NP-EGTA-loaded growth cone extending toward a region of pulsed UV light before and after inhibition of calpain with CPI. Prior to calpain inhibition, this growth cone turns away from the site of locally imposed Ca^{2+} transients; however, after CPI treatment, it is no longer repelled by imposed Ca^{2+} transients.

(B) Cumulative distribution of turning angles for control, NP-EGTA-loaded, and NP-EGTA+CPI growth cones. The average turning angles for the three conditions are indicated at the top of the graph. Turning angles of control and NP-EGTA+CPI-loaded growth cones are not significantly different from the average turning angle of NP-EGTA-loaded growth cones not exposed to UV light (vertical gray line). Data was obtained from a minimum of 16 growth cones in each condition.

that local calpain activation is necessary to stimulate both filopodial adhesion and asymmetric veil protrusion.

Calpain Activity Regulates Phosphotyrosine Levels in Growth Cones and Filopodia

The behaviors observed during Ca^{2+}/calpain-dependent repulsive turning suggest that signals mediating adhesion complex turnover may also regulate the cytoskeletal changes underlying growth cone guidance. However, it is unclear what signaling mechanisms transduce local Ca^{2+} transients into changes in cell motility downstream of calpain activation. Work in motile nonneuronal cells has demonstrated that tyrosine phosphorylation of signaling proteins within adhesion complexes can control cell motility (Zamir et al., 2000). Calpain is known to regulate the activity of many signaling proteins associated with focal contacts and translocates to integrin-containing adhesion complexes upon activation (Glading et al., 2002). Calpain proteolysis regulates the function of protein tyrosine kinases (PTKs) such as focal-adhesion kinase (FAK) and Src (Cooray et al., 1996; Oda et al., 1993), as well as phosphatases, including PTP-1B (Frangioni et al., 1993). Furthermore, active calpain alters the balance between tyrosine kinase and phosphatase activities in Ca^{2+} ionophore-stimulated platelets, resulting in decreased phosphotyrosine (PY) levels (Pain et al., 1999). These findings suggest that calpain activity mediated by Ca^{2+} transients in growth cone filopodia may regulate the turnover of adhesion sites by modulating the phosphorylation state of focal contact proteins.

To determine if calpain activity correlates with PY levels in neurons, we used immunocytochemistry to compare growth cone PY levels on substrata that activate calpain to different degrees. On LN, where calpain activity is low, growth cones and filopodia exhibited intense PY staining (Figure 5A). Acute inhibition of tyrosine kinase activity with Herbimycin A (HA) reduced PY staining (data not shown), indicating the specificity of our antibody. Conversely, on TN (Figure 5B), where calpain activity is high, PY levels were low compared to LN (reduced 2.8- and 2-fold in filopodia and growth cones, respectively). Importantly, both chelation of Ca^{2+} transients and direct inhibition of calpain activity with CPI or ALLM (Figures 5C, 5D, 5J, and 5K; ALLM not shown) increased PY levels in growth cones on TN, although not to the level observed on LN, suggesting calpain-independent factors also regulate tyrosine phosphorylation on these substrata. However, behavioral assays suggest that the Ca^{2+}-activated, calpain-dependent component of tyrosine dephosphorylation may be sufficient to mediate the increased filopodial stability observed on TN (Figure 1) or during imposed high-frequency Ca^{2+} transients (Figure 3). Together these data suggest that tyrosine phosphorylation in growth cones is regulated by substratum-dependent calpain activity downstream of filopodial Ca^{2+} transients.

Calpain is known to cleave pp60Src (Oda et al., 1993), which plays a central role in adhesion site turnover and cell motility via phosphorylation of targets such as FAK. To explore the possibility that calpain activity modulates Src tyrosine kinase signaling, we used a phospho-specific antibody that recognizes the Y418 autophosphorylation site of Src family kinases. Autophosphorylation of this tyrosine residue within the kinase activation loop is a marker of Src activation (Pellicena and Miller, 2002). Consistent with our PY observations, we find increased active phospho-Src in neurons on LN as compared to TN (Figures 5E and 5F). Growth cones and filopodia on LN exhibit 2- and 1.6-fold greater phospho-Src labeling compared to those on TN. Phospho-Src levels were also elevated in growth cones on TN by calpain inhibition or

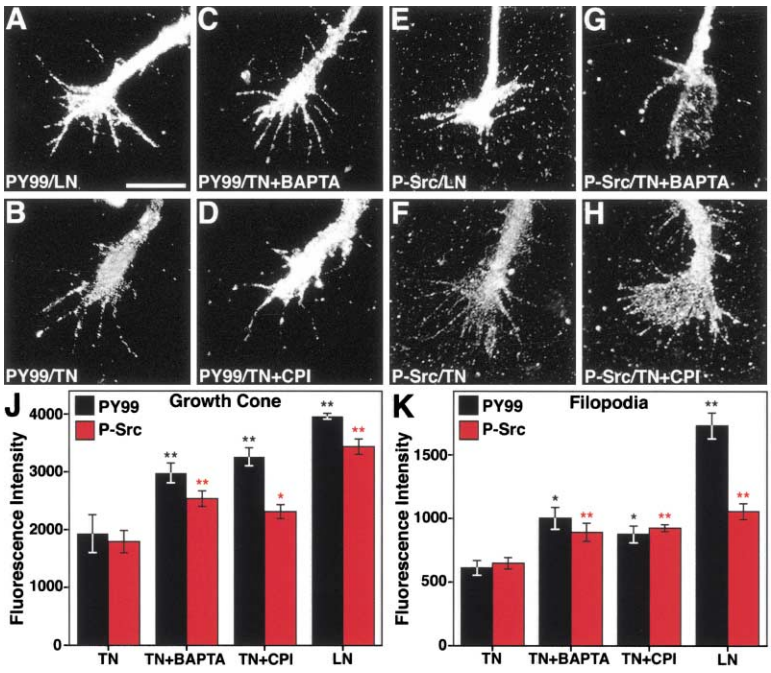

Figure 5. Substratum-Dependent Calpain Activity Regulates Tyrosine Phosphorylation in Growth Cones and Filopodia

(A–D) Representative growth cones immunofluorescently labeled for PY under different experimental conditions. (A) Neurons on LN exhibit high levels of tyrosine phosphorylation throughout growth cones and filopodia. (B) Neurons on TN exhibit low PY staining compared to LN. (C and D) PY staining increases in growth cones and filopodia on TN following Ca^{2+} chelation and 30 min inhibition of calpain with 1 μM CPI.

(E and F) Immunofluorescent staining for phospho-Src shows that Src kinase is highly active in growth cones and filopodia on LN, but not TN.

(G and H) Phospho-Src staining is increased by Ca^{2+} chelation and calpain inhibition.

(J and K) Quantification of PY fluorescence intensities within the central domain and filopodia of growth cones after experimental manipulation. $n > 20$ growth cones and 50 filopodia for each condition (**$p < 0.001$, *$p < 0.05$; p values denote statistical significance relative to values on TN). Scale, 5 μm.

intracellular Ca^{2+} chelation (Figures 5G and 5H). These results suggest that calpain-dependent changes in tyrosine phosphorylation may be due to direct cleavage and downregulation of Src kinases, as previously demonstrated in platelets (Pain et al., 1999).

Filopodial Ca^{2+} Signaling Locally Modulates Tyrosine Phosphorylation

To determine whether a local decrease in PY levels correlates with growth cone turning, both substrata boundary and local caged Ca^{2+} assays were conducted prior to fixation and staining for PY. We previously demonstrated that a majority of *Xenopus* growth cones undergo Ca^{2+}-dependent turning away from a substratum that stimulates a higher frequency of Ca^{2+} transients (Gomez et al., 2001). Consistent with those findings, calpain inhibition with either 1 μM CPI or ALLM decreases the percentage of growth cones on LN that turn away from TN (from 92% to 59% and 62%, respectively; $n \geq 30$ growth cones for each condition). Furthermore, growth cones turning at a LN/TN border exhibit reduced PY levels in regions of filopodia contacting TN (Figure 6). Quantification of fluorescence intensity along individual filopodia spanning a LN/TN boundary shows that PY levels are reduced in regions of filopodia contacting TN relative to actin filaments which remain constant (Figures 6A and 6B). Filopodia on TN exhibit a 38% reduction in PY staining as compared to those from the same growth cone contacting LN (Figure 6E). This decrease in local PY labeling may be due to both Ca^{2+}/calpain-dependent and -independent tyrosine dephosphorylation, as described previously.

To examine if local Ca^{2+}/calpain signals are sufficient to promote asymmetric PY in growth cones on a homogeneous substratum, we used caged Ca^{2+} as described previously in turning assays (Figures 3 and 4). Localized Ca^{2+} transients at a frequency of 6/min were imposed on one side of growth cones on LN for 4–10 min (Figure 6C). These neurons were subsequently fixed and processed for PY immunofluorescence. We find that filopodia stimulated with Ca^{2+} transients (NP-EGTA loaded) exhibit a 38% reduction in PY levels compared to unstimulated control filopodia on the same growth cone (Figures 6D and 6E). The maximum duration of uncaging stimulus used in experimental trials (10 min) had no effect on filopodial PY levels in unloaded control growth cones. Tyrosine dephosphorylation was calpain dependent as it was prevented in the presence of calpain inhibitor (Figure 6E). These results confirm that local calpain activation leads to an asymmetric decrease in PY that may directly promote filopodial stability and inhibit lamellar protrusion.

Inhibition of Src Family Tyrosine Kinases Promotes Filopodial Stability

To determine if PY levels directly correlate with filopodial stability, lifetimes of growth cone filopodia on LN were measured during acute pharmacological inhibition of PTK activity. PTK inhibition resulted in increased stability and length of existing filopodia as well as a rapid loss of growth cone veil (Figure 7A). Bath application of 1 and 10 μM HA resulted in an increase in average filopodial lifetime (Figure 7B). Similarly, inhibition of Src family kinases with PP2 also increased filopodial stability (Figure 7B) and veil retraction on LN (data not shown). If baseline PY is low in growth cones on TN, as shown above, then PTK inhibition should have little effect on neurite outgrowth on TN. Analysis of neurite lengths on LN and TN in the presence of HA confirms this hypothesis, as PTK inhibition reduced neurite outgrowth on LN but not TN (Figure 7C). These data support a model in which extracellular cues generate intracellular Ca^{2+}/calpain signals that regulate cell adhesion and motility

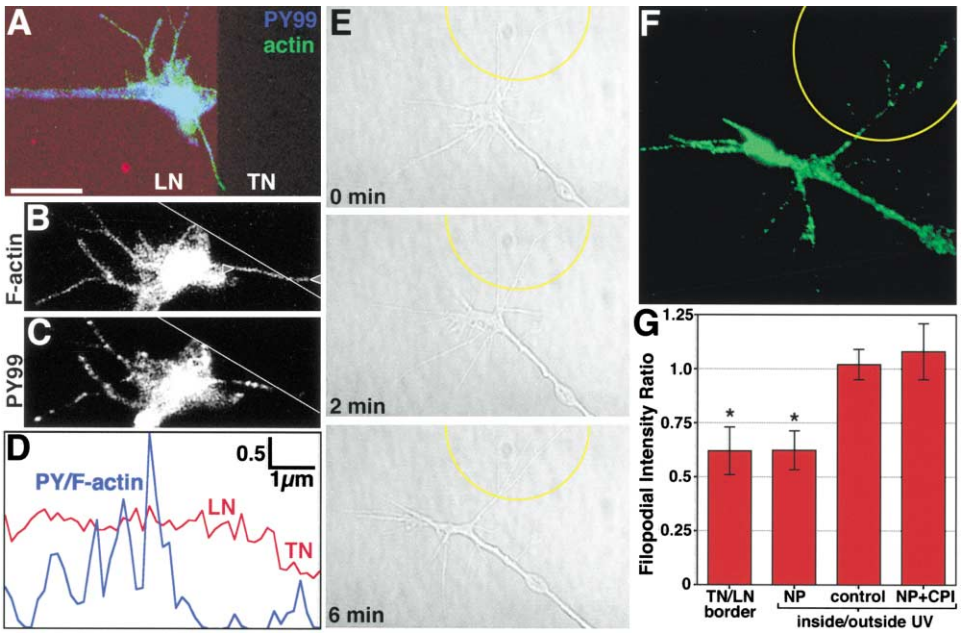

Figure 6. Filopodial Ca^{2+} Transients Locally Decrease Tyrosine Phosphorylation

(A) PY and phalloidin double-labeled growth cone turning at a border between LN and TN.

(B and C) Separate F-actin and PY channels showing that PY staining is selectively reduced in filopodia over TN. Scale in (A), 5 μm; and (B) and (C), 3.8 μm.

(D) Ratiometric intensity plot along the length of the filopodium (between arrowheads in [B]) spanning the LN/TN border. Blue trace represents the ratio of fluorescence intensity of PY staining to phalloidin labeling along the filopodium, and the red trace indicates fluorescence intensity of Alexa-546 dextran used to mark the LN-coated region. Note that reduced filopodial PY staining is not restricted to the region contacting TN, consistent with the spread of Ca^{2+} signals and calpain activity within filopodia.

(E) DIC images of a growth cone during repulsive turning from the site of imposed Ca^{2+} transients in filopodia (100 ms duration, 6/min; yellow circle, 12.5 μm diameter).

(F) PY-labeled growth cone previously turned by locally imposed Ca^{2+} transients.

(G) Quantification of the PY immunofluorescence of growth cone filopodia exposed to local Ca^{2+} transients. Data from LN/TN border and caged Ca^{2+} experiments are presented as the average ratio of PY fluorescence intensity relative to control filopodia (entirely on LN or outside the region of UV exposure). Inhibition of calpain activity with CPI blocks the reduction in PY levels within filopodia in response to locally generated Ca^{2+} transients. $n \geq 8$ growth cones in each condition (*$p < 0.05$).

by modulating the balance between tyrosine kinase and phosphatase activities.

One potential mechanism for PTK-dependent regulation of filopodial motility and veil extension is through the regulation of adhesion complex composition. To address this possibility, we examined growth cones immunofluorescently labeled for five adhesion-related proteins, including an integrin receptor (β1), a tyrosine kinase (FAK), a scaffolding molecule (paxillin), and two actin binding proteins (talin and vinculin). To determine if recruitment of these proteins required PTK activity, we compared fluorescence intensity of growth cone filopodia under control and HA-treated conditions. Among these five proteins, the actin binding protein vinculin was the only component significantly reduced by PTK inhibition (Figure 7E). Vinculin is highly enriched at adhesion complexes, and its accumulation is directly correlated with the generation of traction force in fibroblasts (Galbraith et al., 2002). Consistent with this role, we find vinculin colocalized with PY by immunofluorescence in selective sites within filopodia (Figure 7D).

Vinculin may also couple adhesion sites to membrane protrusion by directly binding proteins of the Arp 2/3 complex (DeMali et al., 2002), which nucleates and branches actin filaments and is required for the formation of lamellipodia (Bailly et al., 2001). In support of this role in growth cones, we find that localization of p16-Arc, a member of the Arp 2/3 complex (Welch et al., 1997), in filopodia requires PTK activity (Figure 7E). Together, these results suggest that spatial patterns of PTK activity generated by extracellular cues control growth cone motility and guidance by regulating the cellular mechanisms underlying force generation and membrane protrusion.

Discussion

In this study, we have identified the Ca^{2+}-sensitive protease calpain as a downstream effector of filopodial Ca^{2+} transients. Local calpain activity is regulated by the frequency of filopodial Ca^{2+} transients and serves to stabilize filopodia and slow axon growth. Calpain activity is greatest on substrata that produce high-frequency filopodial Ca^{2+} transients regardless of integrin engagement. However, inhibition of calpain activity promotes filopodial motility and axon growth only on integrin binding substrata, suggesting that calpain regulates integrin-mediated processes. Active calpain promotes filopodial stability by modulating tyrosine kinase signaling within growth cone filopodia. Localized calpain activity is nec-

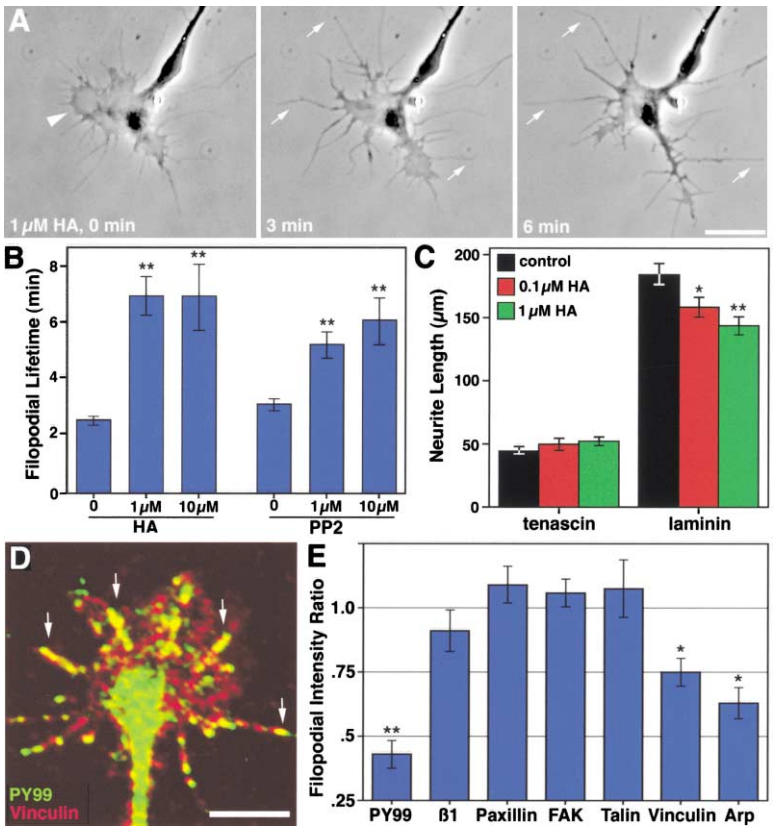

Figure 7. Inhibition of PTKs Leads to Increased Filopodial Stability and Veil Retraction

(A) Time lapse phase contrast images of a growth cone on LN during acute inhibition of PTKs with HA. Before application of HA ($t = 0$), this growth cone is highly lamellipodial (arrowhead) with many short, motile filopodia. Within 3 min of HA treatment there is a dramatic loss of lamella and an increase in filopodial length and stability. By 6 min, most lamella has receded and few stable filopodia remain (arrows). Scale, 10 μm.

(B) PTK inhibition (HA) and Src-kinase inhibition (PP2) increases average lifetime of filopodia on LN (**$p < 0.001$).

(C) Chronic PTK inhibition reduces neurite length in a dose-dependent manner on LN, but not TN (*$p < 0.05$; **$p < 0.001$).

(D) Immunofluorescence labeling for vinculin (red) and PY (green) shows these two antigens colocalize in distinct regions within the peripheral veil and filopodia of a growth cone on LN (arrows). Scale, 5 μm.

(E) PTK inhibition with HA selectively reduces levels of PY, vinculin and an Arp subunit (p16-Arc) in filopodia relative to untreated control levels. $n > 60$ filopodia for each condition.

essary for growth cone turning downstream of filopodial Ca^{2+}-transients, suggesting it may control axon guidance in vivo.

Ca^{2+} Transients Activate Calpain to Reduce Filopodial Motility and Slow Axon Outgrowth

Calpain has varied effects on the adhesion and motility of different cell types (reviewed in Glading et al., 2002). Studies examining the regulation of cell motility by calpain often find that its inhibition leads to decreased cell motility, contrary to our findings that calpain inhibition increases neurite outgrowth on TN. This discrepancy may be due to differences in the molecular composition, physiological regulation, and localization of growth cone adhesive contacts compared to other cell types. In nonneuronal cells, calpain is thought to promote cell motility by increasing the turnover rate of focal adhesions. Focal adhesions are macromolecular complexes that associate with actin stress fibers and mediate adhesion to the ECM (Geiger and Bershadsky, 2001). Immunocytochemical staining of focal adhesion components such as β1-integrin, FAK, and vinculin indicate that growth cones lack large focal adhesion-like structures; however, they do express smaller point contacts reminiscent of focal complexes (Renaudin et al., 1999; Gomez et al., 1996). In fibroblasts, calpain proteolysis may promote cell motility by locally disrupting focal adhesions leading to rear-end detachment from the ECM (Huttenlocher et al., 1997). The effects of calpain on focal complex assembly and disassembly in growth cones are not known. However, if calpain activity stimulated by high-frequency Ca^{2+} transients in filopodia disrupts linkages between the actin cytoskeleton and integrin receptors, the primary effect of this disengagement would likely be a reduction in traction forces required for lamellar protrusion and growth cone translocation. Disruption of the link between the actin cytoskeleton and the substratum within growth cone filopodia has been proposed in the clutch hypothesis to negatively regulate forward movement of elongating axons (Mitchison and Kirschner, 1988; Jay, 2000). A similar process may occur in neutrophils, which have recently been shown to accelerate upon calpain inhibition and also lack classic focal adhesions (Lokuta et al., 2003).

In addition to slowing neurite outgrowth, we also find that calpain stabilizes filopodia, as its inhibition reduces filopodial lifetime. This result is unexpected if calpain fully disrupts actin filament association with integrin receptors, which should lead to filopodial retraction according to the clutch hypothesis (Jay, 2000). However, in growth cones, calpain proteolysis may function primarily to alter the molecular composition of adhesive sites. Both the assembly and dissassembly of focal adhesions are tightly regulated by the composition and phosphorylation state of these macromolecular complexes (Zamir et al., 2000). Loss or dephosphorylation of one or more proteins within adhesion sites may lead to altered function and prevent disassembly, as has been seen in Src and FAK null cells (Ilic et al., 1995; Volberg et al., 2001). This model is consistent with our finding that PTK inhibition results in both inhibition of protrusion and stabilization of some filopodia. Although in this study we used filopodial lifetime as an indicator

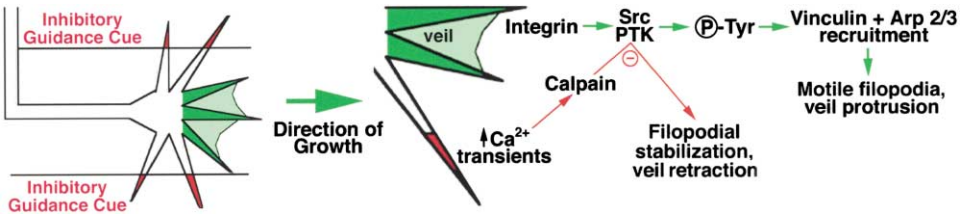

Figure 8. Model for the Regulation of Axon Growth and Guidance by Local Ca^{2+} Signals in Filopodia

Ca^{2+} transients in filopodia stimulated by inhibitory guidance cues lead to growth cone turning through local activation of calpain. Active calpain cleaves and inactivates PTKs such as pp60Src, preventing tyrosine phosphorylation of structural and signaling proteins within filopodia. Reduced PY leads to disengagement of vinculin, p16-Arc and likely other links between the actin cytoskeleton and integrin receptors, resulting in stabilization of filopodia and loss of veil protrusion.

of stability, filopodia also appear to lengthen in a Ca^{2+}-dependent manner (Figures 3A, 4A, and 6C), as has been reported previously for *Helisoma* neurons (Cheng et al., 2002).

Downstream Targets of Calpain in Growth Cones

One of the principal sites of calpain action is believed to be within integrin-containing adhesion complexes. These dynamic structures are expressed in several different forms by cells, varying in spatial, temporal, and molecular organization. Adhesion sites are believed to function both as anchorage points to the ECM, as well as organizing centers for actin polymerization (Zamir et al., 2000). Accordingly, these macromolecular complexes contain both signaling and structural components. Structural proteins such as talin, vinculin, paxillin, α-actinin, zyxin, and ezrin/radixin/moesin (ERM) proteins are involved in direct or indirect linking of actin filaments to the cytoplasmic tail of integrin receptors. In many cases, these linking proteins also function as adaptor molecules that complex several proteins into functional units. Several known regulators of actin polymerization, such as ena/VASP family members, modulators of small GTPases, and acidic phospholipids, are among the molecules recruited to these complexes. Given the diverse and functionally significant roles of adhesion complexes for cell motility, the regulation of their assembly and disassembly is likely to be critical for coordinated axon outgrowth. To this end, signaling proteins such as the tyrosine kinases pp60Src and pp125FAK, serine/threonine kinases, phosphatases, and calpain localize within these adhesive complexes to regulate their formation and turnover.

Many focal adhesion proteins contain Src homology 2 (SH2) domains or are tyrosine phosphorylated, suggesting that phosphorylation regulates focal adhesion assembly by promoting intermolecular SH2-PY interactions. The presence of tyrosine-phosphorylated proteins in neuronal growth cones is well documented (Wu and Goldberg, 1993; Wu et al., 1996), and the importance of these modifications for axon guidance is clear from studies demonstrating axon path finding defects following tyrosine kinase inhibition in vivo (Worley and Holt, 1996; Menon and Zinn, 1998). Tyrosine phosphorylation is stimulated by a variety of means, including the clustering of integrin receptors (Kornberg et al., 1991) and application of mechanical force (Streit et al., 1998). In a recent study, Src kinase-mediated tyrosine phosphorylation was identified in response to tension applied to the surface of growth cones. Increased phosphorylation at sites of initial tension was necessary to support greater traction loads on restrained beads (Suter and Forscher, 2001). These results suggest that Src kinase activities stimulate cytoskeletal rearrangements that strengthen the linkage between the actin cytoskeleton and adhesion receptors. In this study, greater traction forces localized to one side of a growth cone resulted in turning toward the site of increased PY (Suter and Forscher, 2001). Our data are consistent with these findings, as we observe growth cone turning away from sites of decreased PY in filopodia. Local uncoupling of receptor-cytoskeletal linkages by calpain cleavage of Src and other focal contact proteins provides a mechanism whereby filopodial contact with inhibitory guidance cues may initiate growth cone turning.

We find that LN and TN, two integrin binding ECM proteins expressed along the pathway of growing *Xenopus* spinal axons in vivo (Krotoski and Bronner-Fraser, 1991; Somasekhar and Nordlander, 1995), differentially regulate PY levels in growth cones. *Xenopus* growth cones cultured on LN exhibit intense tyrosine phosphorylation and phospho-Src, suggesting that Src and other kinases are highly active on this substratum. Based on the model described by Suter and Forscher (2001), high PY suggests strong receptor-cytoskeletal linkages and enhanced ability to support traction forces, consistent with rapid neurite outgrowth observed on LN. We find that acute inhibition of PTK activity reduces PY in filopodia, as well as vinculin and p16-Arc, a subunit of the Arp2/3 complex (Welch, et al., 1997). Loss of these proteins coincides with diminished filopodial motility and reduced veil protrusion, consistent with their role in adhesion and actin polymerization, respectively. In contrast to LN, growth cones on TN exhibit low levels of PY, stable filopodia, and slow growth. Tyrosine phosphorylation is reduced across growth cones on TN, suggesting either that calpain proteolysis spreads from the sites of local Ca^{2+} transients in filopodia or that calpain is activated by Ca^{2+} signals in other regions of growth cones. Ca^{2+} transients within the veil of growth cones are occasionally detected, however, appear less frequent than filopodial transients (data not shown). Finally, differences between PY levels in filopodia across growth cones at LN/TN boundaries appear sufficient to promote turning away from TN.

Calpain Is Necessary for Ca^{2+}-Mediated Growth Cone Guidance In Vitro

To assess the role of calpain in axon guidance, we imposed local Ca^{2+} transients through targeted release of caged Ca^{2+}. We find that calpain activity is necessary for local Ca^{2+} transient-mediated growth cone turning. Detailed examination of turning responses revealed that local Ca^{2+} transients initiate distinct cellular behaviors that were each dependent on calpain activity. Typically, Ca^{2+}-mediated growth cone turning begins with stabilization of stimulated filopodia followed by loss of veil around stabilized filopodia and veil protrusion on the distal side of the growth cone. Enhanced veil protrusion at the site opposite imposed Ca^{2+} transients was evident, but it is unclear if this is due to local activation or asymmetric inhibition of a stochastic process. The fact that inhibition of calpain prevents these two behaviors suggests it functions as an upstream regulator of multiple processes. Local inhibition of lamellar protrusion by inhibitory guidance factors has been shown to stimulate similar repulsive growth cone turning (Steketee and Tosney, 1999; Fan and Raper, 1995; Zhou and Cohan, 2001). Our results suggest a mechanism in which Ca^{2+} transients in filopodia activate calpain, which cleaves and inactivates Src and possibly other tyrosine kinases, leading to a local loss of PY. This reduction of tyrosine phosphorylated proteins displaces vinculin from focal contacts, leading to a local loss of Arp2/3 activity (Figure 7E). Within this model, the direction of growth cone extension is determined by the location of filopodia that permit veil protrusion (Figure 8).

Concluding Remarks

Our previous work identified three basic functions of filopodial Ca^{2+} transients. First, transients in filopodia function as elemental signals that integrate to produce global growth cone Ca^{2+} transients that slow axon outgrowth. Second, local transients function as autonomous regulators of filopodial motility. Third, filopodial Ca^{2+} transients serve as instructive signals that guide growth cone extension based on the spatiotemporal pattern of activity in filopodia (Gomez et al., 2001). The present study has examined the downstream mechanisms that underlie the regulation of filopodial motility and growth cone guidance by the frequency of local Ca^{2+} signals. We report that Ca^{2+} transients increase filopodial stability through local activation of calpain. Further, we have demonstrated that calpain-dependent modulation of tyrosine phosphorylation mediates the effects of local Ca^{2+} signals. To our knowledge, this is the first study to identify a specific protease in the regulation of growth cone motility. However, this unexpected role for calpain is reminiscent of recent findings implicating degradatory proteolysis downstream of inhibitory guidance cues (Campbell and Holt, 2001). Together, these findings suggest that spatially and temporally precise regulation of intracellular proteolysis may represent a fundamental mechanism allowing growth cones to quickly and accurately respond to environmental cues.

Experimental Procedures

Xenopus Spinal Neuron Culture

Neural tubes from 1-day-old *Xenopus* embryos were isolated and cultured in 2 mM Ca^{2+} containing modified Ringer's solution (MR) at 20°C as described (Gomez et al., 2003). Neural tubes were either dissociated or plated as explants onto acid-washed glass coverslips coated with 25 μg/ml LN (Sigma-Aldrich), 10 μg/mL TN-C (Chemicon), or 100 μg/mL PDL (Sigma-Aldrich). Substratum boundaries were prepared as described (Gomez et al., 2001).

Neurite Outgrowth and Filopodial Lifetime Assays

Phase-contrast images used for neurite length measurements and filopodial lifetime analysis were captured on a Zeiss Axiovert microscope using a 40× objective. Images were collected with a Coolsnap HQ digital camera (Roper Instruments) using MetaMorph software (Universal Imaging) for acquisition and analysis. For some experiments, GFP-γ-actin was expressed in neurons by injection of 1–3 ng of mRNA synthesized with the mMessage Machine Kit (Ambion) into two blastomeres of eight-cell stage embryos. GFP images were collected using a 100× objective on an Olympus Fluoview 500 laser-scanning confocal system mounted on an AX-70 upright microscope. Lifetimes of individual filopodia were measured as described (Gomez et al., 2001). CPI was from Calbiochem and ALLM from Sigma. Herbamycin A and PP2 were from Biomol.

t-BOC Proteolysis Assay

Xenopus neurons were incubated with 10 μM 7-amino-4-chloromethylcoumarin, *t*-BOC-L-leucyl-L-methionine amide (t-Boc; Molecular Probes) in 0.01% pluronic acid/0.1% DMSO in MR. Coumarin fluorescence was monitored using a 40× objective on a Zeiss axiovert microscope equipped with a 75 W Xenon light source filtered through 380 ± 15 nm excitation and 460 ± 25 nm emission filters (Chroma Technology). For experiments using CPI or Ca^{2+}-free solutions, cultures were preincubated for 30–60 min prior to t-BOC loading and imaging. BAPTA-AM (Molecular Probes) was loaded by incubating cultures with 200 nM (Figure 2B) or 4 μM (Figure 2D) BAPTA-AM for 30–45 min followed by a 30–60 min recovery period. Fluorescence intensities of individual growth cones and along filopodia were quantified within user defined regions using MetaMorph imaging software. Ca^{2+} imaging using Fura-2 was conducted using MetaFluor software (Universal Imaging) on a Zeiss axiovert as described (Gomez et al., 1995).

Caged Ca^{2+} Experiments

For caged Ca^{2+} experiments, neurons on LN were loaded for 45–60 min with 4 μM NP-EGTA AM (Molecular Probes) in 0.01% pluronic acid/0.1% DMSO in MR. NP-EGTA was photo-activated using 360 ± 25 nm light filtered from a 100 W mercury light source, attenuated to 25% power using a neutral density filter. The region exposed to UV light was restricted to a 12.5 μm spot using the microscope field diaphragm. A programmable shutter (Uniblitz) controlled the pulse duration (100 ms) and interpulse interval (10 s). Growth cones loaded with NP-EGTA and Fluo-4 were used to calibrate the UV pulse paradigm to mimic the amplitude and kinetics of spontaneously occurring filopodial Ca^{2+} transients as described (Gomez et al., 2001). For turning assays, motile growth cones were positioned so their leading edge was 5 μm from the region of shuttered UV light. Differential interference contrast (DIC) or GFP fluorescence images were acquired every 15 or 30 s using an Olympus Fluoview 500 confocal microscope. Only growth cones that advanced at least 10 μm during imaging were used for analysis of turning using ImageJ software (W. Rasband, NIH).

Immunofluorescent Staining

Cultures were fixed with 4% paraformaldehyde/4% sucrose in 0.1 M phosphate-buffered saline for 10 min at room temperature, then permeabilized with 0.1% triton-X solution in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS) and blocked in 0.2% fish gelatin (Sigma) as described (Gomez et al., 2003). The following antibodies were used: PY99 and FAK (Santa Cruz Biotechnology), phospho-Src (Biosource International), μ-calpain (Chemicon), β1-

integrin (Developmental Studies Hybridoma Bank), paxillin (Transduction Labs), talin and vinculin (Sigma), and a rabbit polyclonal antibody against a peptide fragment of p16-Arc (generously provided by William Bement, University of Wisconsin). Primary antibodies were detected with Alexa-fluor conjugated 2° antibodies (Alexa-488, -546, or -647; Molecular Probes). Alexa-546 phalloidin (Molecular Probes) was included with 2° antibodies in some instances to stain actin filaments.

Immunoblot Analysis

Stage 22 *Xenopus* spinal cords were isolated and denatured in lysis buffer. Proteins isolated from individual neural tubes were run in separate lanes on a Tris-glycine poly-acrylmide gel and analyzed via Western blotting using anti-μ-calpain antibody. HRP-conjugated anti-mouse 2° antibody (Jackson Immuno) was used to detect protein bands via chemiluminescence.

Acknowledgments

We thank Drs. Paul Letourneau and Kate Kalil for comments on the manuscript. This work was supported by NIH NS41564 to T.M.G. and a NSF predoctoral fellowship to E.R.

Received: November 27, 2002
Revised: February 25, 2003
Accepted: April 21, 2003
Published: May 21, 2003

References

Al-Mohanna, F.A., Bolsover, S., and Archer, F.R. (1996). CAMs and FGF cause a local submembrane calcium signal promoting axon outgrowth without a rise in bulk calcium concentration. Brain Res. Dev. Brain Res. *92*, 101–110.

Alderton, J.M., and Steinhardt, R.A. (2000). Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. J. Biol. Chem. *275*, 9452–9460.

Bailly, M., Ichetovkin, I., Grant, W., Zebda, N., Machesky, L.M., Segall, J.E., and Condeelis, J. (2001). The F-actin side binding activity of the Arp2/3 complex is essential for actin nucleation and lamellipod extension. Curr. Biol. *11*, 620–625.

Bandtlow, C.E., Schmidt, M.F., Hassinger, T.D., Schwab, M.E., and Kater, S.B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science *259*, 80–83.

Behar, O., Mizuno, K., Badminton, M., and Woolf, C.J. (1999). Semaphorin 3A growth cone collapse requires a sequence homologous to tarantula hanatoxin. Proc. Natl. Acad. Sci. USA *96*, 13501–13505.

Bentley, D. (1987). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. Dev. Biol. *119*, 454–465.

Bixby, J.L., Grunwald, G.B., and Bookman, R.J. (1994). Ca^{2+} influx and neurite growth in response to purified N-cadherin and laminin. J. Cell Biol. *127*, 1461–1475.

Bray, D., and Chapman, K. (1985). Analysis of microspike movements on the neuronal growth cone. J. Neurosci. *5*, 3204–3213.

Campbell, D.S., and Holt, C.E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron *32*, 1013–1026.

Cheng, S., Geddis, M.S., and Rehder, V. (2002). Local calcium changes regulate the length of growth cone filopodia. J. Neurobiol. *50*, 263–275.

Chien, C.B., Rosenthal, D.E., Harris, W.A., and Holt, C.E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. Neuron *11*, 237–251.

Cooray, P., Yuan, Y., Schoenwaelder, S.M., Mitchell, C.A., Salem, H.H., and Jackson, S.P. (1996). Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. Biochem. J. *318*, 41–47.

Davenport, R.W., Dou, P., Rehder, V., and Kater, S.B. (1993). A sensory role for neuronal growth cone filopodia. Nature *361*, 721–724.

DeMali, K.A., Barlow, C.A., and Burridge, K. (2002). Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. J. Cell Biol. *159*, 881–891.

Fan, J., and Raper, J.A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron *14*, 263–274.

Frangioni, J.V., Oda, A., Smith, M., Salzman, E.W., and Neel, B.G. (1993). Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. EMBO J. *12*, 4843–4856.

Galbraith, C.G., Yamada, K.M., and Sheetz, M.P. (2002). The relationship between force and focal complex development. J. Cell Biol. *159*, 695–705.

Geiger, B., and Bershadsky, A. (2001). Assembly and mechanosensory function of focal contacts. Curr. Opin. Cell Biol. *13*, 584–592.

Gitler, D., and Spira, M.E. (1998). Real time imaging of calcium-induced localized proteolytic activity after axotomy and its relation to growth cone formation. Neuron *20*, 1123–1135.

Glading, A., Lauffenburger, D.A., and Wells, A. (2002). Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol. *12*, 46–54.

Gomez, T.M., and Spitzer, N.C. (1999). In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. Nature *397*, 350–355.

Gomez, T.M., and Spitzer, N.C. (2000). Regulation of growth cone behavior by calcium: new dynamics to earlier perspectives. J. Neurobiol. *44*, 174–183.

Gomez, T.M., Snow, D.M., and Letourneau, P.C. (1995). Characterization of spontaneous calcium transients in nerve growth cones and their effect on growth cone migration. Neuron *14*, 1233–1246.

Gomez, T.M., Roche, F.K., and Letourneau, P.C. (1996). Chick sensory neuronal growth cones distinguish fibronectin from laminin by making substratum contacts that resemble focal contacts. J. Neurobiol. *29*, 18–34.

Gomez, T.M., Robles, E., Poo, M., and Spitzer, N.C. (2001). Filopodial calcium transients promote substrate-dependent growth cone turning. Science *291*, 1983–1987.

Gomez, T.M., Harrigan, D., Henley, J., and Robles, E. (2003). Working with *Xenopus* spinal neurons in live cell culture. Methods Cell Biol., in press.

Gu, X., Olson, E.C., and Spitzer, N.C. (1994). Spontaneous neuronal calcium spikes and waves during early differentiation. J. Neurosci. *14*, 6325–6335.

Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M., and Poo, M. (2000). Calcium signalling in the guidance of nerve growth by netrin-1. Nature *403*, 93–98.

Huttenlocher, A., Palecek, S.P., Lu, Q., Zhang, W., Mellgren, R.L., Lauffenburger, D.A., Ginsberg, M.H., and Horwitz, A.F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. J. Biol. Chem. *272*, 32719–32722.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539–544.

Janmey, P.A. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu. Rev. Physiol. *56*, 169–191.

Jay, D.G. (2000). The clutch hypothesis revisited: ascribing the roles of actin-associated proteins in filopodial protrusion in the nerve growth cone. J. Neurobiol. *44*, 114–125.

Kater, S.B., and Rehder, V. (1995). The sensory-motor role of growth cone filopodia. Curr. Opin. Neurobiol. *5*, 68–74.

Kornberg, L.J., Earp, H.S., Turner, C.E., Prockop, C., and Juliano, R.L. (1991). Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. Proc. Natl. Acad. Sci. USA *88*, 8392–8396.

Krotoski, D., and Bronner-Fraser, M. (1991). Distribution of integrins and their ligands in the trunk of Xenopus laevis during neural crest cell migration. J. Exp. Zool. *253*, 139–150.

Kuhn, T.B., Williams, C.V., Dou, P., and Kater, S.B. (1998). Laminin directs growth cone navigation via two temporally and functionally distinct calcium signals. J. Neurosci. *18*, 184–194.

Lokuta, M.A., Nuzzi, P.A., and Huttenlocher, A. (2003). Calpain regulates neutrophil chemotaxis. Proc. Natl. Acad. Sci. USA *100*, 4006–4011.

Menon, K.P., and Zinn, K. (1998). Tyrosine kinase inhibition produces specific alterations in axon guidance in the grasshopper embryo. Development *125*, 4121–4131.

Mitchison, T., and Kirschner, M. (1988). Cytoskeletal dynamics and nerve growth. Neuron *1*, 761–772.

Oda, A., Smith, M., Salzman, E.W., and Neel, B.G. (1993). pp60src is an endogenous substrate for calpain in human blood platelets. EMBO J. *12*, 4843–4856.

Pain, S., Monstero-Lastres, A., Falet, H., Brohard-Bohn, B., Fraiz, N., Bachelot-Loza, C., Cano, E., and Rendu, F. (1999). Calpain controls the balance between protein tyrosine kinase and tyrosine phosphatase activities during platelet activation. FEBS Lett. *453*, 119–123.

Pellicena, P., and Miller, W.T. (2002). Coupling kinase activation to substrate recognition in SRC-family tyrosine kinases. Front. Biosci. *7*, d256–d267.

Renaudin, A., Lehmann, M., Girault, J., and McKerracher, L. (1999). Organization of point contacts in neuronal growth cones. J. Neurosci. Res. *55*, 458–471.

Sato, K., and Kawashima, S. (2001). Calpain function in the modulation of signal transduction molecules. Biol. Chem. *382*, 743–751.

Shea, T.B., Cressman, C.M., Spencer, M.J., Beermann, M.L., and Nixon, R.A. (1995). Enhancement of neurite outgrowth following calpain inhibition is mediated by protein kinase C. J. Neurochem. *65*, 517–527.

Snow, D.M., Atkinson, P.B., Hassinger, T.D., Letourneau, P.C., and Kater, S.B. (1994). Chondroitin sulfate proteoglycan elevates cytoplasmic calcium in DRG neurons. Dev. Biol. *166*, 87–100.

Somasekhar, T., and Nordlander, R.H. (1995). Differential distributions of HNK-1 and tenascin immunoreactivity during innervation of myotomal muscle in Xenopus. Brain Res. Dev. Brain Res. *88*, 53–67.

Song, D.K., Malmstrom, T., Kater, S.B., and Mykles, D.L. (1994). Calpain inhibitors block Ca(2+)-induced suppression of neurite outgrowth in isolated hippocampal pyramidal neurons. J. Neurosci. Res. *39*, 474–481.

Spitzer, N.C., Lautermilch, N.J., Smith, R.D., Gomez, T.M., and Rohrbough, J. (2000). Coding of neuronal differentiation by calcium transients. Bioessays *22*, 811–817.

Steketee, M.B., and Tosney, K.W. (1999). Contact with isolated sclerotome cells steers sensory growth cones by altering distinct elements of extension. J. Neurosci. *19*, 3495–3506.

Streit, M., Kaiser, R., Herzberg, F., Schirner, M., Schramm, K., Kaufmann, C., Henneken, M., Schafer-Korting, M., Thiel, E., Kreuser, E.D., et al. (1998). Mechanical stressing of integrin receptors induces enhanced tyrosine phosphorylation of cytoskeletally anchored proteins. Int. J. Cancer *76*, 91–98.

Suter, D.M., and Forscher, P. (2001). Transmission of growth cone traction force through apCAM-cytoskeletal linkages is regulated by Src family tyrosine kinase activity. J. Cell Biol. *155*, 327–330.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

Volberg, T., Romer, L., Zamir, E., and Geiger, B. (2001). pp60(c-src) and related tyrosine kinases: a role in the assembly and reorganization of matrix adhesions. J. Cell Sci. *114*, 2279–2289.

Wang, Q., and Zheng, J.Q. (1998). cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. J. Neurosci. *18*, 4973–4984.

Welch, M.D., DePace, A.H., Verma, S., Iwamatsu, A., and Mitchison, T.J. (1997). The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. J. Cell Biol. *138*, 375–384.

Worley, T., and Holt, C. (1996). Inhibition of protein tyrosine kinases impairs axon extension in the embryonic optic tract. J. Neurosci. *16*, 2294–2306.

Wu, D.Y., and Goldberg, D.J. (1993). Regulated tyrosine phosphorylation at the tips of growth cone filopodia. J. Cell Biol. *123*, 653–664.

Wu, D.Y., Wang, L.C., Mason, C.A., and Goldberg, D.J. (1996). Association of beta 1 integrin with phosphotyrosine in growth cone filopodia. J. Neurosci. *16*, 1470–1478.

Zamir, E., Bershadsky, A., Kam, Z., Yamada, K.M., and Geiger, B. (2000). Molecular diversity of cell-matrix adhesions. Mol. Biol. Cell *11*, 1047–1060.

Zheng, J.Q. (2000). Turning of nerve growth cones induced by localized increases in intracellular calcium ions. Nature *403*, 89–93.

Zheng, J.Q., Felder, M., Connor, J.A., and Poo, M. (1994). Turning of nerve growth cones induced by neurotransmitters. Nature *368*, 140–144.

Zheng, J.Q., Wan, J.J., and Poo, M. (1996). Essential role of filopodia in chemotropic turning of nerve growth cone induced by a glutamate gradient. J. Neurosci. *16*, 1140–1149.

Zhou, F.Q., and Cohan, C.S. (2001). Growth cone collapse through coincident loss of actin bundles and leading edge actin without actin depolymerization. J. Cell Biol. *153*, 1071–1084.

Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. Annu. Rev. Physiol. *64*, 355–405.