Growth cone steering by a physiological electric field requires dynamic microtubules, microfilaments and Rac-mediated filopodial asymmetry

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Summary

Electric fields (EFs) resembling those in the developing and regenerating nervous systems steer growth cones towards the cathode. Requirements for actin microfilaments, microtubules and their interactions during EF growth cone steering have been presumed, but remain unproven. Here, we demonstrate essential roles for dynamic microfilaments and microtubules in cathode-directed migration. Cathodal turning of growth cones on cultured *Xenopus* embryonic spinal neurons was attenuated significantly by nanomolar concentrations of the microfilament inhibitor latrunculin, the microtubule-stabilising drug taxol, or the microtubule-destabilising drugs vinblastine or nocodazole. Dynamically, the cathodal bias of filopodia preceded cathodal turning of the growth cone, suggesting an instructive role in EF-induced steering. Lamellipodial asymmetry accompanied turning. Filopodia and lamellipodia are regulated by the GTPases Cdc42 and Rac, respectively, and, as shown in the companion paper in this issue, peptides that selectively prevented effector binding to the CRIB domains of Cdc42 or Rac abolished cathodal growth cone turning during 3 hours of EF exposure. Here, the Rac peptide suppressed lamellipodium formation, increased the number of filopodia, abolished cathodal filopodial orientation, and prevented cathodal steering. The Cdc42 peptide suppressed filopodium formation, increased lamellipodial area and prevented cathodal steering. The cathodal bias of lamellipodia was independent of Cdc42 CRIB activity and was not sufficient for cathodal steering in the absence of filopodia, but the cathodal bias of filopodia through Rac CRIB activity was necessary for cathodal turning. Understanding the mechanism for cathodal growth cone guidance will enhance the emerging clinical effort to stimulate human spinal cord regeneration through EF application.

Supplementary material available online at
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Key words: Filopodia, Lamellipodia, Growth cone, Electric field, Rho GTPases

Introduction

Neurons extend axons towards appropriate targets in the developing and regenerating nervous systems via growth cones, the motile structures at axonal tips. Growth cones navigate within a complex milieu of temporally and spatially regulated guidance cues including gradients of soluble and substratum-bound molecules. Coincident with molecular gradients are naturally occurring voltage gradients driven by the ion transport properties of epithelia, which generate steady, direct current (DC) electrical fields (EFs) within embryonic tissues (e.g. Hotary and Robinson, 1991) (reviewed by McCaig et al., 2005). EF disruption within the nervous system induces profound defects (Borgens and Shi, 1995) and application of EFs to the injured central nervous system (CNS) stimulates axonal regrowth (e.g. Borgens et al., 1993) (reviewed by McCaig et al., 2005), suggesting important roles in nervous system development and regeneration. Growth cones migrate faster towards the cathode (negative electrode) than towards the anode (positive electrode), often retract from the anode, and are steered towards the cathode of an EF in vitro (McCaig, 1987; McCaig, 1989; McCaig et al., 2005) and in vivo (Song et al., 2004), suggesting a role in growth cone path-finding.

The mechanism for growth cone steering by a voltage gradient remains unclear despite identification of several essential membrane receptors and intracellular signalling molecules (reviewed by McCaig et al., 2002; McCaig et al., 2005). Analogous to the mechanism proposed for steering by molecular gradients (Mueller, 1999; Song and Poo, 2001; Patel and Van Vactor, 2002), an electrical gradient is thought to induce selective redistribution or activation of membrane receptors and consequent regional activation of intracellular signals that modify cytoskeletal dynamics. The Rho family of GTPases, including Cdc42, Rac and Rho, regulate the dynamics of actin microfilaments and microtubules, and have been implicated in growth cone steering by molecular and electrical gradients (Giniger, 2002; Rajnicek et al., 2006).

Cdc42 activation in growth cones stimulates formation of dynamic, finger-like filopodia (Kozma et al., 1997) comprising bundles of actin microfilaments. They sense spatial cues within the extracellular environment and are believed to play an instructive role in growth cone guidance because their

depletion (1) causes growth cones to meander (Marsh and Letourneau, 1984), (2) induces path-finding errors in vivo (Bentley and Toroian-Raymond, 1986; Chien et al., 1993; Kaufmann et al., 1998), and (3) prevents turning away from a repellent cue (Challacombe et al., 1997) in vitro. In addition, filopodial asymmetry precedes turning towards attractive cues (O'Connor and Bentley, 1993; Zheng et al., 1996), including the cathode of an EF (McCaig, 1989) and even a single filopodial contact can redirect a growth cone (Caudy and Bentley, 1986), suggesting a causal role [but see Isbister and O'Connor (Isbister and O'Connor, 1999)]. However, the absolute requirement for filopodia in growth cone navigation is contentious. Interfering with filopodial activity through modulation of Cdc42 in *Drosophila* embryos failed to induce significant navigational errors (Kim et al., 2002) and embryonic *Xenopus* growth cones denuded of filopodia with cytochalasin turned towards the cathode of an EF in vitro as usual (McCaig, 1989).

It is possible that, in the absence of filopodia, cathodal growth cone steering is mediated by actin-rich lamellipodia. Indeed, it has been proposed recently that lamellipodia, not filopodia, are essential for growth cone turning (Wang et al., 2003). Lamellipodial dynamics are mediated by Rac, whose activity is required for guidance of *Drosophila* motor neuron growth cones (Kaufmann et al., 1998) and we have implicated Rac activity in steering of *Xenopus* spinal neuron growth cones by an EF (Rajnicek et al., 2006). Here, we test the relative contributions of filopodia and lamellipodia in steering by an EF and relate their directional behaviour to Rac and Cdc42 signalling.

In addition to their roles in modulating microfilaments, Rho GTPases affect microtubules. Their downstream effectors localise to microtubule tips (Gordon-Weeks, 2003; Andersen, 2004), providing a means by which microtubule dynamics can be regulated spatially. Microtubules have been implicated in growth cone steering because damping microtubule dynamics with low nanomolar concentrations of taxol or vinblastine prevented turning in molecular gradients (Challacombe et al., 1997; Williamson et al., 1996; Gordon-Weeks, 2003). Additionally, photactivation of taxol in growth cones stimulated turning towards taxol-stabilised microtubules or away from a source of nocodazole, which destabilised microtubules (Buck and Zheng, 2002). This has led to models (Gordon-Weeks, 2003; Andersen, 2004) in which growth cone steering results from stabilisation of filopodial actin microfilaments on the side of the growth cone facing the attractive cue, and the preferential capture and consequent stabilisation of dynamic microtubules by filopodial actin in the direction of the turn. Dynamic microtubules are not essential for cathodal migration of fibroblasts in an EF (Finkelstein et al., 2004) but their requirement in cathodal growth cone steering has not been tested.

If the model outlined above underpins guidance by an EF then the following predictions emerge: (1) cathodal turning requires actin microfilaments as well as dynamic microtubules; (2) filopodia would be biased towards the cathode in advance of turning; and (3) lamellipodial bias towards the cathode would not be sufficient or essential to induce turning. Here, we provide evidence supporting each of these predictions and verify the proposed link between Cdc42/Rac activity and cytoskeletal dynamics in cathodal steering (Rajnicek et al., 2006). An improved understanding of how growth cones respond to electrical gradients will advance attempts to aid human spinal cord repair using DC EF application (Shapiro et al., 2005).

Results

Actin microfilaments are required for growth cone turning in an EF

Growth cones exposed to an EF for 3 hours turn dramatically towards the cathode but 1 nM latrunculin A (latA) attenuates the extent and frequency of cathodal turning and 10 nM latA abolishes it (Fig. 1A,C). Although latA was dissolved in dimethyl sulphoxide (DMSO), DMSO itself did not affect the extent or the frequency of turning towards the cathode compared with EF-treated growth cones without any drug or solvent (Fig. 3A,C). The EF increased the rate of growth cone advance during 3 hours, as has been reported previously for experiments lasting 5 hours (Rajnicek et al., 2006). In DMSO, rates were 26.6±1.3 μm/hour without an EF (n=79) and 39.5±1.0 μm/hour in an EF (n=485) (P<0.0001). The failure to turn cathodally cannot be attributed to lack of neurite extension because EF-treated growth cones migrated at 27.1±1.0 μm/hour (n=132) in 1 nM latA and at 21.0±1.5 μm/hour (n=89) in 10 nM latA; the same as controls in the absence of an EF or drug (Fig. 1B). Therefore, the EF-induced increase in migration rate was lost by inhibiting the actin cytoskeleton with latA. This was confirmed by examining the migration rates of cathode-facing and anode-facing growth cones. In DMSO, growth cones facing the cathode migrated faster (42.1±1.3 μm/hour, n=327) than those facing the anode (29.1±3.3 μm/hour, n=30) but the rates in 1 or 10 nM latA were the same cathodally (28.0±1.4 μm/hour, n=73 for 1 nM; 20.2±2.0, n=42 for 10 nM) as anodally (25.3±3.5 μm/hour, n=12 for 1 nM; 21.8±3.8, n=18 for 10 nM).

Neurons were stained with Rhodamine-phalloidin to label filamentous actin and an antibody to α-tubulin to determine the consequences of latA treatment on the cytoskeleton. In the absence of any drug, growth cones had numerous, well-developed filopodia and lamellipodia. Microtubules splayed within the central region of the growth cone (Fig. 2A). Growth cones in 1 nM (not shown) or 10 nM (Fig. 2D) latA had few (if any) filopodia, and had few (or very small) lamellipodia. At a higher concentration (25 nM), growth cones had no filopodia or lamellipodia and actin was present only as distinct foci (arrow in Fig. 2E). In 25 nM latA, growth cones failed to respond directionally to the EF, turning only 1.5±5.0° (n=46) during 3 hours of EF exposure and migrating at 19.9±2.4 μm/hour. The microtubule staining in latA-treated growth cones (Fig. 2D,E) was similar to control neurons (Fig. 2A). Therefore, latA treatment disrupted the structure of the actin cytoskeleton selectively and prevented cathodal turning even when microtubules were morphologically (at least grossly) indistinguishable from those in the absence of the drug. This suggests collectively that microtubules in the absence of actin filaments are not sufficient to direct growth cones cathodally.

Microtubules are required for cathodal turning

Dynamic microtubules are required for growth cone steering in molecular gradients (Challacombe et al., 1997; Williamson et al., 1996; Gordon-Weeks, 2003) and they are influenced by Rho GTPases (Gordon-Weeks, 2003; Andersen, 2004), which

underpin cathodal turning (Rajnicek et al., 2006). We therefore tested the role of microtubules in cathodal steering by disrupting their dynamics pharmacologically with taxol, vinblastine or nocodazole.

Taxol (Tax) shifts the equilibrium of microtubule dynamics towards assembly and prevents depolymerisation, therefore preventing dynamic rearrangement within the growth cone. We confirmed that Tax treatment affected microtubules in our cultures by labelling growth cone microfilaments with Rhodamine-phalloidin and microtubules with an antibody to α-tubulin. At 7 nM Tax, there were few microtubules in the central region of the growth cone (Fig. 2B); at 20 nM Tax, some growth cones displayed loops of microtubules in the central region (Fig. 2C) or a swelling on the neurite shaft marking the point where the growth cone was at the time of drug addition (Fig. 2F). Growth cones in 20 nM Tax did not respond directionally to the EF (data not shown) and migration rates (16.5±2.4 μm/hour, n=23) were slower than DMSO controls (39.5±1.0 μm/hour, n=485). 30% (7/23) of growth cones stalled (active filopodia but no further advance) and 14% (3/23) retracted by 3 hours so we used 4 nM Tax for subsequent EF experiments, in which 10/73 (14%) stalled intermittently and no growth cones retracted.

In 4 nM Tax, the angle of cathodal growth cone turning and the frequency of anodal turning were not different than DMSO-treated cells without an EF (Fig. 3A,C) and the frequency of cathodal turning was attenuated significantly (Fig. 3C). The migration rate (24.0±1.0 μm/hour, n=73, Fig. 3B) was reduced compared with DMSO + EF (P<0.0001), with cathode-facing and anode-facing growth cones migrating at the same rates

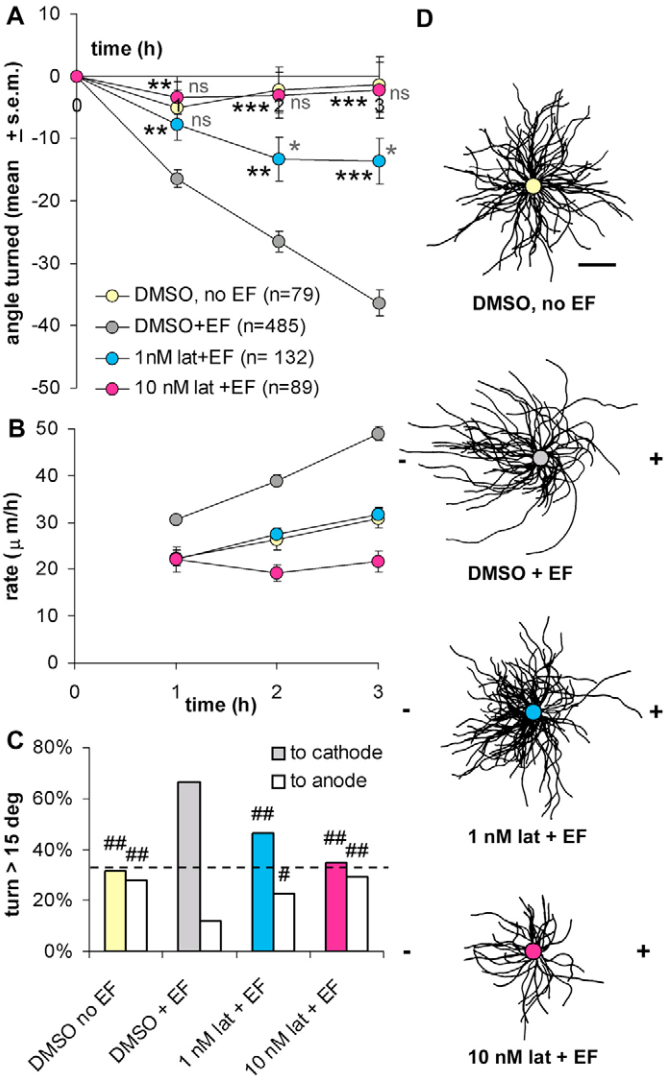


Fig. 1. Actin microfilaments are required for growth cone turning towards the cathode. (A) Mean angle turned in 3 hours; lat, latrunculin; n, number of growth cones measured; ns, not significant. *P<0.05; **P<0.005; ***P<0.0001. Black asterisks indicate comparison with 'DMSO + EF' and grey asterisks indicate comparison with 'DMSO, no EF'. (B) Mean migration rates for the growth cones in A. (C) The frequency of turning towards the cathode (filled bars) and the anode (open bars) for the same population. $^{\#}P$<0.002; $^{\#\#}P$<0.001 compared with DMSO + EF. (D) Composite drawings of individual neurons at 3 hours made by superimposing cell bodies at the coloured dot and tracing individual neurite paths. For EF-treated cells, the EF vector is horizontal with the cathode to the left and the anode to the right. Bar, 100 μm, applies to all.

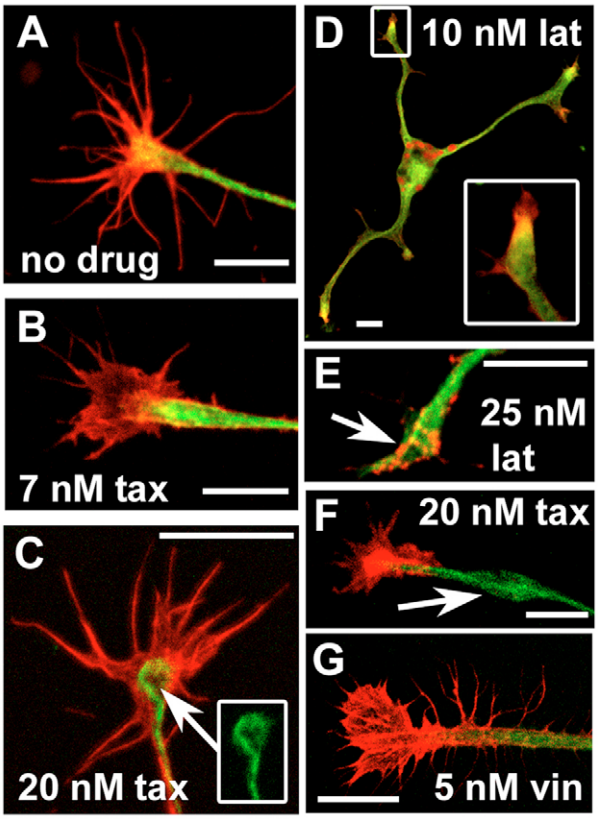


Fig. 2. Actin and microtubule staining of growth cones exposed to an EF of 150 mV/mm (cathode to the left) in the presence of cytoskeleton inhibitors. Confocal images of Rhodamine-phalloidin (red) staining of F-actin and α-tubulin (green). (A) No drug + EF. (B,C,F) Taxol (tax) + EF. Arrow in C indicates looped microtubules in the central region of the growth cone, enlarged in the inset. Arrow in F indicates the swelling that sometimes forms in neurons exposed to >20 nM taxol. It marks the point on the neurite where the growth cone was at the time of drug addition. (D,E) Latrunculin A (lat) + EF. Inset in D shows detail of the growth cone in the white rectangle. Actin staining in 25 nM latrunculin is punctate, indicated by arrow in E. (F) Taxol + EF. (G) Vinblastine + EF, Bars, 10 μm.

(22.0±1.3 μm/hour, *n*=39, and 26.5±2.1 μm/hour, *n*=11, respectively). Therefore, stabilisation of microtubules with Tax abolished the directional EF responses, including cathodal turning and faster cathodal migration rates.

Two other drugs used at low nanomolar concentrations tested further the role of microtubules in cathodal EF guidance. Sub-micromolar concentrations of vinblastine (Vin) or nocodazole (Noc) inhibit dynamic instability and treadmilling (Wilson et al., 1999; Gordon-Weeks, 2003), therefore preventing dynamic exploration of the growth cone periphery by individual microtubules. Vin and Noc had similar affects on cathodal growth cone steering. Cathodal turning was attenuated substantially in 1, 2 or 5 nM Vin (there was no difference in growth cone behaviour within this range so data were pooled) or in 10 nM Noc compared with no drug + EF (Fig. 3A). The frequencies of cathodal and anodal turning were reduced and increased, respectively, by both drugs (Fig. 3C). Vin (31.4±1.3 μm/hour, *n*=199) and Noc (38.8±1.4 μm/hour, *n*=161) did not affect the rates of growth cone advance compared with their respective no drug + EF (31.4±2.3 μm/hour, *n*=85) or DMSO + EF (39.5±1.0 μm/hour, *n*=485) controls (Fig. 3B), so the reduction in turning was not attributable to failure to migrate. Indeed, relatively few growth cones failed to advance in these drugs: 12% (25/199) stalled and 2% (3/199) retracted in Vin, and 7% (12/161) stalled in Noc with none retracting.

Double labelling of growth cone microfilaments and microtubules in Vin-treated neurons showed faint microtubule staining in neurites and growth cones and the usual splayed arrangement was not present in the central region of the growth cone (Fig. 2G). However, numerous actin-rich filopodia were present, especially along the neurite shaft, and growth cone lamellipodia were well developed (Fig. 2G). These data indicate that preventing dynamic instability of microtubules with Tax, Noc or Vin disrupts growth cone guidance by an EF.

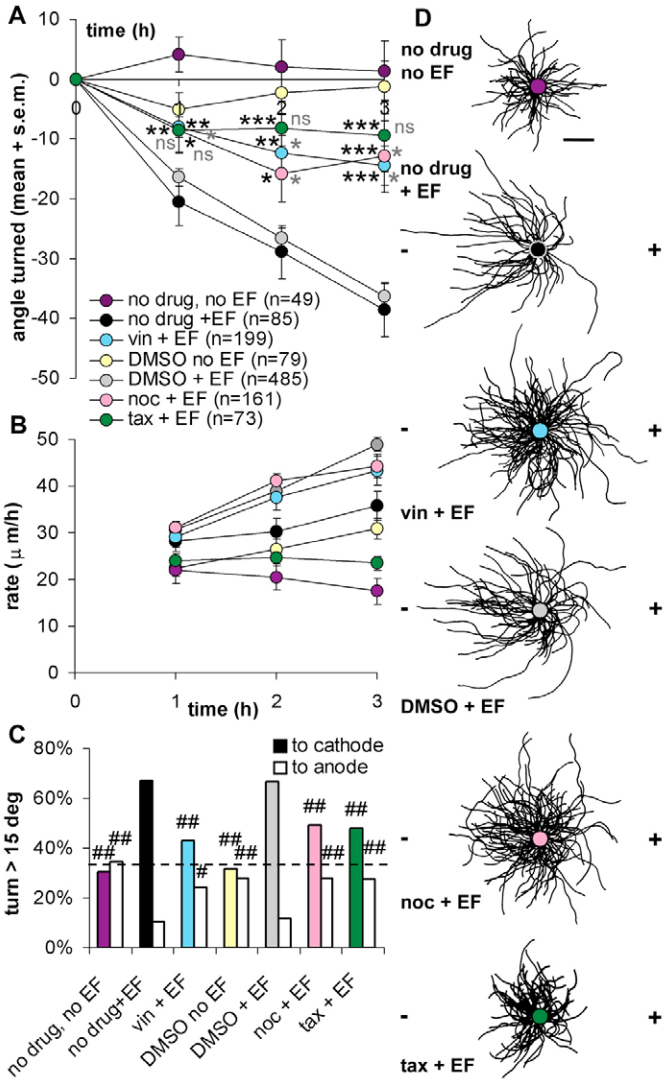


Fig. 3. Microtubules are required for cathodal turning. (A) Mean angle turned in 3 hours; n, number of growth cones; noc, nocodazole; tax, taxol; vin, vinblastine. **P*<0.05; ***P*<0.005; ****P*<0.0001. Black asterisks indicate comparison with 'DMSO + EF' (for noc and tax) or 'no drug + EF' (for vin), and grey asterisks indicate comparison with 'DMSO no EF' control or 'no drug, no EF' control. (B) Mean rate of growth cone advance. (C) Frequency of turning towards the cathode (filled bars) or anode (open bars) during 3 hours. #*P*<0.002; ##*P*<0.001. (D) Composite drawings made from images of individual neurons at the end of 3 hours. Cell bodies have been superimposed and individual neurite paths traced. Bar, 100 μm, applies to all.

Dynamic analysis of growth cone morphology and turning

To relate cytoskeletal architecture to the mechanism for EF guidance, we determined the spatial and temporal relationships between growth cone morphology and orientation in an EF. Images were captured at intervals of 1 minute. The orientation of the growth cone, the number of filopodia facing the cathode and anode, and the area of the lamellipodium on the anode-facing and cathode-facing sides of each growth cone were measured every 10 minutes. As expected for randomly directed growth cones, neurons not exposed to an EF showed no turning response during the 3 hours of observation (*n*=36; Movie 1, supplementary material) but EF-treated growth cones reoriented towards the cathode (*n*=37; Movie 2, supplementary material). The cathodal turning response was statistically significant at every point from 20 minutes (*P*=0.0243 at 20 minutes, 0.0021 at 30 minutes, *P*=0.0032 at 40 minutes, *P*=0.0095 at 50 minutes and *P*<0.0001 at >60 minutes), with most turning occurring within the first 60 minutes (Fig. 4A). The frequency of cathodal turning (Fig. 4B) was also significantly different from the 'no EF' control at 20 minutes (*P*<0.02 between 20 minutes and 50 minutes) and at every point thereafter (*P*<0.001 at >60 minutes).

Filopodia and lamellipodia reorient cathodally in advance of turning

The requirement for filopodia in growth cone steering by molecular gradients has been controversial but our data suggest that guidance by electrical gradients requires cytoskeletal reorganisation. We therefore explored the dynamics of EF-induced rearrangement of filopodia and lamellipodia to determine whether reorientation precedes growth cone turning. Growth cones in the absence of an EF bore, on average, 2.7±0.1 filopodia per growth cone (*n*=29) but this was increased to 3.0±0.1 per growth cone (*n*=32) in an EF (*P*=0.0045).

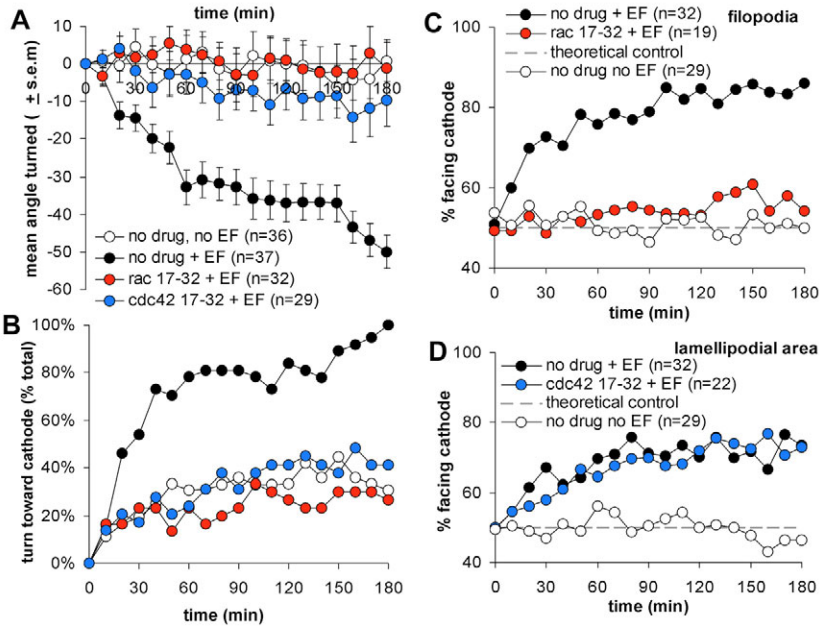


Fig. 4. Dynamic analysis of growth cones in the presence and absence of an EF and inhibitors of the Rac and Cdc42 CRIB domains. (A) The mean angle turned at intervals of 10 minutes; n, number of growth cones. The angle turned in an EF (black spots) is no different than the no EF control (white spots) at 10 minutes; however, by 20 minutes, the angle turned is significantly more cathodal (P=0.0243) and becomes increasingly cathodal with time (P<0.005 from 30-50 minutes, and P<0.0001 from 60-180 minutes). The Rac (red spots) and Cdc42 (blue spots) peptides abolish turning for the entire 3 hours (P>0.05 throughout). (B) Frequency of growth cones that turn >15° towards the cathode. Symbols and number of growth cones are as for A. (C) The percentage of filopodia facing the cathode. The broken line indicates the expected frequency for randomly directed filopodia (50%); n, number of growth cones measured. (D) The percentage of total lamellipodial area facing the cathode. The dotted line represents the theoretical frequency for randomly directed lamellipodia (50%); n, number of growth cones.

Similarly, the EF increased the total lamellipodial area of the same growth cones from 61.1±0.7 μm^2 without an EF to 73.3±2.5 μm^2 in an EF (P<0.001). The EF increased the percentage of filopodia facing the cathode (Fig. 4C); 60% faced cathodally within 10 minutes (P<0.02 compared with anode facing) and 70% of filopodia faced cathodally by 20 minutes (P<0.002 at 20 minutes and P<0.001 for every point thereafter compared with expected frequency of 50% for no EF). By 3 hours, 86% were cathode facing. The frequency of filopodia on the left side of control growth cones (analogous to the cathode for EF treated) ranged from 47-56% at individual time points, averaging 51% over the entire 3 hours, confirming that random filopodial orientation correlates with random directional migration (Fig. 4A,C). Since cathodal filopodial asymmetry was evident within 10 minutes, and significant cathodal reorientation of the growth cone did not occur until 20 minutes, filopodial reorientation preceded growth cone turning, suggesting an instructive role. This is consistent with previous reports for growth cones in electrical or chemical gradients (McCaig, 1989; Zheng et al., 1996).

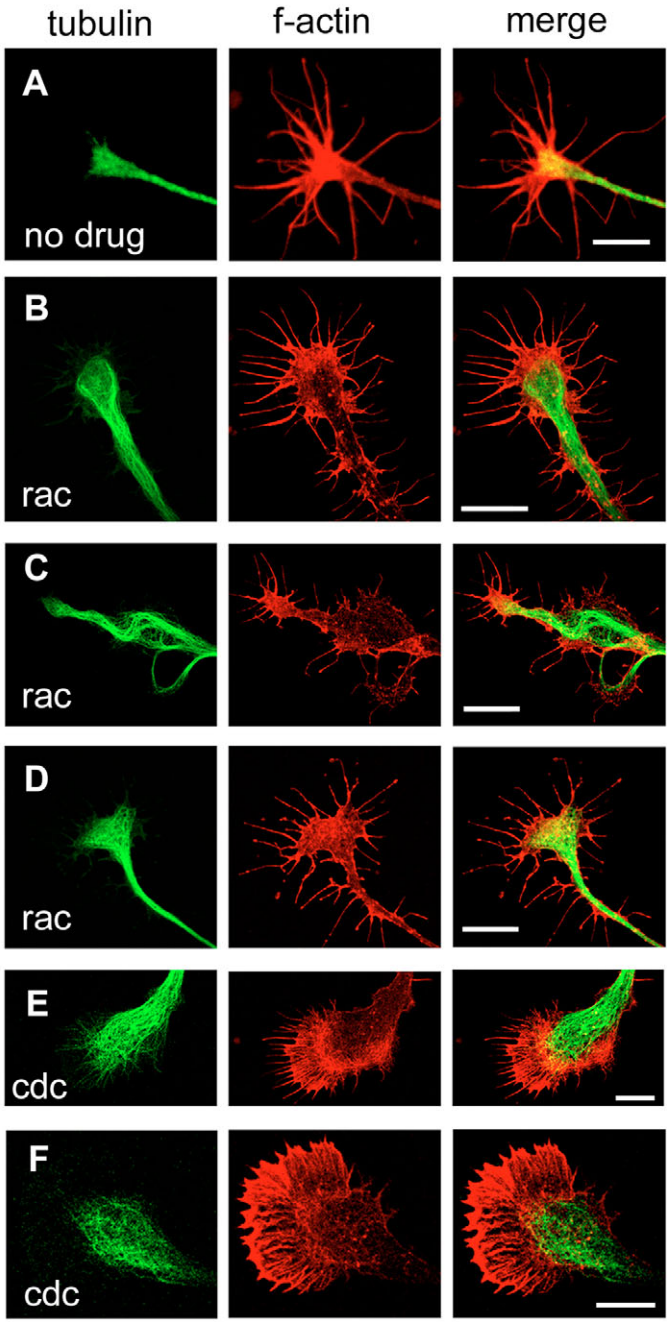
A similar analysis of lamellipodial area indicated that the EF biased lamellipodial distribution towards the cathode (Fig. 4D). Lamellipodial area was increased cathodally relative to the expected frequency of 50% within 20 minutes of EF initiation (P<0.001 at each point >20 minutes). The percentage of lamellipodial area on the left side of control (no EF) growth cones was never significantly different to 50% throughout the 3 hours of observation (Fig. 4D). The total cathode-facing (32.4±4.5 μm^2, n=29) area was not greater than the anode-facing area (27.1±4.6 μm^2) of the same growth cones at 10 minutes but it was significantly increased cathodally (40.9±4.4 μm^2) compared with anodally (25.7±4.1 μm^2) by 20 minutes (P=0.1143) and at every point thereafter. Analysis of the total area on the cathode- and anode-facing halves revealed that the relative shift towards cathodal asymmetry was attributable to an increase in cathodal area rather than a decrease in anodal area since at no point was the anode-facing lamellipodial area of EF-treated growth cones significantly different compared with the right half of control growth cones without an EF (data not shown). Therefore, filopodial reorientation towards the cathode precedes cathodal growth cone reorientation in an EF. Lamellipodial orientation follows filopodial reorientation and is temporally coincident with the onset of cathodal turning (~20 minutes). The cathodal bias of filopodia and lamellipodia is maintained as growth cones migrate cathodally throughout the 3-hour period of EF exposure.

Rac CRIB activity is required for cathodal orientation of filopodia and cathodal turning

We have demonstrated (Rajnicek et al., 2006) that a peptide corresponding to Rac 17-32 [the Cdc42/Rac-1 binding (CRIB) domain] prevents cathodal turning. The present study confirmed and extended this by showing that the peptide prevented the cathodal orientation of filopodia over the entire 3 hours (Fig. 4C and Movie 3, supplementary material), accompanied by loss of cathodal turning (Fig. 4A,B) of the same growth cones. Labelling of EF-treated growth cones for filamentous actin and tubulin (Fig. 5B-D) indicated that the peptide affected cytoskeletal structure. Rac activation stimulates lamellipodial formation (Kozma et al., 1997) so Rac inhibition would be predicted to reduce growth cone lamellipodia but not filopodia, which are initiated by Cdc42 signalling (Ruchhoeft et al., 1999; Kim et al., 2002). Consistent with this idea, growth cones treated with Rac 17-32 had numerous, well-defined filopodia and relatively underdeveloped lamellipodia. Time-lapse analysis of live growth cones in an EF indicated that growth cones treated with Rac 17-32 bore significantly (P<0.0001) more filopodia (4.2±0.2, n=19) than those with no drug (3.0±0.1, n=29). Filopodia in fixed, labelled preparations were not biased towards the cathode of the EF (Fig. 5B-D), supporting our time-lapse observations. Microtubule structure was reminiscent of that in taxol (Fig. 2C), with loops in the central region of the growth cone or in the neurite shaft (Fig. 5B,C).

Cdc42 CRIB inhibition reduces filopodia and prevents turning despite cathodal bias of lamellipodia

Incubation with the Cdc42 17-32 peptide, which binds to the Cdc42 CRIB domain, prevents cathodal orientation during 3 hours of EF exposure (Rajnicek et al., 2006). The present study confirmed this and further revealed that growth cones failed to turn cathodally in the peptide (Fig. 4A,B and Movie 4, supplementary material), even though lamellipodia were biased cathodally (Fig. 4D). Rhodamine-phalloidin staining confirmed that EF-treated growth cones had large, cathodally biased lamellipodia (Fig. 5E,F). Growth cones had fewer, smaller filopodia (Fig. 5F) than without the peptide (Fig. 5A) or in the Rac 17-32 peptide (Fig. 5B-D), so we were unable to perform dynamic analysis of filopodial symmetry in the Cdc42 inhibiting peptide. However, Rhodamine-phalloidin staining revealed that, when rudimentary filopodia were present, they were biased cathodally (Fig. 5E). Microtubule staining in the central region of the growth cone was much more distinct than in the Rac inhibiting peptide (Fig. 5B-D). Microtubules were distinct, extended into the periphery of the growth cone and appeared loosely adherent along their lengths (Fig. 5E,F). Measurements of time-lapse phase contrast images confirmed a significant (P<0.0001) increase in total lamellipodial area for growth cones treated with the peptide + EF (176.4±4.4 μm^2, n=23) compared with an EF alone (73.3±2.5 μm^2, n=29).



Collectively, these data indicate that cathodal bias of filopodia is required for cathodal guidance by an EF and that lamellipodial asymmetry is insufficient to induce cathodal turning in the absence of filopodia. Furthermore, the data suggest a mechanism in which Rac, not Cdc42 CRIB activity, underlies the cathodal filopodial asymmetry that underpins cathodal turning. The role of microtubules in cathodal steering remains unclear but the failure to turn cathodally under conditions in which peripheral actin was lost while preserving microtubules in the centre of the growth cone suggests that actin and microtubules act cooperatively to induce cathodal guidance.

Discussion

We have established roles for the GTPases Cdc42, Rac and Rho in growth cone guidance by EFs and proposed that they mediate cathodal steering through modulation of regional cytoskeletal dynamics (Rajnicek et al., 2006). Here, we have identified roles for the cytoskeleton, Rac and Cdc42 in EF-induced guidance of embryonic *Xenopus* spinal neuron growth cones by: (1) testing the ability of growth cones to turn cathodally in the presence of latA, which selectively disrupts microfilaments; (2) testing the ability to turn cathodally in the presence of Vin, Noc or Tax, which dampen microtubule dynamics; (3) correlating the dynamics of filopodial and lamellipodial growth cone reorganisation with cathodal turning; and (4) selectively inhibiting filopodial (Cdc42-mediated) or lamellipodial (Rac-mediated) dynamics using inhibitory peptides specific for the Cdc42 and Rac CRIB domains that prevent cathodal turning (Rajnicek et al., 2006).

Cathodal growth cone turning requires intact microfilaments

A common theme in proposed mechanisms for growth cone guidance by extracellular gradients is that downstream effector cascades converge to mediate dynamics of the growth cone cytoskeleton (e.g. Giniger, 2002). Structurally, the growth cone comprises a central domain (C-domain) and a peripheral

Fig. 5. Effects of inhibition of Rac or Cdc42 CRIB activity on cytoskeletal morphology. All growth cones were exposed to an EF (cathode at left) for 5 hours. (A) No drug + EF. (B-D) Growth cones treated with Rac 17-32 (rac). Note that microtubules (first column) appear less adherent to each other along their lengths. Numerous, uniformly distributed filopodia are present but lamellipodia are not well formed. (E,F) Growth cones in Cdc42 17-32 peptide (cdc). Microtubules (first column) appear loosely adherent to each other and they splay into the peripheral region of the growth cone more than when treated with Rac 17-32. Filamentous actin staining (second column) reveals large, well-spread lamellipodia and relatively fewer filopodia than when treated with Rac 17-32. When they are present, filopodia face the cathode. Bars, 10 μm.

domain (P-domain) with distinct cytoskeletal components. The C-domain is continuous with the distal neurite and contains bundles of splayed microtubules. Individual microtubules explore the P-domain transiently (Zhou et al., 2002; Zhou et al., 2004; Schaefer et al., 2002) but the main components of the P-domain are actin microfilaments arranged in dynamic, finger-like filopodia and veil-like lamellipodia.

Initial attempts to explain growth cone steering by extracellular gradients centred almost exclusively on microfilaments because they are the main components of the P-domain, which is ideally suited to detect and respond to gradients. In addition, the dynamics of microfilament assembly, disassembly and stabilisation are tightly regulated spatiotemporally within the P-region to dictate the dynamic shape of the growth cone. A fundamental role for microfilaments in growth cone steering was suggested by evidence that pharmacological depletion of actin filaments prevented turning in extracellular molecular gradients (Bentley and Toroian-Raymond, 1986; Chien et al., 1993; Kaufmann et al., 1998; Challacombe et al., 1997). Our data extend this to include extracellular electrical gradients. Depletion of actin filaments with latA significantly inhibited cathodal turning (Fig. 1). Fluorescent staining of filamentous actin revealed a persistent cortical cap of actin at neurite tips in 10 nM latA but only a few punctate spots remained at 25 nM, perhaps explaining why slight cathodal turning persisted at 10 nM but was abolished at 25 nM (Fig. 1A). Our data apparently contradict a previous study in which growth cones stripped of filopodia using cytochalasin turned normally toward the cathode of an EF, but a cortical cap of actin remained in the distal neurite tip of those cells (McCaig, 1989), similar to the one observed here in 10 nM latA (Fig. 2D). It is possible that the residual cytochalasin-resistant actin was sufficient to mediate cathodal turning in the absence of filopodia, perhaps acting as a ‘lamellipodium’. Latrunculin is a more potent inhibitor of microfilament organisation than cytochalasin (Spector et al., 1989), and its ability to abolish turning completely under conditions in which no actin remained (25 nM latA) indicate conclusively that filopodia are essential for cathodal steering. Collectively, the notion that the cytochalasin-resistant ‘lamellipodium’ in the study by McCaig (McCaig, 1989) mediated cathodal turning and our observation that 25 nM latA abolished filopodia, lamellipodia and cathodal turning, raise the possibility that lamellipodia contribute to cathodal steering.

What are the relative roles of filopodia and lamellipodia in growth cone steering by an EF? We approached this question by determining the dynamics of filopodial and lamellipodial reorganisation induced by the EF. Consistent with a previous report (McCaig, 1989), filopodia reoriented towards the cathode (Fig. 4C). We extended this observation by demonstrating that filopodial reorientation precedes growth cone turning, suggesting a causal role in steering by an EF (Fig. 4C,D) analogous to that in growth cone steering by chemical gradients (Zheng et al., 1996; Zhou et al., 2002; Zhou et al., 2004) and showed that lamellipodial asymmetry accompanies turning.

Actin structure is regulated dynamically by Cdc42 (which induces filopodia), by Rac (which induces lamellipodia) and by Rho (which induces collapse). In the companion paper in this issue, we demonstrated requirements for Cdc42 and Rac in growth cone steering by an EF and presented a model in which Rac, Cdc42 and Rho mediated cytoskeletal assembly and disassembly locally, inducing cathodal steering (Rajnicek et al., 2006). Here, we explored the roles of Cdc42 and Rac in dynamic rearrangements of filopodia and lamellipodia that accompany turning. Using peptides that selectively prevent effector binding to the CRIB domain on Cdc42 or Rac, we demonstrated that the Rac peptide depleted lamellipodia and promoted long filopodia (Fig. 5B-D). Filopodia failed to orient cathodally in the EF, and growth cones did not subsequently reorient towards the cathode (Fig. 4A,C). The Cdc42 peptide suppressed filopodial formation but large lamellipodia persisted (Fig. 5E,F), which oriented towards the cathode (Fig. 4D) as usual. However, lamellipodial asymmetry was insufficient to induce cathodal steering in the absence of filopodia (Fig. 4A,D). Therefore, filopodial asymmetry is sufficient to induce cathodal turning in the absence of lamellipodia, but lamellipodial asymmetry in the absence of filopodia is insufficient to induce cathodal turning.

Cathodal steering requires dynamic microtubules

There is increasing evidence that microtubules have a significant role in growth cone steering (Andersen, 2004; Gordon-Weeks, 2003). We used low nanomolar concentrations of drugs that dampen microtubule dynamics (Tax, Noc or Vin) with minimal effects on microtubule structural integrity or polymer mass (Tanaka et al., 1995; Zakharenko and Popov, 1998; Wilson et al., 1999; Suter et al., 2004) to demonstrate an instructive role for dynamic microtubules in cathodal steering by an EF. These drugs permitted growth cone advance but attenuated turning in an EF (Fig. 3), which is consistent with reports of inhibition of chemotropic growth cone guidance (Buck and Zheng, 2002; Suter et al., 2004; Challacombe et al., 1997).

Cathodal steering requires interaction between microfilaments and microtubules

Our data suggest that microfilaments and microtubules act coordinately to produce cathodal steering since growth cones failed to turn cathodally under conditions in which microfilaments were depleted while sparing microtubules (latA), and under conditions that prevented microtubule dynamics but spared P-region microfilaments (Vin and Tax). A model that incorporates this mutual requirement and is consistent with our data for filopodial accumulation cathodally is summarised in Fig. 6. It suggests that filopodial actin microfilaments are stabilised towards the cathode by an unidentified mechanism. Our data implicating Rac CRIB effectors in cathodal bias of filopodia and subsequent steering towards the cathode suggest it might involve a Rac CRIB effector such as p21-activated kinase (Pak1), which mediates growth cone filopodia (Kim et al., 2003). Stabilised filopodial microfilaments can then act as guides for cathodally directed microtubule assembly (Schaefer et al., 2002) or capture individual microtubules as they probe the P-domain dynamically (Gordon-Weeks, 2003). Capture would prevent microtubule collapse cathodally, yielding stable microtubules oriented in the direction of the turn. The stable microtubules would encourage membrane addition on the side of the growth cone facing the cathode through directed membrane vesicle transport along microtubules and microtubule-mediated regional insertion (Zakharenko and Popov, 1998). Preferential membrane addition cathodally might contribute to the relative

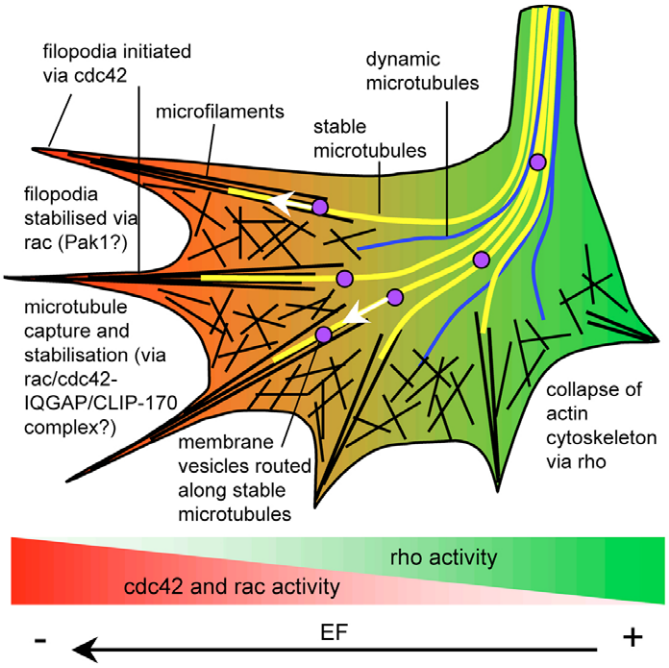


Fig. 6. Hypothetical mechanism by which Rho GTPases mediate growth cone steering towards the cathode. The concentrations of active Rac and Cdc42 (red) are relatively high on the cathode-facing side of the growth cone, but Rho activity (green) is relatively low on that side. Conversely, active Rho is relatively high anodally and active Rac and Cdc42 are low. Some candidate Rac and Cdc42 effectors are suggested. Others effectors, whose involvement has been tested, are presented in the companion paper in this issue (Rajnicek et al., 2006).

increase in lamellipodial area on the cathode-facing sides of EF-treated growth cones.

According to this model, filopodial, not lamellipodial, reorientation would be required for cathodal steering since stabilised microtubules colocalise with filopodial microfilaments, not the intervening lamellipodial regions (Schaefer et al., 2002; Zhou et al., 2002). This notion is supported by evidence that localised filopodial collapse results in local loss of microtubules in the P-domain and turning towards the side of the growth cone that retains microtubules (Zhou et al., 2002). This is consistent with our observation that cathodal bias of lamellipodia in the absence of filopodia was not sufficient to induce cathodal steering (Fig. 4D) and our observation that cathodal filopodial re-orientation precedes lamellipodial orientation and turning. However, it contrasts with the observation that attractive turning towards a source of Tax requires Rho GTPase-mediated stabilisation of microtubules and lamellipodial protrusion in advance of turning (Buck and Zheng, 2002). Perhaps this reflects a failure of the Tax gradient to activate the membrane receptors and intracellular signals that normally initiate cathodal steering.

Roles of Cdc42 and Rac in cathodal steering

Our data confirm that cytoskeletal rearrangements mediated by Cdc42 and Rac underpin cathodal steering as proposed previously (Rajnicek et al., 2006). The differences in growth cone morphology (Fig. 5) and EF-induced cytoskeletal dynamics (Fig. 4) produced by the Rac and Cdc42 peptides suggest that Rac and Cdc42 act through different effector pathways, as has been shown for guidance of *Drosophila* growth cones in vivo (Kim et al., 2003). We show that Rac CRIB activity underpins filopodial asymmetry towards the cathode. Rac induces thick filopodial actin bundles in growth cones in vivo (Matsuura et al., 2004), suggesting that Rac might stabilise cathode-facing filopodia. Lamellipodial asymmetry induced by the EF was independent of Cdc42 activity (Fig. 4D) and was insufficient to induce turning (Fig. 4A), suggesting that Rac-mediated filopodial asymmetry is essential for cathodal guidance. Although Cdc42 induces filopodial formation, it controls activity of growth cone filopodia independently of path-finding (Kim et al., 2002). Our data suggest that, although Cdc42 mediates filopodial extension (Fig. 5E,F), Rac activity controls the filopodial behaviour that underpins EF-induced steering.

We incorporate Rac and Cdc42 activity in our hypothetical model (Fig. 6) by suggesting that Rac and Cdc42 might aid microtubule capture by filopodia. The actin-binding protein IQGAP1, an effector of Cdc42 and Rac, is located at the leading edge of growth cone filopodia and associates with the cytoplasmic linker protein 170 (CLIP-170), which accumulates at the plus-end of microtubules (Fukata et al., 2003). Activated Rac and/or Cdc42 forms a complex with IQGAP and CLIP-170, enhancing the interaction of IQGAP with CLIP-170 (Fukata et al., 2002). Activated Rac/Cdc42 could therefore provide a docking site for microtubules near the filopodial cortex. It is an additionally attractive scheme in terms of cathodal growth cone steering since regional influx of Ca^{2+} is proposed to mediate cathodal steering (see McCaig et al., 2005) and cytoplasmic levels of Ca^{2+} and calmodulin regulate IQGAP and affect Cdc42 activity (Briggs and Sacks, 2003). In terms of growth cone guidance by Efs, this provides a mechanism whereby activated Rac/Cdc42 forms a complex at the plus-ends of microtubules with the potential to regulate microtubule stability locally.

In the present study, we have found that: (1) dynamic microfilaments and microtubules are essential for growth cone guidance by an EF; (2) filopodia reorient cathodally in advance of growth cone turning but lamellipodial reorientation is coincident with turning; (3) Rac CRIB activity, which was implicated previously in cathodal growth cone turning (Rajnicek et al., 2006), is required for cathodal orientation of filopodia; (4) contrary to a previous report (McCaig, 1989), filopodial asymmetry was essential for cathodal turning; (5) lamellipodia reorient cathodally even when Cdc42 CRIB activity is inhibited but lamellipodial asymmetry in the absence of filopodia is not sufficient for cathodal steering; and (6) the organisation of microtubules and actin microfilaments were affected by the Rac and Cdc42 CRIB inhibitors. Collectively, our data are consistent with the idea that Rac/Cdc42-mediated dynamics of microfilaments and microtubules act cooperatively to generate cathodal steering.

This study of embryonic *Xenopus* spinal neurons provides insight into the mechanism for growth cone guidance by naturally occurring EFs because we used an EF of 150 mV/mm, which is a conservative approximation of the EF present naturally in the developing *Xenopus* neural tube (Hotary and Robinson, 1991). Our results also have potential clinical impact. An emerging strategy for the treatment of adult

human spinal cord injuries is DC EF application (Shapiro et al., 2005). The stimulator design and EF polarity and duration used in this clinical trial were based on the EF responses of cultured *Xenopus* neurons (e.g. McCaig, 1987). Our observations of the dynamic responses of growth cones in EFs might therefore contribute to refining future stimulator design and suggest combinations of therapies in light of evidence that modulation of Rho GTPases affects spinal cord regeneration (Fournier et al., 2003).

Materials and Methods

Cell culture and EF application

The procedure for obtaining neurula-stage *Xenopus* embryos, preparation of EF chambers, primary culture of spinal neurons and EF application were identical to Rajnicek et al. (Rajnicek et al., 2006). The EF strength was 150 mV/mm and the EF vector was horizontal in time-lapse images.

Pharmacological inhibitors

The following reagents were dissolved directly into culture medium: vinblastine (Sigma), and peptides Rac 17-32 and Cdc42 17-32 [custom synthesised at the Aberdeen University Proteomics Facility (see Rajnicek et al., 2006)]. DMSO was the solvent for taxol (also called paclitaxel; Sigma), nocodazole (Sigma) and latrunculin A (Molecular Probes). Neurons were pre-incubated in the peptides for at least 1 hour but other drugs were added immediately prior to collecting the first time-lapse image and drugs were present throughout the experiment.

Immunocytochemistry

At the end of the experiment, cultures were fixed and permeabilised simultaneously with 4% (v/v) formaldehyde with 0.1% (v/v) Tween-20 (BDH Biochemicals) for 20-30 minutes followed by three rinses for 5 minutes in phosphate-buffered saline with 0.1% (v/v) Tween-20 (PBST). In preliminary experiments, cells fixed as above but omitting Tween developed substantial blebs on growth cones, so Tween was used routinely to prevent blebbing artefacts and to promote penetration of fixative into the cytoplasm, thereby fixing cytoskeletal structure rapidly. After fixing, cells were double labelled with (1:50) Rhodamine-phalloidin (Molecular Probes) and a FITC-conjugated antibody (1:200) to α-tubulin (Sigma) in PBST. Cells were rinsed three times with PBST, mounted in Vectashield (Vector Labs) and viewed with a BioRad Microradiance (or BioRad 1024) laser-scanning confocal microscope using a 60×/1.3NA water immersion objective.

Time-lapse observation of growth cone turning in an EF

Dissociated neurons were grown in custom-made chambers prepared from 100 mm diameter tissue culture dishes and exposed to an EF as described in Rajnicek et al. (Rajnicek et al., 2006), except that here the experiment duration was three hours. For low temporal resolution, neurons identified by time-lapse analysis were photographed at hourly intervals using an inverted phase contrast microscope (10× objective) equipped with a motorised X-Y stage (Prior Scientific) and Metamorph (Universal Imaging) imaging software. Neurite length and the angle of growth cone turning relative to the horizontal EF vector were measured from printouts of hourly images. For high temporal resolution studies, one image was collected every minute (Metamorph software) and growth cone morphology and orientation were determined from every tenth frame as described below.

Lamellipodial and filopodial orientation

Growth cones in phase contrast images were divided visually into cathode-facing (or left side, when no EF) and anode-facing (or right side when no EF) halves and the filopodia in each half were counted and expressed as a percentage of total. The mean lamellipodial area was calculated by manually tracing the periphery of the lamellipodium on each half of the growth cone using Metamorph software. Turning frequencies were compared using a D-test (Bailey, 1981), and the mean turning angles and mean number of filopodia and lamellipodial areas were compared using a two-tailed Student's *t*-test.

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References

Andersen, S. S. L. (2004). The search and prime hypothesis for growth cone turning. *BioEssays* **27**, 86-90.

Bailey, N. T. J. (1981). *Statistical Methods in Biology* (2nd edn), pp. 38-39. London: Hodder and Stoughton.

Bentley, D. and Toroian-Raymond, A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature* **323**, 712-715.

Borgens, R. B. and Shi, R. (1995). Uncoupling histogenesis from morphogenesis in the vertebrate embryo by collapse of the transneural tube potential. *Dev. Dyn.* **203**, 456-467.

Borgens, R. B., Toombs, J. P., Blight, A. R., McGinnis, M. E., Bauer, M. S., Widmer and W. and Cook, J. R., Jr (1993). Effects of applied electrical field son cases of complete paraplegia in dogs. *Restor. Neurol. Neurosci.* **5**, 305-322.

Briggs, M. W. and Sacks D. B. (2003). IQGAP proteins are integral components of cytoskeletal regulation. *EMBO Rep.* **4**, 571-574.

Buck, K. B. and Zheng, J. Q. (2002). Growth cone turning induced by direct local modification of microtubule dynamics. *J. Neurosci.* **22**, 9358-9367.

Caudy, M. and Bentley, D. (1986). Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. *J. Neurosci.* **6**, 1781-1795.

Challacombe, J. F., Snow, D. M. and Letourneau, P. C. (1997). Dynamic microtubule ends are required for growth cone turning to avoid an inhibitory guidance cue. *J. Neurosci.* **17**, 3085-3095.

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.

Finkelstein, E., Chang, W., Chao, P. H., Gruber, D., Minden, A., Hung, C. T. and Bulinski, J. C. (2004). Roles of microtubules, cell polarity and adhesion in electric-field-mediated motility of 3T3 fibroblasts. *J. Cell Sci.* **117**, 1533-1545.

Fournier, A. E., Takizawa, B. T. and Strittmatter, S. M. (2003). Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci.* **23**, 1416-1423.

Fukata, M., Watanabe, T., Noritake, J., Nakagawa, M., Yamaga, M., Kuroda, M., Matsuura, Y., Iwamatsu, A., Perez, F. and Kaibuchi, K. (2002). Rac1 and cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell* **109**, 873-885.

Fukata, M., Nakagawa, M. and Kaibuchi, K. (2003). Roles of rho-family GTPases in cell polarisation and directional migration. *Curr. Opin. Cell Biol.* **15**, 590-597.

Giniger, E. (2002). How do Rho family GTPases direct axon growth and guidance? A proposal relating signalling pathways to growth cone mechanics. *Differentiation* **70**, 385-396.

Gordon-Weeks, P. R. (2003). Microtubules and growth cone function. *J. Neurobiol.* **58**, 70-83.

Hotary, K. B. and Robinson, K. R. (1991). The neural tube of the Xenopus embryo maintains a potential difference across itself. *Dev. Brain Res.* **59**, 65-73.

Isbister, C. M. and O'Connor, T. P. (1999). Filopodial adhesion does not predict growth cone steering events in vivo. *J. Neurosci.* **19**, 2589-2600.

Kaufmann, N., Wills, Z. P. and Van Vactor, D. (1998). Drosophila Rac1 controls motor axon guidance. *Development* **125**, 453-461.

Kim, M. D., Kolodziej, P. and Chiba, A. (2002). Growth cone pathfinding and filopodial dynamics are mediated separately by cdc42 activation. *J. Neurosci.* **22**, 1794-1806.

Kim, M. D., Kamiyama, D., Kolodziej, P., Hing, H. and Chiba, A. (2003). Isolation of rho GTPase effector pathways during axon development. *Dev. Biol.* **262**, 282-293.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling, relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol. Cell. Biol.* **17**, 1201-1211.

Marsh, L. and Letourneau, P. C. (1984). Growth of neurites without filopodial or lamellipodial activity in the presence of cytochalasin B. *J. Cell Biol.* **99**, 2041-2047.

Matsuura, R., Tanaka, H. and Go, M. J. (2004). Distinct functions of rac1 and cdc42 during axon guidance and growth cone morphogenesis in Drosophila. *Eur. J. Neurosci.* **19**, 21-31.

McCaig, C. D. (1987). Spinal neurite reabsorption and regrowth in vitro depend on the polarity of an applied electric field. *Development* **100**, 31-41.

McCaig, C. D. (1989). Nerve growth in the absence of growth cone filopodia and the effects of a small applied electric field. *J. Cell Sci.* **93**, 715-721.

McCaig, C. D., Rajnicek, A. M., Song, B. and Zhao, M. (2002). Has electrical growth cone guidance found its potential? *Trends Neurosci.* **25**, 354-358.

McCaig, C. D., Rajnicek, A. M., Song, B. and Zhao, M. (2005). Controlling cell behaviour electrically: current views and future potential. *Physiol. Rev.* **85**, 943-978.

Mueller, B. K. (1999). Growth cone guidance: first steps to a deeper understanding. *Annu. Rev. Neurosci.* **22**, 351-388.

O'Connor, T. P. and Bentley, D. (1993). Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues in situ. *J. Cell Biol.* **123**, 935-948.

Patel, B. N. and Van Vactor D. L. (2002). Axon guidance: the cytoplasmic tail. *Curr. Opin. Cell Biol.* **14**, 221-229.

Rajnicek, A. M., Foubister, L. E. and McCaig, C. D. (2006). Temporally and spatially coordinated roles for Rho, Rac, Cdc42 and their effectors in growth cone guidance by a physiological electric field. *J. Cell Sci.* **119**, 1723-1735.

Ruchhoeft, M. L., Ohnuma, S.-I., McNeill, L., Holt, C. E. and Harris, W. A. (1999). The neuronal architecture of Xenopus retinal ganglion cells is sculpted by rho-family GTPases in vivo. *J. Neurosci.* **19**, 8454-8463.

Schaefer, A. W., Kabir, N. and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. *J. Cell Biol.* **158**, 139-152.

Song, B., Zhao, M., Forrester, J. and McCaig, C. (2004). Nerve regeneration and wound healing are stimulated and directed by an endogenous electrical field in vivo. *J. Cell Sci.* **117**, 4681-4690.

Song, H. and Poo, M.-M. (2001). The cell biology of neuronal navigation. *Nat. Cell Biol.* **3**, E81-E88.

Shapiro, S., Borgens, R., Pascuzzi, R., Roos, K., Groff, M., Purvines, S., Rodgers, R. B., Hagy, S. and Nelson, P. (2005). Oscillating field stimulation for complete spinal cord injury in humans: a phase 1 trial. *J. Neurosurg. Spine* **2**, 3-10.

Spector, I., Shochet, N. R., Blasberger, D. and Kashman, Y. (1989). Latrunculins- novel marine macrolides that disrupt microfilament organization and affect cell growth: I comparison with cytochalasin D. *Cell Motil. Cytoskeleton* **13**, 127-144.

Suter, D. M., Schaefer, A. W. and Forscher, P. (2004). Microtubule dynamics are necessary for src family kinase-dependent growth cone steering. *Curr. Biol.* **14**, 1194-1199.

Tanaka, E., Ho, T. and Kirschner, M. W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.* **128**, 139-155.

Wang, F. S., Liu, C. W., Diefenbach, T. J. and Jay D. G. (2003). Modeling the role of myosin 1c in neuronal growth cone turning. *Biophys. J.* **85**, 3319-3328.

Williamson, T., Gordon-Weeks, P. R., Schachner, M. and Taylor, J. (1996). Microtubule reorganisation is obligatory for growth cone turning. *Proc. Natl. Acad. Sci. USA* **93**, 15221-15226.

Wilson, L., Panda, D. and Jordan, M. A. (1999). Modulation of microtubule dynamics by drugs, a paradigm for the actions of cellular regulators. *Cell Struct. Funct.* **24**, 329-335.

Zakharenko, S. and Popov, S. (1998). Dynamics of axonal microtubules regulate the topology of new membrane insertion into the growing neurites. *J. Cell Biol.* **143**, 1077-1086.

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

Zhou, F. Q. and Cohan, C. S. (2004). How actin filaments and microtubules steer growth cones to their targets. *J. Neurobiol.* **58**, 84-91.

Zhou, F. Q., Waterman-Storer, C. M. and Cohan, C. S. (2002). Focal loss of actin bundles causes microtubule redistribution and growth cone turning. *J. Cell Biol.* **157**, 839-849.