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ERM proteins regulate growth cone responses to Sema3A

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Abstract

Axonal growth cones initiate and sustain directed growth in response to cues in their environment. A variety of events such as receptor internalization, kinase activation, and actin rearrangement can be stimulated by guidance cues and are essential for mediating targeted growth cone behavior. Surprisingly little is known about how such disparate actions are coordinated. Our data suggest that ezrin, radixin, and moesin (ERMs), a family of highly homologous, multifunctional proteins may be able to coordinate growth cone responses to the guidance cue, Sema3A. We show that active ERMs concentrate asymmetrically in neocortical growth cones, are rapidly and transiently inactivated by Sema3A, and are required for Sema3A-mediated growth cone collapse and guidance. The FERM domain of active ERMs regulates internalization of the Sema3A receptor, Npn1 and its co-receptor, L1CAM, while the ERM C-terminal domain binds and caps F-actin. Our data support a model in which ERMs can coordinate membrane and actin dynamics in response to Sema3A.

Keywords

ezrin; axon guidance; actin; L1CAM; neuropilin; LPA

INTRODUCTION

Axonal growth cones navigate complex environments by detecting and responding to guidance cues. Cues are often encountered as gradients and minute differences in cue concentration that occur across the distance of a single growth cone can be sufficient to reorient axon extension (Goodman, 1996; Song et al., 1997; Sperry, 1963). The stimulation of guidance cue receptors on the growth cone surface initiates a complex series of events that serve to reflect and amplify cue position and concentration gradients. A variety of critical guidance cue responses have been identified (Buck and Zheng, 2002; Cowan et al., 2005; Fournier et al., 2000; Gomez and Zheng, 2006; Huber et al., 2003; Leung et al., 2006; Lin and Forscher, 1995; Mallavarapu and Mitchison, 1999; Meima et al., 1997; Piper et al., 2005; Suter and Forscher, 2000; Yao et al., 2006), but it remains unclear how disparate

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responses such as receptor internalization and actin reorganization can be coordinated so that growth cones turn, extend or retract appropriately.

Members of the ERM family of proteins (ezrin, radixin, and moesin) have several properties that make them excellent candidates to participate and potentially integrate growth cone responses to guidance cues. They are widely expressed in the developing nervous system and are concentrated in growth cones (Dickson et al., 2002; Gonzalez-Agosti and Solomon, 1996; Goslin et al., 1989; Mintz et al., 2003b; Paglini et al., 1998; Takahashi et al., 1999). They tether the actin cytoskeleton to the plasma membrane by interactions with PIP₂ and transmembrane proteins, such as the axonally polarized cell adhesion molecule, L1CAM (Algrain et al., 1993; Chishti et al., 1998; Turunen et al., 1994). The ERM binding site on L1CAM overlaps with that of the clathrin adapter, AP2 (Cheng et al., 2005; Dickson et al., 2002), suggesting that ERM binding may influence endocytosis and adhesion. Local changes in actin dynamics influence axon guidance (Bentley and Toroian-Raymond, 1986; Chien et al., 1993; Zheng et al., 1996), and findings in cell-free experiments (Tsukita and Hieda, 1989) suggest that ERMs may be able to directly regulate actin stability by capping microfilament barbed ends in growth cones. Finally, ERM actions are known to influence lymphocyte chemotaxis (del Pozo et al., 1998; Lee et al., 2004; Li et al., 2007; Vicente-Manzanares et al., 1999), a process that bears similarities to growth cone guidance (Ibanez, 2004; Meinhardt, 1999).

Sema3A is a secreted guidance cue that generally repels axonal growth cones. Its receptor, neuropilin-1 (Npn1), can form a co-receptor complex with L1CAM, and this interaction is essential for Sema3A-stimulated Npn1 endocytosis in COS cells as well as for cortical axons to respond to Sema3A (Castellani et al., 2000; Castellani et al., 2004). Given the relationship between ERMs and L1CAM, we sought to determine whether ERMs influence Sema3A-mediated axon guidance. We find that ERMs in cortical growth cones respond to Sema3A, and their actions are required for cortical axon targeting and for Sema3A-mediated growth cone collapse. ERMs regulate Sema3A-stimulated Npn1 endocytosis, and they cap actin in growth cones. Together these data suggest that ERMs can modulate both membrane and cytoskeletal dynamics in growth cones in response to Sema3A.

MATERIALS AND METHODS

Culture Preparation

Sprague Dawley rats were used for all of the experiments, and their care and treatment adhered strictly to guidelines adopted by the National Institutes of Health and Mount Sinai's Institutional Animal Care and Use Committee. Embryonic day 18 rat neocortical neurons were dissociated and plated on coverslips coated with poly-L-lysine, laminin or L1-Fc and maintained in Neurobasal (Invitrogen) using protocols described previously (Dickson et al., 2002) that were modified from a method used for the preparation of hippocampal neurons (Goslin and Banker, 1991). All experiments were performed at one to three days *in vitro* (DIV), and the results presented are taken from at least three separate cultures. Numbers of cells varied from experiment to experiment, and "n" values along with statistical tests used are provided in the figure legends.

Immunocytochemistry, Antibodies, and Microscopy

Neurons were fixed with 4% paraformaldehyde/4% sucrose warmed to 37°C, and except when determining surface expression of Npn1, were then permeabilized with 0.25% Triton-X 100, for 5 minutes. Immunocytochemical procedures were carried out as described previously (Anderson et al., 2004) using the following antibodies (see Table 1 for details):

anti-cpERM (1/25), anti-L1CAM (1:20), anti-Npn1 (1:200), anti-alpha adaptin (1:100), anti-GFP which also recognizes YFP (1:4000), and anti- β -Tubulin (1:3000).

The anti-cpERM antibody recognizes the phosphorylated ERM proteins in western blots. Following exposure to Rho kinase (Haas et al., 2007), or in the presence of a ser/thr phosphatase inhibitor (Suppl. Fig. 1), labeling increases in the absence of a change in total ERM levels. An in vitro kinase assay has also shown that phosphorylation of ERMs by Nck-interacting kinase can be detected equally well with autoradiograms or cpERM labeling. An inactive kinase results in no phosphorylation and no cpERM labeling (Baumgartner et al, 2006). Anti-L1CAM (ASCS4) was raised against the entire rat L1CAM molecule and recognizes an epitope between aa 1 and 804 in the extracellular domain (Sweadner, 1983b; Tang et al., 2006). It binds to a single band of the expected molecular weight in western blots from rat whole brain lysates that is the same size as a band identified with a polyclonal antibody directed against an intracellular epitope of L1CAM (Tang et al., 2006), and siRNA-mediated knockdown of L1CAM greatly diminishes ASCS4 immunostaining (Suppl. Fig. 2). We and others have shown that immunostaining is on the surface of neurons, polarized to axons, developmentally regulated, and shows a similar anatomical distribution as other antibodies against L1CAM (Akopians et al., 2003; Mintz et al., 2003a). Anti-Npn1 identifies two bands in western blots of extracts from cultured rat neocortical neurons corresponding to the full length and a cleaved soluble form, and the full-length band (~135 kDa) is the same size as that obtained using an antibody directed against the C-terminal domain of Npn-1 (Gagnon et al., 2000). Immunostaining is evident in dissociated cortical neurons at a time and in a pattern that correlates with its expression pattern *in vivo* (Morita et al., 2006). Consistent with the location of the epitope, we see labeling in cultured neurons in the absence of permeabilization (e.g. Fig. 7). Anti- β -tubulin (Tuj1) has been characterized extensively. It recognizes the sequence CEAQGPK in neuronal β III tubulin and labels a single 50 kDa band in Western blots from a variety of CNS regions (Gass et al., 1990). Anti-AP2 alpha labels a single band of ~110 kD in Western blots of rat brain extracts. Pull down assays from the same tissue using a protein that binds to AP2 (enthoproten) yields a similar band (Wasiak et al, 2002) as does immunoprecipitation using either Numb or anti-AP2 (Santolini et al, 2000). Immunolabeling was visualized using fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch; 1:200). F-actin was labeled using fluorophore conjugated phalloidin (Molecular Probes; 1:100).

Photomicrographs were captured with a Hamamatsu ORCA ER camera mounted on a Nikon Diaphot 300 fluorescent microscope using a 60x 1.4 N.A. objective at a resolution of 1280X1024. The confocal images were collected on a LSM 510 Zeiss microscope using a 100x objective at a resolution of 1024X1024, except for figure panels 7H–M, which were captured at a resolution of 512X512. Exposure conditions were identical across treatment conditions. Images were analyzed using MetaMorph software (Universal Imaging) and then imported into Photoshop (Adobe Systems). In some images, brightness and contrast were adjusted, and for those images covering different treatment conditions, all figures were adjusted identically. Wide-field images were deconvolved using a 2-d deconvolution algorithm (AutoQuant, Media Cybernetics). Outlines of YFP-filled structures were made using Photoshop. Figures were organized and lettering was added using InDesign (Adobe).

Plasmids

Plasmids (3–5 μ g) were introduced into neurons using the Nucleopore device (Amaxa). The plasmids used include the following: (1) YFP: pEYFP-N1 (BD Biosciences); (2) N-Ez: The N-terminus of human ezrin (Dickson et al., 2002) inserted into pEYFP-N1; (3) EzWt: Full length human ezrin inserted into the pTRE vector; (4) EzT567D: EzWt with a single codon mutation such that threonine at 567 is replaced with an aspartic acid (the latter two

were kind gifts of Dr. M. Arpin, Institut Curie, Paris; (Gautreau et al., 2000); (5) RdxWt: Full length mouse radixin inserted into the pDNR-Lib vector (ATCC).

Slice culture overlay guidance assay

The protocol to assay axon guidance was adapted from Ghosh and coworkers (Polleux et al., 1998). Four hundred micrometer thick coronal slices were dissected from the neocortex of postnatal day three rat pups. They were allowed to settle on Costar transwell insert membranes for four hours in a humidified incubator at 37°C with 5% CO₂. Transfected dissociated neurons prepared as described above were applied to the slices, and returned to the incubator. Co-cultures were fixed in 4% paraformaldehyde fifteen-eighteen hours later and immunolabeled with anti-GFP.

Transfected cells were visualized using an Axiophot microscope with a 40X, 1.3 NA objective (Zeiss). The boundaries of the slice and neurons that adhered within the cortical plate were traced using NeuroLucida software (Microbrightfield). The length of axons and the angle of their trajectory relative to the pial surface were determined using functions resident in NeuroInvestigator (Microbrightfield). This information was translated from polar coordinates to Cartesian coordinates such that zero degrees represented a straight ventral trajectory. Quantitative analysis to test for differences between groups was determined using a two-sided Wilcoxon signed rank test that the deviation angle is equal to 90 degrees.

Collapse assay

Media was harvested from COS-7 cells transduced with human Sema3A expressing AAV (kind gift of Dr. S.Kohtz, Mount Sinai School of Medicine, New York (Good et al., 2004). For each batch, growth cone collapse was assessed for several concentrations and compared to the dose response curve we obtained using recombinant Sema3A-Fc (R&D Systems, Fig. 3A). Batches have been very consistent, and for most assays we used a dilution that produces 55% collapse at 10 minutes. For LPA, we used 1μM. For controls we used either conditioned Neurobasal (for recombinant Sema3A or LPA experiments) or media collected from untransfected COS-7 cells (for harvested Sema3A experiments). Transfected cortical neurons were incubated for the specified time intervals, and subsequently fixed and labeled for YFP and F-actin. The number of collapsed, transfected growth cones was counted in the fixed preparations using a 40X objective on a Zeiss Axiophot microscope and reported as the percentage of the total number of growth cones. For statistical analysis we used ANOVA and Bonferroni post tests.

Western Blot assessment of ERM activity

Three day-old cortical neurons were lysed in NP-40 buffer after exposure to control media, Sema3A media, or lysophosphatidic acid LPA (1μM; Sigma) in media. After the DNA was removed, the samples were separated by size in 10% SDS gels. The proteins were then transferred to PVDF membranes, blocked in 5%BSA and blotted with anti-C-terminal phosphorylated ERM (cpERMs) (1:1000), anti-ezrin (Upstate Biotech, 1:1000), and HRP conjugated anti-GAPDH (Abcam, 1:2500). Appropriate HRP- or AP-conjugated secondary antibodies were used and then the membranes were developed using ECL or alkaline phosphatase substrates.

Analysis of cpERM asymmetry

Photomicrographs of cpERM labeled growth cones were divided into quadrants using a Cartesian grid with the center corresponding to the center of the growth cone. They were then color indexed for the intensity of cpERM and F-actin staining--warmer colors indicate higher intensities. The quadrants were ordered with respect to the levels of cpERM labeling

into: first (strongest), second, third and fourth (weakest). We measured the extent of cpERM labeling with respect to F-actin and reported the percent fluorescence in each of the quadrants. The relationship between F-actin and cpERM or alpha adaptin levels was assessed using line scans on overlay images (Image J). A series of lines were drawn from the growth cone center to the periphery and intensities in both channels along the lines were measured and exported to Excel. Correlations were determined in Excel using Pearson's test.

Detecting internalized and surface receptors

L1CAM endocytosis was assayed using a protocol adapted from Lemmon and colleagues (Kamiguchi et al., 1998). Transfected, dissociated neocortical neurons were plated onto glass coverslips coated with human L1CAM-Fc (Dickson et al., 2002). At one or two days in culture neurons were exposed to 1:200 ASCS4 (Developmental Studies Hybridoma Bank; (Sweadner, 1983a), a mouse IgG anti-rat L1CAM extracellular domain specific antibody that does not recognize the human L1 substrate diluted in home media. Cells were fixed and then incubated in an excess (1:20) unconjugated goat anti-mouse IgG (Jackson Laboratories) to saturate the surface. The cells were fixed again and then permeabilized as indicated above. The endocytosed pools of anti-L1CAM were then labeled with fluorescence-tagged secondary antibodies. Results using this strategy were very similar to those obtained after stripping off surface proteins using low concentrations of acid (Mintz, Wang, and Benson, unpublished results). We analyzed surface, rather than internalized Npn1, because anti-Npn1 antibodies could block Sema3A binding. Following exposure to Sema3A or control media, neurons were fixed and labeled for Npn1 in the absence of permeabilization (Piper et al, 2005; Wisco et al, 2003).

To quantify the extent of L1CAM and Npn1 labeling, growth cone area was traced and images of immunolabeling (acquired using the same exposure parameters) were then thresholded within the traced area. The percentage of total growth cone area occupied by labeled pixels was determined. Mean percent internalized L1CAM and surface Npn1 for the YFP, EzT567D and N-Ez was compared using ANOVA and subsequently using Bonferroni post tests.

Neurons were incubated with 2mg/ml Texas Red conjugated dextran with a molecular weight of 10 kDa (Molecular Probes). After 30 minutes of constitutive internalization, neurons were washed five times in PBS, fixed as described above, and the coverslips were mounted on slides.

Actin barbed-end binding protein displacement

For the assessment of general endocytosis neurons were incubated for five minutes at 37°C in media in which cytochalasin D (NP Biomedicals) or latrunculin A (Molecular Probes) were diluted at 900 or 100 nM from stocks (3.33 mg/mL in ethanol and 6.25 mg/mL in DMSO, respectively). Control neurons were incubated in media with the appropriate concentration of diluents. The areas of phalloidin and anti-cpERM labeling were determined using thresholding and tracing functions in MetaMorph, and comparisons between latrunculin A and cytochalasin D groups were made by Student's t-test after normalizing to diluent controls.

RESULTS

Activated ERM proteins are localized asymmetrically in growth cones

We first asked where ERMs might be active in cortical growth cones because ERMs are regulated principally by activation state and not by expression levels (Henry et al., 1995;

Martin et al., 1995). Activation state is controlled by changes in conformation. ERMs have an N-terminal FERM (Band Four Point One and ERM) domain and a C-terminal F-actin binding domain that bind to one another intramolecularly forming a closed, inactive conformation (Gary and Bretscher, 1995; Magendantz et al., 1995; Pearson et al., 2000; Tsukita and Yonemura, 1997). When PIP₂ binds to a site in the FERM domain, the molecule assumes an open and active conformation. A threonine residue in the C-terminal domain becomes phosphorylated and this prevents intramolecular binding (Fievet et al., 2004; Nakamura et al., 1999). Thus, antibodies recognizing the C-terminal phosphorylated form of ERMs (cpERMs) can be used to localize active ERMs selectively (Brown et al., 2003; Gautreau et al., 2000; Matsui et al., 1998).

cpERMs were localized in cultures of dissociated cortical neurons in which growth cones are easily seen and intragrowth cone distribution can be evaluated. We find that only a third of the axonal growth cones label for cpERMs at any one time, and within labeled growth cones, cpERMs concentrate in a subpopulation of filopodia (Fig. 1A – F). This pattern is consistent in a variety of growth cone shapes; we find no predictable relationship between the presence of cpERMs and growth cone morphology. However, filopodia labeled strongly for cpERMs often appear to have less F-actin (Fig. 1A–F). In contrast to cpERMs, total ERM proteins are distributed more symmetrically in the periphery of nearly all cortical axonal growth cones (Fig. 1G – I) (Haas et al., 2004), similar to other types of neurons (Gonzalez-Agosti and Solomon, 1996; Goslin et al., 1989; Paglini et al., 1998). The strong asymmetry seen with cpERMs is common to several agents known to influence axon turning in response to localized guidance cues (Gallo and Letourneau, 2004; Gomez et al., 2001; Henley and Poo, 2004) and suggests that ERMs may also respond to locally produced cues.

ERMs are required for cortical axon guidance

Ezrin, radixin and moesin appear to perform largely overlapping roles (Doi et al., 1999; Paglini et al., 1998), and their collective function has been investigated in most cells by the use of mutants. Given the high homology between them, a single mutant family member can target key functions of all three without causing cell death (Funayama et al., 1991; Lankes and Furthmayr, 1991), a common side effect of triple knockdowns. Here we employed two ezrin mutants, EzT567D and N-Ez. In EzT567D, threonine 567 has been mutated to a phosphomimetic aspartate. This prevents intramolecular binding and renders the protein dominantly active (Fievet et al., 2004; Gautreau et al., 2000; Nakamura et al., 1999; Ng et al., 2001; Speck et al., 2003; Yonemura and Tsukita, 1999). N-Ez contains the ezrin FERM domain alone. It can bind native ERM targets at the membrane, but lacks the C-terminal F-actin binding domain (Amieva et al., 1999; Brown et al., 2003; Crepaldi et al., 1997; Dickson et al., 2002; Martin et al., 1995; Speck et al., 2003).

To test whether ERMs contribute to cortical axon targeting, we employed an overlay guidance assay, developed and characterized by Ghosh and colleagues (Polleux et al., 1998). Cortical neurons are dissociated and plated on slices of neocortex. Within 24 hours, neurons become oriented within the boundaries of the cortical plate and extend their axons ventrally in response to a repulsive signal that includes Sema3A (Behar et al., 1996; Morita et al., 2006; Polleux et al., 1998) (Fig. 2A). We transfected cortical neurons with yellow fluorescent protein (YFP, used as a marker) alone or together with wild type ezrin, EzT567D, or N-Ez, and then plated them on slices of neocortex. The axon trajectories of neurons growing on the cortical plate were measured and plotted as vectors. Consistent with previous work using untransfected cortical neurons (Polleux et al., 1998), 96% of the YFP or EzWt expressing axons extend ventrally (Fig. 2B – F). In stark contrast, EzT567D and N-Ez expressing axons can elongate, but they project in all directions (Fig. 2C, D, G, H). These data indicate that ERM actions are critical for the ventral targeting of cortical axons. Additionally, since both mutants contain a FERM domain, the data strongly suggest that it is

this part of the ERM protein and not the C-terminal actin binding domain that requires appropriate regulation for axon orientation.

ERMs are required for Sema 3A-mediated growth cone collapse

Our results from the overlay assay suggest that ERM proteins may be important for Sema3A-mediated repulsion in cortical growth cones. To more directly assess whether ERM proteins participate in Sema3A-mediated responses, we asked whether ERM proteins influence the Sema3A-induced growth cone collapse. Collapse and repulsive guidance are thought to be closely related, and collapse responses can be readily quantified in large populations of growth cones (Kapfhammer et al., 2007). We first carried out a Sema3A dose-response curve, and then, using the lowest dose producing maximal collapse in cortical axons, we measured the time course of the response (Fig. 3A, B, methods). We then exposed control and mutant ezrin-expressing neurons to Sema3A or equal amounts of media and measured growth cone collapse at ten minutes. In media-exposed neurons, neither N-Ez nor EzT567D expression had any effect on the baseline level of collapse measured in neurons expressing YFP alone. However, Sema3A-stimulated collapse was completely prevented in either N-Ez or EzT567D-expressing neurons (Fig. 3C). Consistent with work in a variety of systems (Fievet et al., 2004, data not shown), overexpressing wild-type ERM proteins did not influence the response to Sema3A (69% collapse in radixin-expressing neurons, $n=128$). These data indicate that normal ERM function, and in particular, appropriate regulation of the FERM domain, is required to mediate collapse in response to Sema3A in cortical neurons.

While neither of the ezrin mutants alters the baseline level of growth cone collapse measured in the absence of Sema3A (Fig. 3C), we considered the possibility that interfering with ERM activity blocks a general mechanism of growth cone collapse. To address this, we measured growth cone collapse in neurons treated with lysophosphatidic acid (LPA), which induces cortical growth cone collapse over a time course similar to Sema3A, but via a different signaling cascade (Fukushima et al., 2002; Jalink et al., 1994). Neither N-Ez nor EzT567D prevents growth cone collapse in response to LPA (Fig. 3D). In fact, growth cone collapse is even more robust in neurons expressing EzT567D, suggesting that ERM activation enhances LPA-mediated collapse. These data indicate that overexpression of the FERM domain common to the two mutants does not generically block collapse. Additionally, the data suggest that ERM proteins may be recruited differentially in response to different repulsive cues.

ERMs in axonal growth cones show a unique and dynamic response to Sema3A

Since the ezrin mutants prevent Sema3A-mediated growth cone collapse, we asked whether Sema3A regulates endogenous ERM activation. Cortical neurons were exposed to Sema3A or control media for two, ten, or thirty minutes—intervals that span the collapse response (Fig. 3B), and the effects on ERM phosphorylation were monitored in Western blots of whole cell lysates. cpERM levels decrease at two minutes, return to baseline at ten minutes, and are sustained thereafter (Fig. 3E). All three ERM proteins appear to be regulated similarly, but the phosphorylated ERM proteins are principally ezrin and radixin (Fig. 3E and data not shown).

To test whether the activation pattern seen following Sema3A stimulation is unique, we repeated the assay using LPA. In contrast to Sema3A, LPA strongly stimulates ERM activation at two minutes, activation is maintained through ten minutes and then reduced by thirty minutes (Fig. 3F). Such strong activation is consistent with the enhanced growth cone collapse we see in neurons expressing the dominant active EzT567D mutant (Fig. 3D). Thus, Sema3A and LPA activate and utilize ERM proteins in distinctly different ways during growth cone collapse.

Since cpERMs are found in growth cones of both axons and dendrites (Haas et al., 2004), we asked whether the changes in cpERM levels seen in whole cell lysates reflects differences in axons. Two minutes following Semaphorin 3A (Sema3A) exposure, the percentage of cpERM-labeled axonal growth cones is reduced to less than half of the control value (Fig. 3G), and by ten minutes, the percentage of labeled axonal growth cones returns to baseline, in parallel to what is observed in Western blots (Fig. 3E). Thus, Sema3A stimulation rapidly and transiently inactivates ERMs in axonal growth cones.

ERMs redistribute independent of F-actin levels in response to Sema3A

Given that cpERMs show an asymmetric distribution in growth cones, we would predict that they would redistribute in response to focal presentation of Sema3A. However, ERM dephosphorylation and re-phosphorylation are rapid following exposure to Sema3A (Fig. 3E), making it difficult to correlate changes in cpERM staining with the relatively slow turning responses that can be provoked by focal Sema3A presentation (e.g. Song et al., 1998). In order to assay a large number of growth cones following a brief and simultaneous exposure to Sema3A, we compared the distribution of cpERMs in naïve growth cones to growth cones following ten minutes of bath applied Sema3A, a time at which our biochemical data indicate ERMs are re-activated (Fig. 3E). To compare distribution patterns, growth cones were divided into quadrants and the percentage of total label in each quadrant was quantified (Fig. 4A–D). In naïve growth cones most cpERMs are confined to a single quadrant, but in growth cones exposed to a uniform application of Sema3A, cpERMs are more evenly distributed among quadrants (Fig. 4A,D,E). Under the same conditions, labeling for total ERMs is not detectably altered (data not shown). While growth cone shape is also changing, these data suggest that ERM activation patterns respond dynamically to Sema3A and possibly reflect cue location.

The C-terminal domain of ERMs binds to F-actin and so the redistribution of cpERMs might simply be following F-actin levels. To assess the relationship between active ERMs and F-actin, we plotted their fluorescence intensity levels along a line drawn from the growth cone center to the periphery (Fig 4F,G). Regions showing intense labeling for cpERMs have relatively less F-actin labeling (correlation coefficient = -0.3245 , $n = 11$, Fig. 4F, G). Ten minutes following Sema3A stimulation, cp-ERMs continue to distribute in regions having less F-actin, even as F-actin generally becomes more concentrated in the center of growth cones as part of the collapse response. Thus, while cpERMs bind to F-actin and redistribute in response to Sema3A, they concentrate in regions where F-actin levels are lower.

Active ERMs bind the barbed ends of F-actin in growth cones

We asked whether the low levels of F-actin in regions enriched with cpERMs might reflect ERM function. Actin filaments are polarized and dynamic polymers in which subunits are added preferentially to the barbed ends and lost preferentially from the pointed ends. Proteins binding to the barbed end can cap F-actin and prevent further polymerization. As subunits continue to be lost from the pointed end, actin capping ultimately results in depolymerization. In cell-free assays, the ERM protein, radixin, caps the barbed ends of F-actin (Tsukita and Hieda, 1989), but *in vitro* assays have not always proven predictive for this type of *in vivo* function (Bear et al., 2000; Lambrechts et al., 2000; Pollard and Borisy, 2003). To determine whether active ERMs bind the barbed ends of actin filaments in growth cones, we examined cpERM distribution in the presence of two actin depolymerizing agents which act by different mechanisms. Cytochalasin D binds to the barbed ends of actin filaments and latrunculin A sequesters G-actin monomers. High doses (900 nM) of either agent for five minutes decrease F-actin staining and nearly eliminate cpERM labeling in growth cones (Fig. 5A–C vs D–I), while immunostaining for total ERMs is largely unchanged (data not shown, Goslin et al., 1989). This is consistent with active ERMs

binding to F-actin. At low doses, cytochalasin D competes with other barbed end-binding proteins without depolymerizing actin filaments (Cooper, 1987; MacLean-Fletcher and Pollard, 1980). Five minutes exposure to a low dose of cytochalasin D (100 nM) produces no detectable alterations in F-actin levels, but almost completely eliminates cpERM labeling relative to untreated controls (Fig. 5J–L, P). By contrast, a low dose of latrunculin A (100 nM) has no effect on cpERM labeling (Fig. 5M–O, P). These data strongly support that ERMs bind to the barbed ends of F-actin in intact growth cones.

If ERMs were binding and capping F-actin, this would be predicted to favor depolymerization and increase turnover in filopodia, which are actin-based structures. To test this, we used time lapse imaging to compare filopodial turnover in neurons overexpressing control vector to those expressing EzT567D, which might be expected to accelerate turnover, and those expressing N-Ez, which without an actin-binding domain, would be predicted to retard turnover. Neurons were imaged once every second for five minutes and filopodia were examined frame by frame. Most filopodia in cortical axonal growth cones turn over rapidly (in approximately 30 seconds), but there is no significant increase in turnover in neurons expressing EzT567D (Fig. 5Q). However at 60 seconds, growth cones expressing N-Ez show a significant increase in the percentage of filopodia that persist (Fig. 5Q, 20.9 ± 3.6 seconds vs. 32.9 ± 4.6 seconds), similar to what has been described in neurons exposed simultaneously to moesin and radixin antisense oligonucleotides (Paglini et al., 1998).

Actin capping would also be predicted to limit filopodial length. On growth cones, we detect no notable differences in length, but neurons expressing N-Ez often exhibit numerous, abnormally long, actin-filled protrusions along axon shafts (Fig. 5R–T), which presumably result from unchecked actin polymerization (Dickson et al., 2002). While EzT567D does not enhance filopodial turnover in growth cones, the rates are very rapid and may be at a limit. Our data show that in live neurons cytochalasin D displaces cpERMs and NEz retards filopodial turnover and generates protrusions. Together with the *in vitro* findings of Tsukita and Hieda (Tsukita and Hieda, 1989), the data strongly support that active ERMs can cap F-actin filaments in growth cones. This activity may account for the low levels of F-actin seen in regions having high concentrations of cp-ERMs and would also be expected to directly impact motility.

ERMs regulate the endocytosis of L1CAM and Npn1

Clathrin-mediated endocytosis regulates responsiveness to Sema3A in COS cells and L1CAM-mediated adhesion in growth cones (Castellani et al., 2004; Kamiguchi, 2003; Kamiguchi et al., 1998). L1CAM and the Sema3A receptor Npn1 bind in cis and contribute to Sema3A-mediated repulsion in cortical neurons (Castellani et al., 2000). ERMs bind L1CAM at two sites, one of which overlaps a docking site for the $\mu 2$ subunit of the clathrin adapter, AP2 (Cheng et al., 2005; Dickson et al., 2002) and thus might be expected to regulate internalization. To test whether ERMs regulate L1CAM endocytosis, we stimulated L1CAM internalization by incubating neurons with anti-L1CAM for 15 minutes at 37°C (Schmid et al., 2000) and asked whether EzWt, EzT567D, or N-Ez altered the ability of growth cones to internalize L1CAM. After fixation, surface epitopes were adsorbed with an excess of unlabeled secondary antibody, and the internalized pool of L1CAM was labeled with a tagged secondary following mild permeabilization (Kamiguchi and Lemmon, 2000). Similar to previous studies of dorsal root ganglion growth cones, cortical neurons internalize L1CAM in the central domain of growth cones (Fig. 6A) (Kamiguchi and Lemmon, 2000). Neurons expressing EzWt behave similar to YFP-expressing controls (Fig. 6B, E). In contrast, neurons expressing either EzT567D or N-Ez fail to internalize L1CAM (Fig. 6C, D, E). This suggests the ezrin N-terminal domain, which is common to the two mutants (as well as active ERM proteins), negatively regulates L1CAM endocytosis.

In COS cells expressing exogenous proteins, L1CAM is required to escort Npn1, which lacks an AP2 binding site, into clathrin-coated pits (Castellani et al., 2004). To determine whether ERMs also affect Npn1 internalization, we quantified the surface pool of Npn1 following Sema3A stimulation in growth cones expressing control or mutant ezrin proteins. Surface expression was monitored rather than internalized pools because Npn1 antibodies can interfere with Sema3A binding (He and Tessier-Lavigne, 1997). In vector-expressing growth cones exposed to control media for two minutes, Npn1 is found in small, evenly distributed clusters (Fig. 7A). This is similar to untransfected, unexposed neurons at steady state (not shown) and to what has been described in other studies (Dent et al., 2004; Piper et al., 2005). Media stimulated neurons expressing EzT567D show a surface distribution similar to YFP controls (Fig. 7A,C). Those expressing N-Ez appear to show somewhat increased levels of surface Npn1 relative to YFP controls, but this is not statistically significant (Fig. 7E, G). After two minutes exposure to Sema3A, when ERM proteins are inactivated, the level of surface Npn1 decreases dramatically in YFP-expressing growth cones (Fig. 7B, G), similar to what has been described in *Xenopus* retinal ganglion cells over the same time period (Piper et al., 2005). In contrast, growth cones expressing either EzT567D or N-Ez exhibit stable surface Npn1 levels following Sema3A stimulation and show no change in the distribution pattern (Fig. 7D,F,G).

Since the ezrin N-terminal domain binds PIP₂ as well as to L1CAM and may have additional targets in growth cones (e.g. Schlatter et al, 2007), we asked whether the ezrin mutants act as general inhibitors of endocytosis. Neurons transfected with ezrin mutants or control vector were incubated with a passive marker of endocytosis, 40 KDa fluorescent dextran, for 30 minutes. Internalized dextran is detected at similar levels in growth cones and cell bodies of neurons expressing YFP, EzT567D, or N-Ez (Fig. 7H–M) indicating that the ERM mutants do not abrogate general mechanisms of endocytosis.

Collectively, our findings support that active ezrin prevents L1CAM and Npn1 internalization, but it is not clear how such endocytosis relates to growth cone collapse. To address this, we assayed Sema3A-mediated growth cone collapse in the presence of monodansyl cadaverine (MDC), a specific inhibitor of clathrin-mediated endocytosis (Nandi et al., 1981; Schutze et al., 1999). MDC prevents Sema3A-mediated growth cone collapse (Fig 7N).

cpERMs and AP2 segregate in growth cones

Since the μ 2 subunit of the AP2 adapter complex and the N-terminal domain of ERM proteins can bind the same tyrosine-based endocytosis motif in L1CAM (YRSLE, Dickson et al., 2002; Kamiguchi et al., 1998), it is possible that they compete directly for binding to L1CAM. As a first step toward addressing this possibility, we compared the distribution of native cpERMs and alpha adaptin, another component of the AP2 complex, in individual growth cones. We find that the proteins occupy segregated growth cone domains. Growth cones having cpERM labeling in the periphery have centrally restricted alpha adaptin (Fig. 8A–D), while growth cones having a peripheral distribution of alpha adaptin lack cpERM labeling entirely (Fig. 8E–H). Line scans from the enter to periphery indicate that within this region intensities are also negatively correlated (correlation coefficient = -0.2679 , $n = 11$). The complete lack of colocalization suggests that the proteins may never come into contact with one another or that they may actively carve out domains.

With this latter possibility in mind, we asked whether cpERM distribution impacts AP2 localization by examining alpha adaptin distribution in neurons exposed to Sema3A for two minutes when cpERM levels are reduced. However, there is no detectable increase in peripheral alpha adaptin even in the absence of cpERMs (Fig. 8 I,K vs J,K,L). Together our data suggest that while ERM activity negatively regulates L1CAM endocytosis and both

ERMs and the AP2 complex can bind to L1CAM, the proteins occupy separate growth cone domains and are unlikely to directly compete. It will be important in future experiments to determine the biological basis for sorting ERMs and the AP2 adapter into different domains within growth cones.

DISCUSSION

Here we show that the ERM family of proteins regulates cortical axon orientation and growth cone collapse in response to Semaphorin 3A. We find that active ERMs are distributed asymmetrically in growth cones and concentrate in filopodia where the C-terminal domain binds to the barbed ends of F-actin filaments and can influence filopodial dynamics. The N-terminal FERM domain binds L1CAM and prevents the clathrin-mediated internalization of L1CAM and Npn1. Exposure to Semaphorin 3A transiently inactivates ERMs, and when ERMs are reactivated, they redistribute. The off/on regulatory cycle appears to be of key importance as in the absence of normal ERM regulation axons extend, but they are not targeted appropriately and their growth cones fail to collapse in response to Semaphorin 3A. Thus, targeted axonal growth in response to Semaphorin 3A demands balanced and dynamic regulation of the ERMs.

ERMs and Endocytosis

ERM inactivation in response to Semaphorin 3A is transient, suggesting that ERMs can regulate the timing of endocytosis of L1CAM and Npn1. It has been known for some time that ERMs participate indirectly in G-protein-coupled receptor desensitization via an interaction with a PDZ binding partner, EBP50 (e.g. Cao et al., 1999). Thus, we propose that ERMs can directly regulate short-term growth cone desensitization to Semaphorin 3A. Endocytosis-mediated short-term desensitization was recently described as an essential component of growth cone adaptation to guidance cues (Piper et al, 2005). Adaptation, or the continual resetting of the baseline sensitivity to a cue, is a prominent feature of bacterial motility in a cue gradient (Baker et al., 2006; Macnab and Koshland, 1972), and appears to also be utilized by growth cones in response to attractive and repulsive cues (Ming et al., 2002; Piper et al., 2005). The timing of ERM inactivation and subsequent reactivation closely parallels the desensitization and resensitization time frame observed in retinal (Piper et al., 2005) and cortical growth cones (unpublished, Carcea and Benson) in response to Semaphorin 3A, and suggests that ERMs may act as a switch by first permitting the endocytic desensitization phase to proceed and then terminating the phase upon reactivation, allowing receptors to re-accumulate on the surface.

Endocytosis can regulate receptor availability and can also initiate signaling pathways important for growth cone responses. An increase in the surface expression of the deleted in colorectal cancer (DCC) receptor enhances netrin-1-stimulated growth (Bouchard et al., 2004), and the selective internalization of another netrin receptor, UNC5A, can convert netrin-1 induced repulsion to attraction (Bartoe et al., 2006; Williams et al., 2003). Thus, ERMs may determine sensitivity to Semaphorin 3A simply by regulating Npn1 removal from the plasma membrane. Internalization is also required for L1CAM dependent MAPK signaling, as L1CAM localizes with ERK only in endocytic vesicles (Schaefer et al., 1999; Schmid et al., 2000). This pathway seems particularly relevant as MAPK signaling downstream of Semaphorin 3A is necessary for a chemotropic response (Campbell and Holt, 2003).

Endocytosis of cell adhesion molecules can also decrease growth cone adhesion to the substrate. Integrins are internalized from the growth cone surface to promote neurite extension on laminin (Condic and Letourneau, 1997). Similarly, as axons extend on L1CAM substrates there is an asymmetric distribution of L1CAM at the growth cone surface that is created by a proximally directed gradient of endocytosis (Kamiguchi and Lemmon, 2000;

Kamiguchi et al., 1998). ERMs may serve a dual role in this context, linking L1CAM and CHL1 (Schatter et al, 2007) to the underlying actin cytoskeleton to transduce traction forces when active and permitting endocytosis for redistributing L1CAM within the growth cone when inactivated.

ERMs and Actin Dynamics

Targeted axon growth requires a dynamic F-actin cytoskeleton (Bentley and Toroian-Raymond, 1986; Dent and Gertler, 2003; Goldberg and Burmeister, 1989) and our data suggest that ERMs may directly influence actin dynamics in growth cone filopodia by capping actin filaments. We find that low levels of cytochalasin D, which bind and shield the barbed or plus ends of F-actin (Cooper, 1987; MacLean-Fletcher and Pollard, 1980), completely displace active ERMs as effectively as depolymerizing F-actin altogether. Additionally, ERM mutants lacking the capping domain increase the lifetime of growth cone filopodia and the length of filopodia along the axon shaft, both consistent with diminished actin-capping activity (see also (Paglini et al., 1998). Together these data indicate that ERMs in living growth cone filopodia are likely to engage in the capping activity first identified using purified radixin and cell-free actin bundles (Tsukita and Hieda, 1989).

Restricted activation of a capping protein in filopodia undoubtedly influences growth cone steering: Focal presentation of low concentrations of cytochalasin asymmetrically produces filopodial retraction followed by growth cone repulsion (Yuan et al., 2003; Zheng et al., 1996). Interestingly, over a time scale that is similar to what we observe with the ERMs, *Sema3A* transiently decreases the activity of ADF/cofilin, which severs and depolymerizes actin filaments (Aizawa et al., 2001; Kuhn et al., 2000). Decreased actin severing and capping might be expected to favor actin assembly, but in this brief period they probably contribute to actin rearrangement as a prelude to collapse (Gallo, 2006). The precise role that ERMs play in growth cone actin dynamics is clearly complex and extends beyond simple predictions of activation or inactivation, turning or collapse, since LPA, which also collapses growth cones, produces a different ERM regulation profile, and the expression of a dominant active ezrin mutant (EzT567D) does not, on its own, cause collapse (Fig. 3).

Integrating Events in the Growth Cone

The execution of a guidance response requires integration and coordination of plasma membrane components, cytoskeletal networks, and signaling pathways. How do these separate components interact to generate a coordinated output? Our data suggest that the ERMs, like MICAL and talin, have the capacity to coordinate growth cone responses to *Sema3A* (Di Paolo et al., 2002; Hemmings et al., 1996; Nadella et al., 2005; Terman et al., 2002; Toyofuku et al., 2005). ERMs mediate diverse functions and associations in a highly regulated manner, they bind directly to transmembrane proteins like L1CAM and to the barbed ends of actin filaments, and they can participate directly in signaling cascades (Lee et al., 2004; Speck et al., 2003; Takahashi et al., 1998). Thus, at the very least, ERMs in growth cones have the capacity to influence actin dynamics, endocytosis, adhesion, and signaling. It will be important in future work to determine whether ERMs can define or segregate particular signaling networks, and to assess how they act in the context of other guidance cues such as LPA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. cpERMs are asymmetrically expressed in selected axonal growth cones

Epifluorescence and confocal images show ERM (A, D, G and green in overlays C, F, I) and F-actin distribution (B, E, H and magenta in overlays C, F, I) in growth cones. Anti-cpERM labeling is often enriched in filopodia where it overlaps almost completely with phalloidin labeling for F-actin (arrow, A–C). Within the same growth cone, cpERM distribution is often asymmetric (A, arrowhead in D), and cpERMs are present in some axonal growth cones (D–F, arrowhead) and not in others (D–F, arrow). Total anti-ERM immunolabeling is more evenly distributed than cpERMs (G–I). Scale bars for A,B,C,G,H,I = 1.4 μ m. Scale bar for D–F = 5 μ m.

Figure 2. Intact ERM function is required for cortical axon guidance

To assay the influence of ERM proteins on cortical axon targeting, dissociated cortical neurons were plated on slices of neocortex, where a marginal zone signal (likely Sema3A) repels the newly sprouted axons ventrally (A). The trajectories of axons expressing YFP, EzWt, EzT567D, and N-Ez are displayed graphically such that dorsal is upwards and numbers on each axis represent μ m (E–H). Maps of the axon trajectories taken from neurons expressing YFP or EzWt, show a predominantly ventral orientation (e.g. B; trajectories shown in E and F). By contrast, both EzT567D and N-Ez transfected neurons elaborate axons in all directions, including dorsal (e.g. C, trajectories shown in G and H). Box plots display the deviation angles of all axons oriented away from a straight ventral direction (D). In each box plot the middle line is the median, the bottom and top of the rectangle box are the 25th and 75th percentile of the deviation angles, respectively. Applying a two-sided Wilcoxon signed rank test where the null hypothesis is that the deviation angle is equal to 90 degrees, p-values for YFP and EzWt are less than 0.01 (indicated by * in the plot). By contrast, both EzT567D and N-Ez axons display dorsal and ventral orientations (p-values are greater than 0.10). n = 200 neurons and 30 slices. Scale bar in C = 5 μ m.

Figure 3. ERMs function in Sema3A induced collapse

The collapse of cortical axonal growth cones to Sema3A is concentration dependent with 150 ng/mL showing a maximal response, $n = 4,231$ growth cones (A). The time course of growth cone collapse in response to an intermediate concentration of Sema3A (100 ng/mL) is shown in (B) ($n = 5,665$ growth cones). In neurons expressing N-Ez or EzT567D, Sema3A does not mediate collapse (C), while all transfected growth cones exposed to control media show similar levels of collapse ($n = 4,447$ growth cones; *indicates $p < 0.01$ for Student's *t*-test). LPA induces collapse in YFP-, N-Ez- and EzT567D-expressing neurons (D) (*indicates $p < 0.05$ with respect to its own control; additionally LPA-EzT567D is significantly different from LPA-YFP). Western blots (E) of lysates from cortical neurons

have reduced levels of cpERMs two minutes following exposure to Sema3A relative to total ezrin and to media controls. cpERM levels recover to baseline at ten minutes and are maintained thereafter (E). The doublet shown corresponds to ezrin and radixin. Moesin bands are much fainter, but can be seen below ezrin and radixin upon longer exposures (not shown). In contrast to Sema3A, LPA strongly stimulates ERM activation at two minutes, activation is sustained at ten minutes, and drops at thirty minutes (F). Changes in ERM activity observed in cortical neuron lysates can be detected by the percentage of labeled axonal growth cones (G). *Indicates $p < 0.01$ for comparison to control at two minutes and Sema3A at ten minutes, ANOVA and Bonferroni post test; $n = 2,387$ neurons growth cones. Blots are representative examples of three separate experiments.

Figure 4. Active ERMs redistribute following Sema3A stimulation

In untreated growth cones (A,B) ERM activation, as measured by anti-cpERM immunolabeling (B), is asymmetrical and does not correlate with differences in F-actin levels (A), determined by phalloidin labeling. This can be seen readily in pseudocolored images where warmer colors represent higher fluorescence intensities. Ten minutes after bath application of Sema3A (C,D), the distribution of cpERMs is more homogeneous. Changes in ERM distribution were compared quantitatively (E) by parceling growth cones into four quadrants (white dotted lines in A–D) and determining the extent of cpERM and actin labeling in each quadrant. The quadrants were ordered such that the first quadrant has the highest anti-cpERM fluorescence intensity per unit area, and the second, third, and fourth quadrants have progressively lower cpERM labeling (as shown in B,D). All values were calculated as percentage of total labeling. After ten minutes bath application of Sema3A, the second, third, and fourth quadrants show significantly more cpERM signal than untreated controls (E). Line scans drawn from the growth cone center to the periphery show that the fluorescence intensity of F-actin (magenta) and cpERMs (green) does not co-vary in control growth cones (F) or following ten minutes of Sema3A (G). $n = 21$ neurons, and * in E indicates $p < 0.01$, ANOVA and Bonferroni post test.

Figure 5. ERMs cap F-actin in growth cones

Fluorescence photomicrographs (A – O) showing F-actin (A,D,G,J,M, magenta in overlay) and cpERM labeling (B,E,H,K,N, green in overlay) in growth cones exposed to various concentrations of actin depolymerizing agents or DMSO. Brief treatment with high doses of cytochalasin D (CyD, D,E,F) or latrunculin A (Lat A, G,H,I) result in the loss of F-actin and cpERM labeling, supporting that activated ERMs bind F-actin in growth cones. Brief treatment with a low dose of CyD shows no effect on F-actin labeling (J), but results in a loss of detectable cpERM (K) compared to DMSO control (B). A low dose of LatA (M–O) shows no effect on F-actin or cpERM labeling (M,N). The area of cpERM labeling as a percentage of the area of F-actin labeling was measured for growth cones treated with low dose CyD or LatA and normalized to diluent controls (P). Low dose CyD greatly reduces cpERM labeling ($23.8\% \pm 7.1\%$), whereas low dose LatA does not ($95\% \pm 20.7\%$), indicating that activated ERMs associate with the barbed ends of F-actin. Bar graph in (Q) compares filopodial persistence over two bins: 0–30 and 31–60 seconds, in YFP (white), N-Ez (gray), and EzT567D (black) transfected neurons. Filopodia in N-Ez transfected cells show a significant increase in filopodial stability when compared to controls (32.9 ± 4.6 seconds vs. 20.9 ± 3.6 seconds, $\text{prob} > |z| < 0.0001$, z-test). Reduced F-actin capping is also consistent with the elaboration of long actin-containing processes seen on axon shafts of N-Ez expressing neurons (arrow, R–T). $n = 123$ growth cones; *indicates $p < 0.01$ for Student's t test; for G, $n = 80$ growth cones; scale bar is $1.25 \mu\text{m}$ (A–O) and $5 \mu\text{m}$ (R–T).

Figure 6. ERMs regulate L1CAM endocytosis

Fluorescent photomicrographs show *internalized* immunofluorescence for L1CAM in growth cones from neocortical neurons transfected with YFP (A), EzWt (B), EzT567D (C), or N-Ez (D). Fluorescent images were inverted so that labeling appears black. In the YFP controls (A), signal for internalized L1CAM is more evident in the central domain of the growth cone than the periphery (the profile of the growth cones in A – D is indicated by the tracing). Growth cones from EzWt-expressing neurons look similar to those expressing YFP (B). By comparison, axonal growth cones of both EzT567D (C) and N-Ez (D) expressing neurons show a dramatic reduction in internalized L1CAM. The bar graph in (E) shows the mean percentage of total area occupied by internalized L1CAM signal for YFP ($4.74\% \pm$

0.47%), EzWt ($4.30\% \pm 0.41\%$), EzT567D ($0.89\% \pm 0.23\%$), and N-Ez ($0.35\% \pm 0.09\%$) expressing neurons. *Indicates $p < 0.01$ for comparison to EzWt, ANOVA and Bonferroni post test; $n = 232$ neurons. Scale bar in (A–D) is $1.93 \mu\text{m}$.

Figure 7. ERMs prevent Sema3A-mediated recycling of Npn1

Fluorescence photomicrographs showing *surface* Npn1 immunolabeling in neurons transfected with YFP (A, B), EzT567D (C, D) or N-Ez (E, F) following two minutes exposure to media (A,C,E) or Sema3A (B,D,F). Images were prepared as in 6, so that label appears black within tracings showing the outline of growth cones or cell bodies. Growth cones exposed to control media have multiple clusters of Npn1 on the surface (A,C,E). Percent growth cone area occupied by Npn1 label appears slightly higher in neurons expressing N-Ez (E) than YFP (A), but this difference is not significant (G, ANOVA, $p = 0.08$). Two minutes following exposure to Sema3A, surface levels of Npn1 decrease dramatically in YFP transfected neurons (A,B), but remain stable in EzT567D and N-Ez

expressing neurons (C–F). Results from these experiments were quantified by comparing surface Npn1 labeling per unit area relative to YFP, media-stimulated controls (G). Surface levels in media-stimulated controls are similar with all three constructs, and Sema3A exposure decreases Npn1 surface labeling significantly only in YFP expressing neurons ($51.7\% \pm 7\%$ of control levels; $n = 307$ growth cones; *indicates $p < 0.01$ for ANOVA and Bonferroni post test). Confocal images show internalized fluorescent dextran in neurons expressing YFP, N-Ez, and EzT567D (H–M). Neurons expressing ezrin mutants internalize dextran as well as YFP expressing neurons, both in cell bodies (H–J) and in axonal growth cones (K–M). A median filter was applied to these images. In (N), MDC prevents cortical growth cone collapse in response to Sema3A (*indicates $p < 0.01$ for ANOVA and Bonferroni post test). Scale bar in E = $2.32 \mu\text{m}$ in A–F. Scale bar in J = $3.1 \mu\text{m}$ in H–J and $6.7 \mu\text{m}$ in K–M.

Figure 8. Active ERMs and AP2 occupy separate domains

Confocal images show immunolabeling for cpERMs (green in overlay), the alpha subunit of the AP2 adapter complex (blue in overlay) and phalloidin staining for F-actin (red in overlay) (A–H; K–M). cpERMs are enriched in the distal growth cone, while AP2 concentrates centrally (A–D; arrow indicates border between the two labels). Growth cones lacking detectable cpERM labeling show more AP2 in the periphery (E–H). Compared to growth cones exposed to media (K), two minutes exposure to Sema3A decreased the levels of cpERM in the periphery, but did not redistribute AP2 (L,M). Representative line scan plots of fluorescence intensity running from the growth cone center to periphery show no

difference in the distribution of AP2 (turquoise) relative to F-actin (red) between control (I) and Sema3A treated growth cones (J). Scale bar = 1.1 μm (A–H), 2 μm (K–M).

Table 1

Primary Antibodies

Antibody	Host, isotype	Immunogen	Clone number	Source	Cat. number	Lot number
Tuj1	Mouse Ig G	Rat brain microtubules	Tuj1	Covance	MMS-435P	139018002
cpERM	Rabbit	KLH-coupled synthetic peptide surrounding Thr567 of human ezrin	NA	Cell Signaling	3141	2
L1CAM	Mouse IgG1, kappa light chain	Sympathetic neurons, rat SCG	ASCS4	Developmental Studies Hybridoma Bank (Paul Patterson)	NA	NA
AP2 α subunit	Mouse IgG1	aa 38–215 of mouse α -adaptin	8	BD Transduction Laboratories	610501	27858
GFP	Chicken IgY	Recombinant GFP containing a 6 His tag	NA	Chemicon	AB16901	0608036905
Neuropilin 1	Rabbit	Synthetic peptide from the N-terminus of the human Nrp1 (aa 22–41)	ZMD.272	Zyomed	36–1400	60203707