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RACK1 Is Necessary for the Formation of Point Contacts and Regulates Axon Growth

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Abstract

Receptor for activated C kinase 1 (RACK1) is a multifunctional ribosomal scaffolding protein that can interact with multiple signaling molecules concurrently through its seven WD40 repeats. We recently found that RACK1 is localized to mammalian growth cones, prompting an investigation into its role during neural development. Here, we show for the first time that RACK1 localizes to point contacts within mouse cortical growth cones. Point contacts are adhesion sites that link the actin network within growth cones to the extracellular matrix, and are necessary for appropriate axon guidance. Our experiments show that RACK1 is necessary for point contact formation. Brain-derived neurotrophic factor (BDNF) stimulates an increase in point contact density, which was eliminated by RACK1 shRNA or overexpression of a nonphosphorylatable mutant form of RACK1. We also found that axonal growth requires both RACK1 expression and phosphorylation. We have previously shown that the local translation of β -actin mRNA within growth cones is necessary for appropriate axon guidance and is dependent on RACK1. Thus, we examined the location of members of the local translation complex relative to point contacts. Indeed, both β -actin mRNA and RACK1 colocalize with point contacts, and this colocalization increases following BDNF stimulation. This implies the novel finding that local translation is regulated at point contacts. Taken together, these data suggest that point contacts are a targeted site of local translation within growth cones, and RACK1 is a critical member of the point contact complex and necessary for appropriate neural development.

Keywords

RACK1; growth cone; point contacts; axon growth; local translation

INTRODUCTION

During neural development, both diffusible and substrate-bound cues guide axons to their synaptic targets. Growth cones, located at the tips of developing axons, are highly motile sensory and motor structures that underlie this pathfinding process. Filopodia, finger-like protrusions at the leading edge of growth cones, and lamellipodia, sheet-like veils between filopodia, are both composed of filamentous actin (F-actin). Changes in actin dynamics,

such as polymerization and retrograde flow, help steer the axon in the appropriate direction (Lowery and Van Vactor, 2009; Nichol et al., 2016).

Point contacts are multiprotein, integrin-dependent adhesion sites that play a critical role in axon outgrowth and guidance. Point contacts are composed of a number of molecules including $\beta 1$ integrin, talin, vinculin, and paxillin (Gomez et al., 1996; Renaudin et al., 1999). Thus far, the major role identified for point contacts is to provide an adherence point between intracellular actin and the extracellular matrix, which results in the stabilization of new growth cone protrusions. Although point contacts provide stability to growth cones, they are also dynamic structures, as there is a constant flow of their assembly and disassembly during growth cone advance and pathfinding. Recent studies have started to shed light on these important structures (Robles and Gomez, 2006; Woo and Gomez, 2006; Kurklin-sky et al., 2011; Myers and Gomez, 2011; Santiago-Medina et al., 2013; Nichol et al., 2016), but there is much about point contacts that is still not understood. However, some insight can be gained from focal adhesions, which have been the focus of numerous studies and are partly analogous to point contacts.

Here, we present the novel finding that receptor for activated C kinase 1 (RACK1) localizes at point contacts. RACK1 is a seven WD40 domain ribosomal scaffolding protein that is involved in numerous signaling pathways (Gandin et al., 2013; Gallo and Manfrini, 2015). Almost nothing is known about RACK1 during neural development, but its signaling is vital for mammalian development as mice lacking RACK1 are gastrulation lethal (Volta et al., 2013). Although RACK1 is highly expressed throughout the developing mouse brain (Ashique et al., 2006) only a single study has investigated the role of RACK1 in growth cones; this study revealed that RACK1 is necessary for axon guidance in *Caenorhabditis elegans* (Demarco and Lundquist, 2010). RACK1's seven WD40 domains can simultaneously interact with multiple signaling molecules including both soluble signaling proteins and cytosolic domains of membrane-bound receptors. Specifically, RACK1 can interact with several integrin β subunit tails, and regulate signaling that is necessary for migration of Chinese Hamster Ovary (CHO) cells (Buensuceso et al., 2001; Cox et al., 2003). RACK1 has also been shown to regulate focal adhesions through its binding to Src. Blocking Src phosphorylation of RACK1 significantly inhibits directional migration of fibroblasts in response to media containing growth factors (Cox et al., 2003) and prevents central focal adhesion formation in CHO cells (Doan and Huttenlocher, 2007). Furthermore, a study in 3T3 fibroblast cells showed that blockade of RACK1 expression by antisense oligonucleotides led to a suppression of cell spreading (Hermanto et al., 2002). Taken together, these studies suggest an important role for RACK1 in the regulation of adhesion and cell motility.

Local translation is a highly tuned, multistep process by which mRNA-binding proteins bind to specific sequences within the untranslated region of mRNAs in the soma; mRNAs are then transported to subcellular regions and translated at the appropriate spatial location in response to specific signaling cues. Integrin binding has been shown to result in the localization of mRNA to focal adhesions in endothelial cells (Chicurel et al., 1998). Furthermore, our recent study demonstrated that binding of RACK1 to the β -actin mRNA/ZBP1 complex is critical for the release and local translation of β -actin mRNA in growth

cones (Ceci et al., 2012). Taken together, these findings suggest that RACK1 may regulate local translation at adhesion sites within cells. Furthermore, these studies provide a strong basis to examine how RACK1 regulates point contacts in growth cones and functional aspects of axon guidance. Indeed, we demonstrate here that RACK1 is required for point contact formation and axon outgrowth. We also provide strong evidence that RACK1 regulates local translation at point contacts.

METHODS

Cell Culture and Transfection

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Kent State University. C57BL/6J mice (The Jackson Laboratory) were bred to produce timed pregnant animals. The day of the plug was considered embryonic day 0 (E0). Cortices were dissected from E17 embryos of either sex. Dissociated cortical cells were plated in MEM with 10% FBS on acid-rinsed coverslips (Carolina Biological) pretreated with 100 µg/mL poly-L-lysine (Sigma-Aldrich) and 10 µg/mL laminin (Life Technologies). After cells adhered to the coverslips (approximately 2 h), the media were changed to Neurobasal with Glutamax and B27 (all Life Technologies) and cells were cultured for 2 DIV at 37°C in 5% CO₂. For transfections, neurons were transfected with 1 µg of GFP, GFP-RACK1-WT, GFP-RACK1-Y246F, nonsilencing control shRNA, or RACK1 shRNAs using calcium-phosphate transfection (Jiang and Cheng, 2006). The nonsilencing control shRNA and shRNAs were purchased from GE Dharmacon (see the “shRNA Constructs” section for additional details); GFP, GFP-RACK1-WT, and GFP-RACK1-Y246F have been used previously (Ceci et al., 2012). Cells that underwent transfection were cultured an additional 2 days before being used for experimentation. When comparing basal and stimulated conditions, cells were starved by removing B27 from the medium for 3 h and then treated with 100 ng/mL BDNF (PeproTech) or vehicle control for 30 min at 37°C. In all experiments, cells were fixed with 4% paraformaldehyde in phosphate buffered saline. Only cells clearly expressing GFP were included in the analyses.

shRNA Constructs

Commercially available and validated shRNAs for RACK1 and a nontargeting control shRNA were purchased from GE Dharmacon's GIPZ construct library. The human cytomegalovirus promoter (hCMV) drives the expression of the shRNAs. The GIPZ constructs also contain GFP as a marker of shRNA expression. Only cells clearly expressing GFP were included in analyses. The mature antisense sequences used to knockdown RACK1 were: sh1 (V3LMM_447148): TCTGAATGACTCTCATCCT and sh2 (V3LMM_447149): AACTTTATGGTCTTGTCTC. The non-targeting mature antisense sequence was: CTTACTCTCGCCCAAGCGA GAG.

Combination Fluorescent In Situ Hybridization (FISH) and Immunofluorescence (IF)

Custom Stellaris FISH probe sets, containing 42 directly labeled probes, were used to detect β -actin mRNA. Stellaris probes label mRNAs with single-molecule specificity (Raj et al., 2008; Raj and Tyagi, 2010). mRNAs can only be visualized (as a bright punctate spot) when multiple probes bind to one mRNA; off-target binding generates very weak fluorescence that

is below the limit of detection (Raj et al., 2008). A modified FISH protocol was used to perform FISH and IF simultaneously (Welshhans and Bassell, 2011). Briefly, fixed neurons were rinsed in 0.1 MPBS/0.2 mM MgCl₂ and equilibrated in 1× saline sodium citrate (SSC). They were then transferred to 10% formamide/1× SSC before being pre-incubated in hybridization buffer (25% dextran sulfate, 2× SSC, 0.4% bovine serum albumin, 20 mM ribonucleoside vanadyl complex, 10 mM MPBS, salmon sperm DNA, and tRNAs) for 1.5 h. The neurons were then hybridized with Stellaris probes in hybridization buffer at 37°C overnight. We modified the protocol here by including primary antibodies to the protein of interest along with the Stellaris probes. The following primary antibodies were used: mouse anti-paxillin antibody (1:500; BD Biosciences) or mouse anti-vinculin antibody (1:500; Sigma-Aldrich). Secondary antibodies were applied during subsequent washes on the following day. The first and second 20 min washes both contained 10% formamide/1× SSC and one of the following secondary antibodies: goat anti-mouse Alexa 488 or donkey anti-mouse Alexa 647 (all from Jackson ImmunoResearch). Rhodamine phalloidin or Alexa Fluor 350 phalloidin (Life Technologies) was also added in some experiments during the secondary antibody incubation. After stringent washes with 1× SSC, cells were mounted onto glass slides using ProLong® Gold antifade mountant (ThermoFisher).

Immunofluorescence Experiments (IF)

IF experiments were performed as previously described (Welshhans and Bassell, 2011), and the following primary antibodies were used: mouse anti-paxillin antibody (1:500), mouse anti-vinculin antibody (1:500), rabbit anti-RACK1 antibody (1:500; Santa Cruz Biotechnology), mouse anti-RACK1 antibody (1:500; Santa Cruz Biotechnology), rabbit anti-ribosomal protein S6 antibody (1:100; Santa Cruz Biotechnology), mouse anti-ribosomal protein S6 antibody (1:100; Santa Cruz Biotechnology), and mouse anti-ribosomal RNA 5.8s Y10b antibody (1:100, Abcam). The following secondary antibodies were used: goat anti-mouse Alexa 488, goat anti-rabbit Alexa 488, goat anti-mouse Cy3 and donkey anti-mouse Alexa 647 (all from Jackson ImmunoResearch). Rhodamine phalloidin or Alexa Fluor 350 phalloidin (Life Technologies) was also added in experiments during the secondary antibody incubation to stain F-actin for visualization of growth cone area.

Image Acquisition and Analysis

All experiments were conducted in a blinded fashion by assigning random numbers as the labels for both the slides and the acquired images. Experiments were only unblinded following analysis. IF images were visualized on an Olympus IX81 microscope using either an Olympus UPlanSApo 20X (0.75 NA) or an Olympus PlanApo 60X (1.4 NA) in combination with the microscope's 1.6 multiplier (total magnification 96×). Images were captured with a Sensi-Cam QE CCD camera using Slidebook software (3i: Intelligent Imaging Innovations). To quantify fluorescence, all experimental and image acquisition parameters were kept constant throughout each experiment. Any images containing overexposed pixels were excluded from analysis. Total internal reflection fluorescent (TIRF) images were acquired using an Olympus IX81 TIRF microscope, an Olympus UApoN 100X TIRF objective (1.49 NA), and an Andor iXon3 EMCCD camera.

Pearson's correlation coefficients (PCC) of a region of interest (ROI) were quantified using the Coloc2 plugin of Fiji software (Schindelin et al., 2012). Pearson's correlation coefficients measure the linear correlation between two variables. A PCC value of 0 signifies no meaningful relationship, a value of 1 signifies a perfect, linear positive correlation, and a value of negative 1 signifies a perfect, negative linear correlation (Adler and Parmryd, 2010). We ran Costes' method, which is included with the Coloc2 plugin, on every image to statistically confirm true colocalization (100 randomizations per image) (Costes et al., 2004). This method shuffles one of the two channels being compared and then determines the PCC of the shuffled image versus the other channel. For a robust analysis, we set the Costes' randomizations value to 100 so that this process was repeated 100 times. Only images with Costes' significance values of 0.95 or greater were included in the analysis, meaning that all included images had a 95% or greater statistical probability of true colocalization. Any images that failed Costes' method by having a colocalization significance test value below the significance threshold of 0.95 were excluded from analysis. As an additional control, we also calculated PCC for each set of images, but rotated one of the images 180°. This rotation should result in a loss of colocalization (Dunn et al., 2011; Kanda et al., 2011).

Axon length, growth cone area, point contact density, and fluorescence intensity were quantified using ImageJ tools within the Fiji software (Schindelin et al., 2012). We can reliably define the axon as the longest neurite, which extends at least three times the length of the next longest neurite. Axon length was determined by measuring the length of the primary axon (i.e., longest neurite) from the cell body to the center of the axonal growth cone. Axon length does not include the length of any branches coming off the main axon. Growth cones were defined based on phalloidin staining of F-actin. The brightness and contrast were adjusted in each image following acquisition to easily visualize the entire growth cone, including the central domain, lamellipodia and filopodia. Growth cone area was then calculated as the area defined by enclosing the entire perimeter of the growth cone including central domain, lamellipodia, and filopodia, but not the axon. This method is in line with previous studies (Myers and Gomez, 2011; Sasaki et al., 2011). Point contact density was determined by counting the number of fluorescently labeled paxillin puncta within a growth cone and then dividing that value by growth cone area. Point contacts were counted by hand in growth cones with few puncta; otherwise, they were counted using the particle analysis tools in Fiji (Schindelin et al., 2012). A preliminary analysis was performed to verify that the puncta counts from the computerized particle analysis method did not differ from puncta counted by hand. To determine the mean fluorescent intensity in a ROI, the structure of interest (e.g., growth cone) was first outlined. The mean fluorescent intensity was background subtracted in each image by measuring the fluorescent intensity in a region near the ROI, but excluding the ROI. Only cells with a pyramidal morphology were included in these analyses. Each experiment was repeated a minimum of three times.

Statistical Analysis

All statistical analyses were conducted in GraphPad Prism. Significance was set at $P = 0.05$. The specific statistical test used for each experiment is given in its figure legend. Error bars represent the standard error of the mean from a minimum of three independent experiments unless otherwise indicated.

RESULTS

RACK1 Is Localized to Point Contacts within Growth Cones

We recently found that RACK1 is localized to mammalian growth cones and plays a role in the local translation of β -actin mRNA (Ceci et al., 2012). Thus, we investigated whether RACK1 was localized to point contacts within developing mouse cortical neurons. Here we employed two well-described markers of point contacts, paxillin and vinculin (Robles and Gomez, 2006; Woo and Gomez, 2006; Woo et al., 2009; Myers and Gomez, 2011). Paxillin and vinculin are adaptor proteins that play a critical role in connecting actin to the intracellular domain of integrin receptors (Myers et al., 2011). Although a portion of paxillin and vinculin colocalize in growth cones, there are also subsets of point contacts that label only for paxillin and not vinculin, and vice versa (Woo and Gomez, 2006). Using double label immunocytochemistry, we find that RACK1 colocalizes with paxillin in growth cones (Fig. 1A). We quantified this colocalization using Pearson's correlation coefficient (PCC) and found a PCC value of 0.38 ± 0.03 (Fig. 1B). We then confirmed this finding by performing double label immunocytochemistry for RACK1 and vinculin (Fig. 1C,D) and found a similar result. RACK1 and vinculin colocalize with a PCC value of 0.59 ± 0.03 . As a control, we rotated one of the images in the pair 180° and then calculated PCC for each set again. This resulted in only random colocalization. We also ran Costes' method when analyzing each set of images to verify true colocalization (see the "Methods" section for additional details and controls). Thus, RACK1 colocalizes with two separate markers of point contacts, suggesting that it may be an integral member of the point contact complex.

Point contacts occur at the substrate–membrane interface and are usually localized in the peripheral region of growth cones (Renaudin et al., 1999; Short et al., 2016). To confirm that RACK1 is expressed near the membrane, we performed immunocytochemistry for RACK1 and stained the F-actin cytoskeleton with phalloidin. Images acquired using total internal reflection fluorescence (TIRF) microscopy show that RACK1 colocalizes with F-actin near the membrane (Fig. 1E). More specifically, we find that RACK1 localizes in filopodia along actin filaments (Fig. 1E, arrows). Furthermore, RACK1 is found along actin filament bundles that are projecting into the central domain (Fig. 1E, arrowheads designate two sets of these puncta). Taken together, these data provide further evidence that RACK1 may be a component of point contacts.

RACK1 Is Required for Point Contact Formation in Growth Cones

The observation that RACK1 localizes to growth cone point contacts (Fig. 1) suggests that RACK1 may play a critical role in the function of point contacts. Thus, we examined whether point contact formation requires RACK1 expression. Although a RACK1 knockout mouse has been created, the embryos do not survive gastrulation (Volta et al., 2013). Thus, we used shRNA to knockdown RACK1. We tested multiple RACK1 shRNAs and identified two commercially available and validated RACK1 shRNAs that result in significant knockdown of RACK1 within cultured cortical neurons, but without causing growth cone loss. Mouse cortical neurons were transfected at 2 DIV with either nonsilencing control shRNA or one of the two RACK1 shRNAs. At 4 DIV, cells were fixed and quantitative immunocytochemistry was performed to label RACK1. Measurements of fluorescence

intensity within pyramidal neurons demonstrated that both shRNAs knockdown RACK1 expression significantly in both somas (Fig. 2A) and growth cones (Fig. 2B–E).

A previous study demonstrated that stimulation with brain-derived neurotrophic factor (BDNF) results in a significant increase in paxillin-containing point contact density in *Xenopus* spinal neurons (Myers and Gomez, 2011). We repeated this experiment in mouse cortical neurons and found the same result. BDNF stimulation for 30 min results in an increase in paxillin-containing point contact density in growth cones (Fig. 3A, nonsilencing control shRNA). Here, point contact density is defined as the number of puncta/ μm^2 , and growth cone area is defined as the area including the central domain, lamellipodia, and filopodia, but not the axon. Next, to examine the role of RACK1 in point contact density, cortical neurons expressing either a nonsilencing control shRNA or one of the two RACK1 shRNAs were stimulated with BDNF or vehicle control for 30 min and then fixed. Immunocytochemistry was performed to label paxillin, a marker of point contacts. Knockdown of RACK1 resulted in significantly lower point contact density in growth cones versus the nonsilencing control shRNA under basal conditions (Fig. 3A,C,D). Furthermore, the BDNF-induced increase in paxillin-containing point contact density seen under control conditions (i.e., nonsilencing control shRNA) was lost in the presence of RACK1 shRNAs. We then conducted the same experiment using vinculin, which is also a marker of point contacts. BDNF stimulation did not increase vinculin-containing point contact density in any condition, but RACK1 knockdown significantly lowered point contact density versus the nonsilencing control shRNA (Fig. 3B). These data indicate that RACK1 is necessary for paxillin-containing and vinculin-containing point contact formation under basal conditions, and is necessary for paxillin-containing point contact formation under growth factor stimulated conditions.

RACK1 Expression Is Required for BDNF-Induced Growth Cone Spreading and Axon Growth

Given that RACK1 is required for the formation of point contacts, we next asked whether RACK1 is required for functional aspects of neuronal development, such as growth cone spreading and axon outgrowth. Previous studies have demonstrated that BDNF stimulation results in an increase in growth cone area (i.e., growth cone spreading) in *Xenopus* spinal neurons (Myers and Gomez, 2011). We repeated this experiment in mouse cortical neurons and found the same result. BDNF stimulation for 30 min results in a significant increase in growth cone spreading (Fig. 4A,B, nonsilencing control shRNA). Knockdown of RACK1 did not significantly alter growth cone area under vehicle-treated conditions; however, BDNF stimulation no longer resulted in an increase in growth cone area in these groups (Fig. 4A,B). Taken together, these data indicate that an appropriate level of RACK1 is necessary for BDNF-induced growth cone spreading.

Because RACK1 is critical to the formation of point contacts, this suggests that it may also hold an important role in the regulation of axon outgrowth. Cortical neurons were transfected at 2 DIV with a nonsilencing control shRNA or shRNA to RACK1, fixed at 4 DIV, and axon length was compared between the groups. RACK1 knockdown significantly

impairs axon outgrowth versus the nonsilencing control shRNA (Fig. 4C,D). Thus, here we find that RACK1 is a critical regulator of axon outgrowth in mammalian neurons.

Appropriate Levels of RACK1 and Its Phosphorylation Regulate Point Contact Formation in Growth Cones

Because RACK1 is required for point contact density in growth cones (Fig. 3A), we next investigated the mechanism underlying its regulation of this process. Research in human embryonic kidney cells has shown that RACK1 regulates focal adhesion morphology in a Src dependent manner (Doan and Huttenlocher, 2007). RACK1 is phosphorylated by Src at residue 246, thus we investigated whether this site may be involved in the regulation of point contact density. In order to investigate this possibility, we overexpressed a nonphosphorylatable mutant form of RACK1, RACK1-Y246F, in cortical neurons. This mutant form of RACK1 cannot be phosphorylated at the Y246 Src binding site (Cox et al., 2003).

Cortical neurons were transfected with GFP, GFP-RACK1-WT, or GFP-RACK1-Y246F. GFP-RACK1-WT and GFP-RACK1-Y246F are expressed at similar levels ($P = 0.449$, data not shown). Furthermore, GFP-RACK1-WT colocalizes with paxillin labeled point contacts similarly to the endogenous RACK1 colocalization shown in Figure 1B (PCC for GFP-RACK1-WT and paxillin = 0.36, PCC for endogenous RACK1 and paxillin = 0.38, data not shown). Cells were transfected at 2 DIV and at 4 DIV cells were treated with either BDNF or vehicle for 30 min and then fixed. Quantitative immunocytochemistry was performed to label paxillin, a marker of point contacts. In control conditions (i.e., transfection with GFP), BDNF stimulation led to a significant increase in point contact density (measured as puncta/ μm^2 ; Fig. 5A,B). Overexpression of the wild type form of RACK1 led to an increase in point contact density in vehicle-treated conditions that was significantly higher than the point contact density in GFP vehicle-treated conditions. However, BDNF stimulation did not result in a further point contact density increase in RACK1-WT expressing neurons, suggesting a ceiling effect of RACK1-WT overexpression (Fig. 5A,B). This suggests that the level of RACK1 in growth cones is critical to the regulation of point contact density. Interestingly, overexpression of the nonphosphorylatable mutant form of RACK1, RACK1-Y246F, did not significantly affect point contact density under vehicle-treated conditions (as compared to GFP transfected, vehicle-treated neurons). However, BDNF-stimulation resulted in a significant decrease in point contact density in RACK1-Y246F expressing neurons (Fig. 5A,B), suggesting a complete dysregulation of point contact number in this condition. Taken together, these data suggest that appropriate levels of RACK1, as well as Src phosphorylation of RACK1, may be involved in the regulation of point contact formation in growth cones.

To further determine the potential role of Src in BDNF-induced point contact formation, we inhibited Src kinase and examined the effect on point contact density. At 2 DIV, cortical neurons were exposed to either PP2, a Src inhibitor, or PP3, an inactive control, and treated with vehicle or BDNF for 30 min. We then fixed the cells and performed immunocytochemistry for point contacts (paxillin) and RACK1. As in previous experiments, BDNF stimulation in control conditions (i.e., PP3) significantly increases point contact

density. However, Src inhibition eliminates this significant increase (Fig. 5C). Furthermore, under control conditions, colocalization of RACK1 with point contacts significantly increases following BDNF treatment, suggesting that BDNF stimulation recruits RACK1 to point contacts (Fig. 5D). However, inhibition of Src eliminates the BDNF-induced increase in colocalization (Fig. 5D). Thus, Src is required for the BDNF-induced increase in both point contact formation and RACK1 localization to the point contact complex.

Appropriate Levels of RACK1 and Its Phosphorylation Regulate Growth Cone Spreading and Axon Outgrowth

We next examined whether overexpression of RACK1-WT or the nonphosphorylatable mutant form of RACK1, RACK1-Y246F, has a functional effect on neuronal development. First, we examined the effect of these constructs on growth cone spreading. As expected, BDNF stimulation resulted in an increase in growth cone area in cells expressing the control GFP construct (Fig. 6A,B). Expression of RACK1-WT or RACK1-Y246F significantly increased growth cone area under vehicle-treated conditions as compared to GFP vehicle-treated conditions. However, BDNF stimulation did not result in a further increase in growth cone area in neurons expressing either of these constructs (Fig. 6A,B), suggesting a ceiling effect resulting from overexpression. These data indicate that appropriate levels of RACK1, and Src phosphorylation of RACK1, may regulate growth cone area and spreading.

Next, we examined the effect of these constructs on axon outgrowth. Overexpression of either the wild-type form of RACK1 or the nonphosphorylatable mutant form of RACK1, RACK1-Y246F, significantly impairs axon outgrowth as compared to GFP control (Fig. 6C,D). Taken together, these data suggest that both an appropriate level of RACK1 and Src phosphorylation of RACK1 regulate axon outgrowth.

Members of the Local Translation Complex Colocalize with Point Contacts in Growth Cones

Previous studies have demonstrated that the local translation of multiple mRNAs, including β -actin mRNA, in growth cones is necessary for appropriate axon guidance (Yoon et al., 2009; Jung et al., 2012). However, the specific substructural localization of this translation has yet to be investigated. Point contacts have a major role in the regulation of axon growth and guidance (Robles and Gomez, 2006; Woo and Gomez, 2006; Myers and Gomez, 2011) and thus would be an optimal site for local translation, but this has not been tested. Because we have recently shown that RACK1 is an integral member of the local translation complex in growth cones (Ceci et al., 2012) and localizes to point contacts (Fig. 1), we sought to determine whether point contacts might be sites of local translation in mammalian growth cones. We first examined whether members of the local translation complex colocalize with point contacts in growth cones. To this aim, mouse cortical neurons were cultured for 2 days and then fixed. Double label immunocytochemistry and/or fluorescent in situ hybridization were performed for a member of the local translation complex (either a ribosomal subunit, RACK1, or β -actin mRNA) and a marker of point contacts (paxillin or vinculin). We then determined the Pearson's correlation coefficient between the members of the local translation complex and point contacts within growth cones of cortical pyramidal neurons. Here we find that ribosomal subunits, RACK1 and β -actin mRNA all colocalize with a

marker of point contacts (paxillin) under basal conditions (Fig. 7A,B). Similar results were also obtained with a second marker of point contacts, vinculin (Fig. 7C).

Thus, we next examined if this colocalization would be altered following stimulation with BDNF. We have previously demonstrated that BDNF stimulation results in local translation in growth cones. Specifically, BDNF increased RACK1 binding of the ZBP1/ β -actin mRNA complex on ribosomes. Phosphorylation of RACK1 then facilitates the release of β -actin mRNA from ZBP1 and its subsequent translation (Sasaki et al., 2011; Ceci et al., 2012). Thus, if point contacts are the sites of local translation, we would expect an increase in the localization of RACK1, β -actin mRNA, and ribosomes at point contacts following BDNF stimulation. BDNF treatment results in a significant increase in the colocalization of both RACK1 and β -actin mRNA with paxillin-containing point contacts (Fig. 7A,B). However, these data could potentially be confounded by a BDNF-induced increase in β -actin mRNA in growth cones, which would result in a nonmeaningful increase in Pearson's correlation coefficient. We find that BDNF stimulation for 30 min does not cause an increase in β -actin mRNA in growth cones ($P = 0.099$, data not shown), confirming that this is a functionally meaningful increase in colocalization. Thus, these data suggest that point contacts are potential sites of local translation within growth cones.

Next, we investigated whether these increases were exclusive to paxillin-containing point contacts. We found that treatment with BDNF also causes a significant increase of RACK1 and β -actin mRNA colocalization at vinculin-containing point contacts (Fig. 7C). We found no change in the localization of the S6 ribosomal subunit at paxillin or vinculin-containing point contacts following BDNF-stimulation (Fig. 7A–C). We also examined a second ribosomal rRNA subunit, 5.8S ribosomal RNA. Again, there was no change in the localization of the 5.8S ribosomal subunit at paxillin-containing point contacts following BDNF stimulation (Fig. 7A,B). However, these data are not unexpected if point contacts are a site of local translation for a variety of mRNAs; ribosomes would remain localized at point contacts to mediate the translation of many mRNAs. Taken together, these data suggest that point contacts are likely a site of local translation within growth cones.

Finally, we examined whether RACK1 is required for β -actin mRNA localization at point contacts. Mouse cortical neurons were cultured for 2 days and then transfected with either nonsilencing control shRNA or RACK1 shRNA as in previous experiments. A combination of immunocytochemistry and fluorescent in situ hybridization were performed at 4 DIV for paxillin and β -actin mRNA. We then determined the Pearson's correlation coefficient between paxillin and β -actin mRNA. We found that RACK1 is required for the BDNF-induced increase in the localization of β -actin mRNA at paxillin-containing point contacts (Fig. 7D). These data provide further support that RACK1 may be required for BDNF-induced local translation at point contacts.

DISCUSSION

Regulation of Point Contacts by RACK1

Most of our knowledge about RACK1-regulated cell migration and adhesion comes from studies examining its role as a member of the focal adhesion complex in nonneuronal cells

(Buensuceso et al., 2001; Hermanto et al., 2002; Cox et al., 2003; Doan and Huttenlocher, 2007; Vomastek et al., 2007; Kiely et al., 2009; Serrels et al., 2010). Previous studies have shown that RACK1 is localized to a subset of focal adhesions in CHO cells (Cox et al., 2003). However, focal adhesions are not present in growth cones. Point contacts and focal adhesions are similar in that they are both multi-protein complexes that form connections to the extracellular matrix. However, point contacts are distinct from focal adhesions in both structure and function. Differences exist between point contacts and focal adhesions in terms of protein composition, size, distribution, and effects on motility (Renaudin et al., 1999). Thus, previous studies on RACK1 and focal adhesions in nonneuronal cells have some relevance for the current studies on point contacts in neurons, but the differences between these adhesion types must also be considered.

Here we demonstrate the novel finding that RACK1 is localized to point contacts within growth cones. Point contacts have been shown to localize with F-actin (Renaudin et al., 1999). Using TIRF microscopy, we also show that RACK1 localizes with F-actin bundles (Fig. 1E). Specifically, RACK1 is localized within almost every filopodia (Fig. 1E, arrows). Paxillin localizes within filopodia in a very similar manner (Nichol et al., 2016), which is expected because paxillin and RACK1 are significantly colocalized in growth cones (Fig. 1B). Furthermore, we find that RACK1 colocalizes with two separate markers of point contacts, paxillin and vinculin. Although RACK1 and point contact markers (paxillin and vinculin) are colocalized under basal conditions (Fig. 1A–D), there is also a proportion that is not colocalized. This is not surprising because RACK1 is a multifunctional protein involved in a number of cellular signaling processes (Adams et al., 2011). Thus, it is likely that a proportion of RACK1 regulates signaling processes in areas of the growth cone outside of point contacts. Our TIRF images also show a population of RACK1 within the central domain (Fig. 1E). Therefore, it is likely that some fraction of RACK1 is present within the cytoplasm and not specifically at point contacts. Future experiments will employ live cell TIRF microscopy to examine only the colocalized population that occurs at adhesion sites at the membrane–substrate interface.

In this study, we also show that RACK1 is required for point contact formation. Knockdown of RACK1 significantly lowers basal point contact density within growth cones (Fig. 3A). A previous study in fibroblasts demonstrates that knockdown of RACK1 causes a decrease in focal adhesion disassembly (Vomastek et al., 2007). Although this article did not quantify disassembly, this suggests an overall increase in focal adhesion number, which is the opposite of our current data (Fig. 3A–D). However, these differences could be attributed to a number of factors, including cell type or differences in adhesion structure, as described earlier. However, it is important to note that the functional outcome of these two seemingly disparate studies is similar; RACK1 knockdown led to decreased motility in fibroblasts and decreased axon length in neurons (Fig. 4D). We find that overexpression of RACK1 also affects point contact density, leading to a significantly elevated basal density of point contacts (Fig. 5B). In line with this, overexpression of RACK1 increases focal adhesion number in CHO cells (Buensuceso et al., 2001; Cox et al., 2003). Additionally, we found that overexpression of RACK1 prevents a BDNF-induced increase in paxillin-containing point contact density. Paxillin-containing point contacts are highly dynamic (Woo and Gomez, 2006). Little is known about vinculin-containing point contacts in growth cones, but

one study suggests that there is a population located in the central domain of growth cones that regulate more stable adhesions (Renaudin et al., 1999). We show here (Fig. 3) that BDNF has differential effects on paxillin and vinculin-containing point contacts when levels of RACK1 are not manipulated. However, these data also show that the level of RACK1 within growth cones is critical for the regulation of both paxillin and vinculin containing point contacts. Taken together, these studies suggest that an appropriate concentration of RACK1 in the growth cone is required to properly modulate point contact density under both basal and BDNF-stimulated conditions.

Our data suggest that RACK1 phosphorylation by Src may not be necessary for point contact formation under basal conditions (Fig. 5B). This is in contrast to focal adhesions, where blocking RACK1 phosphorylation by Src decreases the percent of cells with central focal adhesions (Cox et al., 2003). Surprisingly, we find that overexpression of the mutant form of RACK1 results in a significant decrease in point contact density following BDNF application (Fig. 5B). Prior studies in unmanipulated *Xenopus* spinal neurons demonstrate that BDNF stimulates point contact turnover (Myers and Gomez, 2011). Furthermore, studies in nonneuronal cells have demonstrated that an increase in Src activity leads to a decrease in focal adhesion density (Fincham et al., 1995; Frame et al., 2002). Both BDNF stimulation and RACK1-Y246F likely result in an increase in Src activity; thus, this combination may result in a very large change in the point contact turnover rate. If the disassembly rate of point contacts is greater than the assembly rate of new point contacts, then this could cause the significant drop in point contact density following BDNF stimulation in neurons expressing RACK1-Y246F (Fig. 5B). To clarify this mechanism, future experiments will investigate the role of RACK1 in point contact formation and dynamics in living cells using TIRF microscopy.

We demonstrate that Src inhibition eliminates the BDNF-induced increase in point contact density (Fig. 5C). This is in line with previous findings in *Xenopus* retinal ganglion cells (Woo et al., 2009). Furthermore, Src inhibition disrupts the localization of RACK1 at point contacts following BDNF stimulation (Fig. 5D). Taken together, these data suggest that Src activity is necessary for the regulation of point contact density and localization of RACK1 at point contacts under BDNF stimulation. In the future, we will further examine the Y246 site, as well as other phosphorylation sites on RACK1, to elucidate their role in BDNF stimulated point contact dynamics.

RACK1 and Point Contacts Are Necessary for Appropriate Neural Development

Here, we show that RACK1 is required for functional aspects of nervous system development, including axon outgrowth and growth cone spreading. Indeed, RACK1 signaling is crucial for mammalian development as mice lacking RACK1 are embryonic lethal, which occurs at gastrulation (Volta et al., 2013). However, very few studies have examined the role of RACK1 during neural development. The paralog of RACK1, RACK-1, is necessary for appropriate nervous system development in *C. elegans*. Specifically, this study demonstrated that RACK-1 is required for commissural axon pathfinding and may directly regulate the cytoskeleton (Demarco and Lundquist, 2010). In vertebrates, RACK1 is highly expressed in the nervous system throughout development (Ashique et al., 2006), but

only a handful of studies have examined the role of RACK1 in this process. RACK1 is known to regulate neural tube closure (Wehner et al., 2011) and is downregulated in Down syndrome (Peyrl et al., 2002). In addition, one study examining PC12 cells demonstrated that the interaction of RACK1 with FAK is necessary for cells to initially produce a neurite (Dwane et al., 2014).

Here, we expand our understanding of RACK1 during neural development. Knockdown of RACK1 significantly decreases axon outgrowth (Fig. 4C,D). Overexpression of RACK1-WT also results in a decrease in axon length (Fig. 6D), suggesting that there is a level of RACK1 that leads to optimal outgrowth; increases or decreases from this amount both lead to a decrease in axon outgrowth. We can also correlate this finding with point contact density, such that too few or too many point contacts both lead to a decrease in axon length, due to destabilization or overstabilization of the growth cone, which is in line with previous studies (Woo and Gomez 2006; Woo et al. 2009). However, it is also possible that RACK1 regulates axon growth independently from its regulation of point contact density.

Blocking RACK1 phosphorylation by Src significantly decreases axon outgrowth as well (Fig. 6C,D); however, this decrease is not as pronounced as RACK1 knockdown. This may suggest that RACK1 regulation of axon outgrowth is not controlled exclusively by Src. Previous studies have demonstrated that RACK1 phosphorylation is required for multiple processes, including cell growth and adhesion (Cox et al., 2003; Nilsson et al., 2004; Doan and Huttenlocher, 2007; Kiely et al., 2009; Adams et al., 2011). Specifically, phosphorylation of RACK1 on Tyr-52 is required for cell adhesion and focal adhesion formation in MCF-7 cells (Kiely et al., 2009), as well as initial neurite extension of PC12 cells (Dwane et al., 2014). However, phosphorylation of RACK1 on Tyr-246 is required for cell protrusion and chemotactic migration in CHO cells (Cox et al., 2003). Thus, future studies will further examine the role of additional phosphorylation sites in the regulation of axon growth and guidance.

Previous studies have demonstrated that growth cone morphology is regulated by a complex of the large GTPase, dynamin, and the actin binding protein, cortactin, at point contacts (Kurklinsky et al., 2011). The current study demonstrates that RACK1 regulates growth cone morphology as well, specifically BDNF-induced growth cone spreading (Fig. 4B). RACK1 knockdown eliminates the BDNF-induced significant increase in growth cone area and results in lower overall point contact density. Although RACK1-WT and RACK1-Y246F overexpression lead to a significant increase in growth cone area under basal conditions (Fig. 6B), there is no further increase in growth cone area following BDNF stimulation, suggesting a possible ceiling effect resulting from overexpression of these constructs. Taken together, these results suggest that RACK1 plays a critical role in mediating growth cone morphology.

Here, we define RACK1 as a novel member of the point contact complex. Our current findings are in line with earlier studies demonstrating that the loss of members of the point contact complex disrupts normal nervous system development (Beggs et al., 2003; Clegg et al., 2003; Myers et al., 2011). Point contacts are regulated by Rho GTPases (Woo and Gomez, 2006), focal adhesion kinase (Robles and Gomez, 2006; Myers and Gomez, 2011),

and P21-activated kinase (Santiago-Medina et al., 2013). Furthermore, a recent study elegantly demonstrated a direct relationship between F-actin retrograde flow and point contacts that regulates the axon growth rate (Nichol et al., 2016). This study also demonstrated that BDNF stimulation leads to large decrease in the retrograde flow rate of neurons plated on laminin, which is correlated with an increase in point contact formation and a faster rate of axon growth (Myers and Gomez, 2011; Nichol et al., 2016). Taken together, these studies suggest that point contacts are highly regulated adhesion structures that help modulate axon guidance during neural development.

RACK1 in Cellular Signaling and Local Translation

RACK1 is a scaffolding protein that was originally identified as a receptor for activated PKC. However, RACK1 is also localized on ribosomes (Link et al., 1999; Sengupta et al., 2004) and found as a part of messenger ribonucleoprotein complexes (Angenstein et al., 2002). Much research on RACK1 thus far has focused on its role as a scaffolding protein that is important for cellular signaling. However, a critical role of RACK1 is its function as a linker between cellular signaling and translation (Gibson, 2012). Put in the context of our data, we suggest a critical role for RACK1 in the modulation of local translation specifically at point contacts.

In the current study, we demonstrate that RACK1 localizes at point contacts. Because RACK1 is a ribosomal scaffolding protein, we therefore hypothesized that other members of the local translation complex also reside at point contacts. Indeed, we found that ribosomal subunits and β -actin mRNA also localize to point contacts. BDNF stimulates the local translation of β -actin mRNA (Yao et al., 2006); thus, it is expected that this localization would increase following BDNF stimulation. Our data show that application of BDNF significantly increases the localization of RACK1 and β -actin mRNA at point contacts (Fig. 7B,C), suggesting its local translation at that subcellular location. Furthermore, our data also show that RACK1 is required for this BDNF-induced increase in localization of β -actin mRNA at point contacts (Fig. 7D). Data from previous studies suggest the possibility of local translation at focal adhesions, but this has yet to be directly shown. For example, a previous study demonstrated the dwelling of β -actin mRNA at focal adhesions in fibroblasts, and that forcibly tethering β -actin mRNA at these adhesions affected adhesion dynamics and cell motility (Katz et al., 2012). Furthermore, ribosomes and translation initiation factors are enriched at focal adhesions in actively migrating fibroblasts (Willett et al., 2010) and integrin binding results in the localization of mRNA to focal adhesions in endothelial cells (Chicurel et al., 1998). Taken together, local translation at adhesion sites (e.g., point contacts) would enable the translation of specific mRNAs, such as β -actin, where it can have a direct effect on growth cone motility. This has yet to be directly demonstrated, but will be investigated in future studies. The current data highly suggest that local translation occurs at point contacts, thus revealing their role as important signaling centers within the developing nervous system.

It is important to note that although there is significant colocalization of RACK1 and β -actin mRNA with point contacts under basal conditions (Fig. 7), there is also a portion of these proteins that are not colocalized. We do not expect complete colocalization of RACK1 and

point contacts for multiple, not mutually exclusive, reasons. First, RACK1 regulates multiple aspects of cell physiology and signaling. Thus, we would expect only a certain fraction of RACK1 to be colocalized with point contacts in a signaling and/or translational role. Second, it may be that there are other sites of local translation in growth cones. This would mean that only a certain percentage of local translation takes place at point contacts, and RACK1 may also facilitate local translation at other sites in growth cones. Third, paxillin and vinculin may each only label a subset of point contacts in growth cones. Although paxillin and vinculin mostly colocalize in growth cones, there are subsets of point contacts that label only for paxillin and not vinculin, and vice versa (Woo and Gomez, 2006). Furthermore, paxillin is also a component of invadosomes, adhesive structures that both bind and degrade the extracellular matrix. Growth cones contain similar structures that reside primarily in the central domain (Santiago-Medina et al., 2015). Lastly, it is likely that some fraction of paxillin and vinculin is present within the cytoplasm and not specifically at point contacts. The use of live cell TIRF microscopy in future experiments will investigate only the population of proteins of interest that are localized at adhesion sites at the membrane–substrate interface. In addition, we will examine local translation at point contacts in greater detail and determine if local translation takes place differentially at point contact subtypes. Live cell imaging and local translation reporters will provide definitive evidence as to whether local translation occurs at point contacts and further examine how this local translation is regulated.

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Figure 1.

RACK1 is found in mammalian growth cones and is localized to point contacts. (A) RACK1 colocalizes with paxillin, a marker of point contacts. In composite images, paxillin is shown in red and RACK1 is shown in green. Arrows in composite images designate regions of colocalization. Dotted lines outline an axonal growth cone. Note that dotted lines also include the axon for ease of visualization, but axons were not included in the analyses. Scale bars, 5 μ m. (B) RACK1 colocalizes with paxillin-containing point contacts (PCC = 0.38 ± 0.03). As a negative control, there is almost a complete loss of colocalization if one of the channels is rotated 180°. * $P = 0.05$, Kolmogorov–Smirnov. Normal, $n = 50$; rotated, $n = 50$. (C) RACK1 colocalizes with vinculin, a marker of point contacts. In composite images, vinculin is shown in red and RACK1 is shown in green. Arrows in composite images designate regions of colocalization. Scale bars, 5 μ m. (D) RACK1 colocalizes with vinculin-containing contacts (PCC = 0.59 ± 0.03). As a negative control, there is almost a complete loss of colocalization if one of the channels is rotated 180°. * $P = 0.05$, Student's t -test. Normal, $n = 30$; rotated, $n = 30$. Note that in this figure the brightness and contrast were evenly adjusted throughout the entirety of each image, so that puncta would be easily visible. This results in the appearance of overexposure in some regions. (E) Images acquired using TIRF microscopy show that RACK1 colocalizes with F-actin near the membrane in growth cones. Left and middle images show staining of F-actin and RACK1, respectively. Right image (composite): F-actin is shown in red and RACK1 is shown in green. Arrows and arrowheads in composite image designate regions of colocalization. Arrows designate colocalization of RACK1 with F-actin in filopodia, while sets of arrowheads (open and outlined) designate colocalization of RACK1 with F-actin bundles projecting into the central domain. Note that F-actin bundles can be seen more clearly in the F-actin image than the composite image. Scale bar is 1 μ m in (E) and 5 μ m in (A) and (C). [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2.

Validation of RACK1 shRNAs. (A and B) Validation of RACK1 shRNAs in neuronal culture. Two separate RACK1 shRNAs were tested, and both significantly reduce RACK1 expression versus control shRNA in both somas (A) and growth cones (B). * $P < 0.05$, ANOVA with Dunnett's multiple comparisons. Post hoc analysis for somas: nonsilencing control shRNA versus shRNA 1, * $P < 0.001$; nonsilencing control shRNA versus shRNA 2, * $P < 0.001$. Post hoc analysis for growth cones: nonsilencing control shRNA versus shRNA 1, * $P < 0.001$; nonsilencing control shRNA versus shRNA 2, * $P < 0.001$. Nonsilencing control shRNA, $n = 22$; shRNA 1, $n = 20$; shRNA 2, $n = 20$. (C–E) Immunocytochemistry for RACK1 demonstrates that shRNA 1 and shRNA 2 significantly decrease RACK1 in growth cones. Dotted lines outline axonal growth cones and also include the axon, but axons were not included in the analyses. Note that the brightness and contrast were evenly adjusted throughout the entirety of each image, so that puncta would be easily visible. This results in the appearance of overexposure in some regions. Scale bars, 5 μm .

Figure 3.

RACK1 is required for point contact density. (A) RACK1 knockdown significantly reduces paxillin-labeled point contact density (puncta/ μm^2) in growth cones versus nonsilencing control shRNA. RACK1 knockdown also eliminates the BDNF-induced increase in paxillin-containing point contact density. * P 0.05, two-way ANOVA with Dunnett's multiple comparisons. Post hoc analysis: nonsilencing control shRNA vehicle versus shRNA 1 vehicle, * P < 0.001; nonsilencing control shRNA vehicle versus shRNA 2 vehicle, * P < 0.001; nonsilencing control shRNA vehicle versus nonsilencing control shRNA BDNF, * P = 0.031; shRNA 1 vehicle versus shRNA 1 BDNF, P = 0.482; shRNA 2 vehicle versus shRNA 2 BDNF, 0.437. Nonsilencing control shRNA vehicle, n = 26; nonsilencing control shRNA BDNF, n = 44; shRNA 1 vehicle, n = 25; shRNA 1 BDNF, n = 25; shRNA 2 vehicle, n = 30; shRNA 2 BDNF, n = 25. (B) Expression of RACK1 shRNAs significantly reduces vinculin-labeled point contact density (puncta/ μm^2) in growth cones versus nonsilencing control shRNA. * P 0.05, two-way ANOVA with Dunnett's multiple comparisons. Post hoc analysis: nonsilencing control shRNA vehicle versus shRNA 1 vehicle, * P = 0.006; nonsilencing control shRNA vehicle versus shRNA 2 vehicle, * P = 0.002; nonsilencing control shRNA vehicle versus nonsilencing control shRNA BDNF, P = 0.837; shRNA 1 vehicle versus shRNA 1 BDNF, P = 0.888; shRNA 2 vehicle versus shRNA 2 BDNF, 0.330. Nonsilencing control shRNA vehicle, n = 27; nonsilencing control shRNA BDNF, n = 29; shRNA 1 vehicle, n = 30; shRNA 1 BDNF, n = 28; shRNA 2 vehicle, n = 28; shRNA 2 BDNF, n = 29. (C and D) Immunocytochemistry for paxillin, a marker of point contacts, demonstrates that knockdown of RACK1 significantly decreases point contact density within growth cones. Dotted lines outline axonal growth cones and also include the axon, but axons were not included in the analyses. Note that the brightness and contrast were evenly adjusted

throughout the entirety of each image, so that puncta would be easily visible. This results in the appearance of overexposure in some regions. (E) Phalloidin staining of F-actin was used to visualize the growth cone and define growth cone area for all analyses. Scale bars, 5 μm .

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Figure 4.

RACK1 is required for growth cone spreading and axon length. (A and B) Neurons expressing either nonsilencing control shRNA or a RACK1 shRNA were exposed to either vehicle or BDNF for 30 min. BDNF stimulation results in a significant increase in growth cone area in nonsilencing control shRNA, whereas this increase is lost following knockdown of RACK1 with shRNA 1 or shRNA 2. F-actin was labeled with phalloidin and dotted lines outline axonal growth cones. Note that dotted lines also include the axon for ease of visualization, but axons were not included in the analyses. Scale bars, 5 μ m. * P < 0.05, two-way ANOVA with Dunnett's multiple comparisons. Post hoc analysis: nonsilencing control shRNA vehicle versus nonsilencing control shRNA BDNF, * P = 0.021; shRNA 1 vehicle versus shRNA 1 BDNF, P = 0.283; shRNA 2 vehicle versus shRNA 2 BDNF, P = 0.139. No significant differences are observed between nonsilencing control vehicle growth cone area and shRNA vehicle growth cone area. Nonsilencing control shRNA vehicle, n = 36; nonsilencing control shRNA BDNF, n = 44; shRNA 1 vehicle, n = 25; shRNA 1 BDNF, n = 25; shRNA 2 vehicle, n = 30; shRNA 2 BDNF, n = 25. (C) Knockdown of RACK1 (shRNA

1) results in shortened axon length, as compared to neurons expressing a nonsilencing control shRNA. In these experiments, F-actin was labeled with phalloidin and dotted lines denote the axon in each image. Note that the brightness and contrast were evenly adjusted throughout the entirety of each image, so that the axon would be easily visible. This results in the appearance of overexposure in the soma. Scale bars, 25 μm . (D) Knockdown of RACK1 results in a significant decrease in axon length. $*P = 0.05$, ANOVA with Dunnett's multiple comparisons. Post hoc analysis: nonsilencing control shRNA versus shRNA 1, $*P = 0.004$; nonsilencing control shRNA versus shRNA 2, $*P < 0.001$. Nonsilencing control shRNA, $n = 20$; shRNA 1, $n = 21$; shRNA 2, $n = 23$.

Figure 5.

RACK1 concentration and phosphorylation regulate point contact density. (A) Immunocytochemistry for paxillin demonstrates that a BDNF-induced increase in point contact density requires appropriate levels of RACK1 and RACK1 phosphorylation. Dotted lines outline axonal growth cones and also include the axon, but axons were not included in the analyses. Note that the brightness and contrast were evenly adjusted throughout the entirety of each image, so that puncta would be easily visible. Scale bars, 5 μm . (B) There is a significant increase in point contact density (puncta/ μm^2) following BDNF stimulation in GFP expressing neurons (control). Overexpression of RACK1-WT results in a significantly higher basal density of point contacts, and there is no further increase in density following BDNF stimulation. Overexpression of the nonphosphorylatable mutant form of RACK1, RACK1-Y246F, results in a basal density that is not significantly different from control basal levels, but there is a significant decrease in point contact density following BDNF stimulation. * $P < 0.05$, two-way ANOVA with Fisher's LSD. GFP vehicle versus GFP BDNF, * $P = 0.013$; RACK1-WT vehicle versus RACK1-WT BDNF, $P = 0.805$; RACK1-Y246F vehicle versus RACK1-Y246F BDNF, * $P = 0.005$. GFP vehicle versus RACK1-WT vehicle, * $P = 0.049$; GFP vehicle versus RACK1-Y246F vehicle, $P = 0.326$. RACK1-WT vehicle versus RACK1-Y246F vehicle, $P = 0.363$. GFP vehicle, $n = 26$; GFP BDNF, $n = 28$; RACK1-WT vehicle, $n = 29$; RACK1-WT BDNF, $n = 27$; RACK1-Y246F vehicle, $n = 28$;

RACK1-Y246F BDNF, $n = 26$. (C) Neurons were exposed to PP2, a Src inhibitor, or PP3, an inactive control, as well as either vehicle or BDNF for 30 min. Neurons were then fixed, and ICC was used to label paxillin. Immunocytochemistry for paxillin demonstrates that Src is required for the BDNF-induced increase in point contact density. $*P = 0.05$, ANOVA with Dunnett's multiple comparisons. Post hoc analysis: PP3 vehicle versus PP3 BDNF, $*P = 0.015$; PP2 vehicle versus PP2 BDNF, $P = 0.312$; PP3 vehicle versus PP2 vehicle, $P = 0.984$. PP3 vehicle, $n = 30$; PP3 BDNF, $n = 30$; PP2 vehicle, $n = 29$; PP3 BDNF, $n = 30$. (D) Neurons were exposed to either PP2, a Src inhibitor, or PP3, an inactive control, as well as either vehicle or BDNF for 30 min. Neurons were then fixed, and ICC was used to label paxillin and RACK1. Under control conditions, there is a significant increase in RACK1 colocalization with paxillin following BDNF stimulation. Src inhibition eliminates this increase. $*P = 0.05$, ANOVA with Dunnett's multiple comparisons. Post hoc analysis: PP3 vehicle versus PP3 BDNF, $*P = 0.029$; PP2 vehicle versus PP2 BDNF, $P = 0.513$; PP3 vehicle versus PP2 vehicle, $P = 0.979$. PP3 vehicle, $n = 30$; PP3 BDNF, $n = 30$; PP2 vehicle, $n = 29$; PP3 BDNF, $n = 30$.

Figure 6.

RACK1 concentration and phosphorylation regulate growth cone spreading and axon length. (A and B) Neurons expressing GFP, RACK1-WT, or RACK1-Y246F were exposed to either vehicle or BDNF for 30 min, fixed and F-actin was labeled with phalloidin. BDNF stimulation results in a significant increase in growth cone area of GFP transfected neurons, as compared to vehicle treated neurons. Expression of RACK1-WT or RACK1-Y246F significantly increased growth cone area under vehicle-treated conditions as compared to GFP vehicle-treated conditions. However, BDNF stimulation does not result in a further increase in growth cone area in neurons overexpressing RACK1-WT or RACK1-Y246F. Dotted lines outline the axonal growth cones and also include the axon, but axons were not included in the analyses. Scale bars, 5 μ m. * P 0.05, Holm-Sidak's multiple comparisons. GFP vehicle versus GFP BDNF, * P = 0.004; RACK1-WT vehicle versus RACK1-WT BDNF, P = 0.876; RACK1-Y246F vehicle versus RACK1-Y246F BDNF, P = 0.680. GFP vehicle versus RACK1 vehicle, * P = 0.007; GFP vehicle versus RACK1-Y246F vehicle, * P = 0.049. GFP Vehicle, n = 48; GFP BDNF, n = 50; RACK1-WT vehicle, n = 49; RACK1-WT BDNF, n = 49; RACK1-Y246F vehicle, n = 50; RACK1-Y246F BDNF, n = 47. (C) Neurons overexpressing RACK1-WT or RACK1-Y246F have significantly shorter axons than GFP expressing neurons. F-actin was labeled with phalloidin and dotted lines denote the axon. Note that the brightness and contrast were evenly adjusted throughout the entirety of each image, so that axons would be visible. This results in the appearance of

overexposure in the soma. Scale bars, 25 μm . (D) Overexpression of RACK1-WT or RACK1-Y246F results in a significant stunting of axon length. $*P = 0.05$, ANOVA with Dunnett's multiple comparisons. GFP versus RACK1-WT, $*P = 0.027$; GFP versus RACK1-Y246F, $*P = 0.006$. GFP, $n = 23$; RACK1-WT, $n = 19$; RACK1-Y246F, $n = 18$.

Figure 7.

Members of the local translation complex localize to point contacts. (A) Members of the local translation complex (RACK1, ribosomal subunits, β -actin mRNA) colocalize with a marker of point contacts, paxillin. In composite images, paxillin is shown in red and RACK1, ribosomal subunits (S6 or 5.8S), or β -actin mRNA is shown in green. Arrows in composite images designate regions of colocalization. Dotted lines outline axonal growth cone and also include the axon, but axons were not included in the analyses. Note that the brightness and contrast were evenly adjusted throughout the entirety of each image, so that puncta would be easily visible. Scale bars, 5 μ m. Both β -actin mRNA and RACK1 colocalize with paxillin-labeled point contacts (B) and vinculin-labeled point contacts (C). Furthermore, this colocalization significantly increases following BDNF stimulation. Ribosomal subunits colocalize with point contacts, but this colocalization does not increase following BDNF stimulation. These data suggest that local translation may occur at point contacts. * $P < 0.05$, Kolmogorov–Smirnov. (B) RACK1 vehicle versus RACK1 BDNF, * $P = 0.002$; ribosomes (S6) vehicle versus ribosomes (S6) BDNF, $P = 0.739$; ribosomes (5.8S) vehicle versus ribosomes (5.8S) BDNF, $P = 0.138$; β -actin mRNA vehicle versus β -actin mRNA BDNF, * $P = 0.013$. RACK1 vehicle, $n = 50$; RACK1 BDNF, $n = 50$; ribosomes (S6) vehicle, $n = 50$; ribosomes (S6) BDNF, $n = 59$; ribosomes (5.8S) vehicle, $n = 80$; ribosomes (5.8S) BDNF, $n = 80$; β -actin mRNA vehicle, $n = 60$; β -actin mRNA BDNF, $n = 60$. C: RACK1 vehicle versus RACK1 BDNF, * $P < 0.001$; ribosomes (S6) vehicle versus ribosomes (S6) BDNF, $P = 0.445$; β -actin mRNA vehicle versus β -actin mRNA BDNF, * $P =$

0.004. RACK1 vehicle, $n = 30$; RACK1 BDNF, $n = 28$; ribosomes vehicle, $n = 47$; ribosomes BDNF, $n = 45$; β -actin mRNA vehicle, $n = 48$; β -actin mRNA BDNF, $n = 47$. (D) RACK1 knockdown eliminates the BDNF-induced increase in β -actin mRNA colocalization with point contacts. These data suggest that RACK1 is required for local translation at point contacts. $*P = 0.05$, ANOVA with Dunnett's multiple comparisons. Post hoc analysis: nonsilencing control shRNA vehicle versus nonsilencing control shRNA BDNF, $*P < 0.001$; shRNA 2 vehicle versus shRNA 2 BDNF, $P = 0.888$; nonsilencing control shRNA vehicle versus shRNA 2 vehicle, $P = 0.774$. Nonsilencing control shRNA vehicle, $n = 32$; nonsilencing control shRNA BDNF, $n = 40$; shRNA 2 vehicle, $n = 39$; shRNA 2 BDNF, $n = 39$. [Color figure can be viewed at wileyonlinelibrary.com]