MOCA Induces Membrane Spreading by Activating Rac1*

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The modifier of cell adhesion protein (MOCA), or Dock3, initially identified as presenilin-binding protein (PBP), belongs to the Dock180 family of proteins and is localized specifically in neurons. Here we demonstrate that MOCA binds to Rac1 and enhances its activity, which leads to the activation of c-Jun NH₂-terminal kinase (JNK) and causes changes in cell morphology. Farnesylated MOCA, which is localized in the plasma membrane, enhances the activation of Rac1 and JNK more markedly than wild-type MOCA, and cells expressing farnesylated MOCA show flattened morphology similar to those expressing a constitutive active mutant of Rac1, Rac1Q61L. On poly-D-lysine-coated dishes, endogenous MOCA is concentrated on the leading edge of broad membrane protrusions (lamellipodia) where actin filaments are co-localized. MOCA is also concentrated with actin on the growth cone in primary cultures of cortical neurons. These observations suggest that MOCA may induce cytoskeletal reorganization and changes in cell adhesion by regulating the activity of Rac1.

MOCA has a 40% sequence homology with Dock180, which has an SH3 domain and two CrkII-binding motifs, and has been proposed as an upstream regulator of a small GTPase, Rac1 (6–8). Small GTPases of the Rho family (RhoA, Rac1, and Cdc42) transduce signals from extracellular stimuli and are key regulators of the actin cytoskeleton that cause changes in cell morphology, including neurite extension and retraction (9, 10). Cell migration induced by overexpression of Dock180 is inhibited by co-expression of a dominant negative form of Rac1 (11, 12). Rac1 also controls membrane ruffling and formation of the lamellipodia, which is a sheetlike structure consisting of a cross-linked meshwork of actin filaments that appears at the leading edge of the cell.

Integrins comprise a large family of cell surface receptors that recognize several extracellular matrix proteins, such as fibronectin, laminin, vitronectin, and collagen. Integrin-mediated Rac1 activation induces the activation of JNK, which is involved in cell movement, proliferation, and survival by activating transcription factors in response to stress stimuli such as UV irradiation and inflammatory cytokines (13, 14). Over-expression of dominant negative JNK1 suppresses the movement of cells (15).

The present study shows that MOCA interacts with Rac1 and induces the GTP loading of Rac1. Membrane-targeted MOCA induces membrane spreading by enhancing the activity of Rac1 more markedly than wild-type MOCA. MOCA is co-localized with actin filaments at the growth cone in primary cultures of cortical neurons. These observations suggest that MOCA is involved in cytoskeletal reorganization by regulating the activity of Rac1.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Plasmids encoding Rac1 and CrkII cDNA were generous gifts from Dr. H. Matsuda. Plasmids encoding Rhotekin and CRIB cDNA were kindly provided by Dr. M. Negishi. MOCA mutant cDNAs (MOCA67, MOCA1060, MOCA1413, MOCA1777, and del-MOCA) were amplified by PCR using a full-length MOCA cDNA as a template and ligated into pcDNA expression vector. His-tagged MOCA C-terminal cDNA generated by PCR was ligated into pET32 expression vector. HA-tagged Cdc42, RhoA, Grb2, and His-tagged JNK cDNAs were amplified and ligated into a pcDNA expression vector. Anti-HA polyclonal antibody against MOCA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-CrkII, anti-Grb2, and monoclonal anti-Rac1 antibodies were from BD Biosciences (Franklin Lakes, NJ); and anti-phospho-c-Jun and anti-JNK polyclonal antibodies were from Cell Signaling (Beverly, MA).

Immunohistochemistry—The whole body of a C57BL/6 mouse at embryonic day 16 was immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). 10-μm sections were cut using a cryostat, incubated overnight with an affinity-purified antibody against MOCA at a concentration of 0.5 μg/ml at 4 °C, and further incubated with a peroxidase-conjugated second antibody for 30 min (Histofine simple stain rabbit MAX-PO kit, Nichirei, Tokyo, Japan). Immunoreactivity was visualized with 3,3′-diaminobenzidine and nickel.

For double labeling immunofluorescence, sections were incubated overnight at 4 °C with rabbit anti-MOCA (1.0 μg/ml) and with mouse anti-MAP2 (1.500) antibodies (Sigma). Immunoreactivity to the antibody against MOCA was visualized with Alexa Fluor 488 anti-rabbit IgG (Molecular Probes, Eugene, OR), and immunoreactivity to the antibody against MAP2 was visualized with Alexa Fluor 568 anti-mouse IgG for 1 h (1:500).

Cell Culture and Transfection—COS-7, NIH 3T3, and SY5Y cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO₂ atmosphere. Transient transfection was performed using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. Primary cultures of mouse cerebral cortices were prepared as described previously (16).
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Briefly, 16-day-old mouse embryos were killed by decapitation, and the cerebral cortices were dissected in L-15 medium, treated with 0.25% trypsin (0.25% trypsin, 0.5% glucose, and 1% DNase I in phosphate-buffered saline (PBS)) for 30 min, washed, and dissociated by pipetting. The resulting dissociated cells were plated on a poly-d-lysine-coated cover glass and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Binding Assay for Small G-Proteins—GST-Rac1, GST-Cdc42, and GST-RhoA were purified from bacterial lysates using glutathione-agarose (Amersham Biosciences). After 24 h of transfection with pCIneo-MOCA or pCIneo-delMOCA, COS-7 cells were washed twice with PBS and lysed with EIDTA buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, and 1 mM Na3VO4 containing a protease inhibitor mixture (Roche Applied Science)), and centrifuged. The resulting supernatants were incubated with agarose bead-associated GST fusion proteins for 1 h at 4 °C, and washed four times. MOCA bound to small GTPase proteins was resolved by SDS-PAGE, and assessed by Western blotting with an anti-MOCA antibody.

Measurement of the Activity of Rho Family Proteins—The activities of Rac1, Cdc42, and RhoA were measured as described previously (17, 18). GST-CRIB and GST-Rhotekin were purified from bacterial lysates using glutathione-agarose. After 24 h of transfection, COS-7 cells were washed twice with PBS and lysed with lytic buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, and 10 mM MgCl2 containing protease inhibitor mixture), and centrifuged. The resulting supernatants were incubated with agarose bead-associated GST fusion proteins for 45 min at 4 °C. The beads were washed four times with lysis buffer, the bound small GTPase proteins were subjected to SDS-PAGE, and Western blot analysis was performed with an anti-HA antibody.

Identification of Proteins Associated with a C-terminal Fragment of MOCA—The C-terminal fragment of MOCA (amino acids 184–1433) was purposed with eTag labeled poly-L-lysine-coated c-Jun. The cells were cultured on a cover glass that was coated with poly-L-lysine (BD Biosciences), fibronec- tone (30 μg/ml), or laminin (30 μg/ml). NIH 3T3 and SY5Y cells were transfected with expression vector containing the MOCA cDNA and incubated for 20 h. After extensive washing, proteins bound to the MOCA fragment were eluted with elution buffer (50 mM Tris (pH 7.4), 600 mM NaCl, 5% urea, and 15% thio) and subjected to SDS-PAGE followed by Western blot analysis.

MOCA Localizes to the Central Nervous System—We have demonstrated by Northern blot analysis and in situ hybridization that MOCA is expressed in the cerebral cortex (2, 3). To further examine the localization of MOCA, immunohistochemical analysis was performed by using a specific antibody against MOCA. Bright-field photographs of immunoreactivity for MOCA in the sagittal sections of 16-day-old mouse embryos showed that MOCA is localized mainly to the brain and spinal cord (Fig. 1A). Immunostaining with antibodies against MOCA and MAP2 (a neuronal marker) showed that MOCA was colocalized mainly with MAP2, suggesting that MOCA is specifically localized to neurons (Fig. 1, B–D). To confirm these observations, we used immunohistochemical staining methods. The localization of MOCA was examined using tissues from an embryonic day-16 mouse by Western blot analysis with an antibody against MOCA.
findings, we performed Western blot analysis with homogenates from several tissues of embryonic day-16 mice using an antibody against MOCA (Fig. 1E). MOCA is expressed in the brain and spinal cord, but its expression is below a level that is detectable in the heart, lung, gut, liver, and kidney.

**MOCA Binds to and Activates Rac1**—Because the Dock180 family of proteins interacts with and activates the Rho family of small GTPases (1, 8, 19, 20), we investigated whether MOCA can interact with Rho family proteins. GST fusion of small GTPases Rac1, Cdc42, or RhoA was mixed with lysates of COS-7 cells, which overexpress MOCA, and were subjected to Western blot analysis with an antibody against MOCA. After 24 h of transfection, GTP-Rac1 was pulled down from lysates with GST-CRIB (B), GTP-Rho with GST-Rhotekin (C), or GTP-Cdc42 with GST-CRIB (D), and precipitates were assessed by Western blotting with an antibody against the HA epitope. The amount of the transfected Rac1, RhoA, and Cdc42 was determined by Western blotting with antibodies against HA.

**FIG. 2. MOCA binds to and activates Rac1.** A, MOCA binds specifically to Rac1. GST-Rac1, GST-RhoA, GST-Cdc42, and GST alone were mixed and incubated with lysates of cells expressing MOCA. GST-small GTPases were pull-down assayed, and Western blot analysis was performed with an antibody against MOCA. B–D, MOCA promotes GTP loading of Rac1. HA-tagged Rac1 (B), RhoA (C), and Cdc42 (D) were transiently transfected into COS-7 cells with or without MOCA. MOCA binds to Rac1 but not to Cdc42 and RhoA (Fig. 2A).

**Rac1 Activation Domain of MOCA**—To determine the region of MOCA required for the activation of Rac1, we constructed several deletion mutants of MOCA and measured their activity for GTP loading of Rac1 (Fig. 3A). Each deletion mutant of MOCA was co-expressed with Rac1, and the amounts of GTP-bound Rac1 were measured with GST-CRIB (Fig. 3B). An SH3 deletion mutant (MOCA67) greatly enhanced GTP loading of Rac1, similarly to the wild-type MOCA. The MOCA1060 mutant, which lacks the N-terminal 1059 amino acids, also enhanced GTP loading of Rac1 but slightly less efficiently than the wild type. Three other deletion mutants, MOCA1413 (lacking the N-terminal 1412 amino acids), MOCA1777 (lacking the N-terminal 1776 amino acids), and delMOCA (lacking amino acids 1061–1392) had no effect on GTP loading of Rac1. These

**MOCA Is an Upstream Regulator of Rac1**—Because the Dock180 family of proteins interacts with and activates the Rho family of small GTPases (1, 8, 19, 20), we investigated whether MOCA can interact with Rho family proteins. GST fusion of small GTPases Rac1, Cdc42, or RhoA was mixed with lysates of COS-7 cells, which overexpress MOCA, and were subjected to Western blot analysis with an antibody against MOCA. MOCA binds to Rac1 but not to Cdc42 and RhoA (Fig. 2A). To examine whether MOCA replaces GDP with GTP for Rac1, the levels of GTP-bound Rac1 were measured using GST-CRIB, which specifically binds to GTP-bound Rac1 and Cdc42, in the presence or absence of MOCA. GST-Rhotekin was used to measure the levels of GTP-bound RhoA. In the presence of MOCA the amount of GTP-bound Rac1 was increased, whereas GTP-bound RhoA or Cdc42 did not show any change (Fig. 2B–D). These observations indicate that MOCA specifically promotes guanine nucleotide exchange for Rac1.

**FIG. 3. A region of MOCA required for the activation of Rac1.** A, a schematic representation is shown of the MOCA constructs used for the Rac1 activation assay. B, the effect is shown of MOCA mutants on GTP loading of Rac1. Expression plasmids containing wild-type MOCA, MOCA67, MOCA1060, MOCA1413, MOCA1777, or delMOCA cDNA were co-transfected with a Rac1 expression plasmid. Twenty-four hours after transfection, cells were extracted and incubated with the GST-CRIB, and precipitates were assessed by Western blotting with an antibody against the HA epitope. The amount of transfected Rac1 was determined by Western blotting with an antibody against Rac1. C, a region of MOCA required for the binding to Rac1 is shown. Wild-type MOCA or delMOCA expressed in COS-7 cells was mixed with GST-Rac1, and the binding of MOCA to Rac1 was detected by Western blotting with an antibody against MOCA.
Farnesylated MOCA enhances GTP loading of Rac1 and JNK activation. A, farnesylated MOCA enhances GTP loading of Rac1 more markedly than the wild-type MOCA. Lysates of cells expressing the wild-type (WT MOCA) or farnesylated MOCA (F-MOCA) were mixed with GST-CRIB and precipitated. The precipitates were subjected to SDS-PAGE and Western blot analysis performed with an antibody against Rac1. Total Rac1 was determined by Western blotting with an antibody against Rac1. B, farnesylated MOCA enhances the activity of JNK. COS-7 cells were co-transfected with a His-tagged JNK expression plasmid and an expression plasmid containing a cDNA of wild-type MOCA or farnesylated MOCA (F-MOCA). After 24 h of transfection, cells were extracted, and His-tagged JNK was purified using TALON resin. Kinase buffer containing c-Jun was added to the TALON beads, and phosphorylated c-Jun was detected by Western blotting with an antibody against phosphorylated c-Jun. C, delMOCA weakly activated JNK. Transfection with a delMOCA expression plasmid was performed as described in B.

These observations suggest that the amino acids 1061–1392 of MOCA are required for promoting GTP loading of Rac1.

To confirm the above observations, we tested whether this region of MOCA is required for the binding to Rac1. GST-Rac1 was mixed with lysates of cells expressing wild-type MOCA or delMOCA, and pull-down assays were performed. Wild-type MOCA interacted with Rac1, whereas delMOCA did not (Fig. 3C). These observations indicate that the amino acid 1061–1392 region of MOCA is required for the binding and GTP loading of Rac1.

Farnesylated MOCA Enhances GTP Loading of Rac1 and JNK Activation—Because Rac1 is activated at the plasma membrane by prenylation of its C terminus (21), it is possible that Rac1 can be efficiently activated when the localization of MOCA shifts to the membrane. To examine this possibility, the C terminus of Ki-Ras, which contains the CAAX farnesylation signal (22), was attached to MOCA, and its effect on GTP loading of Rac1 was examined. Farnesylated MOCA enhanced GTP loading of Rac1 more efficiently than the wild-type MOCA (Fig. 4A). This observation suggests that MOCA enhances GTP loading of Rac1 at the plasma membrane.

Because GTP-bound Rac1 activates JNK (23, 24), we tested whether or not MOCA activates JNK. His-tagged JNK was co-expressed with the wild-type or farnesylated MOCA, and JNK activity was measured in vitro with c-Jun as a substrate. Farnesylated MOCA increased the phosphorylation of c-Jun more efficiently than the wild-type MOCA (Fig. 4B). We also tested the effect of delMOCA or farnesylated delMOCA on the phosphorylation of c-Jun (Fig. 4C). Overexpression of both mutants caused phosphorylation of c-Jun less effectively than the wild-type MOCA. These observations suggest that Rac1 activation mediated by MOCA induces JNK activation.

Interaction of MOCA with Adapter Proteins—MOCA has three proline-rich PXE motifs (2), which interact with the SH3 domain, in its C termini. Because MOCA binds to CrkII (2), the interaction of the C-terminal fragment of MOCA (amino acids 1841–2028) attached to Affigel 10/15 with other SH3 domain-containing adapter proteins was examined. The MOCA fragment interacts with two adapter proteins, CrkII and Grb2 (Fig. 5A). The interaction of full-length MOCA was also examined by immunoprecipitation assays. Full-length MOCA was co-immunoprecipitated with CrkII and Grb2 (Fig. 5B and C). These observations show that MOCA interacts with CrkII and Grb2.

Because JNK activation mediated by the Dock180-Rac1 pathway is enhanced by CrkII (8), it is possible that JNK activation mediated by the MOCA-Rac1 pathway is also enhanced by CrkII or Grb2. Neither adapter protein, however, had any effect on the JNK activation mediated by MOCA (data not shown).

Effect of MOCA on Cell Morphology—Because MOCA regulates cell adhesion (5), it is possible that cells expressing MOCA may have specific morphological characteristics. To test this...
against MOCA (Rac1). NIH 3T3 cells transfected with plasmids containing the wild-type and mutant MOCA were fixed and stained with an antibody against MOCA (A, B, and D–F) or against GFP (C) or Rac1 (G). Cells were cultured on non-coated (A–C and G) or on fibronectin-coated cover glasses (D–F). Scale bar, 10 μm. H, dependence of the effect of F-MOCA on extracellular matrix is shown. The percentage of cells expressing F-MOCA with morphological changes relative to the number of those with normal morphology is shown.

possibility, the effects of the wild-type and farnesylated MOCA on cell morphology were examined using NIH 3T3 cells. Although there was no significant change in morphology in cells expressing the wild-type MOCA, membrane spreading was observed in cells expressing farnesylated MOCA (Fig. 6, A and B). When cells were cultured on fibronectin-coated slides, the expression of farnesylated MOCA caused the round, flattened shape characteristic of the morphology observed in cells in which Rac1 is constitutively activated (Rac1Q61L) (25) (Fig. 6, E and G). Similar results were observed when the cells were cultured on laminin (Fig. 6H). To further examine whether Rac1 activation is involved in the morphological changes, the effect of farnesylated delMOCA, which does not activate Rac1 (Fig. 4), on morphology was investigated. No significant change in morphology was observed in cells expressing farnesylated delMOCA (Fig. 6F). These observations suggest that MOCA may promote GTP loading of Rac1 at the plasma membrane and cause morphological changes.

MOCA Co-localizes with F-actin at the Leading Edge of Membrane Protrusion—Because farnesylated MOCA causes changes in cell morphology (Fig. 6), MOCA may be involved in the rearrangement of the cytoskeleton. The localization and distribution of MOCA at the location of cell spreading were examined using a human neuroblastoma cell line, SY5Y, which endogenously expresses MOCA (3). The localization of MOCA

was examined 50 min after the inoculation of cells onto culture dishes. When cells exhibited extensive spreading and formed broad membrane protrusion, MOCA was translocated to the leading edge of the broad membrane protrusions (lamellipodia) and co-localized with actin filaments (Fig. 7, D–F). Similar results were obtained with cells overexpressing MOCA (Fig. 7, G–I). These observations suggest that MOCA may regulate cell spreading by reorganizing actin filaments through the activation of Rac1.

MOCA Co-localizes with GAP43 and Actin Filaments at the Growth Cone—Because MOCA is a neuron-specific protein (Fig. 1, and Ref. 2), it is possible that MOCA is involved in axonal elongation. To examine this possibility, the localization of MOCA in primary cultures of cortical neurons was investigated. Analysis of the immunoreactivity for MOCA showed that MOCA co-localized with GAP43, which was expressed in growth cones of elongating axon (Fig. 8, A–F) (26, 27). At the growth cones, MOCA was also co-localized with actin filaments (Fig. 8, G–L). These observations suggest that MOCA may be involved in axon elongation where actin filaments are reorganized by regulating Rac1 activation.

FIG. 6. MOCA causes morphological changes by activating Rac1. NIH 3T3 cells transfected with plasmids containing the wild-type and mutant MOCA were fixed and stained with an antibody against MOCA (A, B, and D–F) or against GFP (C) or Rac1 (G). Cells were cultured on non-coated (A–C and G) or on fibronectin-coated cover glasses (D–F). Scale bar, 10 μm. H, dependence of the effect of F-MOCA on extracellular matrix is shown. The percentage of cells expressing F-MOCA with morphological changes relative to the number of those with normal morphology is shown.

FIG. 7. MOCA is localized to the leading edge of membrane protrusions. A–C, localization of MOCA (A, D, and G) and F-actin (B, E, and H) is shown in SY5Y cells. Cells cultured on non-coated cover glasses were fixed and stained with an antibody against MOCA (A) or with phalloidin conjugated with rhodamine, which visualizes F-actin (B). D–F, co-localization of MOCA with F-actin at lamellipodia is shown. After being detached by trypsinization, SY5Y cells that endogenously expressed MOCA (D–F) or cells transfected with an HA-tagged MOCA plasmid (G–I) were plated on poly-λ-lysine-coated cover glasses and cultured for 50 min. Cells were fixed and stained with an antibody against MOCA (D), HA (G), or phalloidin (E and H). Arrows indicate that MOCA concentrated on the leading edge of broad membrane protrusions (lamellipodia). Scale bar, 10 μm.

FIG. 8. MOCA localization in growth cones. A–F, localization of MOCA and GAP43 in the corresponding region ofDock180 (called Docker) with the corresponding region of Dock180. B, HA (G), or phalloidin (E and H). Arrows indicate that MOCA concentrated on the leading edge of broad membrane protrusions (lamellipodia). Scale bar, 10 μm.

Discussion

The present study showed that MOCA binds to and activates a small GTPase (Rac1), leading to the enhancement of JNK activation. Although MOCA lacks the tandem Dbl and pleckstrin homology domains found in most guanine nucleotide exchange factors that activate the Rho family of GTPases (28), we have identified the domain (amino acid 1061–1393) that is required for the binding and activation of Rac1. The fact that the corresponding region of Dock180 (called Docker (amino acids 1111–1657) or DHR-2 (amino acids 1111–1636) domain) is required for Dock180 to bind to and activate Rac1 suggests that a peptide fragment containing the DHR-2 domain of MOCA fails to activate Rac1 (1). This discrepancy may have been caused by the different constructions of MOCA used, whereas our observations were obtained with full-length MOCA.

Membrane-targeted MOCA produced by farnesylation increased the GTP loading of Rac1 and enhanced the JNK activ-
Farnesylated MOCA caused membrane spreading even when cells were cultured on non-coated cover glasses (Fig. 6). On fibronectin-coated dishes, cells overexpressing farnesylated MOCA displayed a round, flattened shape similar to that of cells overexpressing constitutively active Rac1 (Fig. 6). These observations suggest that integrin signaling enhances the effect of farnesylated MOCA, which activates Rac1 to cause membrane spreading. Because wild-type MOCA does not induce morphological changes even on fibronectin-coated dishes, the interaction of MOCA with the plasma membrane may be required for effective transduction of integrin signaling. Alternatively, considering the fact that Dock180 forms complexes with CrkII and p130Cas to transduce integrin signaling to Rac1 (35), MOCA may also form a complex with unknown protein(s) at the plasma membrane to transduce integrin signaling to fully activate Rac1.

Neuroblasts expressing a dominant-negative mutant of Rac1 do not extend neurites, and primary cultures of neurons expressing a dominant negative mutant of Rac1 do not form lamellipodia (36, 37). MOCA is concentrated with actin on the growth cone of primary cultures of neurons, and it is also concentrated on lamellipodia when SY5Y cells adhere to dishes (Figs. 7 and 8). These observations suggest that Rac1 activation by MOCA may be involved in the reorganization of actin filaments at lamellipodia in neuroblastoma cells as well as at the growth cone in primary cultures of neurons.

In conclusion, MOCA binds to Rac1 and promotes GTP loading of Rac1, which leads to the activation of JNK. Farnesylated MOCA is concentrated with actin filaments on lamellipodia and growth cones. These observations suggest that MOCA may be involved in cell morphological changes at neural extension by regulating actin reorganization.

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FIG. 8. MOCA is concentrated on the growth cone in cortical neurons. Primary cultures of mouse cerebral cortical neurons were incubated on a poly-D-lysine-coated cover glass for 18 h, then fixed and stained with antibodies against MOCA (A, D, G, and J), GAP43 (B and E), or with phallolidin conjugated with rhodamine to visualize F-actin (H and K). MOCA is concentrated on growth cones and co-localized with GAP43 (C and P) and F-actin (J and L). D, E, F, J, K, and L show higher magnification views of areas indicated by arrows in A, B, C, G, H, and I, respectively. Scale bars, A–C and G–I, 10 μm; D–F and J–L, 2 μm. Arrows indicate growth cones and arrowheads the filopodia.
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