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Recommended Citation

Zhang Y, Qin Y, Chopp M, Li C, Kemper A, Liu X, Wang X, Zhang L, and Zhang ZG. Ischemic Cerebral Endothelial Cell-Derived Exosomes Promote Axonal Growth. Stroke 2020; 51(12):3701-3712.

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Stroke

BASIC AND TRANSLATIONAL SCIENCES

Ischemic Cerebral Endothelial Cell–Derived Exosomes Promote Axonal Growth

Yi Zhang, PhD*; Yi Qin, PhD*; Michael Chopp, PhD; Chao Li, PhD; Amy Kemper, BS; Xianshuang Liu, MD, PhD; Xinli Wang, PhD; Li Zhang, MD; Zheng Gang Zhang, MD, PhD

BACKGROUND AND PURPOSE: Cerebral endothelial cells (CECs) and axons of neurons interact to maintain vascular and neuronal homeostasis and axonal remodeling in normal and ischemic brain, respectively. However, the role of exosomes in the interaction of CECs and axons in brain under normal conditions and after stroke is unknown.

METHODS: Exosomes were isolated from CECs of nonischemic rats and is chemic rats (nCEC-exos and isCEC-exos), respectively. A multicompartmental cell culture system was used to separate axons from neuronal cell bodies.

RESULTS: Axonal application of nCEC-exos promotes axonal growth of cortical neurons, whereas isCEC-exos further enhance axonal growth than nCEC-exos. Ultrastructural analysis revealed that CEC-exos applied into distal axons were internalized by axons and reached to their parent somata. Bioinformatic analysis revealed that both nCEC-exos and isCEC-exos contain abundant mature miRNAs; however, isCEC-exos exhibit more robust elevation of select miRNAs than nCEC-exos. Mechanistically, axonal application of nCEC-exos and isCEC-exos significantly elevated miRNAs and reduced proteins in distal axons and their parent somata that are involved in inhibiting axonal outgrowth. Blockage of axonal transport suppressed isCEC-exo–altered miRNAs and proteins in somata but not in distal axons.

CONCLUSIONS: nCEC-exos and isCEC-exos facilitate axonal growth by altering miRNAs and their target protein profiles in recipient neurons.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: axons ■ cell body ■ endothelial cells ■ exosomes ■ microRNAs

Stroke is a leading cause of morbidity. Neurovascular coupling, including stroke-induced angiogenesis and axonal remodeling, is one of the key contributors to brain repair processes after stroke, which leads to spontaneous and most often incomplete functional recovery.[1,2] Angiogenesis is an orchestrated process that requires a switch of relatively quiescent cerebral endothelial cells (CECs) to activated phenotype in the peri-infarct area where the survival neurons often undergo spontaneous axonal outgrowth.[3,4] Communications between stroke-activated CECs and axonal sprouting have not been fully investigated, although sprouting cerebral blood vessels and neurites share the same group of genes that guide axonal remodeling.[5,6] Elucidating cellular and molecular mechanisms that underlie this communication may provide new therapeutic targets for facilitating neurovascular remodeling, consequently resulting in improvement of neurological function during stroke recovery.

Exosomes (diameter of ≈30–100 nm), small extracellular vesicles (<100 nm), are nanovesicles originating from the fusion of endosomes and multivesicular bodies with the cell plasma membranes.[7,8] Exosomes

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The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.120.031728.

For Sources of Funding and Disclosures, see page 3711.

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Nonstandard Abbreviations and Acronyms

CEC	cerebral endothelial cell
CSPG	chondroitin sulfate proteoglycan
nCEC-exo	exosome derived from nonischemic cerebral endothelial cells
isCEC-exo	exosome derived from ischemic cerebral endothelial cells
PCR	polymerase chain reaction
premiRNA	precursor miRNA

are essential components of cell-cell communication by transferring their cargo of proteins, lipids, and RNAs between source and recipient cells.[9,10] Emerging data indicate that exosomes derived from glial cells and mesenchymal stromal cells regulate neuronal function by transferring cargo of proteins and miRNAs.[11–14] Fruhbeis et al[12] demonstrated that exosomes from oligodendrocytes can be internalized by distal axons of embryonic cortical neurons and thereby improve neuronal viability under conditions of cell stress. We have shown that exosomes derived from mesenchymal stromal cells transfer miRNAs including the miR-17-92 cluster to distal axons of cortical neurons and promote axonal growth even in the presence of axonal inhibitory chondroitin sulfate proteoglycans (CSPGs).[14] These data suggest that distal axons can internalize exosomes and that transfer of exosome miRNAs may modulate axonal function. However, it is unknown whether exosomes derived from CECs activated by stroke play a role in axonal growth.

In the present study, we used cortical neurons cultured in a multicompartmental culture device as a model system to investigate the effect of exosomes derived from nonischemic and ischemic CECs (nCEC-exos and isCEC-exos, respectively) on axonal growth and on changes of endogenous miRNA profiles within recipient neurons. Our findings indicate that both nCEC-exos and isCEC-exos significantly promote axonal growth; however, isCEC-exos exhibit a more robust effect on axonal growth by modulating axonal and somal miRNAs and their target proteins that are involved in mediating axonal growth.

MATERIALS

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. The data that support the findings of this study are available from the corresponding author, upon reasonable request. Please see Materials in the Data Supplement for expanded Methods.

Cortical Neurons Cultured in Microfluidic Devices

Cortical neurons were harvested from embryonic day-18 Wistar rats (Charles River), in which the sex cannot be identified, according to published protocols.[14,15]

Two types of axonal microfluidic chamber devices were used: (1) Standard Device (SND450; Xona Microfluidics; Figure IA in the Data Supplement)[16] and (2) Triple Chamber Neuron Device (TCND500; Figure IIA and IIB in the Data Supplement).[17]

Animal Model and Primary Culture of Rat CECs

Young adult male Wistar rats (3 months old, 270–300 g; Charles River) were subjected to transient (1 hour) middle cerebral artery occlusion according to our published protocols.[18,19] Animals were euthanized 7 days after transient (1 hour) middle cerebral artery occlusion when angiogenesis is at a peak.[19] Male rats were used based on data that angiogenesis in the ischemic brain has been well characterized in male but not in female ischemic rats.[18,20] CECs were isolated from nonischemic adult male rats (n=4) or rats subjected to 7 days of transient (1 hour) middle cerebral artery occlusion (n=4), respectively, according to published protocols, and >90 % of isolated CECs exhibited phenotypes of endothelial cells.[20,21] The CECs were cultured in CEC growth medium for 4 to 7 days when CECs reached ≈60% confluence. Then, fetal bovine serum was replaced with exosome-depleted fetal bovine serum (System Biosciences) for additional 48 to 72 hours. After that, the conditioned medium was collected for isolation of exosomes.

Isolation and Characterization of Exosomes From CECs

Exosomes were isolated from the conditioned medium according to our published protocol.[22] The particle numbers and size of CEC-exos were analyzed by nanoparticle tracking analysis of Nanosight (NS300; Malvern Panalytical).

Ultrastructural morphology and proteins in collected exosomes were examined by means of transmission electron microscope (EM208; Phillips) and Western blotting, respectively.

Experimental Protocol of Axonal Growth

To examine the effect of exosomes on axonal growth, exosomes were placed into the axonal compartment of the SND450 and TCND500 devices on 3 days in vitro and 5 days in vitro, respectively, and the total length of axons and growth cone extension were measured according to our published protocols.[15,22,23]

To analyze growth cone extension, a time-lapse microscope was used.

To assess the effects of CSPGs and the soluble form of Sema6A (Semaphorin 6A) (Sema6A-Fc) on axonal growth in the presence of exosomes, CSPGs at 2 μg/mL[23] (MilliporeSigma) or Sema6A-Fc at 10 nM[24] (R&D Systems) along with exosomes was applied to the axonal compartment of SND450 on 3 days in vitro for 24 hours.

To examine the effect of exosomes on axonal transport, the movement of endosomes/lysosomes labeled by lysotracker (ThermoFisher Scientific) within the axons was analyzed.[25]

To block the axonal transport, emetine (2 μM) was added into the proximal axon compartment (proximal axon; Figure II in

the Data Supplement) for 4 hours on 5 days in vitro and then removed. After that, exosomes were added into the distal axon compartment (distal axon; Figure II in the Data Supplement). The growth cone extension was measured by means of the time-lapse microscope. The RNA and protein samples were collected accordingly at the end of experiments.

Exosome Labeling and Immunogold Staining

Two sets of exosome labeling were used. To label fresh harvested exosomes, an Exo-Fect exosome transfection kit (System Biosciences, CA) was used as reported previously.[22] To specifically label exosomes, CEC-exos with the presence of GFP proteins (CEC–GFP-exos) were generated according to our published protocol.[26] To track axonal internalization of CEC-GFP-exos at the ultrastructural level, we performed immunogold staining according to our published protocol.[26]

Knockdown of Dicer

To examine the effect of CEC-exo cargo miRNAs on axonal growth, we transfected CECs with shRNA against Dicer. Levels of Dicer and miRNAs in dp-Dicer-exo cargo were examined by means of Western blot and quantitative reverse transcription PCR, respectively.

Fluorescent In Situ Hybridization and Immunocytochemistry

Locked nucleic acid probes specifically against rat miR-27a, miR-19a, and U6 snRNA and scramble probes (Exiqon) were used for hybridization to detect mature miRNAs according to a published protocol.[27] Immunofluorescent staining was performed and analyzed as described previously.[15]

Western Blot Analysis

Total proteins in the cell body or in axonal compartments were extracted. Western blots were performed according to our published protocol.[15,22]

miRNA Polymerase Chain Reaction Array and Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total RNA in axons and cell bodies of cortical neurons or in CEC-exos was isolated using the miRNeasy Mini kit (Qiagen) as reported.[15,22,23]

MiRNA profiles were analyzed using a miRNA polymerase chain reaction (PCR) array kit (MIRN-107ZE-1; Qiagen).[22,23]

TaqMan miRNA assays were performed to verify miRNAs detected by the miRNA PCR array.

miScript Precursor Assay (Qiagen, Valencia, CA) was used to determine the precursor miRNA (premiRNA) levels. Analysis of gene expression was performed using the $2^{(-\Delta\Delta Ct)}$ method.[28]

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism 8 (version 8.2.1). One-way ANOVA with Tukey multiple comparisons test was used when comparing >2 groups. Student *t* test was used when comparing 2 groups. Values presented are expressed as mean±SEM. A $P<0.05$ was considered to be significant.

RESULTS

CEC-Exos Applied to Distal Axons Promote Axonal Growth of Cortical Neurons

We first characterized extracellular vesicles isolated from primary nonischemic and ischemic CECs (nCEC-exos and isCEC-exos, respectively). Our data (Figure III in the Data Supplement) indicate that extracellular vesicles isolated from CEC supernatant are enriched with exosomes according to the Minimal Information for Studies of Extracellular Vesicles 2018 guideline[29] and that ischemic CECs do not alter the morphological and characteristic properties of their released exosomes.

We then assessed the effects of nCEC-exos and isCEC-exos on axonal growth of cortical neurons cultured in microfluidic device SND450 (Figure IA in the Data Supplement). The axonal application of nCEC-exos significantly increased axonal growth in a dose-dependent manner with a maximum effect at a dose of 3×10^7 particles/mL (Figure IB in the Data Supplement). In addition, nCEC-exos significantly enhanced the speed of growth cone extension with leveling off at 4 hours after treatment (Figure IC in the Data Supplement). Compared with nCEC-exos, isCEC-exos further significantly enhanced axonal growth and growth cone extension (Figure 1).

CEC-Exos Applied to Axons Alter Endogenous miRNAs and Their Target Genes in Recipient Neurons

Exosomes transfer their cargo miRNAs into recipient cells and mediate cell function.[30,31] The PCR miRNA array analysis of CEC-exo cargo showed that 24 enriched miRNAs were found to be >2× higher in isCEC-exos than in nCEC-exos (Figure 2A; Tables I through III in the Data Supplement; Figure IV in the Data Supplement). Taqman miRNA assay verified the levels of 6 miRNAs (miR-19a, miR-27a, miR-298, miR-125b, miR-34a, and miR-195) were significantly higher in isCEC-exos than nCEC-exos (Figure 2A). We then examined the effect of CEC-exos on axonal miRNAs with PCR miRNA array and found that compared with nontreated axons, the 6 exosome-enriched miRNAs were among upregulated miRNAs in axons by CEC-exo treatment (Figure 2B; Tables IV through VI in the Data Supplement; Figure IV in the Data Supplement). Quantitative RT-PCR analysis showed that compared with nCEC-exos, isCEC-exo treatment significantly increased 4 miRNAs (miR-19a, 27a, 298, and 195) in axons, whereas miR-34a and miR-125b did not significantly increase (Figure 2B).

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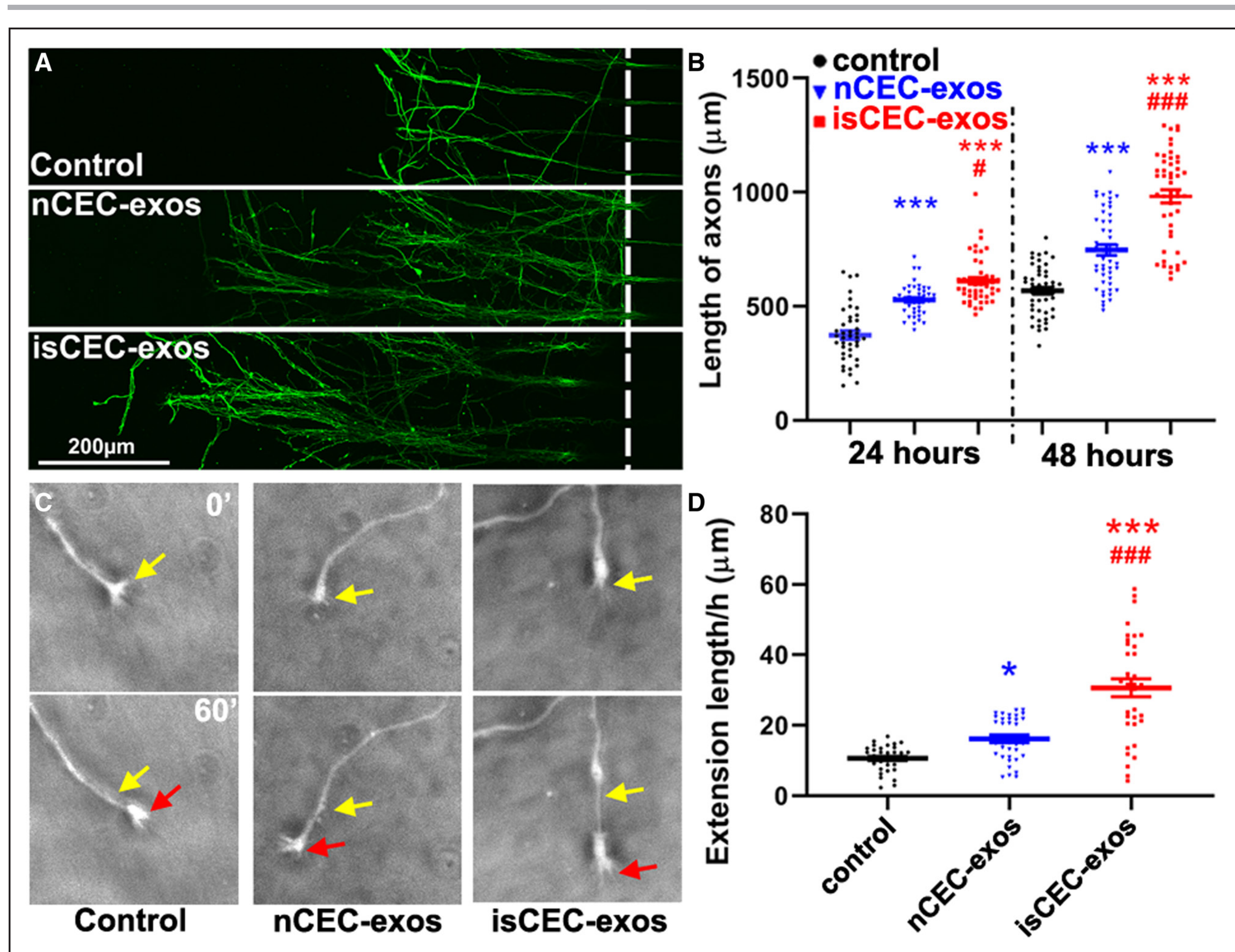

Figure 1. Cerebral endothelial cell (CEC)–exosomes (exos) applied to distal axons promote axonal growth.
Representative confocal microscopic images show axonal growth at 24 h (**A**) and time-lapse microscopic images of growth cone extension (**C**) and quantitative data of distal axonal growth at 24 and 48 h (**B**) and growth cone extension during a 24-h period (**D**), respectively. Yellow and red arrows in **C** indicate the start (0′) and end positions (60′), respectively. isCEC-exos indicates exosomes isolated from cerebral endothelial cells of ischemic rats. **P*<0.05, ****P*<0.001 vs control; #*P*<0.05, ###*P*<0.001 vs exos isolated from CECs of nonischemic rats (nCEC-exos).

Fluorescent in situ hybridization analysis further verified isCEC-exos substantially increased miR-27a and miR-19a in axons and growth cones (Figure VA in the Data Supplement). These data indicate that the increased 4 miRNAs in axons were associated with their enrichment within CEC-exos.

It is possible that the augmented miRNAs could be endogenously induced by axons of neurons upon CEC-exo uptake, rather than transferred by CEC-exos. Mature miRNAs are derived from Dicer-cleaved premiRNAs, while Dicer and premiRNAs are present in distal axons of neurons.[15,32] We thus examined premiRNA levels of the 4 miRNAs within CEC-exos and in axons and somata of cortical neurons. Quantitative RT-PCR analysis did not detect these 4 premiRNAs within nCEC-exos and isCEC-exos (Table VII in the Data Supplement). However, application of nCEC-exos into the axonal compartment for 4 hours increased the levels of 4 premiRNAs in distal axons and their parent somata, with more robust elevation of these premiRNAs in samples collected from the cell body compartment (Figure 2C) than in samples from the axon compartment (Figure 2D). Compared with nCEC-exos, isCEC-exos further elevated these premiRNAs in axons and their somata (Figure 2C and 2D). Moreover, nCEC-exos and isCEC-exos significantly increased mature forms of these 4 miRNAs in the cell bodies (Figure VB in the Data Supplement). Western blotting analysis showed that application of CEC-exos into the axonal compartment for 4 hours did not alter the levels of Dicer and Ago2 (Argonaute2) in distal axons and in their parent somata, suggesting that CEC-exos did not affect levels of the miRNA synthesis machinery proteins (Figure VC in the Data Supplement). Collectively, these data suggest that in addition to transferring their cargo miRNAs to recipient neurons, CEC-exos applied into the distal axons trigger endogenous miRNA synthesis in the somata of cortical neurons, leading to increased premiRNAs and mature miRNAs.

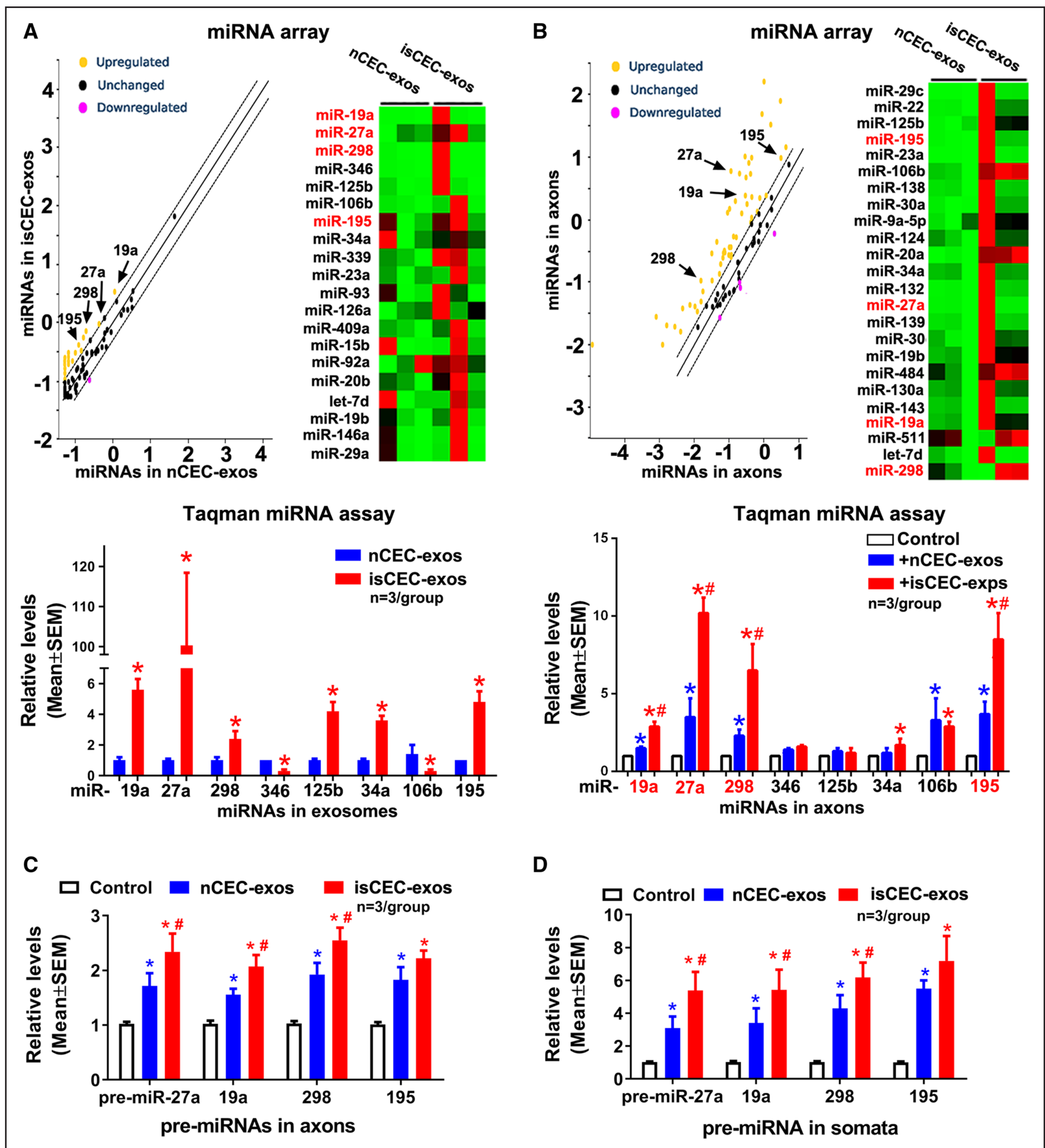

Figure 2. The effect of cerebral endothelial cell (CEC)–exosomes (exos) on levels of mature and precursor miRNAs and miRNA machinery proteins of cortical neurons.

Scatterplot and heat map of miRNA polymerase chain reaction (PCR) array data demonstrate the differential miRNAs in exos (**A**, **top**), and the differential miRNAs in axons after axonal application of CEC-exos (**B**, **top**). Quantitative reverse transcription PCR data show the mature miRNAs in exos (**A**, **bottom**), mature (**B**, **bottom**) and precursor miRNAs (**C**) in axons, and mature miRNAs in somata (**D**) after the axonal application of CEC-exos, respectively. The heat map images only listed partial miRNAs, and please view all miRNAs measured in Figure IV in the Data Supplement. isCEC-exos indicates exosomes isolated from cerebral endothelial cells of ischemic rats. *P<0.05 vs exos isolated from CECs of nonischemic rats (nCEC-exos) in **A** vs control in **B**–**D**; #P<0.05 vs nCEC-exos in **B**–**D**.

Axonal miRNAs regulate axonal growth by locally modulating protein composition.[15,23,33] We thus performed bioinformatics analysis by means of Ingenuity Pathway Analysis, which revealed a network of miR-27a, 19a, 298, and 195 and their putative target genes (Figure 3A) that include well-known axon-inhibitory

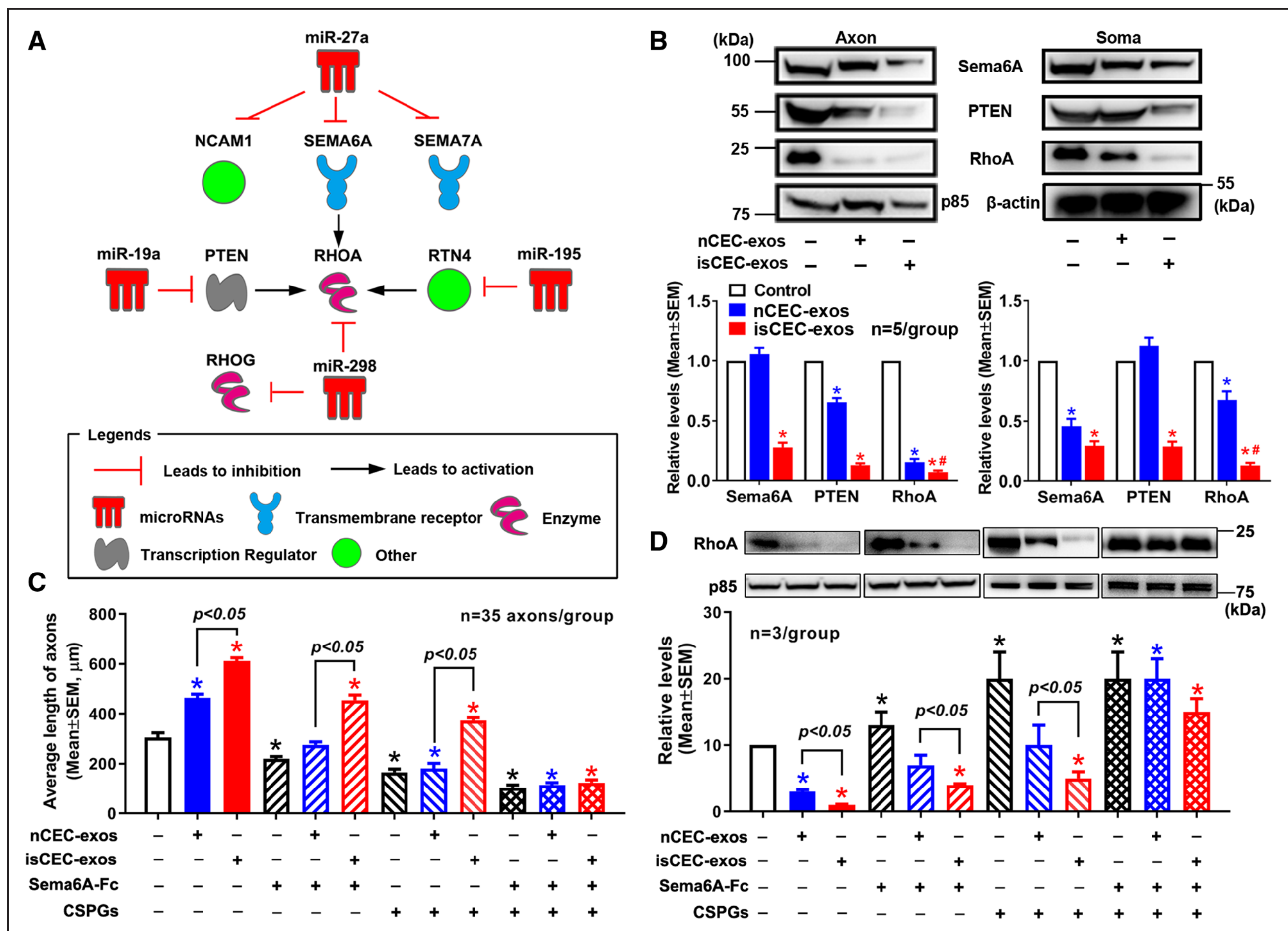

Figure 3. The effect of cerebral endothelial cell (CEC)–exosomes (exos) on levels of proteins in cortical neurons.
A miRNA/target genes network generated by Ingenuity Pathway Analysis (**A**). Representative Western blot images and quantitative data (**B**, n=5/group) show axonal application of exos isolated from CECs of nonischemic rats (nCEC-exos) or exos isolated from CECs of ischemic rats (isCEC-exos) on levels of Sema6A (semaphorin 6A), PTEN, and RhoA in distal axons (Axon) and cell bodies (Soma). Quantitative data of axon length (**C**), representative Western blot images, and their quantitative data (**D**) show axonal application of Sema6A-Fc or chondroitin sulfate proteoglycans (CSPGs) in the presence or absence of nCEC-exos or isCEC-exos, respectively, on axonal growth (**C**) and the axonal levels of RhoA (**D**). NCAM1 indicates neural cell adhesion molecule 1; PTEN, phosphatase and tensin homolog; RhoG, ras homolog family member G; RTN, reticulon; and SEMA7A, semaphorin 7A. *$P<0.05$ vs control; #$P<0.05$ vs nCEC-exos.

proteins, Sema6A, PTEN (Phosphatase and tensin homolog), and RhoA (Ras homolog family member A).[15,34–36] Western blotting analysis showed that treatment of axons with nCEC-exos or isCEC-exos reduced neuronal levels of PTEN, RhoA, and Sema6A in axons and somata, whereas treatment with isCEC-exos induced a significantly greater reduction of these proteins than treatment with nCEC-exos (Figure 3B). These results suggest that the CEC-exos-elevated the 4 miRNAs could potentially target genes encoding these axonal inhibitory proteins in axons and somata. We thus examined whether CEC-exosomal cargo miRNAs contribute to the effect of CEC-exos on axonal growth. CEC-exos isolated from CECs transfected with shRNA against Dicer (dp-Dicer-exos) had a broad reduction of Dicer-related miRNAs compared with cargo miRNAs of CEC-exos derived from CECs transfected with control shRNAs (con-exos; Figure VIA and VIB in the Data Supplement). Treatment of axons with dp-Dicer-exos did not significantly enhance axonal growth (Figure VIC in the Data Supplement), indicating that CEC-exo cargo miRNAs are required for promoted axonal growth.

RhoA is a center node among genes in the miRNA/target network (Figure 3A). We thus further examined the effect of RhoA on CEC-exo–enhanced axonal growth. Application of nCEC-exos into the axonal compartment in the presence of CSGPs that are known to activate RhoA[23] in axons abolished nCEC-exo–augmented axonal growth, which was associated with an increase of RhoA (Figure 3C and 3D). Moreover, soluble Sema6A-Fc also inhibited nCEC-exo–enhanced axonal growth and increased RhoA protein (Figure 3C and 3D). However, individually adding CSPGs or soluble Sema6A-Fc into the axonal compartment did not significantly affect isCEC-exo–augmented axonal growth and did not alter isCEC-exo–reduced RhoA, although CSPGs or soluble

Sema6A-Fc by themselves significantly inhibited axonal growth and increased RhoA (Figure 3C and 3D). In contrast, when they were added together, CSPGs and soluble Sema6A-Fc blocked isCEC-exo–enhanced axonal growth and significantly increased RhoA (Figure 3C and 3D). These data suggest that reduction of RhoA is critical to nCEC-exo–enhanced and isCEC-exo–enhanced axonal growth.

Axonal Transport Contributes to Axon-Applied CEC-Exo–Induced Endogenous miRNA Regulation

Aforementioned data that premiRNAs and mature miRNAs in axons and somata increased by axonal application of CEC-exos suggest that there is a communication between distal axons and their parent somata. To examine the effect of CEC-exos on this communication, a triple-compartment device (TCND500) was used (Figure IIA and IIB in the Data Supplement). We found axonal application of nCEC-exos promotes axonal transport. However, the isCEC-exos exhibited further enhancement of axonal transport than nCEC-exos (Figure II in the Data Supplement). Emetine is a global protein synthesis inhibitor and has been widely used to study axonal transport.[37,38] Transient application of emetine alone for 4 hours to the proximal axon compartment inhibited bidirectional axonal transport up to 4 hours (Figure VII in the Data Supplement). We thus assessed whether transient blockage of axonal transport affects the endogenous miRNA expression induced by isCEC-exos. isCEC-exos applied into distal axons after emetine removal (Figure 4A) for 2 hours did not significantly increase the selected premiRNAs in distal axons and somata (Figure 4B and 4C). In contrast, levels of mature miRNAs in the distal axons were significantly increased at 2 hours after isCEC-exos treatment (Figure 4B, bottom), whereas levels of these mature miRNAs in the somata did not significantly change (Figure 4C, bottom). These data suggest that blockage of axonal transport between distal axons and their parent cell bodies affects premiRNA but not mature miRNA levels in distal axons altered by isCEC-exos. However, 4 hours after the isCEC-exos application, a significant augmentation of premiRNAs was detected in somata (Figure 4C, top) but not in distal axons (Figure 4B, top). By 12 hours, these premiRNAs were significantly elevated in both distal axons (Figure 4B, top) and somata (Figure 4C, top). Western blot analysis showed that when isCEC-exos were applied into the distal axon for 2 hours after emetine removal (Figure 5A), isCEC-exos reduced protein levels of Sema6A, PTEN, and RhoA only in distal axons but did not alter these protein levels in somata (Figure 5A). At 4

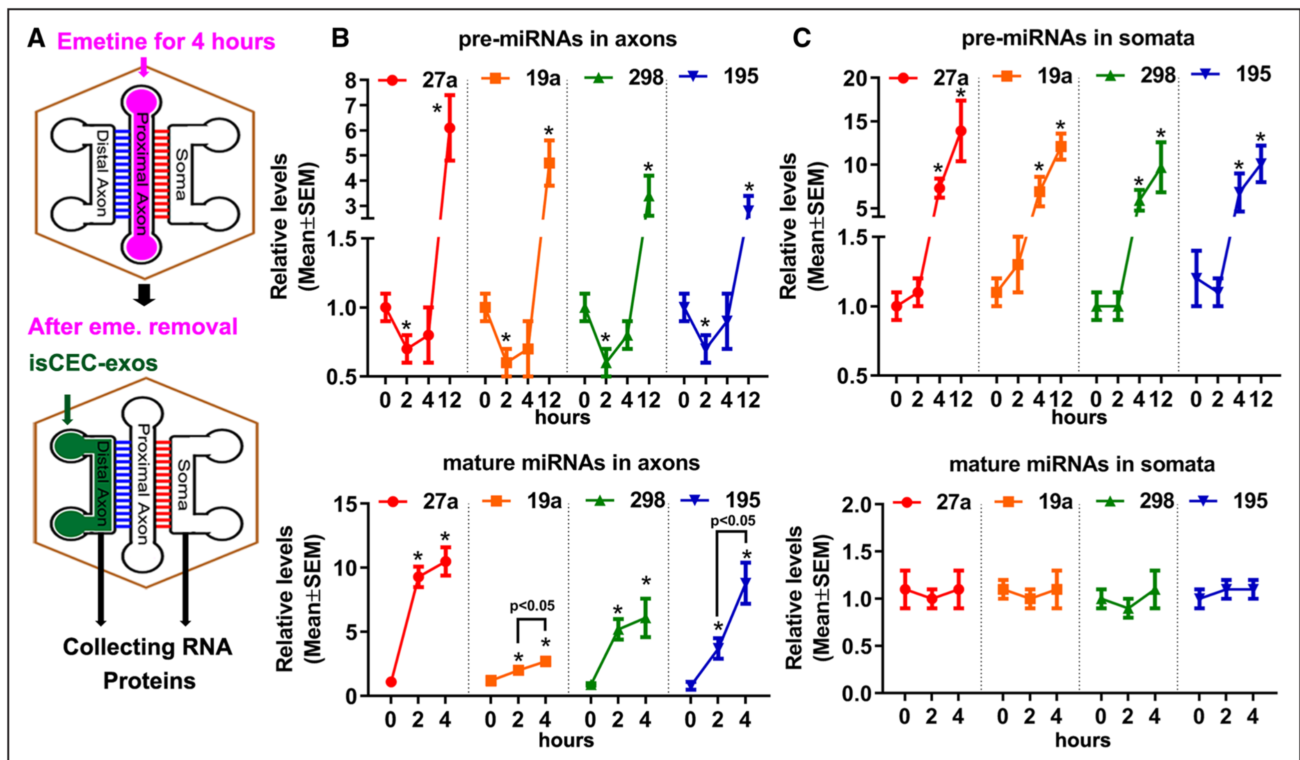

Figure 4. The transient blockage of axonal transport on exosomes isolated from cerebral endothelial cells of ischemic rat (isCEC-exo)–altered miRNAs in cortical neurons.

A schematic (**A**) shows a workflow of collecting samples in distal axons and somata in TCND500 after transient proximal axonal application of emetine and followed distal axonal application of isCEC-exos. Quantitative reverse transcription PCR data show levels of selected precursor (**top**) and mature (**bottom**) miRNAs in the distal axons (**B**) and somata (**C**) after the distal axonal application of isCEC-exos for 0, 2, 4, and 12 h, respectively, following emetine removal. *P<0.05 vs control.

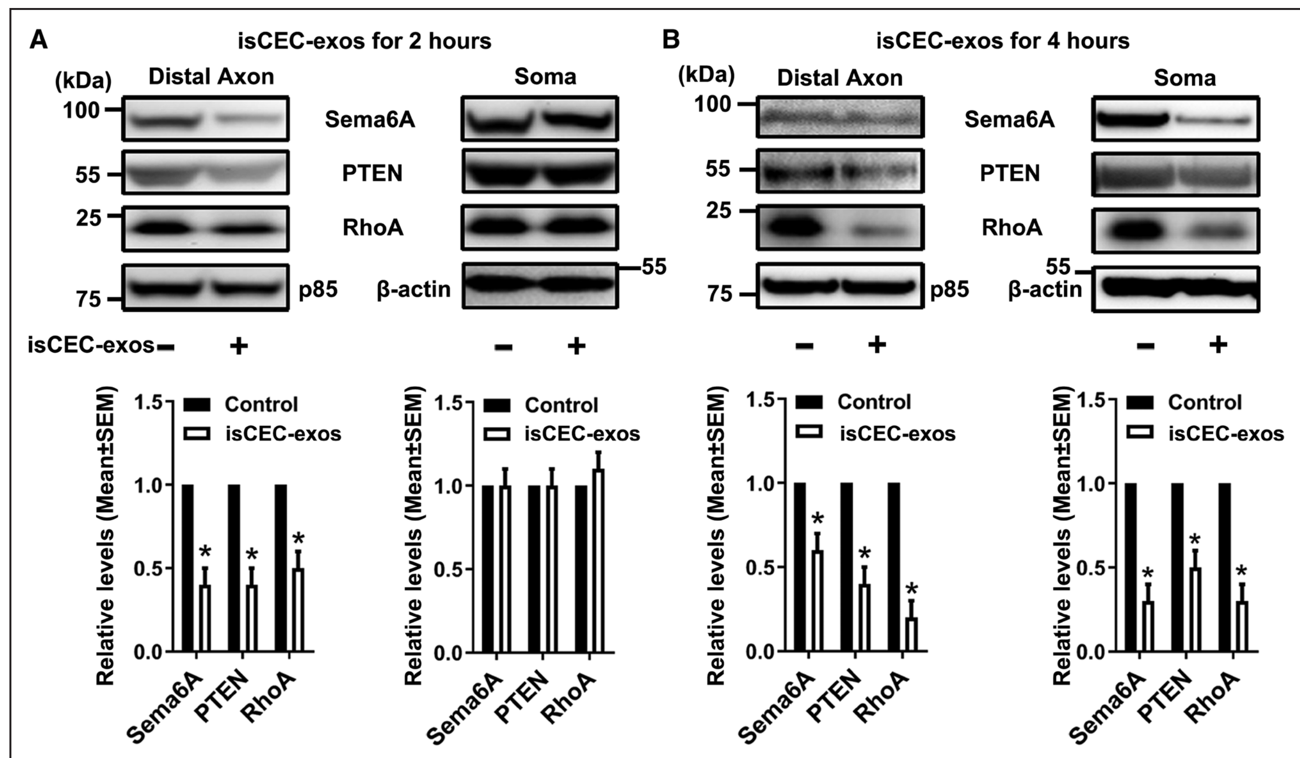

Figure 5. The transient blockage of axonal transport on exosomes isolated from cerebral endothelial cells of ischemic rat (isCEC-exo)–altered proteins in cortical neurons.
Representative Western blot images and quantitative data show the levels of Sema6A, PTEN, and RhoA in distal axons (Distal Axon) and cell bodies (Soma) after the distal axon application of isCEC-exos for 2 h (**A**) or 4 h (**B**), respectively, following emetine removal. PTEN indicates phosphatase and tensin homolog; RhoA, ras homolog family member A; and Sema6A semaphorin 6A. *$P<0.05$ vs control.

hours, significant decreases of Sema6A, PTEN, and RhoA proteins were detected in both distal axons and somata (Figure 5B). Furthermore, we found transient application of emetine alone significantly decreased the speed of axonal growth in the distal axon compartment, which gradually recovered 12 hours after removing emetine (Figure VIII in the Data Supplement). Application of isCEC-exos in distal axons after emetine removal did not significantly increase axonal growth until application for 4 hours (Figure VIII in the Data Supplement). Transient application of emetine alone to the proximal axon compartment did not significantly change levels of premiRNAs and mature miRNAs in the distal axon and cell body compartments (Figure IX in the Data Supplement). These data suggest that a network of miRNAs and proteins regulated by CEC-exos in recipient neurons is involved in CEC-exos–enhanced axonal growth, which likely occurs via a communication between distal axons and their parent somata.

CEC-Exos Are Internalized by Distal Axons and Reach to Parental Cell Bodies

To examine whether CEC-exos are internalized by axons, we imaged Texas Red–labeled nCEC-exos applied to the axonal compartment. Confocal microscopic images showed red fluorescent signals were detected within GFP (green fluorescent protein)-positive axons and growth cones (Figure 6A), suggesting the axonal internalization of nCEC-exos.

To further examine whether exogenous CEC-exos are internalized by axons and reach to their parent cell bodies, we generated GFP carrying CEC-GFP-exos (Figure X in the Data Supplement) and applied CEC-GFP-exos into the axonal compartment for 4 hours. Transmission electron microscope analysis revealed GFP-positive gold particles within neurofilaments and mitochondria of the treated axons (Figure 6B and 6C), whereas GFP-positive gold particles were not detected when the primary antibody against GFP was omitted (Figure XI in the Data Supplement), indicating that CEC-exos are internalized by axons. Moreover, GFP-positive gold particles were also detected in cytoplasm and nucleus of neuronal cell bodies (Figure 6D), suggesting that CEC-exos internalized by axons reach to their parent cell bodies.

DISCUSSION

The present study demonstrated that exosomes derived from nonischemic and ischemic CECs enhanced axonal growth. More importantly, the CEC-exos internalized by distal axons triggered upregulation of miRNAs, which was associated with targeted reduction of axonal inhibitory proteins in recipient neurons. These novel data suggest that

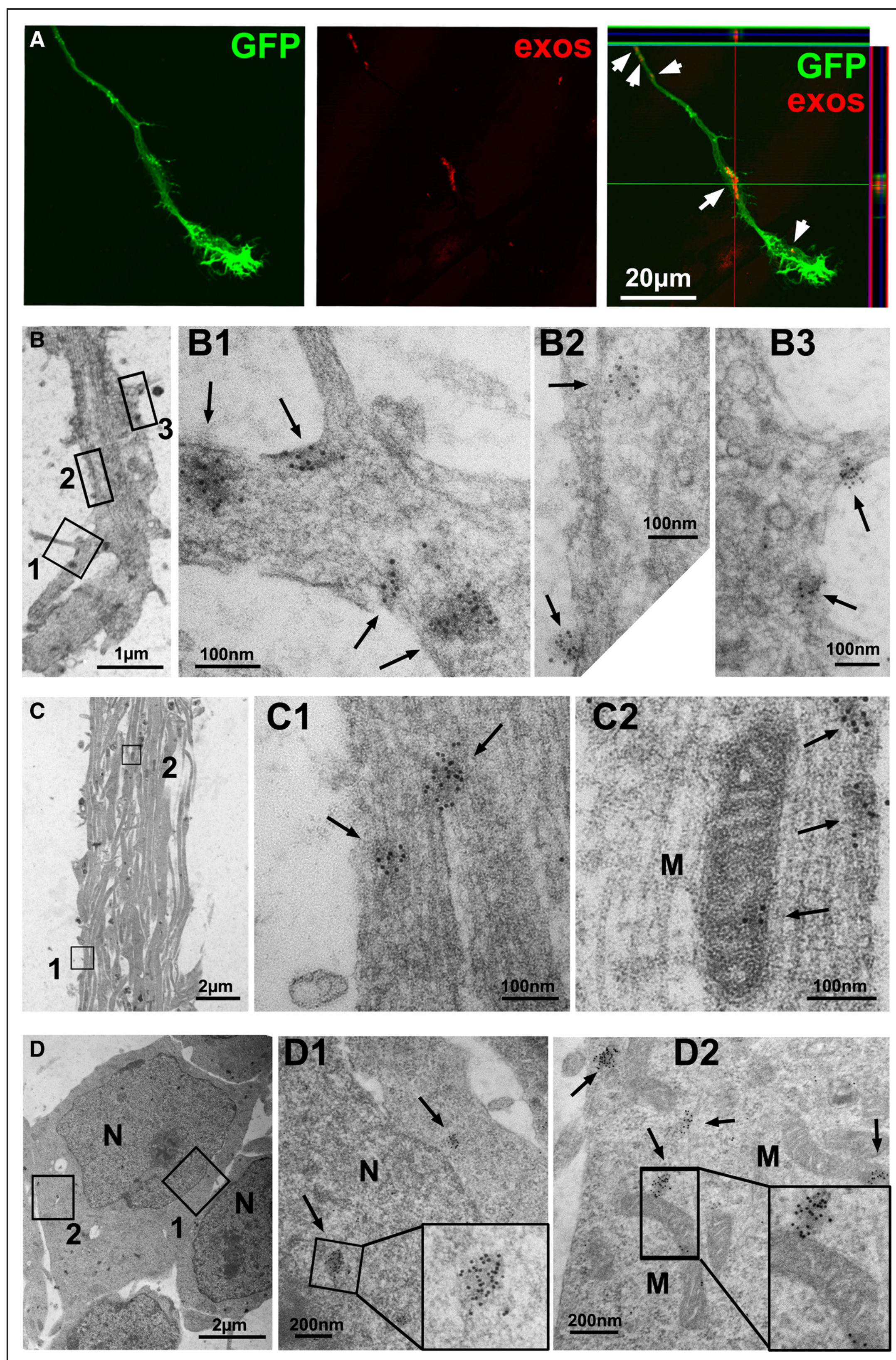

Figure 6. Axonal application of cerebral endothelial cell (CEC)–exosomes (exos) is internalized by axons and reach to their parent cell bodies.

Confocal microscopic images show the internalization of Texas Red–labeled exos isolated from CECs of nonischemic rats in axons and growth cone (exos, white arrows). Representative transmission electron microscope images show the presence of GFP-positive gold particles (black arrows) in axonal growth cone in the axonal compartment (**B**), axon bundles in microgrooves (**C**), and neuronal cell bodies in the soma compartment (**D**). B1, B2, B3 and C1, C2, C3 are magnified regions in **B** and **C**, respectively. GFP indicates green fluorescent protein; M, mitochondria; and N, nucleus.

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CEC-released exosomes play an important role in mediating axonal homeostasis and axonal plasticity under physiological and ischemic conditions, respectively. This is so particularly in light of the fact that activated CECs contribute to improvement of neurological function, while axonal remodeling in ischemic brain is required for stroke recovery.[39,40]

The dynamic interaction between CECs and neurons in the neurovascular unit plays an essential role in the maintenance of brain homeostasis.[41,42] Exosomes mediate communication among brain cells that include neurons, glia, and blood vessel cells.[43–45] CECs release exosomes[46,47]; however, how the endothelium-derived exosomes communicate with brain parenchymal cells, in particular with neurons, remains unknown. The present in vitro study provides evidence that exosomes derived from nonischemic endothelial cells promote axonal growth. Furthermore, exosomes derived from ischemic endothelial cells have a more robust effect on promoting axonal growth. Limited axonal growth has been demonstrated in peri-infarct regions after stroke.[1,48] We previously demonstrated that primary CECs isolated from the ischemic brain exhibit distinct RNA and protein profiles and angiogenic activity compared with CECs harvested from nonischemic brain, although the isCECs were cultured under the normoxia condition.[20] The present findings suggest that isCEC-exos contribute to axonal remodeling in ischemic brain. In addition to axons, our data show that CEC-exos internalized by distal axons reached to their cell bodies, suggesting that CEC-exos could affect dendritic plasticity, which warrants further investigation. Together, data from the present study and others suggest that in addition to factors released by CECs, the endothelium-generated exosomes mediate neuronal function and that administration of CEC-exos could potentially enhance neuronal remodeling in ischemic brain.

Exosomes mediate intercellular communication by transferring their cargo including proteins and miRNAs between source and recipient cells and consequently regulate biological function of recipient cells.[9,10] Emerging data indicate that exosomes affect axonal function by delivering their cargo[30,49]; however, there are few studies that investigate how exosomal cargo alters gene and protein profiles that eventually determine the biological function of recipient neurons. Stroke alters miRNA expression in CECs,[50,51] but it remains unknown whether stroke changes miRNA profiles in CEC-exos. Using multiple approaches, the present study suggests that exosomes internalized by distal axons regulate a network of miRNA/target locally in distal axons and remotely in their cell bodies, which impact axonal growth. We first demonstrated that CEC-exos were rapidly internalized by distal axons, which led to elevation of CEC-exo–enriched mature miRNAs, initially in the distal axons and later in somata, indicating that CEC-exos elevate axonal miRNAs. We then showed that in addition to mature miRNAs, precursors of mature miRNAs were increased in somata and distal axons. premiRNAs are synthesized in the nucleus and are then exported to cytoplasm where they are processed into mature miRNAs by Dicer.[32,52] Since CEC-exos only contained mature miRNAs, elevated premiRNAs are likely transported anterogradely from neuronal cell bodies to the axons. Indeed, our ultrastructural data showed that CEC-exos were internalized by distal axons and reached the cytoplasm and nucleus of neuronal cells, which provide strong evidence to support that the CEC-exo cargo regulates miRNA expression in recipient neurons. Moreover, transient blocking of axonal transport resulted in reduction of premiRNA levels in distal axons, while resuming transiently blocked axonal transport led to elevation of premiRNAs in distal axons treated with CEC-exos. In addition, premiRNAs and Dicer were present in distal axons, whereas CEC-exos only contained mature miRNAs. Augmentation of mature miRNAs and reduction of premiRNAs in distal axons by CEC-exos under conditions of axonal transport blockage suggest that increased mature miRNAs either from CEC-exos or from preexisting premiRNAs in the axon that have been locally converted into mature miRNAs by Dicer. Studies have shown that multivesicular bodies and mitochondria regulate axonal transport.[53–55] Our ultrastructural imaging data showed that nCEC-exos were localized to mitochondria of axons after axonal internalization (Figure 6). Thus, the roles of multivesicular bodies and mitochondria in mediating CEC-exo–altered axonal transport warrant further investigation.

Selectively increased miRNAs in distal axons and their cell bodies were inversely related to their target gene encoded proteins, Sema6A, PTEN, and RhoA. These proteins have been demonstrated as intrinsically inhibitory proteins within neurons that suppress axonal growth.[35,36,56] We and others have reported that the CSGPs activate RhoA in axons, leading to inhibition of axonal growth, and that inhibition of RhoA increases the regeneration of axons.[23,36] Using CSPGs and Sema6A-Fc that activate RhoA, the present study suggests that reduction of RhoA plays an important role in mediating CEC-exos–enhanced axonal growth. Others have shown that suppression of axonal miR-338 leads to augmentation of mRNA and protein in one of its target genes, mitochondrial cytochrome C oxidase IV, in axons and somata as early as 4 hours after transfecting.[57] Collectively, the present study suggests that in addition to transferring cargo miRNAs, CEC-exos regulate endogenous miRNAs and their putative target protein profiles in recipient neurons, leading to CEC-exo–enhanced axonal growth.

ARTICLE INFORMATION

Received July 24, 2020; final revision received September 26, 2020; accepted October 7, 2020.

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Acknowledgments

We thank Maria Ericsson from The Harvard Medical School Electron Microscopy Facility for the guidance and assistance on transmission electron microscope sample processing and imaging.

Sources of Funding

This work was supported by the National Institutes of Health grants, R01 NS111801 (Z.G. Zhang), and American Heart Association 16SDG29860003 (Y. Zhang).

Disclosures

None.

Supplemental Materials

Expanded Materials and Methods
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