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Ischemic cerebral endothelial cells-derived exosomes promote axonal growth

Yi Zhang, PhD[1,‡,*], **Yi Qin, PhD**[1,‡], **Michael Chopp, PhD**[1,3], **Chao Li, PhD**[1], **Amy Kemper, BS**[2], **Xianshuang Liu, MD, PhD**[1], **Xinli Wang, PhD**[1], **Li Zhang, MD**[1], **Zheng Gang Zhang, MD, PhD**[1,*]

[1)]Department of Neurology, Henry Ford Hospital, Detroit, Michigan, 48202

[2)]Department of Pathology, Henry Ford Hospital, Detroit, Michigan, 48202

[3)]Department of Physics, Oakland University, Rochester, Michigan, 48309

Abstract

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 are unknown.

Methods: Exosomes were isolated from CECs of non-ischemic rats (nCEC-exos) and ischemic rats (isCEC-exos), respectively. A multi-compartmental cell culture system was employed 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.

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

Graphical Abstract

[*]Correspondence should be addressed to: Dr. Yi Zhang, yzhang3@hfhs.org, Dr. Zheng Gang Zhang, Henry Ford Hospital, Department of Neurology. 2799 W Grand Blvd, Detroit, MI 48202., zzhang1@hfhs.org, zhazh@neuro.hfh.edu.
Current address of Yi Qin: State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing.
[‡]both authors contribute equally to this manuscript.

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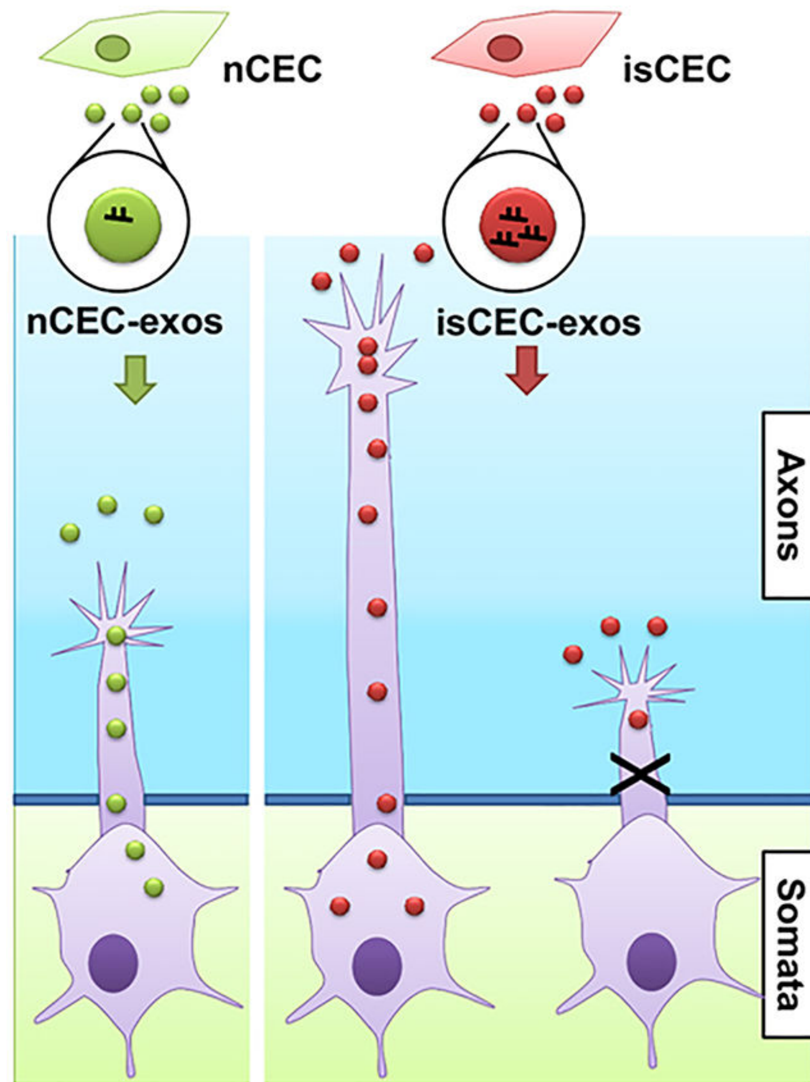

Keywords

stroke; endothelial cells; exosomes; microRNA; axons

Introduction

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

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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 to 100nm), small extracellular vesicles (EVs, <100nm), are nano-vesicles originating from the fusion of endosomes and multivesicular bodies (MVB) with the cell plasma membranes [7, 8]. Exosomes 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 (MSCs) regulate neuronal function by transferring cargo of proteins and miRNAs [11–14]. Fruhbeis et al 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[12]. We have shown that exosomes derived from MSCs 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 exosomes 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 employed cortical neurons cultured in a multi-compartmental culture device as a model system to investigate the effect of exosomes derived from non-ischemic 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.

Methods and materials

All experimental procedures were carried out in accordance with the NIH 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 supplemental materials 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, Supplemental Fig. IA) [16]. 2) Triple Chamber Neuron Device (TCND500, Supplemental Fig. II AB)[17].

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Animal model and primary culture of rat CECs

Young adult male Wistar rats (3-months old, 270–300g, Charles River) were subjected to transient (1 hour) middle cerebral artery occlusion (tMCAO) according to our published protocols[18, 19]. Animals were sacrificed 7 days after tMCAO when angiogenesis is at a peak [19]. Male rats were employed 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 non ischemic adult male rats (n=4) or rats subjected to 7 days of tMCAO (n=4), respectively, according to published protocols and more than 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 approximately 60% confluence. Then, fetal bovine serum (FBS) was replaced with exosome depleted FBS (System Biosciences) for additional 48~72 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 (NTA) of Nanosight (NS300, Malvern Panalytical).

Ultra-structural morphology and proteins in collected exosomes were examined by means of transmission electron microscope (TEM, Phillips, EM208) 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 DIV3 and DIV5, 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 employed.

To assess the effects of CSPGs and the soluble form of Sema6A (Sema6A-Fc) on axonal growth in the presence of exosomes, CSPGs at 2μg/ml[23] (MilliporeSigma) or Sema6A-Fc at 10nM [24] (R&D system) along with exosomes were applied to the axonal compartment of SND450 on DIV3 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 were analyzed[25].

To block the axonal transport, emetine (2μM) was added into the proximal axon compartment (Proximal Axon, Supplemental Fig. II) for 4 hours on DIV5 and then removed. After that, exosomes were added into the distal axon compartment (Distal Axon, Supplemental Fig. II). 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.

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Exosome labeling and Immunogold staining

Two sets of exosomes labeling were employed. To label fresh harvested exosomes, an Exo-fect exosome transfection kit (System Bioscience, CA) was used as previously reported[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 ultra-structural 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 qRT-PCR, respectively.

Fluorescent In situ hybridization (FISH) 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 previously described [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 PCR 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 PCR array kit (MIRN-107ZE-1, Qiagen) [22, 23].

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

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

Statistical analysis.

All statistical analysis was performed using the GraphPad Prism 8 (version 8.2.1). One-way ANOVA with Tukey's multiple comparisons test was used when comparing more than two groups. Student's t test was used when comparing two groups. Values presented are expressed as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered to be significant.

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Results

CEC-exos applied to distal axons promote axonal growth of cortical neurons

We first characterized EVs isolated from primary non-ischemic and ischemic cerebral endothelial cells (nCEC-exos and isCEC-exos, respectively). Our data (Supplemental Fig. III) indicate that EVs isolated from CEC supernatant are enriched with exosomes according to the MISEV 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 (Supplemental Fig. I A). The axonal application of nCEC-exos significantly increased axonal growth in a dose dependent manner with a maximum effect at a dose of 3×10^7particles/mL (Supplemental Fig. I B). In addition, nCEC-exos significantly enhanced the speed of growth cone extension with leveling off at 4 hours after treatment (Supplemental Fig. I C). Compared with nCEC-exos, isCEC-exos further significantly enhanced axonal growth and growth cone extension (Fig. 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 more than 2 times higher in isCEC-exos than in nCEC-exos (Fig. 2A, Supplemental Tables I–III, Supplemental Fig. IV). 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 (Fig. 2A). We then examined the effect of CEC-exos on axonal miRNAs with PCR miRNA array and found that compared with non-treated axons, the 6 exosome-enriched miRNAs were among upregulated miRNAs in axons by CEC-exo treatment (Fig. 2B, Supplemental Tables IV–VI, Supplemental Fig. IV). Quantitative RT-PCR analysis showed that compared with nCEC-exos, isCEC-exos treatment significantly increased four miRNAs (miR-19a, 27a, 298 and 195) in axons, while miR-34a and miR-125b did not significantly increase (Fig. 2B). FISH analysis further verified isCEC-exos substantially increased miR-27a and miR-19a in axons and growth cones (Supplemental Fig. V A). These data indicate that the increased four 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-exos uptake, rather than transferred by CEC-exos. Mature miRNAs are derived from Dicer-cleaved precursor miRNAs (pre-miRNA), while Dicer and pre-miRNAs are present in distal axons of neurons[15, 32]. We thus examined pre-miRNA levels of the four miRNAs within CEC-exos and in axons and somata of cortical neurons. Quantitative RT-PCR analysis did not detect these 4 pre-miRNAs within nCEC-exos and isCEC-exos (Supplemental Table VII). However, application of nCEC-exos into the axonal compartment for 4 hours increased the levels of 4 pre-miRNAs in distal axons and their parent somata, with more robust elevation of these pre-miRNAs in samples collected from the cell body compartment (Fig. 2C) than in samples from the axon compartment (Fig. 2D). Compared

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with nCEC-exos, isCEC-exos further elevated these pre-miRNAs in axons and their somata (Fig. 2C, D). Moreover, nCEC-exos and isCEC-exos significantly increased mature forms of these four miRNAs in the cell bodies (Supplemental Fig. V B). 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 in distal axons and in their parent somata, suggesting that CEC-exos did not affect levels of the miRNA synthesis machinery proteins (Supplemental Fig. V C). 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 pre- and mature miRNAs.

Axonal miRNAs regulate axonal growth by locally modulating protein composition[15, 23, 33]. We thus performed bioinformatics analysis by means of Ingenuity Pathway Analysis (IPA), which revealed a network of miR-27a, −19a, −298 and −195 and their putative target genes (Fig. 3A) that include well-known axon-inhibitory proteins, Sema6A, PTEN, and RhoA[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 (Fig. 3B). These results suggest that the CEC-exos-elevated the four 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 to cargo miRNAs of CEC-exos derived from CECs transfected with control shRNAs (con-exos, Supplemental Fig. VI AB). Treatment of axons with dp-Dicer-exos did not significantly enhance axonal growth (Supplemental Fig. VI C), indicating that CEC-exo cargo miRNAs are required for promoted axonal growth.

RhoA is a center node among genes in the miRNA/target network (Fig. 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[37] in axons abolished nCEC-exo-augmented axonal growth, which was associated with an increase of RhoA (Fig. 3CD). Moreover, soluble Sema6A-Fc also inhibited nCEC-exo-enhanced axonal growth and increased RhoA protein (Fig. 3CD). 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 (Fig. 3CD). In contrast, when they were added together, CSPGs and soluble Sema6A-Fc blocked isCEC-exo-enhanced axonal growth and significantly increased RhoA (Fig. 3CD). These data suggest that reduction of RhoA is critical to nCEC-exo- and isCEC-exo-enhanced axonal growth.

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Axonal transport contributes to axon-applied CEC-exo-induced endogenous miRNA regulation

Aforementioned data that pre-miRNAs 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 employed (Supplemental Fig. II AB). We found axonal application of nCEC-exos promotes axonal transport. However, the isCEC-exos exhibited further enhancement of axonal transport than nCEC-exos (Supplemental Fig. II). Emetine is a global protein synthesis inhibitor and has been widely used to study axonal transport [38, 39]. Transient application of emetine alone for 4h to the proximal axon compartment inhibited bidirectional axonal transport up to 4 hours (Supplemental Fig. VII). 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 (Fig. 4A) for 2 hours did not significantly increase the selected pre-miRNAs in distal axons and somata (Fig. 4BC). In contrast, levels of mature miRNAs in the distal axons were significantly increased at 2 hours after isCEC-exos treatment (Fig. 4B, lower), whereas levels of these mature miRNAs in the somata did not significantly change (Fig. 4C, lower). These data suggest that blockage of axonal transport between distal axons and their parent cell bodies affects pre-miRNA, but not mature miRNA levels in distal axons altered by isCEC-exos. However, 4 hours after the isCEC-exos application, a significant augmentation of pre-miRNAs was detected in somata (Fig. 4C, upper), but not in distal axons (Fig. 4B, upper). By 12 hours, these pre-miRNAs were significantly elevated in both distal axons (Fig. 4B, upper) and somata (Fig. 4C, upper). Western blot analysis showed that when isCEC-exos were applied into the distal axon for 2 hours after emetine removal (Fig. 5A), isCEC-exos reduced protein levels of Sema6A, PTEN, and RhoA only in distal axons, but did not alter these protein levels in somata (Fig. 5A). At 4 hours, significant decreases of Sema6A, PTEN, and RhoA proteins were detected in both distal axons and somata (Fig. 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 (Supplemental Fig. VIII). Application of isCEC-exos in distal axons after emetine removal did not significantly increase axonal growth until application for 4 hours (Supplemental Fig. VIII). Transient application of emetine alone to the proximal axon compartment did not significantly change levels of pre-miRNAs and mature miRNAs in the distal axon and cell body compartments (Supplemental Fig. IX). 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 axon 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 positive axons and growth cones (Fig. 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 (Supplemental Fig. X) and applied CEC-GFP-exos into the axonal compartment for 4 hours. TEM analysis revealed GFP positive gold particles within neurofilaments and mitochondria of the treated axons (Fig. 6B,C), whereas GFP-gold particles were not detected when the primary antibody against GFP was omitted (Supplemental Fig. XI), indicating that CEC-exos are internalized by axons. Moreover, GFP positive gold particles were also detected in cytoplasm and nucleus of neuronal cell bodies (Fig. 6D), suggesting that CEC-exos internalized by axons reach to their parent cell bodies.

Discussion

The present study demonstrated that exosomes derived from non-ischemic 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 CECs 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 contributes to improvement of neurological function, while axonal remodeling in ischemic brain is required for stroke recovery[40, 41].

The dynamic interaction between CECs and neurons in the neurovascular unit plays an essential role in the maintenance of brain homeostasis[42, 43]. Exosomes mediate communication among brain cells that include neurons, glia and blood vessel cells[44–46]. CECs release exosomes[47, 48]; however, how the endothelial-derived exosomes communicate with brain parenchymal cells, in particular with neurons, remains unknown. The present in vitro study provides evidence that exosomes derived from non-ischemic 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, 49]. 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 non-ischemic 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 endothelial 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, 50]; however, there are few studies that investigate how exosomal cargo alters gene and protein profiles that eventually determine biological function of recipient neurons. Stroke alters miRNA expression in CECs[51, 52], but it remains

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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. Precursor miRNAs are synthesized in the nucleus and are then exported to cytoplasm where they are processed into mature miRNAs by Dicer [32, 53]. Since CEC-exos only contained mature miRNAs, elevated pre-miRNAs 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 pre-miRNA levels in distal axons, while resuming transiently blocked axonal transport led to elevation of pre-miRNAs in distal axons treated with CEC-exos. In addition, pre-miRNAs and Dicer were present in distal axons, whereas CEC-exos only contained mature miRNAs. Augmentation of mature miRNAs and reduction of pre-mRNAs in distal axons by CEC-exos under conditions of axonal transport blockage suggest that increased mature miRNAs either from CEC-exos and/or from preexisting pre-miRNAs in the axon that have been locally converted into mature miRNAs by Dicer. Studies have shown that multivesicular bodies (MVB) and mitochondria regulate axonal transport[54–56]. Our ultrastructural imaging data showed that nCEC-exos were localized to mitochondria of axons after axonal internalization (Fig. 6). Thus, the roles of MVB and mitochondria in mediating CEC-exos 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, 57]. 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[37, 58]. 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 cxidase IV, in axons and somata as early as 4 hours after transfecting[59]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

CECs	cerebral endothelial cells
MVBs	multivesicular bodies
MSCs	mesenchymal stromal cells
CSPGs	chondroitin sulfate proteoglycans
nCEC-exos	exosomes derived from non-ischemic CECs
isCEC-exos	exosomes derived from ischemic CECs
NTA	nanoparticle tracking analysis
MCAO	middle cerebral artery occlusion
TEM	transmission electron microscope
FISH	fluorescent in situ hybridization

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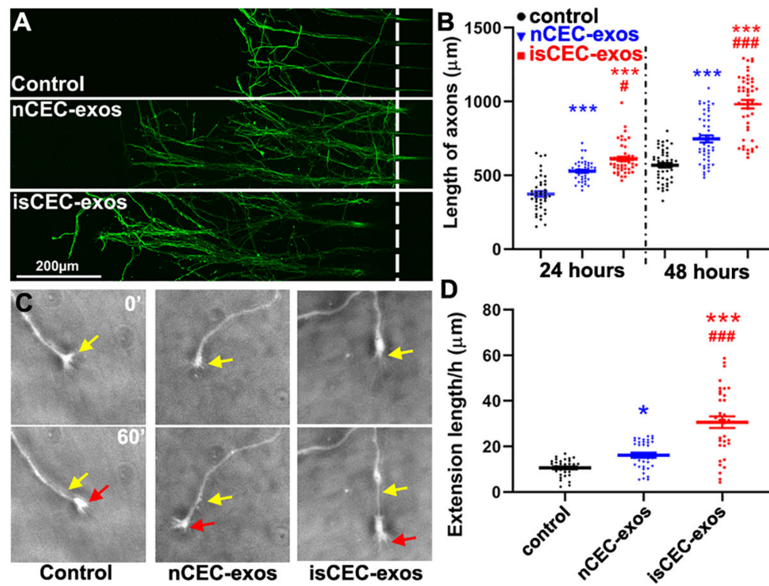

Figure 1. CEC-exos applied to distal axons promote axonal growth.

Representative confocal microscopic images show axonal growth at 24 hours (**A**) and time-lapse microscopic images of growth cone extension (**C**) and quantitative data of distal axonal growth at 24 hours and 48 hours (**B**) and growth cone extension during a 24 hours period (**D**), respectively. Yellow and red arrows in panel **C** indicate the start (0') and end positions (60'), respectively. * $p<0.05$, *** $p<0.001$ vs control; #, $p<0.05$, ###, $p<0.001$ vs nCEC-exos.

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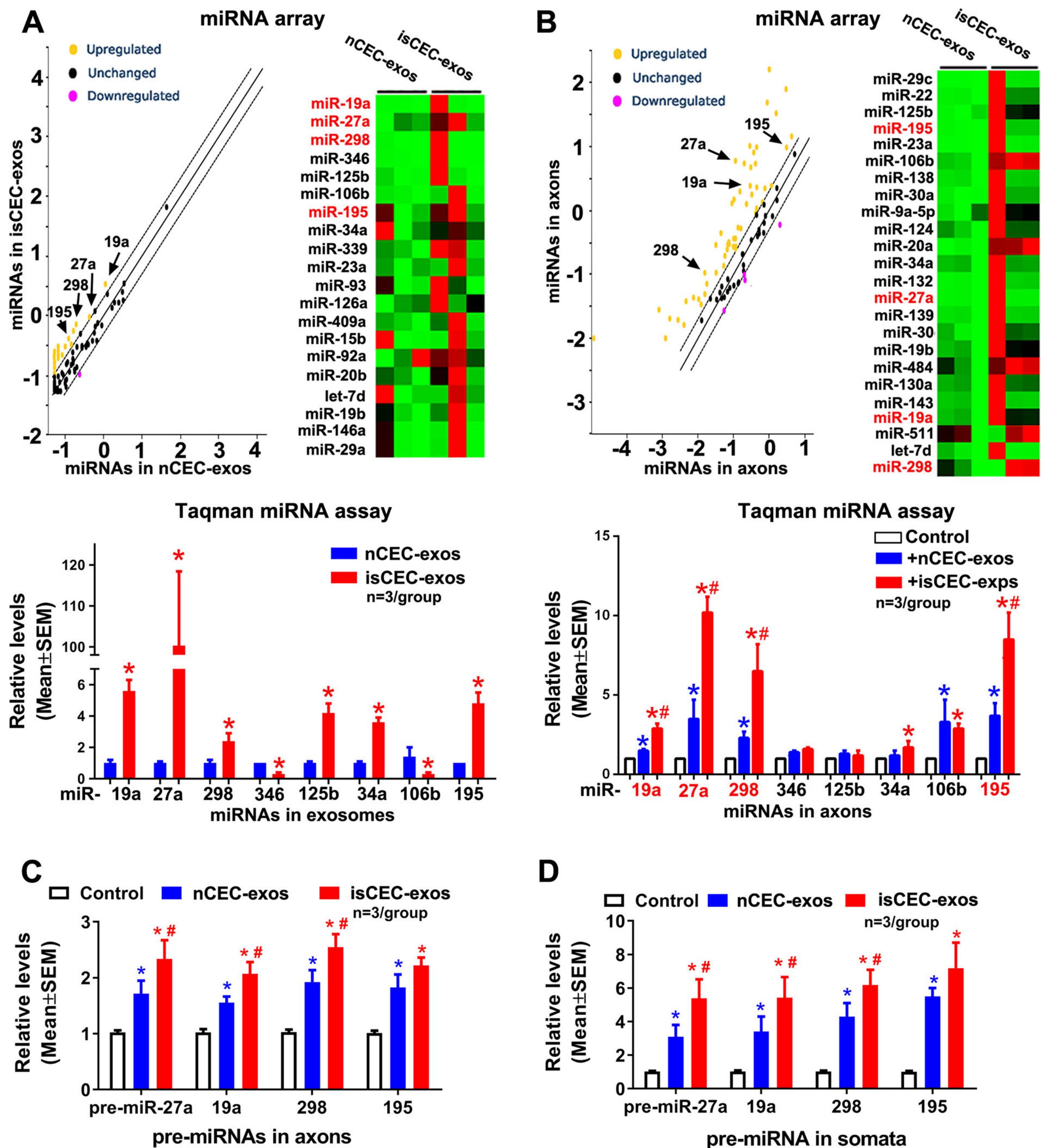

Figure 2. The effect of CEC-exos on levels of mature and precursor miRNAs and miRNA machinery proteins of cortical neurons.

Scatter plot and heatmap of miRNA PCR array data demonstrate the differential miRNAs in exosomes (**A,** upper), and the differential miRNAs in axons after axonal application of CEC-exos (**B,** upper). Quantitative RT-PCR data show the mature miRNAs in exosomes (**A,** lower), mature (**B,** lower) and precursor miRNAs (**C**) in axons and mature miRNAs in somata (**D**) after the axonal application of CEC-exos,. respectively. *, p<0.05 vs nCEC-exos

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in A, vs control in B-D; #, $p<0.05$ vs nCEC-exos in B-D. The heatmap images only listed partial miRNAs and please view all miRNAs measured in Supplemental Figure IV.

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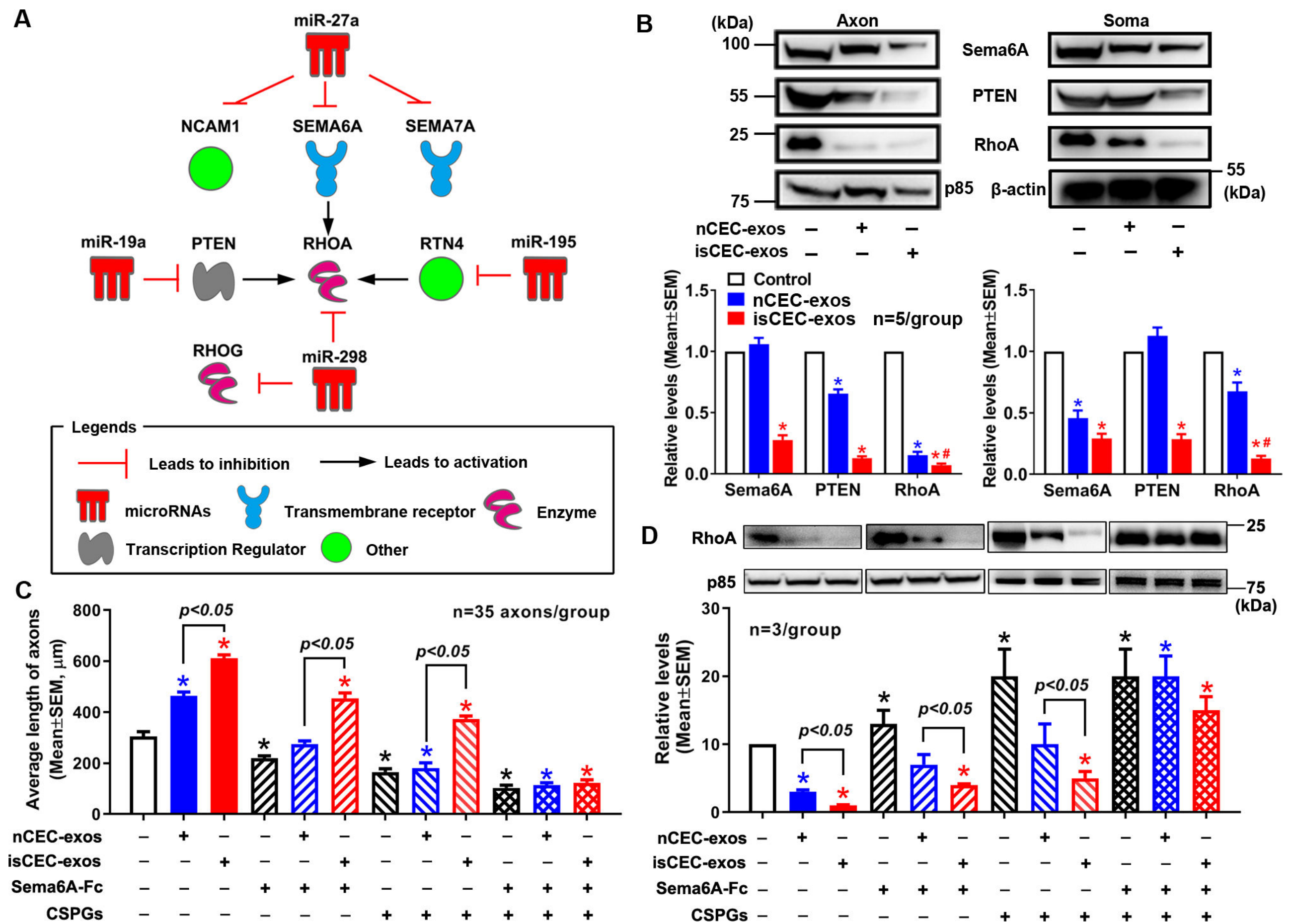

Figure 3. The effect of CEC-exos on levels of proteins in cortical neurons.

A miRNA/target genes network generated by IPA (**A**). Representative Western blot images and quantitative data (**B,** n=5/group) show axonal application of nCEC- or isCEC-exos on levels of Sema6A, PTEN, 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 CSPGs in the presence or absence of nCEC-exos or isCEC-exos, respectively, on axonal growth (**C**) and the axonal levels of RhoA (**D**). * p<0.05 vs control; #, p<0.05 vs nCEC-exos.

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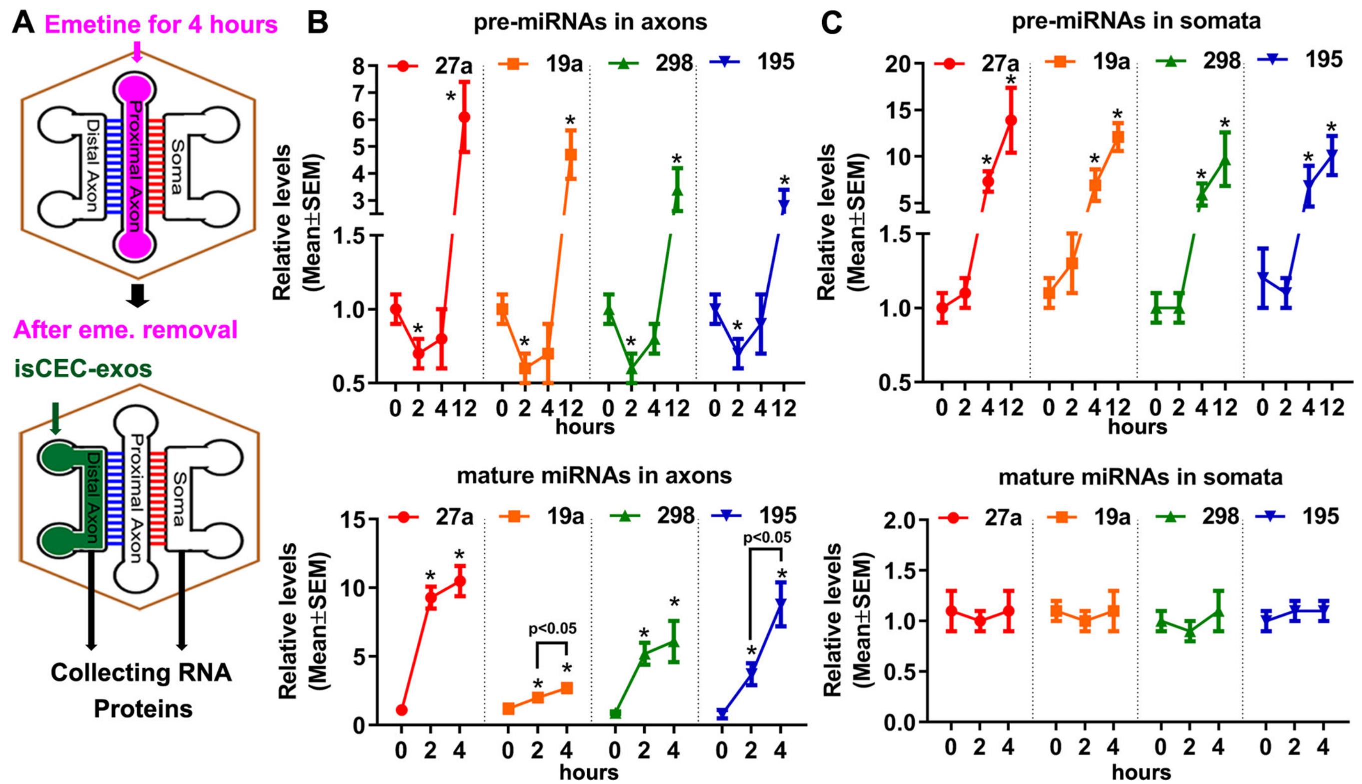

Figure 4. The transient blockage of axonal transport on isCEC-exo-altered miRNAs in cortical neurons.

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 RT-PCR data show levels of selected precursor (upper panels) and mature (lower panels) miRNAs in the distal axons (**B**) and somata (**C**) after the distal axonal application of isCEC-exos for 0, 2, 4 and 12 hours, respectively, following emetine removal. * $p<0.05$ vs control.

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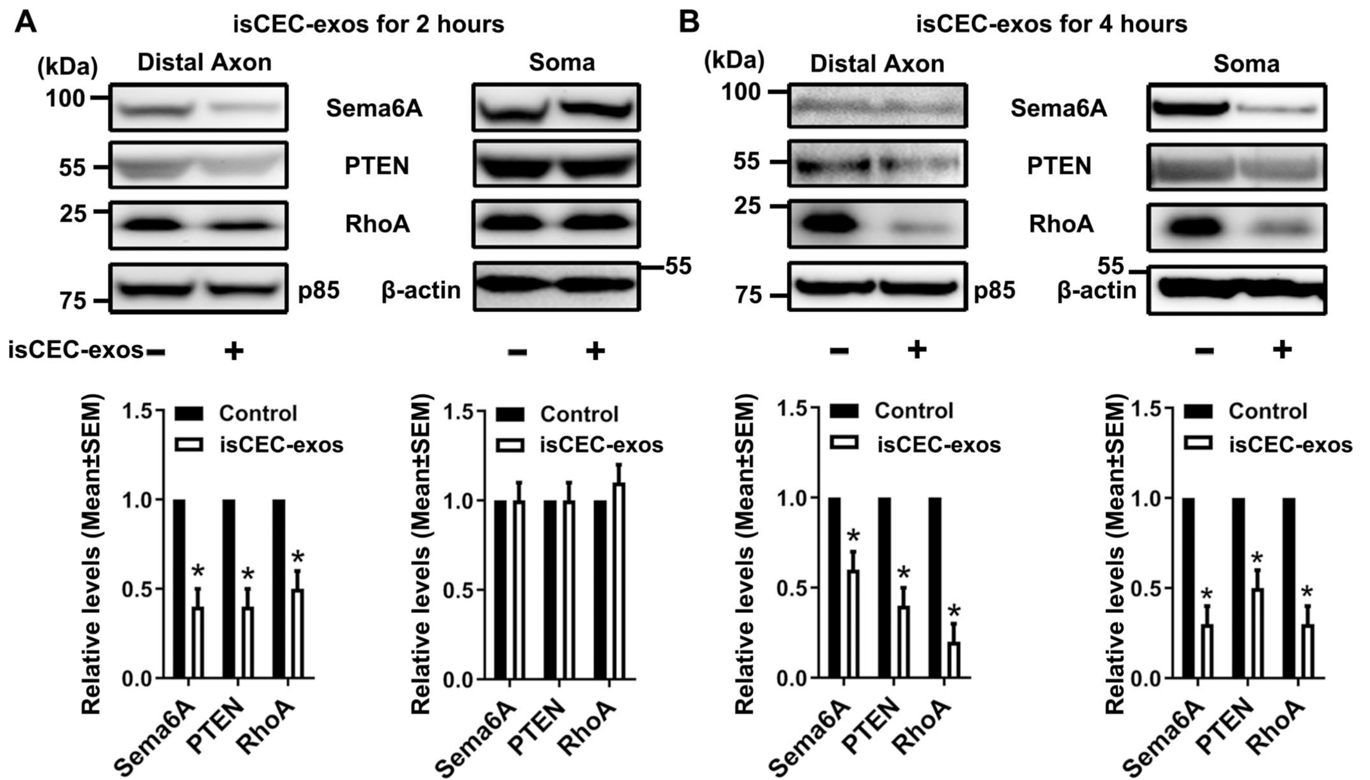

Figure 5. The transient blockage of axonal transport on isCEC-exo-altered proteins in cortical neurons.

Representative Western blot images and quantitative data show the levels of Sema6A, PTEN, RhoA in distal axons (Distal Axon) and cell bodies (Soma) after the distal axon application of isCEC-exos for 2 hours (**A**) or 4 hours (**B**), respectively, following emetine removal. * $p<0.05$ vs control.

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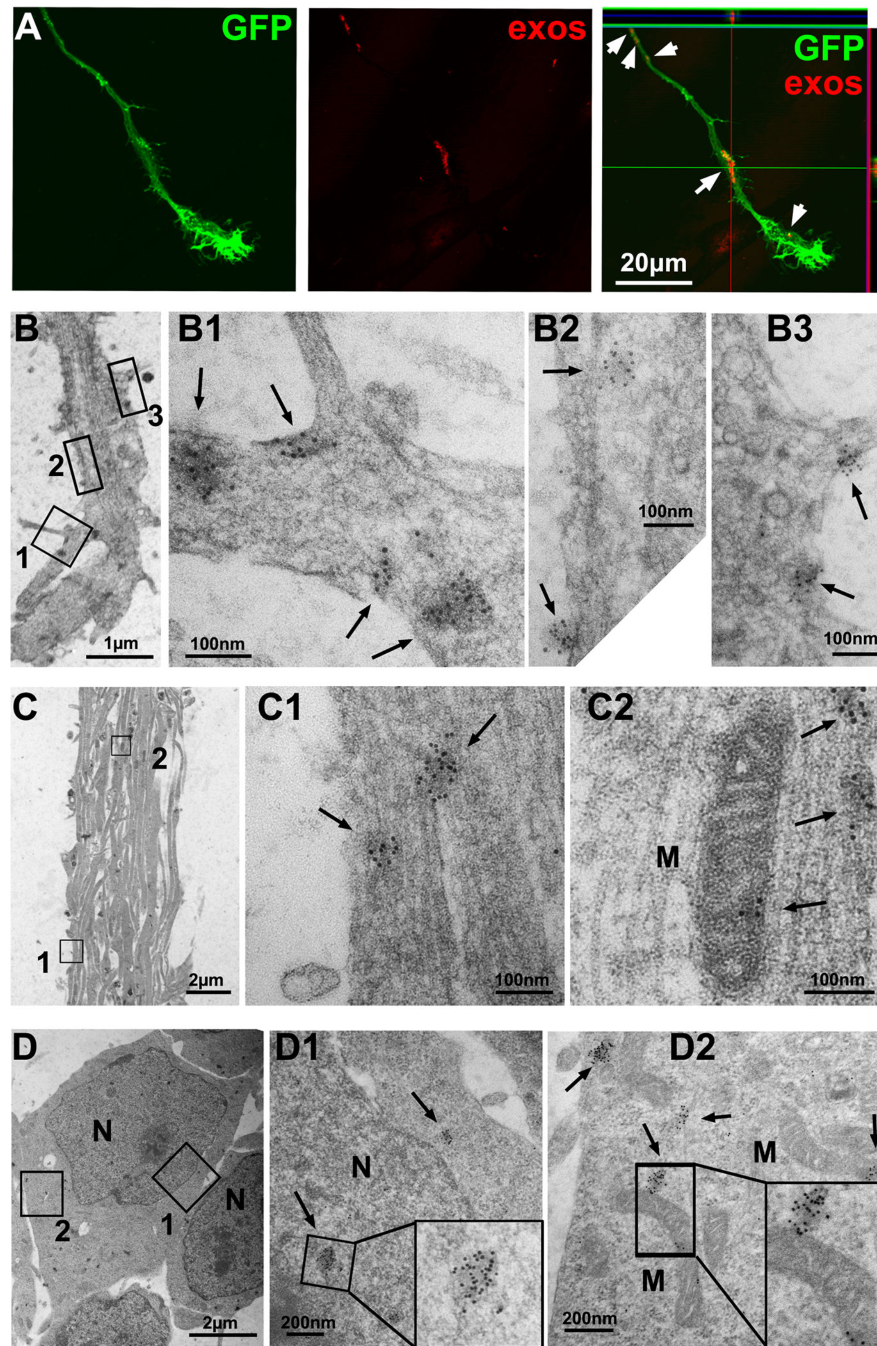

Figure 6. Axonal application of CEC-exos are internalized by axons and reach to their parent cell bodies.

Confocal microscopic images show the internalization of Texas-red labeled nCEC-exos in axons and growth cone (exos, white arrows). Representative TEM 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**). M, mitochondria; N, nucleus.