Vamp3-dependent secretion of endocytic BDNF from astrocytes

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Research Article

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Abstract

Brain-derived neurotrophic factor (BDNF) regulates diverse brain functions via TrkB receptor signaling. Due to the expression of TrkB receptors, astrocytes can internalize extracellular BDNF proteins via receptor-mediated endocytosis. Endocytosed BDNF can be re-secreted upon stimulation, but the molecular mechanism underlying this phenomenon remains unrecognized. Our study reveals that vesicle-associated membrane protein 3 (Vamp3) selectively regulates the endocytic release of BDNF from astrocytes. By using quantum dot (QD)-conjugated mature BDNF (QD-BDNF) as a proxy for the extracellular BDNF protein, we monitored the uptake, transport, and secretion of BDNF from cultured cortical astrocytes. Our data showed that endocytic QD-BDNF particles were enriched in Vamp3-containing vesicles in astrocytes and that ATP treatment sufficiently triggered either the antero- or retrograde transport and exocytosis of QD-BDNF-containing vesicles. Downregulation of Vamp3 expression disrupted endocytic BDNF secretion from astrocytes but did not affect uptake or transport. Collectively, these results provide evidence of the selective ability of astrocytic Vamp3 to control endocytic BDNF secretion during BDNF recycling.

Introduction

Brain-derived neurotrophic factor (BDNF) regulates diverse brain functions, including cell survival, differentiation, synaptic connectivity, and cognitive processes^{1–3}. Secretion of either the pro-form of BDNF (proBDNF) or the mature form of BDNF (mBDNF) from dense-core vesicles depends on the Ca²⁺-mediated actions of vesicular exocytosis machineries such as Soluble NSF Attachment protein Receptor (SNARE) proteins^{4,5}. Extracellular pro-BDNF and mBDNF bind to pan-neurotrophin receptor p75 (p75NTR) and tropomyosin-related kinase B (TrkB), respectively² and can reside in endosomal compartments in nearby target cells after receptor-mediated endocytosis. While the BDNF-TrkB complex in neuronal endosomes can be retrogradely transported or remain active in the form of a “signaling endosome”, extracellular BDNF can also be recycled by re-secretion in response to neuronal activity^{6–8}.

Astrocytes are also thought to recycle extracellular BDNF proteins. ProBDNF was shown to be internalized through p75NTR-dependent endocytosis, and this endocytosed neuronal proBDNF appeared to be re-secreted as mBDNF^{9,10}. The maintenance of long-term potentiation (LTP) and memory acquisition requires the astrocytic secretion of endocytic BDNF^{9,10}. On the other hand, mBDNF seems to be absorbed by astrocytes due to their strong expression of TrkB^{11,12}; however, the re-secretion of endocytic mBDNF has not yet been directly assessed. Neurons require complexin-1/2 and synaptotagmin 6 for the activity-dependent re-secretion of endocytic mBDNF⁷, but the molecular mechanisms underlying the recycling of mBDNF in astrocytes are unknown.

Astrocytes respond to neurotransmitters or active substances, such as glutamate and ATP, displaying the increase in the intracellular Ca²⁺ concentration by the activation of corresponding receptors^{13,14}. Because vesicular exocytosis is dependent on Ca²⁺-dependent SNARE proteins, the astrocytic Ca²⁺-dependent actions of SNARE machinery could feasibly be involved in the release of mBDNF from astrocytes. Diverse SNARE machinery proteins, such as vesicle-associated membrane proteins 2, 3, and 7 (Vamp2, Vamp3, and Vamp7, respectively)¹⁵, are expressed in astrocytes, but which astrocytic SNARE proteins are implicated in endocytic BDNF secretion remains unknown. In this study, we provide direct evidence that Vamp3 is a key SNARE protein controlling endocytic mBDNF release from astrocytes. Monitoring the direct uptake, transport and activity-dependent exocytosis of endocytic mBDNF in astrocytes utilizing recombinant mBDNF proteins linked to quantum dots (QDs) revealed that Vamp3 was selectively involved in the exocytosis of endocytic mBDNF. Our QD-linked mBDNF sensor was sufficient for examining the transport and activity-dependent secretion of endosomes, as reported previously^{7,16,17}, due to the excellent photostability and high signal-to-noise ratio of QDs in live cells. These results support the notion that mBDNF recycling in astrocytes serves as an additional source of extracellular BDNF, which is crucial for activity-dependent synaptic plasticity.

Methods

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Brain Research Institute (IACUC-2017-0047). All experiments were carried out in accordance with the approved guidelines and regulations.

Primary astrocyte culture

We utilized an AWESAM astrocyte culture protocol as reported previously²¹ with minor modifications to acquire cultured astrocytes that had an *in vivo*-like morphology. Cortical astrocytes were prepared from embryos from wild-type C57BL/6 mice on days E17–18. Cortices were dissected in dissection medium (10 mM HEPES in HBSS) at 4°C and then incubated in 0.25% trypsin-EDTA in a 37°C water bath for 20 min with gentle inversion every 5 min. After trypsinization, the tissue was washed in dissection medium at 4°C five times and then triturated with 1 ml of NB + medium (2% B27 supplement, 2 mM GlutaMax, 5,000 U/ml penicillin and 5,000 µg/ml streptomycin in neurobasal medium). Dissociated cells were filtered through a cell strainer and plated on 0.04% polyethylenimine (PEI)-coated cell culture dishes (4 × 10⁶ cells/60 mm dish) in culture media (10% FBS, 5,000 U/ml penicillin and 5,000 µg/ml streptomycin in DMEM). Seven days after plating the dissociated cells, the dishes were shaken at 110 rpm for 6 hours. The cells were then washed with 1x PBS three times, treated with 0.25% trypsin, and plated on 0.04% PEI-coated glass-bottom dishes (3 × 10⁴ cells/dish) or 18 mm coverslips in a 12-well plate (1 × 10⁴ cells/well) in NB + medium containing HBEGF (50 µg/ml).

Transfection of DNA and siRNAs

DNA and siRNA constructs were transfected into cultured astrocytes with Lipofectamine 2000 at 10–11 DIV according to the manufacturer's protocol. To generate pCMV-TeLC-P2A-EYFP, TeLC-P2A-EYFP fragments were amplified from pAAV-hSyn-FLEX-TeLC-P2A-EYFP-WPRE (Addgene plasmid #135391) with

a specific set of primers (Key Resources Table) and then subcloned into a pcDNA3.1 vector by using the HindIII-XhoI site.

To screen *Vamp3* siRNA, C8-D1A (mouse type 1 astrocyte cell line) cells were cultured in DMEM supplemented with 10% FBS at 37°C under 5% CO₂. Each siRNA (100 nM) was transfected into C8-D1A cells using RNAi Max according to the manufacturer's protocol. Two days after transfection, samples were analyzed by western blotting with an anti-Vamp3 primary antibody or β -actin-HRP and HRP-conjugated anti-rabbit secondary antibody. The screening of Vamp3 siRNAs revealed that siVamp3 #1 effectively diminished the level of endogenous Vamp3 (Fig. S2). Therefore, only siVamp3 #1 was employed in the experiments.

TrkB-targeting shRNA lentiviral particles were purchased from Sigma (shRNA-pLKO.1-hPGK-puro-CMV-tGFP). The shRNA target sequences are described in the Key Resources Table. To assess the knockdown efficiency of *TrkB* shRNA, cortical neurons from E17-18 C57BL/6 mouse embryos were cultured. Each Lenti-shTrkB particle was transduced into cortical neurons at 5 DIV. Three days after transduction, total RNA was extracted using TRIzol reagent. Each RNA sample (0.3 μ g) was reverse transcribed into cDNA by using SuperScript III reverse transcriptase. To determine the reduction in TrkB RNA levels, PCR was performed using TrkB and β -actin primers. Because shTrkB #1 reduced the level of endogenous TrkB more effectively than shTrkB #2 (Fig. S1), only shTrkB #1 was used in the experiments.

Immunocytochemistry

To determine the localization of QD-BDNF, cultured astrocytes were incubated with 2 nM QD-BDNF for 20 min and then fixed with 4% paraformaldehyde (PFA). For immunostaining, the cells were permeabilized with 0.1% Triton X-100 for 10 min and then blocked with 5% normal goat serum for 1 hour at room temperature. After blocking, the cells were incubated with anti-Rab5, anti-Rab7, anti-Rab11, anti-Lamp1, anti-Vamp3, or anti-chromograninB for 1 hour and then incubated with an anti-Alexa 488 secondary antibody for 1 hour at room temperature.

QD imaging

For monitoring endocytic BDNF, 50 nM biotinylated mature BDNF (bt-BDNF) or 50 nM biotinylated bovine serum albumin (bt-BSA) was incubated with 50 nM streptavidin-conjugated quantum dot 655 (st-QD655) at 4°C overnight at a ratio of 2:1. QD-BDNF or QD-BSA was then filtered with a 100 kDa Amicon filter to remove unconjugated mBDNF, BSA, or QDs, and 1% BSA containing PBS was added to the filtrates. Astrocytes were incubated with QD-BDNF or QD-BSA on 12–13 DIV, and the medium was then replaced with an extracellular solution (in mM; 119 NaCl, 2.5 KCl, 20 HEPES, 2 CaCl₂, 30 glucose, and 2 MgCl₂, pH 7.4) containing 4 μ M QSY21. Time-lapse images were taken by using a confocal laser scanning microscope (TCS SP8, Leica) at a 1 Hz rate using a 63 X oil objective. ATP (100 μ M) or ionomycin (1 μ M)-containing extracellular solution was added to stimulate the astrocytes. QD655 fluorescence was excited with a 561 nm laser and assessed with a HyD (hybrid) detector in the range of 650–695 nm.

Image and statistical analyses

Image processing and analysis were performed using ImageJ/FIJI software (NIH, USA). To analyze the kinetics or secretion of BDNF particles, regions of interest (ROIs) of astrocytic processes were manually selected and linearized. The linearized time-lapse images were transformed into kymographs using the KymographBuilder plugin in ImageJ/FIJI. After extracting the X and Y coordinate data for each particle from the kymograph, the direction, distance, and velocity were determined.

Colocalization of QD-BDNF and endosomal markers was analyzed by using the Colocalization Threshold plugin in ImageJ/FIJI. To analyze the complexity of astrocytes induced by BDNF, the shape index ($\text{perimeter}^2/\text{area} - 4\pi$) was utilized as described previously^{12,22}.

Statistical analyses were performed using Prism 8.0 software (GraphPad). Statistically significant differences between two groups were determined using Student's unpaired t-test, and three or more groups were compared using one-way ANOVA with Dunnett's multiple comparisons test. The Kolmogorov-Smirnov test was used to examine the statistical significance of the percentages of cumulative distribution between the two groups. All data were from three independent batches of cultured astrocytes and are indicated as the mean $\pm$ standard error of the mean (SEM).

Table 1
Key resource table

Reagent type or resources	Source or reference	Identifiers	Additional information
Antibodies			
Rabbit polyclonal anti-Rab5	Abcam	ab13253	IF 1:200
Mouse monoclonal anti-Rab7	Abcam	ab50533	IF 1:200
Rabbit polyclonal anti-Rab11	Santa Cruz Biotechnology	sc-9020	IF 1:200
Rabbit polyclonal anti-Lamp1	Abcam	ab24170	IF 1:200
Rabbit polyclonal anti-chromograninB	Abcam	ab12242	IF 1:400
Rabbit polyclonal anti-Vamp3	Novus	NB300-510	IB 1:5,000 IF 1:200
β -Actin (13E5) rabbit mAb (HRP- conjugated)	Cell Signaling Technology	5125	IB 1:10,000
HRP-conjugated anti-rabbit antibody	Bio-Rad	1706515	IB 1:10,000
Goat anti-mouse IgG (H + L) Alexa Fluor 488	Thermo Fisher Scientific	A11029	IF 1:200
Goat anti-rabbit IgG (H + L) Alexa Fluor 488	Thermo Fisher Scientific	A11034	IF 1:200
Goat anti-rabbit IgG (H + L) Alexa Fluor 568	Thermo Fisher Scientific	A11011	IF 1:200
Virus strains and DNA			
pLKO.1-puro eGFP shRNA control target sequence: TACAACAGCCACAACGTCTA	Sigma-Aldrich	SHC005V	
shTrkB #1 (shRNA-pLKO.1-hPGK-puro-CMV-tGFP) target sequence: CATTCCAAGTTTGCGATGAAA	Sigma-Aldrich	SHCLNV-NM_008745	TRCN0000023703
shTrkB #2 (shRNA-pLKO.1-hPGK-puro-CMV-tGFP) target sequence: CCACGGATGTTGCTGACCAAA	Sigma-Aldrich	SHCLNV-NM_008745	TRCN0000023701
pEGFP-hVAMP3	Addgene	42310	Gift from Thierry Galli
pCMV-TelC-P2A-EYFP	This paper	N/A	
pCAG-EGFP	Addgene	89684	Gift from Wilson Wong
Chemicals and solutions			
HEPES	Thermo Fisher Scientific	15630080	
HBSS	Thermo Fisher Scientific	14170112	
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	25200056	
B-27™ Supplement (50X), serum-free	Thermo Fisher Scientific	17504044	
Penicillin-streptomycin (5,000 U/mL)	Thermo Fisher Scientific	15070063	
Neurobasal™ Medium	Thermo Fisher Scientific	21103049	
Polyethylenimine (PEI)	Sigma-Aldrich	P3143	
Fetal bovine serum, ultra-low IgG	Thermo Fisher Scientific	16250-078	
DMEM	HyClone	SH30243.01	
HBEGF	Sigma-Aldrich	E4643	
Lipofectamine 2000	Thermo Fisher Scientific	11668027	
Lipofectamine RNAiMax	Thermo Fisher Scientific	13778100	
TRIzol™ LS Reagent	Thermo Fisher Scientific	10296028	
SuperScript™ III Reverse Transcriptase	Thermo Fisher Scientific	18080044	

Reagent type or resources	Source or reference	Identifiers	Additional information
Human BDNF-Biotin	Alomone Labs	B-250-B	
Bovine serum albumin (BSA), biotinylated	Vector Laboratories	B-2007	
Qdot™ 655 streptavidin conjugate	Thermo Fisher Scientific	Q10121MP	
QSY™ 21 carboxylic acid, succinimidyl ester	Thermo Fisher Scientific	Q20132	
4% Paraformaldehyde solution (PFA)	Biosesang	PC2031-100-00	
Normal goat serum	Jackson Immunoresearch	005-000-121	
MitoTracker™ Red CMXRos	Thermo Fisher Scientific	M7512	
Mounting Medium with DAPI	Vector Laboratories	H-1200-10	
Adenosine 5'-triphosphate magnesium salt (ATP)	Sigma-Aldrich	A9187	
Ionomycin calcium salt	Sigma-Aldrich	I3909	
BAPTA-AM	Sigma-Aldrich	A1076	
Strains and Cell Lines			
Mouse: C57BL/6N	Koatech Co., Korea	N/A	
Cell line: C8-D1A	ATCC	CRL-2541	
Oligonucleotides			
TeLC-P2A-EYFP forward: CCCAAGCTTGCCACCATGCCGATCACCATCAACAAC	This paper	N/A	For subcloning
TeLC-P2A-EYFP reverse: CCGCTCGAGTTACTTGTACAGCTCGTCCATG			
siSCR-sense: UAAGGCUAUGAAGAGAUACUU	Ref. 18	N/A	
siSCR-antisense: AAGUAUCUCUUAUAGCCUUA			
siVamp3 #1-sense: CCAAGUUGAAGAGAAAGTAUU	TRC Library Database	TRCN0000110516	https://portals.broadinstitute.org/gpp/public
siVamp3 #1-antisense: AAUACUUUCUCUUAACUUGG			
siVamp3 #2-sense: GUCAAUGUGGAUAAGGUGUUA		TRCN0000110517	
siVamp3 #2-antisense: UAACACCUUAUCCACAUUGAC			
siVamp3 #3-sense: AGGUGCCUCGCAGUUUGAAAC		TRCN0000436473	
siVamp3 #3-antisense: GUUCAAACUGCGAGGCACCU			
siVamp3 #4-sense: UCAGUGUCCUGGUGAUCAUUG		TRCN0000311406	
siVamp3 #4-antisense: CAAUGAUCACCAGGACACUGA			
TrkB-sense: GCGCTTCAGTGGTCTACAA	This paper	N/A	For RT-PCR
TrkB-antisense: TTGGGTTTGTCTCGTAGTC	Ref. 19	N/A	
β-actin-sense: TGTTACCAACTGGGACGACA	Ref. 20	N/A	

Reagent type or resources	Source or reference	Identifiers	Additional information
β-actin-antisense: GGGGTGTTGAAGGTCTCAAA			
Software and Algorithms			
ImageJ	https://imagej.nih.gov/ij		
Prism 8.0	GraphPad	N/A	
Others			
100 μm Cell strainer	BD Falcon	352360	
Amicon Ultra-0.5 Centrifugal Filter Unit	Sigma-Aldrich	UFC510096	
Glass-bottom dish	SPL	101350	

HRP, horseradish peroxidase; mAB, monoclonal antibody; HBSS, Hank’s Balanced Salt Solution; DMEM, Dulbecco’s Modified Eagle Medium; HBEGF, Heparin Binding EGF Like Growth Factor; DAPI, 4’,6-diamidino-2-phenylindole; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; C8-D1A, mouse astrocyte type 1 clone cell line; TeLC, tetanus toxin light chain;

Results

Monitoring endocytic BDNF in cultured astrocytes using QD-BDNF

To directly monitor endocytic BDNF in astrocytes, we utilized biotinylated recombinant mature BDNF directly associated with streptavidin-QDs as described previously (ref. ⁷; see Methods for detailed information). With this method, the fluorescence of the extracellular QD-conjugated mature BDNF complex (QD-BDNF; Fig. 1A) could be cancelled by a hydrophilic fluorescence quencher, QSY21 (4 μM), in the extracellular media, but QD-BDNF fluorescence was recovered after endocytosis (Fig. 1A, C). Under our imaging conditions, the smallest and most observable two-dimensional size of purified QD-BDNF was approximately 0.3 μm², indicating a single QD-BDNF particle (Fig. 1B). The intracellular uptake of QD-BDNF particles into astrocytes was mediated by receptor-mediated endocytosis, as (1) QD-BSA treatment resulted in no intracellular QD particles (Fig. 1C), and (2) the number of intracellular QD-BDNF particles (Fig. 1D) from astrocytes was significantly reduced by shRNA-mediated genetic knockdown (KD) of *TrkB* expression (Fig. S1). Moreover, our QD-BDNF particles were bioactive, because cultured astrocytes showed more complex morphology after QD-BDNF treatment (Fig. 1E), consistent with a previous report ¹². Since astrocytic TrkB.T1-dependent structural complexity is important for the structural and functional maturation of astrocytes ¹², QD-BDNF uptake under our conditions appeared to be mediated by TrkB.T1.

We next explored the ideal concentration and incubation time for the QD-BDNF treatment of cultured astrocytes to track single QD particles. QD-BDNF (0.5 ~ 5 nM) was applied to cultured astrocytes for 5 minutes (min) up to 4 hours. Treatment with 2 nM QD-BDNF for 20 min resulted in the most intracellular single QD-BDNF particles (Fig. 1F-I), and all QD-BDNF tracking and secretion experiments were therefore carried out under this condition.

ATP triggers the transport and secretion of endocytic BDNF in astrocytes

We next monitored intracellular QD-BDNF particles in astrocytes to investigate the transport and secretion of endocytic mBDNF. Since astrocytes can be stimulated by extracellular ATP due to the expression of diverse P2 receptors ²³, 100 μM ATP was added to QD-BDNF-containing astrocytes expressing EGFP (Fig. 2A, B) to induce the activity-dependent transport and secretion of QD-BDNF. Most QD-BDNF particles remained immobile (stationary mode) before ATP treatment (Fig. 2C). However, ATP stimulation triggered either the anterograde or retrograde transport of QD-BDNF (Fig. 2C), leading to an increase in the distance of QD-BDNF trafficking (Fig. 2D) despite the ATP-insensitive speeds of QD-BDNF transport (Fig. 2E). These results suggest that the transport of endocytic BDNF is activity-dependent.

We next assessed whether ATP stimulation evokes endocytic BDNF release in astrocytes. The exocytosis of endocytic QD-BDNF could be detected by the disappearance of QD-BDNF fluorescence due to the exposure of QD-BDNF to the QSY21 quencher via opened vesicle pores ⁷. Despite a few spontaneous QD-BDNF exocytosis events (5.28 ± 1.76 %), QD-BDNF exocytosis was significantly increased (19.37 ± 4.75 %; Fig. 2F) after the ATP treatment, consistent with another study ²⁴. This ATP-induced QD-BDNF secretion was abolished by the expression of the tetanus toxin light chain (TLC) in astrocytes (Fig. 2F), supporting the idea that endocytic BDNF release is SNARE-dependent. Preincubation with BAPTA-AM also largely reduced the ATP-induced QD-BDNF secretion, indicating that ATP-induced Ca²⁺ elevation is required for endocytic BDNF secretion (Fig. 2F). However, direct Ca²⁺ elevation by ionomycin treatment did not trigger QD-BDNF secretion (Fig. 2F). These results indicate that intracellular Ca²⁺ is necessary for QD-BDNF secretion but suggest that the cooperative actions of Ca²⁺ signaling with other signaling pathways are critical for the exocytosis of endocytic BDNF-containing vesicles. Finally, as reported in neurons ⁷, BDNF secretion events were frequently observed in immobile vesicles before ATP treatment (Fig. 2G), suggesting that the arrival of endocytic BDNF vesicles at secretion sites is a prerequisite for exocytosis events.

Subcellular localization of endocytic BDNF in astrocytes

Because endocytosed QD-BDNF showed activity-dependent transport and secretion, we next sought to determine the localization of QD-BDNF after endocytosis. To examine vesicular fractions containing QD-BDNF, immunocytochemistry was performed using antibodies labeling selective vesicular

fractions such as Rab5 (early endosomes), Rab7 (late endosomes), Rab11 (recycling endosomes), Lamp1 (lysosomes), and chromogranin B (ChgB; secretory granules) (Fig. 3A). Vamp3 was also assessed due to its high expression in astrocytes^{15,25}.

QD-BDNF particles were widely detected in all the tested vesicular fractions (Fig. 3A, B). Of note, colocalization of QD-BDNF with Vamp3 was prominent (Fig. 3B), suggesting that internalized BDNF molecules were preferentially sorted into Vamp3-positive vesicles. To further characterize the Vamp3-positive QD-BDNF-containing vesicles, additional immunocytochemistry analyses of astrocytes with both QD-BDNF particles and Vamp3-EGFP were performed with vesicular marker antibodies (Fig. 3C). Regardless of whether QD-BDNF particles were detected, Vamp3-positive vesicles were enriched in vesicles containing Rab5, Rab7, or ChgB (Fig. 3C, D). However, Vamp3-positive vesicles with QD-BDNF were more colocalized with Rab5- or Lamp1-positive vesicles than those without QD-BDNF (Fig. 3C, D). Given that astrocytic Vamp3-containing vesicles are implicated in the exo- and endocytotic cycling of endosomes²⁵, our results suggest that Vamp3 participates in endocytic BDNF recycling in astrocytes.

Vamp3 is required for ATP-induced endocytic BDNF secretion from astrocytes

Since our results showed that endocytic BDNFs were enriched in Vamp3-containing astrocytic vesicles (Fig. 3), ATP-induced BDNF secretion may frequently occur at Vamp3-positive vesicles. We thus compared the fraction of QD-BDNF particles displaying the exocytosis event from Vamp3 (+) vesicles to that from Vamp3-negative (-) vesicles (Fig. 4A). Few very spontaneous QD-BDNF secretion events were observed regardless of the presence of Vamp3 in QD-BDNF-containing vesicles (Fig. 4B), indicating that spontaneous endocytic BDNF release does not involve Vamp3. However, ATP-induced QD-BDNF secretion events was observed from both Vamp3-positive and Vamp3-negative vesicles (Fig. 4B). Moreover, QD-BDNFs in Vamp3-positive vesicles were secreted more frequently than those in Vamp3-negative vesicles (Fig. 4C). These results indicate that activity-induced endocytic BDNF secretion primarily occurs via Vamp3-positive vesicles.

Next, we tested whether Vamp3 directly participates in endocytic BDNF exocytosis by using the siRNA mediated KD method (Fig. S2C). We first assessed whether the endocytosis or transport of QD-BDNF was affected by *Vamp3* KD (Fig. 5A). *Vamp3* KD failed to alter the endocytosis (Fig. 5B) or ATP-induced antero- or retrograde transport of QD-BDNF (Fig. 5C-E). Although why ATP stimulation resulted increased endocytic BDNF transport remains unclear (Fig. 2C, D), the modification of vesicle trafficking or sorting by P2 receptor-mediated Ca^{2+} or lipid signaling may be implicated²⁶⁻²⁸. By contrast, astrocytes with *Vamp3* KD showed significantly reduced ATP-triggered QD-BDNF secretion (Fig. 5F). This reduced QD-BDNF exocytosis was successfully restored by the delivery of the siRNA-insensitive *Vamp3* construct together with *Vamp3* siRNAs (Fig. 5F). Together, these results indicate that Vamp3 selectively controls endocytic BDNF exocytosis in astrocytes.

Discussion

In this work, we showed the direct uptake and recycling of mBDNF in astrocytes by utilizing QD-BDNF as a proxy for the extracellular BDNF protein. After secreted from source cells, neurotrophin proteins seem to be internalized by binding to corresponding Trk receptors on nearby target cells, but direct monitoring of endogenous neurotrophin has been hampered due to their relatively low concentration in live cells. Because QD is a fluorescent nanoparticle with an excellent photostability and could stably tracked in live cells with a high signal-to-noise ratio, the QD-linked neurotrophin sensor has been widely used to examine the transport and activity-dependent secretion of neurotrophin-containing endosomes in live cells^{7,16,17}. Using QD-linked mBDNF, a previous study founds TrkB-dependent mBDNF internalization, as well as complexin 1/2 (Cpx1/2) / synaptotagmin 6 (Sytx6)-dependent re-secretion of endocytic mBDNF⁷. However, it has not examined whether mBDNF is directly internalized and recycled in astrocytes and what molecular mechanisms handle endocytic mBDNF secretion from astrocytes, although astrocytic p75NTR-dependent endocytosis of neuronal proBDNF and its re-secretion were reported¹⁰.

When treated with purified QD-BDNF particles, there was an increase in the complexity of astrocytic morphology (Fig. 1E), as found from other studies showing TrkB.T1-dependent structural complexity and maturation of astrocytes^{11,12}. Given that TrkB-shRNA expression diminished QD-BDNF internalization (Fig. 1D), QD-linked mBDNF endocytosis and morphological changes seem to be mediated by TrkB.T1. Because ATP stimulation of astrocytes was sufficient for triggering Ca^{2+} and SNARE-dependent release of endocytic QD-BDNF (Fig. 2F), our study proposes that neuronal mBDNF directly takes part in the process of astrocytic modulation of extracellular BDNF concentration, in addition to TrkB.T1-dependent regulation of astrocyte functions.

We revealed a key molecular mechanism, Vamp3-dependent exocytosis, that controls activity-dependent endocytic mBDNF secretion from astrocytes. Among all tested vesicular pools, Vamp3-positive vesicles in the early endosomes or lysosome fraction contained most endocytic QD-BDNFs (Fig. 2). However, other vesicular fractions such as Rab7 or Rab11-positive endosomes or ChgB-positive secretory granules appear to contain a portion of endocytic BDNF, because we found significant colocalization of QD-BDNF in both Vamp3-positive and -negative vesicles with corresponding vesicular markers but no significant colocalization with MitoTrackers (Fig. 2D). Because Vamp3 is an enriched vSNARE in astrocytes²⁹ and involved in endosome recycling²⁵, it is possible that recycling of endocytic BDNF-containing vesicles in astrocytes requires the role of Vamp3. Indeed, our findings support this notion; we observed the secretion of QD-BDNF by ATP stimulation frequently from Vamp3-EGFP-containing vesicles (Fig. 4). *Vamp3* KD was successful in diminishing ATP-induced QD-BDNF exocytosis (Fig. 5), supporting the idea that Vamp3 mediates exocytosis of endocytic BDNF-containing vesicles. However, neither endocytosis nor transports of QD-BDNF requires Vamp3, as shown by no changes in QD-BDNF uptake and transports by astrocytic *Vamp3* KD (Fig. 5). These results indicate Vamp3 selectively controls exocytosis of endocytic BDNF-containing vesicles. It is unclear how ATP stimulation of astrocytes caused increased the antero- or retrograde transport of endocytic BDNF-containing vesicles, but modification of vesicle trafficking or sorting by P2 receptor-mediated Ca^{2+} or lipid signaling²⁶⁻²⁸ may be implicated.

Our work also uncovered the complex molecular nature underlying endocytic BDNF secretion from astrocytes. We discovered that chelation of ATP-induced Ca^{2+} elevation significantly reduces QD-BDNF exocytosis, whereas a direct increase in intracellular Ca^{2+} concentration cannot evoke QD-BDNF exocytosis (Fig. 3F). These findings imply the requirement of additional signaling pathway for full exocytosis of endocytic BDNF-containing vesicles. For example, modification of cAMP concentration through P2 receptor activation^{30,31} or A2 receptors³², may influence endocytic BDNF release by activating cAMP-dependent signaling pathways important for vesicle docking or exocytosis^{29,33}. Moreover, Vamp3-independent mechanisms may also be implicated in regulating endocytic BDNF release, because we observed a significant number of ATP-triggered QD-BDNF release events from Vamp3 (-) vesicles (Fig. 4B). These findings support the notion that astrocytic mBDNF recycling involves multiple but differential signaling pathways. Additional studies will further explore the other aspects of molecular events regulating BDNF recycling in astrocytes and their physiological functions in synaptic plasticity and cognitive functions.

Declarations

Data availability

All materials, data, and associated protocols in this study will be available upon requests. Please contact the corresponding author (phj2@kbri.re.kr).

Competing interests

The authors have no competing interests to declare.

Acknowledgments

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Author contributions

J.H. and H.P. conceived the experiment (s). J.H. and S.Y. conducted the experiment(s), analyzed the data, and performed the statistical analysis and figure generation. J.H. and H.P. wrote the manuscript. All authors reviewed the manuscript.

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Figures

Figure 1

QD-BDNF as a tool for monitoring endocytic BDNF in astrocytes. A. Schematic diagram of biotinylated mBDNF conjugated with streptavidin-QD655 (QD-BDNF). B. Left: Representative fluorescence image of purified QD-BDNFs (2 nM). Scale bar= 10 μ m, inset scale bar= 5 μ m. Right: distribution of the 2D sizes of QD-BDNF particles (1,073 particles from 40 cells). C. Representative images of EGFP-expressing astrocytes treated with QD-BSA or QD-BDNF. Scale bar= 10 μ m. Below: magnified views of the indicated locations (numbers). Scale bar= 10 μ m. D. Representative images of endocytic QD-BDNFs in astrocytes expressing scrambled shRNA (Control), TrkB-shRNA #1 (shTrkB #1), or #2 (shTrkB #2). Scale bar= 10 μ m. Inset: magnified view of the indicated location (white box). Scale bar= 5 μ m. Bar graphs: average QD-BDNF densities under each condition. **P < 0.01. N= 10 cells for each group. E. Average shape indices of QD-BSA- and QD-BDNF-treated astrocytes. *P < 0.05. N= 5 or 16 cells. F. Average QD-BDNF densities at each incubation time. *P < 0.05. N= 10 cells in each condition. G. Representative images of astrocytes treated with 0.5, 1, 2, or 5 nM QD-BDNF. Scale bar= 10 μ m, inset scale bar= 5 μ m. H. Average QD-BDNF densities with minimum (0.3 μ m²) or larger sizes (> 0.3 μ m²). *P < 0.05, **P < 0.01. I. Average fractions of QD-BDNF with minimum or larger sizes among total intracellular QD-BDNF. *P < 0.05. N= 9-10 cells in each condition.

Figure 2

ATP stimulation results in the Ca²⁺-dependent exocytosis of endocytic BDNF. A. Left: Representative fluorescence image of EGFP-expressing astrocytes containing QD-BDNF. Right: QD fluorescence image of the cell in A. Yellow line: cell boundary determined by EGFP signals. White boxes: linearized segments used to generate the kymographs in B. Scale bar= 10 μ m. B. Representative kymographs indicated in A. Red bar: ATP (100 μ M) treatment. Arrow heads: disappearances of QD-BDNF fluorescence. C. Average QD-BDNF fractions showing immobility (St) or anterograde (An)/retrograde (Re) transport. *P < 0.05, **P < 0.01. D. Cumulative distributions of the QD-BDNF transport distances at baseline and after ATP stimulation (ATP). ****P < 0.0001. E. Average velocities of mobile QD-BDNF particles. n.s., not significantly different. N= 173 particles from 14 cells for each group. F. Average percentages of secreted QD-BDNF. TLC: tetanus toxin light chain. Ionomyc: ionomycin (1 μ M). *P < 0.05, **P < 0.01. N= 5-11 cells for each group. G. Average secreted QD-BDNF fractions showing immobility or An/Re transport before exocytosis. Green: 60 sec before exocytosis. Blue: immediately prior to ATP treatment. *P < 0.05, ****P < 0.0001.

Figure 3

Subcellular localization of endocytic BDNF in cultured astrocytes A. Representative fluorescence images of the colocalization of QD-BDNF with endogenous vesicular markers. ChgB: chromogranin B. Scale bar= 2 μ m. White arrowheads: representative colocalization of QD-BDNF with the corresponding markers. B. Average colocalization ratios (# colocalized QD-BDNF/# total QD-BDNF). Dotted line: average colocalization ratio between QD-BDNF and MitoTracker (Mito.; negative control). ** $P < 0.01$ (Vamp3 vs. others), ## $P < 0.01$ (Mito. vs. others). N= 42-45 cells for vesicular markers; N= 16 cells for Mito. C. Representative fluorescence images of the colocalization of QD-BDNF, Vamp3-EGFP, and other vesicular markers. Scale bar= 2 μ m. White arrowheads: representative triple colocalization among QD-BDNF, Vamp3-EGFP, and the corresponding vesicular markers. D. Above: average colocalization ratio between Vamp3-EGFP and each vesicular marker. * $P < 0.05$, ** $P < 0.01$. Below: average colocalization ratio of each vesicular marker with QD-BDNF with Vamp3-EGFP (Vamp3(+)) or without Vamp3-EGFP (Vamp3(-)). ** $P < 0.01$. N= 9-10 cells.

Figure 4

ATP-induced secretion of endocytic BDNF from Vamp3-containing vesicles A. Representative fluorescence images of astrocytic processes containing QD-BDNF and Vamp3-EGFP. Black arrow heads: Vamp3-positive QD-BDNF particles. Empty arrowheads: Vamp3-negative QD-BDNF particles. Red bar: ATP treatment. White sharp arrowheads: disappearance of QD-BDNF particles. Black bar= 30 sec. B. Average percentages of ATP-induced QD-BDNF secretion events from vesicles with (+) or without Vamp3 (-). ** $P < 0.01$, *** $P < 0.001$. C. Average fractions of secreted QD-BDNF particles with (+) or without Vamp3 (-) among total secreted QD-BDNFs. **** $P < 0.0001$. N= 15 cells.

Figure 5

Vamp3 is necessary for ATP-induced endocytic BDNF secretion. **A**. Representative QD-BDNF kymographs from astrocytes with GFP- (CTL), siSCR-, siVamp3-, or siVamp3 + human Vamp3 (siVamp3 + rescue). White arrowheads: disappearance of QD-BDNF particles. **B**. Average intracellular QD-BDNF densities under each condition. $N = 9-18$ cells. **C**. Average QD-BDNF fractions showing immobility (St) or anterograde (An)/retrograde (Re) transport. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. $N = 10-16$ cells. **D**. Cumulative distributions of the QD-BDNF transport distances at baseline and after ATP stimulation (ATP) in each group. $****P < 0.0001$. **E**. Average velocities of QD-BDNF transport. $N = 165$ and 302 particles for the siSCR and siVamp3 groups, respectively. **F**. Average percentages of QD-BDNF secretion events after the indicated treatments. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. $N = 6-16$ cells.

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