Journal of Visualized Experiments

Improved protocol to purify and directly mono-biotinylate recombinant BDNF in a tube for cellular trafficking studies in neurons

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE61262R2
Full Title:	Improved protocol to purify and directly mono-biotinylate recombinant BDNF in a tube for cellular trafficking studies in neurons
Section/Category:	JoVE Neuroscience
Keywords:	BDNF, mono-biotinylation, quatum dots, axonal trafficking, protein purification, endosome dynamics, in vivo tracking
Corresponding Author:	Francisca C Bronfman, Ph.D. Universidad Andres Bello Facultad de Medicina Santiago, CHILE
Corresponding Author's Institution:	Universidad Andres Bello Facultad de Medicina
Corresponding Author E-Mail:	francisca.bronfman@unab.cl
Order of Authors:	Nicolas Stuardo
	Guillermo Moya-Alvarado
	Carolina Ramirez
	Giampietro Schiavo
	Francisca C Bronfman
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

TITLE:

An Improved Protocol to Purify and Directly Mono-Biotinylate Recombinant BDNF in a Tube for Cellular Trafficking Studies in Neurons

AUTHORS & AFFILIATIONS:

Nicolás Stuardo[1,2], Guillermo Moya-Alvarado[1,3], Carolina Ramírez[1,3], Giampietro Schiavo[4], Francisca C Bronfman[1,3]

1. Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile
2. Department of Molecular and Cellular Biology, Faculty of Biological Sciences, Center for Aging and Regeneration (CARE UC), Pontificia Universidad Católica de Chile, Santiago, Chile
3. Institute of Biomedical Sciences. Faculty of Medicine and Faculty of Life Sciences. Universidad Andrés Bello, Santiago, Chile
4. Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology and UK Dementia Research Institute at UCL, University College London Campus, London, UK

nstuardo@bio.puc.cl
gmoya@bio.puc.cl
cramireza@bio.puc.cl
giampietro.schiavo@ucl.ac.uk

Corresponding author:
Francisca C Bronfman
francisca.bronfman@unab.cl

KEYWORDS:

BDNF, mono-biotinylation, quatum dots, axonal trafficking, protein purification, endosome dynamics, in vivo tracking

SUMMARY:

Recombinant BDNF containing an Avi sequence (BDNFAvi) is produced in HEK293 cells in a cost-effective manner and is purified by affinity chromatography. BDNFavi is then directly mono-biotinylated with the enzyme BirA in a tube. BDNFavi and mono-biotinylated BDNFavi retain their biological activity when compared to commercially available BDNF.

ABSTRACT:

Recombinant BDNF containing an Avi sequence (BDNFAvi) is produced in HEK293 cells and then cost-effectively purified by affinity chromatography. We developed a reproducible protocol to directly mono-biotinylate BDNFavi with the enzyme BirA in a tube. In this reaction, mono-biotinylated BDNFAvi retains its biological activity.

Neurotrophins are target-derived growth factors playing a role in neuronal development and maintenance. They require rapid transport mechanisms along the endocytic pathway to allow

long-distance signaling between different neuronal compartments. The development of molecular tools to study the trafficking of neurotrophins has enabled the precise tracking of these proteins in the cell using in vivo recording. In this protocol, we developed an optimized and cost-effective procedure for the production of mono-biotinylated BDNF. A recombinant BDNF variant containing a biotinylable avi sequence (BDNFAvi) is produced in HEK293 cells in the microgram range and then purified in an easily scalable procedure using affinity chromatography. The purified BDNF can then be homogeneously mono-biotinylated by a direct in vitro reaction with the enzyme BirA in a tube. The biological activity of the mono-biotinylated BDNF (mbtBDNF) can be conjugated to streptavidin-conjugated to different fluorophores. BDNF Avi and mbtBDNF retain their biological activity demonstrated through the detection of downstream phosphorylated targets using western blot and activation of the transcription factor CREB, respectively. Using streptavidin-quantum dots, we were able to visualize mbtBDNF internalization concomitant with activation of CREB, which was detected with a phospho-CREB specific antibody. In addition, mbtBDNF conjugated to streptavidin-quantum dots was suitable for retrograde transport analysis in cortical neurons grown in microfluidic chambers. Thus, in tube produced mbtBDNF is a reliable tool to study physiological signaling endosome dynamics and trafficking in neurons.

INTRODUCTION:

Neurons are the functional units of the nervous system possessing a complex and specialized morphology that allows synaptic communication, and thus, the generation of coordinated and complex behavior in response to diverse stimuli. Neuronal projections such as dendrites and axons are critical structural features involved in neuronal communication, and neurotrophins are crucial players in determining their morphology and function(s)[1]. Neurotrophins are a family of secreted growth factors that include NGF, NT-3, NT-4, and brain-derived neurotrophic factor (BDNF)[2]. In the central nervous system (CNS), BDNF participates in diverse biological processes including neurotransmission, dendritic arborization, maturation of dendritic spines, long-term potentiation, among others[3,4]. Therefore, BDNF plays a critical role in regulating neuronal function.

Diverse cellular processes regulate BDNF dynamics and function. On the neuronal surface, BDNF binds the tropomyosin receptor kinase B (TrkB) and/or the p75 neurotrophin receptor (p75). BDNF-TrkB and BDNF-p75 complexes are endocytosed and sorted in different endocytic organelles[5-8]. Correct intracellular trafficking of the BDNF/TrkB complex is required for proper BDNF signaling in different neuronal circuits[9-11]. For this reason, a deep understanding of BDNF trafficking dynamics and its alterations found in pathophysiological processes is essential to understand BDNF signaling in health and disease. The development of novel and specific molecular tools to monitor this process will help to drive this field forward and allow a better grasp of the regulatory mechanisms involved.

There are several tools available for the study of BDNF trafficking in neurons. A commonly used methodology involves the transfection of recombinant BDNF tagged with fluorescent molecules such as green fluorescent protein (GFP) or the monomeric fluorescent red-shifted variant of GFP mCherry[12,13]. However, a major shortcoming of BDNF overexpression is that it eliminates the

possibility of delivering known concentrations of this neurotrophin. Also, it may result in cellular toxicity, obscuring the interpretation of results[14]. An alternative strategy is the transfection of an epitope-tagged TrkB, such as Flag-TrkB. This methodology allows the study of TrkB internalization dynamics[15], but it also involves transfection, which might result in altered TrkB function and cellular toxicity. To overcome these methodological hurdles, recombinant variants of NGF and BDNF containing an Avi sequence (BDNFAvi), which can be mono-biotinylated by the biotin-ligase enzyme BirA, were developed[16,17]. Biotinylated recombinant BDNF can be coupled to different streptavidin-bound tools, which include fluorophores, beads, paramagnetic nanoparticles among others for detection. In terms of live-cell imaging, quantum dots (QD) have become frequently used fluorophores, as they have desirable characteristics for single-particle tracking, such as increased brightness and resistance to photobleaching when compared to small molecule fluorophores[18].

The production of mono-biotinylated BDNF (mbtBDNF) using BDNFAvi has been achieved by co-transfection of plasmids driving the expression of BDNFAvi and BirA, followed by the purification of the recombinant protein by affinity chromatography with a yield of 1-2 µg of BDNF per 20 mL of HEK293-conditioned culture media[17]. Here, we propose a modification of this protocol that allows for BDNFAvi purification from 500 mL of HEK293-conditioned media, which seeks to maximize protein recovery in a chromatography-column based protocol for ease of manipulation. The used transfection agent, polyethyleneimine (PEI), ensures a cost-effective method without sacrificing transfection yield. The mono-biotinylation step has been adapted to an in vitro reaction to avoid the complications associated with co-transfections and to ensure homogeneous labeling of BDNF. The biological activity of the mbtBDNF was demonstrated by western blot and fluorescence microscopy experiments, including activation of pCREB and live cell imaging to study retrograde axonal transport of BDNF in microfluidic chambers. The use of this protocol allows for optimized, high-yield production of homogenous mono-biotinylated and biologically active BDNF.

PROTOCOL:

All experiments were carried out in accordance with the approved guidelines of CONICYT (Chilean National Commission for Scientific and Technological Research). The protocols used in this study were approved by the Biosecurity and Bioethical and Animal Welfare Committees of the P. Catholic University of Chile. Experiments involving vertebrates were approved by the Bioethical and Animal Welfare Committee of the P. Catholic University of Chile.

NOTE: The following protocol was designed to purify BDNFAvi from a total volume of 500 mL of conditioned medium produced in HEK293 cells. The amount of conditioned medium that is produced and processed to purify BDNFAvi can be up or downscaled as needed. However, further optimization may be necessary under these circumstances. The composition of the culture media and buffers used throughout the protocol can be found in supplementary materials.

1. Production and purification of BDNFAvi from HEK293-conditioned media

1.1. Transfection of HEK293 cells

1.1.1. Grow HEK293 cells to 70% confluence in supplemented DMEM medium (10% bovine fetal serum, 1x glutamate supplement, 1x antibiotic/antimycotic) in 15 cm culture dishes at 37 °C.

1.1.2. Change the medium to transfection buffer.

1.1.3. Prepare the PEI-DNA mixture for transfection. Use two different 15 cm conical tubes to dilute DNA and PEI 25 K, respectively. Dilute 20 µg of plasmid DNA in a final volume of 500 µL in one tube. Dilute 60 µg of linear PEI 25K in a final volume of 500 µL in the other tube. Incubate at room temperature for 5 min.

1.1.4. Carefully pipette the DNA solution into the PEI tube, mixing once by an up-down motion. Incubate at room temperature for 25 min.

1.1.5. Drip 1 mL of the PEI-DNA mixture throughout each 15 cm dish. Incubate the cells with the PEI-DNA mixture for 3 h at 37 °C.

1.1.6. Change the medium to fresh incubation buffer.

1.2. Media collection and storage

1.2.1. Collect the medium from all the dishes 48 h after the transfection of HEK293 cells. Prepare concentrated stocks of the solutions described in the "supernatant modification buffer" section of **Supplemental File 1** and add them to the HEK293 supernatant to achieve the listed final concentrations.

NOTE: Cells can be discarded or recovered for further analysis.

1.2.2. Incubate the medium in ice for 15 min.

1.2.3. Aliquot the medium into centrifuge tubes.

1.2.4. Centrifuge the medium at 10,000 x *g* for 45 min in a 4 °C centrifuge. This step allows the elimination of cell debris and dead cells suspended in the media.

1.2.5. Collect the supernatants, add BSA at a final concentration of 0.1%. and then store at -20 °C. The media can be aliquoted before freezing for faster thawing during the purification step.

NOTE: Storage times of frozen conditioned media of up to 2 months have yielded positive results, longer storage times have not been evaluated.

1.3. Media concentration and purification

1.3.1. Thaw the media in a 37 °C thermoregulated bath.

1.3.2. Aliquot the media into centrifuge tubes.

1.3.3. Centrifuge the medium for 1 h at 3,500 x *g* in a 4 °C cooled centrifuge. This step allows the elimination of remaining cell debris to ensure adequate flow through the chromatography column.

1.3.4. Use the protein concentrators with a 10 kDa cutoff to reduce the media from 500 mL to 100 mL. Follow the manufacturer's recommended centrifugation parameters for optimal concentration.

1.3.5. Add 500 µL of Ni-NTA agarose beads to the concentrated media and incubate overnight at 4 °C in a rocker.

1.3.6. Assemble the chromatography apparatus and pour the media into it. Let it rest for 5 min and then open the 2-way stopcock to let the medium flow through.

1.3.7. Wash the beads with 5 mL of wash buffer for 5 min. Make sure to resuspend the beads in the column. Drain the wash buffer by opening the 2-way stopcock. Repeat 3 times.

1.3.8. Add 1 mL of elution buffer to the column. Make sure to resuspend the beads in the column. Incubate for 15 min, and then collect the eluate in a 1.5 mL microcentrifuge tube. Repeat this step 3 times for complete elution of BDNFAvi.

1.3.9. Load 5 µL of each eluate and different concentrations of commercially available BDNF (40-160 ng) in a 15% polyacrylamide gel. Detect the purified protein by western blotting using an anti-BDNF antibody.

1.3.10. Determine the concentration of the purified BDNFAvi in each eluate using the concentration curve prepared with the commercially available BDNF.

1.3.11. Aliquot and store the purified BDNFAvi at -80 °C.

2. In vitro mono-biotynilation of BDNFAvi using the BirA enzyme

2.1. In vitro mono-biotinylation reaction

2.1.1. Prepare concentrated stock solutions of the biotinylation buffer reagents. The use of concentrated stocks will minimize the dilution of the recombinant protein.

2.1.2. Take an aliquot of 800 ng of BDNFAvi and add the biotinylation buffer reagents and the enzyme BirA in a 1:1 molar relation to BDNF. For example, for a 200 µL final reaction volume add: 100 µL of solution containing 800 ng of BDNFAvi, 20 µL of 0.5 M Bicine pH 8.3, 20 µL of 100 mM

ATP, 20 µL of 100 mM MgOAc, 20 µL of 500 µM d-biotin, 0.8-1 µg to 1 µL of BirA-GST, and complete to 200 µL with ultrapure water.

NOTE: Successful biotinylation reactions have been performed with aliquots of 400 µL containing a concentration of about 30 ng/µL BDNFAvi, resulting in a homogeneously biotinylated BDNFAvi to a final concentration of ~20 ng/µL in the final reaction.

2.1.3. Incubate the mixture at 30 °C in a hybridization oven for 1 h. Mix the content by tube inversion every 15 min.

2.1.4. Add the same volume of ATP and BirA as in step 2.1.2 and repeat step 2.1.3.

2.1.5. Store at -80 °C for future analyses or keep on ice for immediate use (e.g., biotinylation quality control).

2.2. Biotinylation analysis

2.2.1. Block 30 µL of streptavidin magnetic beads per BDNF sample in 1 mL of blocking buffer. Incubate at room temperature for 1 h in a microcentrifuge tube rotator.

2.2.2. Precipitate the magnetic beads using a magnetic separation rack for 3 to 5 minutes or until the buffer appears completely cleared of the beads and discard the blocking buffer.

2.2.3. Add 50 µL of fresh blocking buffer and 80 ng of mono-biotinylated BDNFAvi (mbtBDNF) sample to the beads, making sure to resuspend them completely by pipetting.

2.2.4. Incubate at 4 °C for 1 h in a microcentrifuge tube rotator spinning at approximately 20 RPM.

2.2.5. Collect the beads using the magnetic separation rack for 3 to 5 minutes, and collect the supernatant, keeping a 30 µL aliquot for analysis.

2.2.6. Wash the beads one time with 500 µL of PBS, and then collect them using the magnetic separation rack for 3 to 5 minutes. Recover the supernatant and keep a 30 µL aliquot for analysis.

2.2.7. Add 10 µL of 4x loading buffer to the beads.

2.2.8. Heat the samples to 97 °C for 7 min to eluate the mbtBDNF.

2.2.9. Detect mbtBDNF using an anti-BDNF specific antibody[19].

3. Verification of mbtBDNF biological activity

3.1. Detection of pTrkB and pERK by western blot

3.1.1. Seed 2 million rat cortical neurons in 60 mm culture dishes.

3.1.2. Culture the neurons for 7 days (DIV7) at 37 °C. Then, change the medium to non-supplemented neurobasal mediun when starting the experiment.

3.1.3. One hour after medium change, add mbtBDNF to a final concentration of 50 ng/mL. Incubate for 30 min at 37 °C. Keep a negative control dish (non-stimulated with BDNF) and a positive control dish (treated with 50 ng/mL of commercially available BDNF).

3.1.4. Collect the medium and gently wash every dish with 1x PBS. Collect and discard the 1x PBS.

3.1.5. Place the dishes on ice and add 50-80 µL of lysis buffer to each dish. Use a cell scraper to lyse the cells.

NOTE: The lysis step should be performed as quickly as possible to avoid protein dephosphorylation and degradation. 1-2 minutes of vigorous scraping are enough to visualize the proteins of interest by western blotting.

3.1.6. Collect the lysis buffer and stir in a vortex mixer at highest speed for 5 s.

3.1.7. Centrifuge the lysis buffer at 14,000 x *g* (4 °C) for 10 min. Collect the supernatant.

3.1.8. Quantify the protein content of the supernatant by BCA protein quantification protocol[20].

3.1.9. Add loading buffer to an aliquot containing 30-50 µg of protein per condition and load it in a 12% polyacrylamide gel for western blotting. Detect pTrkB and pERK using specific phosphor-antibodies to verify BDNFAvi biological activity.

3.2. Verification of BDNF-QD biological activity by pCREB immunofluorescence.

3.2.1. Seed 40,000 rat cortical neurons in 10 mm coverslips, previously autoclaved and treated with poly-L-lysine as described previously[21].

3.2.2. Culture the neurons for 7-8 days in neuronal maintenance buffer (see **Supplemental Materials**) at 37 °C.

3.2.3. To start the experiment, change the medium to unsupplemented neurobasal medium and incubate at 37 °C for 1 h.

3.2.4. Prepare mbtBDNF conjugated to quantum dots (BDNF-QD) by adding to a mbtBDNF aliquot, the necessary volume of quantum dot streptavidin conjugate (streptoavidein-QD) to

achieve a 1:1 BDNF-QD molar ratio. Then, dilute to 20 µL with neurobasal medium. Wrap the tube in aluminum foil to protect it from the light.

NOTE: Prepare another tube with the same volume of quantum dot streptavidin conjugate and dilute it to 20 µL with neurobasal medium as a negative control.

3.2.5. Incubate the mbtBDNF/streptavidin-QD mixture for 30 min at room temperature in a rocker.

3.2.6. Dilute the BDNF-QD to the desired final concentration (200 pM and 2 nM) in neurobasal medium.

3.2.7. After 1 h of incubation with non-supplemented neurobasal medium, stimulate the neurons with BDNF-QD or streptavidin-QD (control) to a final concentration of 200 pM and 2 nM of BDNF for 30 min at 37 °C.

3.2.8. Wash the coverslips 3 times with 1x PBS (37 °C) and fix the cells for 15 min by treating the coverslip with 4% paraformaldehyde solution containing phosphatase inhibitors.

3.2.9. Wash the cells 3 times with PBS, and then incubate with blocking/permeabilization buffer (BSA 5%, Triton X-100 0.5%, 1x phosphatase inhibitor) for 1 h.

3.2.10. Incubate with anti-pCREB antibody 1:500 (in 3% BSA, 0.1% Triton X-100) overnight at 4 °C.

3.2.11. The following day, wash 3 times with 1x PBS, and incubate for 1 h with the secondary antibody 1:500 (3% BSA, 0.1% Triton X-100).

3.2.12. Wash 3 times with 1x PBS. Add Hoechst nuclear stain solution (5 µg/mL) for 7 min.

3.2.13. Wash 3 times with 1x PBS and mount.

3.3. Visualization of retrograde axonal transport of BDNF-QD in live neurons

3.3.1. Prepare microfluidic chambers and seed neurons as described previously[16].

3.3.2. After 7-8 days in culture, change the medium to non-supplemented neurobasal medium.

3.3.3. Prepare mbtBDNF conjugated to quantum dots (BDNF-QD) by adding to a mbtBDNF aliquot, the necessary volume of quantum dot streptavidin conjugate (streptavidin-QD) to achieve a 1:1 BDNF-QD molar ratio. Then, dilute to 20 µL with neurobasal medium. Wrap the tube in aluminum foil to protect it from the light.

NOTE: Prepare another tube with the same volume of quantum dot streptavidin conjugate and dilute it to 20 µL with neurobasal medium as a control.

3.3.4. Incubate the mbtBDNF/streptavidin-QD mixture for 30 min at room temperature in a rocker.

3.3.5. Dilute the BDNF-QD to the desired final concentration (2 nM).

3.3.6. After 1 h of incubation with non-supplemented neurobasal medium add the BDNF-QD or the control mixture to the axonal compartments of the microfluidic chamber. Incubate for 210 min at 37 ˚C to ensure a net retrograde transport of BDNF-QD.

3.3.7. For live-cell imaging, visualize axonal retrograde transport in the segment of the microgrooves that is proximal to the cell body compartment using a 100x objective using a microscope suitable for these purpose (37 °C and 5% CO_2). Acquire images at 1 frame/s.

REPRESENTATIVE RESULTS:

The use of a chromatographic column-based protocol allows the processing of significant volumes of HEK293 conditioned media. In **Figure 1**, the results of the purification of BDNFAvi from 500 mL of conditioned media are shown. Consecutive elutions of BDNFAvi from the Ni-NTA agarose beads yield decreasing concentrations of BDNFAvi (**Figure 1A**). After four consecutive elutions (each lasting 15 min), the majority of the BDNF captured by the beads is recovered. The concentrations of the eluates range from 6 to 28 ng/µL, and the total yield amounted to approximately 60 µg of BDNFAvi (**Table 1**). The produced BDNFAvi was then efficiently biotinylated by an in vitro reaction mediated by BirA-GST, as demonstrated by the lack of non-biotinylated BDNFAvi in the supernatant (**Figure 1B**). Please note that the biotinylation presented in **Figure 1B** corresponds to an aliquot of the total BDNF produced, but the reaction can be scaled up for bigger volumes.

Then, the biological activity of mbtBDNF was evaluated using 2 different experimental approaches. First, cortical neurons seeded in 60 mm plates (2 million neurons, DIV7) were stimulated with 50 ng/mL of mbtBDNF for 30 min, and then proteins were prepared for western blot analysis. The biological activity of the mbtBDNF was quantified by detecting pTrkB (Y515) and pERK (T202/Y204). Binding of BDNF to TrkB triggers the activation of the receptor through an autophosphorylation reaction in its intracellular domain, and ERK is a known target of the BDNF signaling pathway[22]. The bands for both phosphorylated proteins had a similar intensity in neurons treated with commercial BDNF and mbtBDNF, and both showed a stronger signal than control condition (**Figure 2A**). Then, the biological activity of mbtBDNF coupled to streptavidin-QD was evaluated to demonstrate that they can be used in live imaging experiments. Cortical neurons were seeded in 10 mm covers (40,000 cells per cover, DIV7) and treated with a final concentration of 200 pM or 2 nM BDNF-QD for 30 min before fixing and staining for pCREB. CREB is a transcription factor which is targeted by activated ERK1/2 in cortical neurons[22,23]. Stimulating neurons with increasing concentrations of BDNF-QD resulted in a dose-dependent increase of phosphorylation of CREB and presence of QD particles surrounding the nucleus (**Figure 2B**),

indicating that the BDNF-QD particles were endocytosed and triggered the activation of signaling pathways associated with BDNF-mediated TrkB activation. A twofold increase in pCREB signal was detected when stimulating neurons with a low concentration of BDNF-QD (200 pM), whereas stimulating with 2 nM resulted in a 3.5-fold increase in the pCREB signal (**Figure 2C**). These results demonstrate that the biotinylated BDNFAvi is biologically active, and that it does not lose its activity when coupled to streptavidin-QD, making it suitable for immunofluorescence and live cell imaging.

Finally, the imaging potential of BDNF-QD was evaluated in compartmentalized cultures using microfluidic chambers. Cortical neurons were seeded in microfluidic chambers (15 mm covers, 50,000 neurons per microfluidic chamber, DIV7) to separate the axonal and somatodendritic compartments and were stimulated with 2 nM BDNF-QD for 3.5 h. Live cell microscopy was performed, and the resulting kymographs were used to quantify the speed of BDNF-QD containing organelles (**Figure 3A**). An average moving speed of 0.91 µm/s was detected (**Figure 3B**), which is in line with previous analyses of cytoplasmic dynein-mediated transport[7,16]. Microfluidic chambers treated with 2 nM streptavidin-QD did not show moving QDs in the microgrooves, as shown by the kymograph (**Figure 3A**). Cells grown under the same conditions were stimulated with 500 pM or 2 nM BDNF-QD for 210 min, and then fixed and labelled with a nuclear staining. As shown in **Figure 3C**, neurons show a dose-dependent accumulation of BDNF-QD in all the analyzed sub-compartments, including the proximal and distal portions of the microgroove and the somatodendritic compartment. In contrast, control neurons showed almost no QD signal throughout the chamber. Therefore, the BDNF-QD can be detected in live and fixed cells in microfluidic chambers.

FIGURE AND TABLE LEGENDS:

Figure 1: Production and mono-biotinylation of BDNFAvi in HEK293 cells. HEK293 cells were transfected using the PEI reagent and a BDNFAvi encoding plasmid and the conditioned media was collected after 48 h. BDNFAvi contains a 6x Histidine tag allowing purification using nickel-nitrilotriacetic acid (Ni-NTA) chromatography. Commercially available recombinant human BDNF has an expected molecular weight of ~13 kDa, whereas BDNFAvi displays a molecular weight of ~18 kDa. BDNFAvi bound to the resin was fully eluted with four consecutive elution steps. (**A**) Western blot using anti-BDNF antibodies to detect in house prepared recombinant BDNF and commercial BDNF. Aliquots containing known amounts of commercially available human BDNF and 5 µL of each eluate were loaded into an SDS-PAGE gel for detection of BDNFAvi using an antibody against BDNF. **Table 1** indicates the concentrations of BDNFAvi present in each eluate. The amount and concentration of BDNF in each eluate was obtained by densitometric analysis and interpolation from the concentration curve of commercially available BDNF. (**B**) Verification of BDNFAvi biotinylation. Eighty nanograms of biotinylated BDNFAvi (mbtBDNF) were incubated with 30 µL of streptavidin coupled to magnetic beads (20% slurry) for 1 h at 4 °C. Then, magnetic beads were isolated using a magnetic separator. The streptavidin beads were heated with loading buffer to elute the biotinylated BDNFAvi (beads lane). The supernatant (SN lane) was also treated with loading buffer, heated and loaded in the gel (SN lane).

Figure 2: Verification of mbtBDNF biological activity. (**A**) DIV7 cortical neurons were serum starved for 1 h, and then stimulated with 50 ng/mL of commercially-available BDNF or mbtBDNF for 30 min. Proteins were extracted and loaded in an SDS-PAGE gel for analysis of TrkB and ERK1/2 phosphorylation using phospho-specific antibodies and compared to the total levels of the protein using antibodies against total TrkB and ERK1/2. (**B**) DIV7 cortical neurons were serum starved for 1 h, and then stimulated with a final concentration of 200 pM or 2 nM of mbtBDNF coupled to streptavidin-QD (BDNF-QD) for 30 min. Then, cells were fixed and pCREB was labelled for fluorescence microscopy analysis. (**C**) Quantification of nuclear pCREB fluorescence intensity. The results correspond to 90 neurons pooled together from 3 independent experiments, shown as mean ± SEM. The statistical analysis corresponds to a one-way ANOVA with Tukey's multiple comparisons test (****$p < 0.0001$).

Figure 3: Visualization of BDNF-QD in live and fixed cells. (**A**) DIV7 cortical neurons grown in microfluidic chambers were stimulated in the axonal compartment with a final concentration of 2 nM BDNF-QD for 3.5 hrs, and then the proximal portion of the microgrooves was imaged using a live cell microscopy setting. Representative kymographs for control condition (treated with streptavidin-QD) and upon treatment with BDNF-QD are shown. (**B**) Quantification of the speed of moving BDNF-QD. Mobile puncta were defined as those that moved more than 10 µm in the 120 s of recording. (C) DIV7 cortical neurons grown in microfluidic chambers were stimulated in the axonal compartment with a final concentration of BDNF-QD of 500 pM or 2 nM for 3.5 hrs, and then fixed and labelled with Hoechst to visualize the nuclei. Representative images of the somatodendritic compartment and the distal and proximal portions of the microgrooves are shown.

Table 1: Quantification of BDNFAvi purification yield (related to Fig. 1A). HEK293 cells were transfected with a plasmid driving BDNFAvi expression, and the protein was purified by Ni-NTA affinity chromatography. Protein concentration and final yield was calculated by densitometric analysis and interpolation in the known concentration curve of commercially available recombinant human BDNF.

Supplemental File 1: Culture media and buffer components

DISCUSSION:

In this article, an optimized methodology for the production and purification of mbtBDNF in an affinity chromatography-based procedure is described, based on the work of Sung and collaborators[17]. The optimizations include the use of a cost-effective transfection reagent (PEI) while maintaining the efficiency of more expensive transfection methods such as lipofectamine. This optimization translates into a significant cost reduction in the protocol, allowing for scalability while maintaining high cost-effectiveness. The protocol also includes ease of use considerations, including the freezing of conditioned media for up to 2 months. These optimizations make the procedure adaptable to each laboratory's needs, improve cost-effectiveness, and yield homogeneous and biologically active recombinant BDNF. The protocol can also be adapted to smaller scale productions by replacing the use of the chromatography apparatus with gravitational precipitation of the beads in conical tubes. This constitutes a viable

methodology, but it is less time-efficient and has resulted in lower yields in our experience. The biotin-labeled BDNF can then be coupled to different streptavidin-bound probes, including fluorophores and paramagnetic nanoparticles, making it a valuable tool to perform diverse types of experiments for the analysis of BDNF post-endocytic trafficking. Therefore, an optimized and simple production protocol for this protein is highly useful to laboratories working in this field.

Production of recombinant proteins with complex post-translational modifications, such as BDNF[24], in prokaryotic systems often results in proteins that are not correctly folded and thus have poor biological activity[25]. Therefore, expression in mammalian cells is necessary to obtain a bioactive protein. The use of PEI has been described previously as a viable alternative for large-scale production of recombinant proteins in transfected mammalian cells[25,26], and its efficiency in the transfection of the HEK293 cells in the context of academic laboratories has been highlighted[27]. Therefore, the use of this cell line represents a valid option to produce BDNFAvi on a scale that can be managed by an academic laboratory. The proposed protocol could be optimized further by the generation of a HEK293 cell line stably transfected with BDNFAvi, which would eliminate the transient transfection step, thus saving time and resources. Another potential source of optimization is the use of cells in suspension instead of adherent cells. HEK293 cells can be maintained in suspension, generating significant amounts of recombinant protein in the range of grams per liter[28].

Another improvement in the protocol is the biotinylation of the BDNFAvi protein using an in vitro strategy, replacing the previous in vivo co-transfection protocol. Transient co-transfection can have unexpected results in terms of the expression of the constructs, as has been demonstrated in multiple cell lines and with several transfection reagents[29]. Various factors can affect the expression of transfected proteins in a co-transfection context, including vectors, cell types and plasmid concentration. This multiplicity of factors makes optimization and reproducibility a complex task. On the other hand, an in vitro methodology allows for better control over the conditions in which the biotinylation reaction takes place. This methodology results in reproducible and homogeneous labeling of recombinant BDNF.

As demonstrated by the biological activity verification experiments, the mbtBDNF produced using this protocol is comparable to commercially-available recombinant human BDNF in terms of BDNF-TrkB signaling pathway activation. The data also shows that coupling BDNF to streptavidin-QD does not interfere with BDNF-TrkB signaling. In addition, we showed that BDNF-QD can be detected by epifluorescence microscopy in live and fixed cells. Therefore, mbtBDNF represents a valuable tool for studying retrograde axonal trafficking and it presents significant advantages over alternative probes, such as BDNF-GFP[16].The protocol described in this article provides a reliable and consistent methodology for the production of mbtBDNF, which can then be used in post-endocytic dynamics studies in different neuronal models expressing TrkB or p75. BDNF signaling has potent effects on neuronal morphology and function[3,4,21], and has been recently proposed as a potential therapeutic tool to enhance neuronal regeneration[30,31], making its study relevant in the fields of cellular biology and biomedicine. The study of the effects of BDNF signaling and trafficking will further advance our understanding of neuronal cell biology and may allow for the harnessing of its regenerative potential in clinical settings.

ACKNOWLEDGMENTS:

The authors gratefully acknowledge financial support from Fondecyt (1171137) (FCB), the Basal Center of Excellence in Science and Technology (AFB 170005) (FCB), Millenium-Nucleus (P07/011-F) (FCB), the Wellcome Trust Senior Investigator Award (107116/Z/15/Z) (GS) and a UK Dementia Research Institute Foundation award (GS). This work was supported by the Unidad de Microscopía Avanzada UC (UMA UC).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Huang, E., Reichardt, L. Neurotrophins: Roles in Neuronal Development and Function. *Annual Review of Neuroscience*. **24**, 677-736 (2001).
2. Skaper, S.D. The neurotrophin family of neurotrophic factors: an overview. *Methods in Mollecular Biology.* **846**, 1-12 (2012).
3. Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billault, C., Bronfman, F.C. Cellular and molecular mechanism regulating neuronal growth by brain-derived neurotrophic factor. *Cytoskeleton*. **73** (10), 612-628 (2016).
4. Cunha, C., Brambilla, R., Thomas, K. A simple role for BDNF in learning and memory? *Frontiers in Mollecular Neuroscience*. **3**, 1 (2010).
5. Bronfman, F.C., Lazo, O.M., Flores, C., Escudero, C.A. Spatiotemporal intracelular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. In: Lewin G, Carter B (eds) Neurotrophic Factor. Handbook of Experimental Pharmacology, vol 220. Springer, Berlin, Heidelberg (2014).
6. Ascano, M., Bodmer, D., Kuruvilla, R. Endocytic trafficking of neurotrophins in neural development. *Trends in Cell Biology*. **22** (5), 266-273 (2012).
7. Deinhardt, K. et al. Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. *Neuron*. **52** (2), 293-395, 10.1016/j.neuron.2006.08.018 (2006).
8. Escudero, C.A. et al. c-Jun N-terminal kinase (JNK)-dependent internalization and Rab5-dependent endocytic sorting medaited long-distance retrograde neuronal death induced by axonal BDNF-p75 signaling. *Scientific Reports*. **9**, 6070 (2019).
9. Vrabec, J.P., Levin, L.A. The neurobiology of cell death in glaucoma. *Eye*. **21 Suppl 1**, S11-4 (2007).
10. Liot, G. et al. Mutant huntingtin alters retrograde transport of TrkB receptors in striatal dendrites. *Journal of Neuroscience*. **33** (15), 6298-6309 (2013).
11. Zhou, B., Cai, Q., Xie, Y., Sheng, Z.H. Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. *Cell Reports.* **2** (1), 42-51 (2012).
12. Haubensak, W., Narz, F., Heumann, R., Lessmann, V. BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. *Journal of Cell Science*. **111** (11), 1483-1493 (1998).
13. Adachi, N. et al. Glucocorticoid affects dendritic transport of BDNF-containing vesicles. *Scientific Reports*. **5**, 12684 (2015).

14. Biocompare: The Buyer's Guide for Life Scientists [Internet]. Mirus Bio. Cellular Toxicity Caused by Transfection: Why is it important?. 2012 Sep 17 [cited 2018 Dec 5]; [about 4 screens]. Available from: https://www.biocompare.com/Bench-Tips/121111-Cellular-Toxicity-Caused-by-Transfection-Why-is-it-important/ (2012).
15. Zhao, L. et al. Mechanism underlying activity-dependent insertion of TrkB into the neuronal surface. *Journal of Cell Science*. **122** (17), 3123-3136, 10.1242/jcs.047712 (2009).
16. Zhao, X. et al. Real-time imaging of axonal transport of quantum dot-labeled BDNF in primary neurons. *Journal of Visualized Experiments*. (91), 51899 (2014).
17. Sung, K., Maloney, M., Yang, J., Wu, C. A novel method for producing mono-biotinylated, biologically active neurotrophic factors: an essential reagent for single molecule study of axonal transport. *Journal of Neuroscience Methods.* **200** (2), 121-128 (2011).
18. Deerinck, T. The application of fluorescent quantum dots to confocal, multiphoton and electron microscopic imaging. *Toxicologic Pathology*. **36** (1), 112-116 (2008).
19. Unsain, N., Nuñez, N., Anastasia, A., Mascó, D.H. Status epilepticus induces a TrkB to p75 neurotrophin receptor switch and increases brain-derived neurotrophic factor interaction with p75 neurotrophon receptor: an initial event in neuronal injury induction. *Neuroscience*. **154** (3), 978-993 (2008).
20. Walker, J.M. The bicinchoninic acid (BCA) assay for protein quantitation. *Methods in Molecular Biology.* **32**, 5-8 (1994).
21. Moya-Alvarado, G., Gonzalez, A., Stuardo, N., Bronfman, F.C. Brain-derived neurotrophic factor (BDNF) regulates Rab5-positive early endosomes in hippocampal neurons to induce dendritic branching. *Frontiers in Cellular Neuroscience*. **12**, 493 (2018).
22. Sasi, M., Vignoli, B., Canossa, M., Blum, R. Neurobiology of local and intercellular BDNF signaling. *Pflugers Archiv European Journal of Physiology*. **469** (5), 593-610 (2017).
23. Gonzalez, A., Lazo, O.M., Bronfman, F.C. The Rab5-Rab11 endosomal pathway is required for BDNF-induced CREB transcriptional regulation in neurons. Pre-print at https://www.biorxiv.org/content/10.1101/844720v1 (2019).
24. Mowla, S. J. et al. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. *Journal of Biological Chemistry*. **276** (16), 12660-12666 (2001).
25. Longo, P., Kavran, J., Kim, M.S., Leahy, D. Transient Mammalian Cell Transfection with Polyethyleneimine (PEI). *Methods in Enzymology*. **529**: 227-240 (2013).
26. Raymond, C. et al. A simplified polyethyleneimine-mediated transfection process for large-scale and high-throughput applications. *Methods.* **55** (1), 44-51 (2011).
27. Dalton, A., Barton, W. Over-expression of secreted proteins from mammalian cell lines. *Protein Science*. **23** (5), 517-525 (2014).
28. Hunter, M., Yuan, P., Vavilala, D., Fox, M. Optimization of protein expression in mammalian cells. *Current Protocols in Protein Science*. **95** (1), e77 (2019).
29. Stepanenko, A.A., Heng, H.H. Transient and stable vector transfection: Pitfalls, off-target effects, artifacts. *Mutation Research*. **773**, 91-103 (2017).
30. Guerzoni, L.P., Nicolas, V., Angelova, A. In vitro modulation of TrkB receptor signaling upon sequential delivery of curcumin-DHA loaded carriers towards promoting neuronal survival. *Pharmaceutical Research.* **34** (2), 492-505 (2017).
31. Angelova, A., Angelov, B. Dual and multi-drug delivery nanoparticles towards neuronal survival and synaptic repair. *Neural Regeneration Research*. **12** (6), 886-889 (2017).

Figure 1

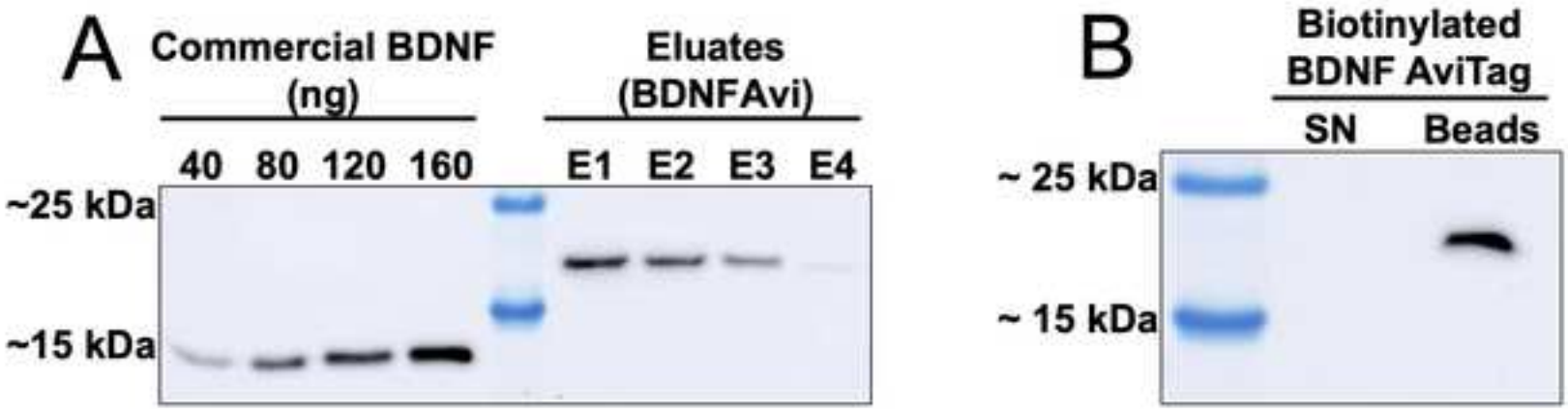


Figure 2

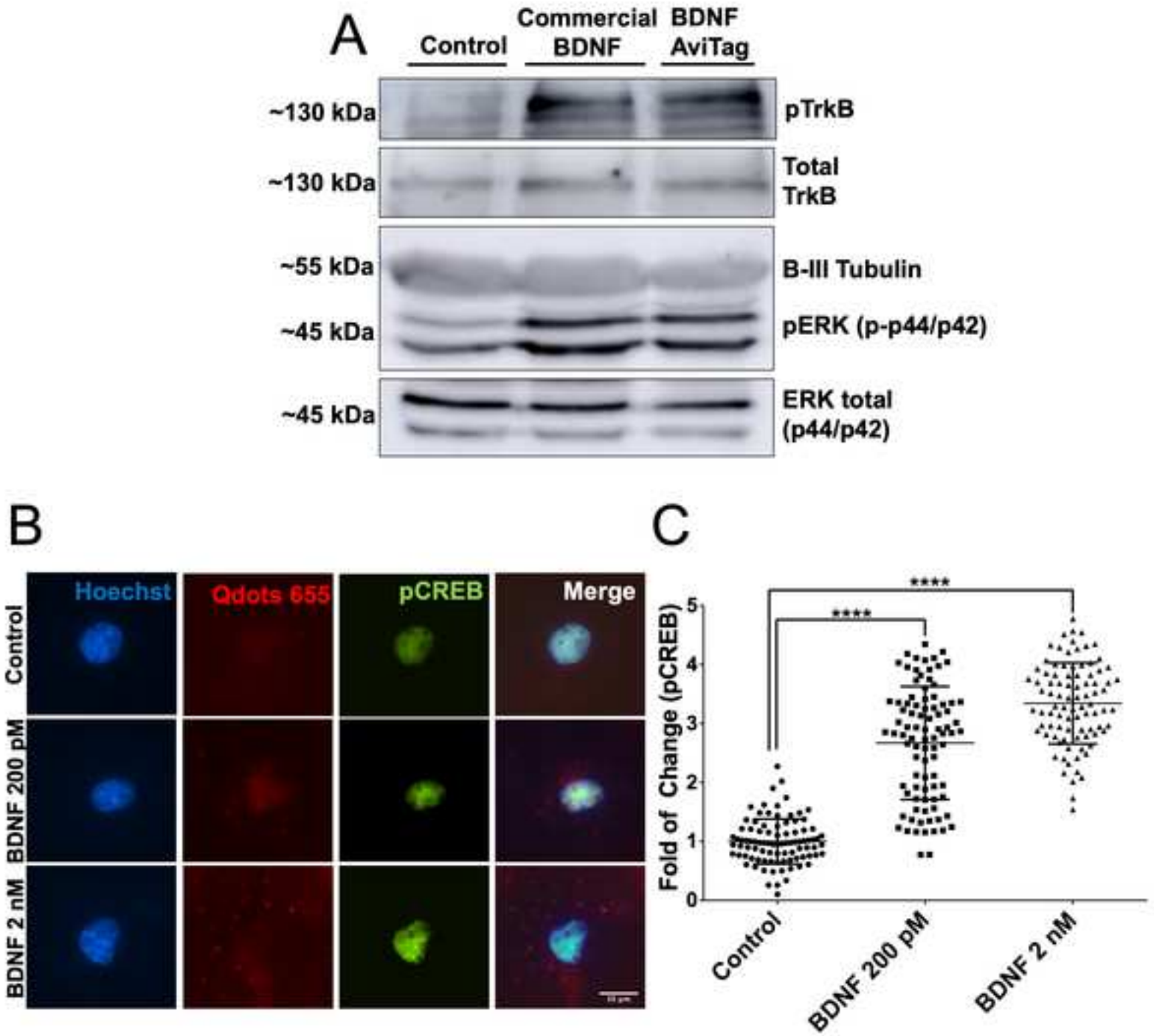
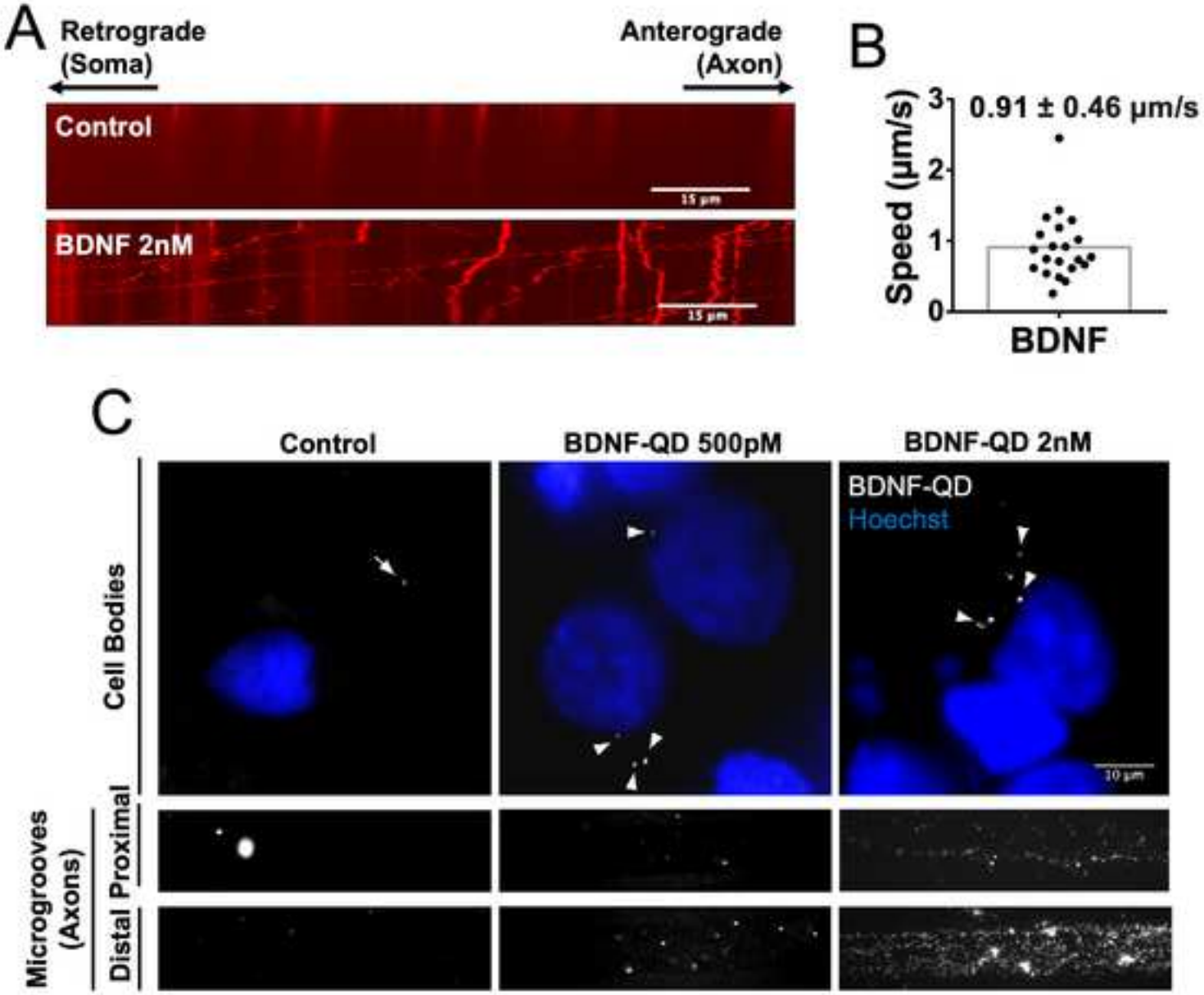


Figure 3



Name of Material/Equipment	Company	Catalog Number	Comments/Description
2 way stopcock	BioRad	7328102	Chromatography apparatus component
2-mercaptoethanol	Sigma	M6250	BDNF elution buffer
Acrylamide/Bisacrylamide	BioRad	1610154	SDS-PAGE gel preparation
Amicon Ultra-15 10K	Millipore	UFC901024	BDNF concentration
Ammonium Persulfate	Sigma	A9164	SDS-PAGE gel preparation
anti B-III-Tubulin antibody	Sigma	T8578	Western blot assays for BDNF biological activity detection
anti BDNF antibody	Alomone	AGP-021	Western blot assays for BDNF quantification
anti BDNF antibody	Alomone	ANT-010	Western blot assays for BDNF quantification
Anti ERK antibody	Cell Signaling	9102	Western blot assays for BDNF biological activity detection
anti pCREB antibody (S133)	Cell Signaling	9198	Western blot assays for BDNF biological activity detection
anti pERK antibody (T202, Y204)	Cell Signaling	4370	Western blot assays for BDNF biological activity detection
anti pTrkB antibody (Y515)	Abcam	ab109684	Western blot assays for BDNF biological activity detection
Antibiotic/Antimycotic	Gibco	15240-062	HEK293 maintenance
ATP	Sigma	A26209	BDNF monobiotinylation buffer
B-27 Supplement	Gibco	17504-044	Neuron maintenance

Bicine	Sigma	B3876	BDNF monobiotinylation buffer
BirA-GST	BPS Bioscience	70031	Enzyme for BDNF AviTag monobiotinylation
Bovine Fetal Serum	HyClone	HC.SH30396.02	HEK293 maintenance
Bovine Serum Albumin	Jackson ImmunoResearch	001-000-162	BDNF buffer modification component, blocking buffer for western blot and immunofluorescence
D-Biotin	Sigma	B4639	BDNF monobiotinylation buffer
Dithiothreitol	Invitrogen	15508-013	
DMEM High Glucose Medium	Gibco	11965-092	Neuron seeding
DMEM Medium	Gibco	11995-081	HEK293 maintenance
Econo Column Funnel	BioRad	7310003	Chromatography apparatus component
EDTA	Merck	108418	
EZ-ECL Kit	Biological Industries	1633664	Protein detection by western blotting
Glutamax	Gibco	35050-061	Neuron and HEK293 maintenance
Glycerol	Merck	104094	BDNF elution buffer, lysis buffer for western blot assays
Hettich Rotina 46R Centrifuge	Hettich	Discontinued	Centrifuge used for clearing the medium of debris
Hettich Universal 32R Centrifuge	Hettich	Discontinued	Centrifuge used for protein concentrator centrifugation

Horse Serum	Gibco	16050-122	Neuron seeding
ImageQuant LAS 500	GE Healthcare Life Sciences	29005063	Western blot image acquisition
Imidazole	Sigma	I55513	BDNF buffer modification component
KCl	Winkler	BM-1370	PBS component
KH_2PO_4	Merck	104873	PBS component
Laminin	Invitrogen	23017-015	Cover coating for compartmentalized neurons
Luer Tubing Adaptor	BioRad	7323245	Chromatography apparatus component
Luminata™ Forte Western HRP Su	Millipore	WBLUF0100	Protein detection by western blotting
$Mg(CH_3COO)_2$	Merck	105819	BDNF monobiotinylation buffer
Mowiol 4-88	Calbiochem	475904	Mounting reagent for immunofluorescence assays
MyOne C1 Streptavidin Magnetic Beads	Invitrogen	65001	Biotinylation verification
Na_2HPO_4	Merck	106586	BDNF buffer modification component
NaCl	Winkler	BM-1630	PBS component, BDNF buffer modification component
NaH_2PO_4	Merck	106346	BDNF buffer modification component
Neurobasal Medium	Gibco	21103-049	Neuron maintenance
Ni-NTA Agarose Beads	Qiagen	30210	BDNF AviTag purification

Nikon Ti2-E	Nikon		Microscope for fluorescence imaging
Nitrocellulose Membrane	BioRad	1620115	Protein transfer for western blotting
ORCA-Flash4.0 V3 Digital CMOS camera	Hamamatsu	C13440-20CU	Camera for epifluorescence imaging
P8340 Protease Inhibitor Cocktail	Sigma	P8340	BDNF buffer modification component
Paraformaldehyde	Merck	104005	Fixative for immunofluorescence assays
Penicillin/Streptomycin	Gibco	15140-122	Neuron maintenance
Poli-D-Lysine	Corning	DLW354210	Cover coating for compartmentalized neurons
Poli-L-Lysine	Millipore	P2363	Cover coating for non-compartmentalized neurons
Poly-Prep Chromatography Column	BioRad	7311550	Chromatography apparatus component
Polyethyleneimine 25K	Polysciences Inc.	PLY-0296	HEK293 transfection
Quantum Dots 655 streptavidin conjugate	Invitrogen	Q10121MP	Monobiotinylated BDNF AviTag label for live and fixed cell experiments
Saponin	Sigma	S4521	Detergent for immunofluorescence assays
Sucrose	Merck	107687	
Syldgard 184 silicone elastomer base	Poirot	4019862	Microfluidic chamber preparation
TEMED	Sigma	T9281	SDS-PAGE gel preparation

Tris	Winkler	BM-2000	Lysis buffer component
Triton X100	Merck	108603	Cell permeabilization in immunofluorescence and western blot assays
Trypsin-EDTA 0.5%	Gibco	15400-054	HEK293 passaging

TITLE:

An Improved Protocol to Purify and Directly Mono-Biotinylate Recombinant BDNF in a Tube for Cellular Trafficking Studies in Neurons

AUTHORS & AFFILIATIONS:

Nicolás Stuardo[1,2], Guillermo Moya-Alvarado[1,3], Carolina Ramírez[1,3], Giampietro Schiavo[4], Francisca C Bronfman*[1,3]

1. Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile
2. Department of Molecular and Cellular Biology, Faculty of Biological Sciences, Center for Aging and Regeneration (CARE UC), Pontificia Universidad Católica de Chile, Santiago, Chile
3. Institute of Biomedical Sciences. Faculty of Medicine and Faculty of Life Sciences. Universidad Andrés Bello, Santiago, Chile
4. Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology and UK Dementia Research Institute at UCL, University College London Campus, London, UK

nstuardo@bio.puc.cl
gmoya@bio.puc.cl
cramireza@bio.puc.cl
giampietro.schiavo@ucl.ac.uk

*Corresponding author:
Francisca C Bronfman
francisca.bronfman@unab.cl

KEYWORDS:

BDNF, mono-biotinylation, quatum dots, axonal trafficking, protein purification, endosome dynamics, in vivo tracking

SUMMARY:

Recombinant BDNF containing an Avi sequence (BDNFAvi) is produced in HEK293 cells in a cost-effective manner and is purified by affinity chromatography. BDNFavi is then directly mono-biotinylated with the enzyme BirA in a tube. BDNFavi and mono-biotinylated BDNFavi retain their biological activity when compared to commercially available BDNF.

ABSTRACT:

Recombinant BDNF containing an Avi sequence (BDNFAvi) is produced in HEK293 cells and then cost-effectively purified by affinity chromatography. We developed a reproducible protocol to directly mono-biotinylate BDNFavi with the enzyme BirA in a tube. In this reaction, mono-biotinylated BDNFAvi retains its biological activity.

Neurotrophins are target-derived growth factors playing a role in neuronal development and maintenance. They require rapid transport mechanisms along the endocytic pathway to allow

long-distance signaling between different neuronal compartments. The development of molecular tools to study the trafficking of neurotrophins has enabled the precise tracking of these proteins in the cell using in vivo recording. In this protocol, we developed an optimized and cost-effective procedure for the production of mono-biotinylated BDNF. A recombinant BDNF variant containing a biotinylable avi sequence (BDNFAvi) is produced in HEK293 cells in the microgram range and then purified in an easily scalable procedure using affinity chromatography. The purified BDNF can then be homogeneously mono-biotinylated by a direct in vitro reaction with the enzyme BirA in a tube. The biological activity of the mono-biotinylated BDNF (mbtBDNF) can be conjugated to streptavidin-conjugated to different fluorophores. BDNF Avi and mbtBDNF retain their biological activity demonstrated through the detection of downstream phosphorylated targets using western blot and activation of the transcription factor CREB, respectively. Using streptavidin-quantum dots, we were able to visualize mbtBDNF internalization concomitant with activation of CREB, which was detected with a phospho-CREB specific antibody. In addition, mbtBDNF conjugated to streptavidin-quantum dots was suitable for retrograde transport analysis in cortical neurons grown in microfluidic chambers. Thus, in tube produced mbtBDNF is a reliable tool to study physiological signaling endosome dynamics and trafficking in neurons.

INTRODUCTION:

Neurons are the functional units of the nervous system possessing a complex and specialized morphology that allows synaptic communication, and thus, the generation of coordinated and complex behavior in response to diverse stimuli. Neuronal projections such as dendrites and axons are critical structural features involved in neuronal communication, and neurotrophins are crucial players in determining their morphology and function(s)[1]. Neurotrophins are a family of secreted growth factors that include NGF, NT-3, NT-4, and brain-derived neurotrophic factor (BDNF)[2]. In the central nervous system (CNS), BDNF participates in diverse biological processes including neurotransmission, dendritic arborization, maturation of dendritic spines, long-term potentiation, among others[3,4]. Therefore, BDNF plays a critical role in regulating neuronal function.

Diverse cellular processes regulate BDNF dynamics and function. On the neuronal surface, BDNF binds the tropomyosin receptor kinase B (TrkB) and/or the p75 neurotrophin receptor (p75). BDNF-TrkB and BDNF-p75 complexes are endocytosed and sorted in different endocytic organelles[5-8]. Correct intracellular trafficking of the BDNF/TrkB complex is required for proper BDNF signaling in different neuronal circuits[9-11]. For this reason, a deep understanding of BDNF trafficking dynamics and its alterations found in pathophysiological processes is essential to understand BDNF signaling in health and disease. The development of novel and specific molecular tools to monitor this process will help to drive this field forward and allow a better grasp of the regulatory mechanisms involved.

There are several tools available for the study of BDNF trafficking in neurons. A commonly used methodology involves the transfection of recombinant BDNF tagged with fluorescent molecules such as green fluorescent protein (GFP) or the monomeric fluorescent red-shifted variant of GFP mCherry[12,13]. However, a major shortcoming of BDNF overexpression is that it eliminates the

possibility of delivering known concentrations of this neurotrophin. Also, it may result in cellular toxicity, obscuring the interpretation of results[14]. An alternative strategy is the transfection of an epitope-tagged TrkB, such as Flag-TrkB. This methodology allows the study of TrkB internalization dynamics[15], but it also involves transfection, which might result in altered TrkB function and cellular toxicity. To overcome these methodological hurdles, recombinant variants of NGF and BDNF containing an Avi sequence (BDNFAvi), which can be mono-biotinylated by the biotin-ligase enzyme BirA, were developed[16,17]. Biotinylated recombinant BDNF can be coupled to different streptavidin-bound tools, which include fluorophores, beads, paramagnetic nanoparticles among others for detection. In terms of live-cell imaging, quantum dots (QD) have become frequently used fluorophores, as they have desirable characteristics for single-particle tracking, such as increased brightness and resistance to photobleaching when compared to small molecule fluorophores[18].

The production of mono-biotinylated BDNF (mbtBDNF) using BDNFAvi has been achieved by co-transfection of plasmids driving the expression of BDNFAvi and BirA, followed by the purification of the recombinant protein by affinity chromatography with a yield of 1-2 µg of BDNF per 20 mL of HEK293-conditioned culture media[17]. Here, we propose a modification of this protocol that allows for BDNFAvi purification from 500 mL of HEK293-conditioned media, which seeks to maximize protein recovery in a chromatography-column based protocol for ease of manipulation. The used transfection agent, polyethyleneimine (PEI), ensures a cost-effective method without sacrificing transfection yield. The mono-biotinylation step has been adapted to an in vitro reaction to avoid the complications associated with co-transfections and to ensure homogeneous labeling of BDNF. The biological activity of the mbtBDNF was demonstrated by western blot and fluorescence microscopy experiments, including activation of pCREB and live cell imaging to study retrograde axonal transport of BDNF in microfluidic chambers. The use of this protocol allows for optimized, high-yield production of homogenous mono-biotinylated and biologically active BDNF.

PROTOCOL:

All experiments were carried out in accordance with the approved guidelines of CONICYT (Chilean National Commission for Scientific and Technological Research). The protocols used in this study were approved by the Biosecurity and Bioethical and Animal Welfare Committees of the P. Catholic University of Chile. Experiments involving vertebrates were approved by the Bioethical and Animal Welfare Committee of the P. Catholic University of Chile.

NOTE: The following protocol was designed to purify BDNFAvi from a total volume of 500 mL of conditioned medium produced in HEK293 cells. The amount of conditioned medium that is produced and processed to purify BDNFAvi can be up or downscaled as needed. However, further optimization may be necessary under these circumstances. The composition of the culture media and buffers used throughout the protocol can be found in supplementary materials.

1. Production and purification of BDNFAvi from HEK293-conditioned media

1.1. Transfection of HEK293 cells

~~1.1.1.~~ Grow HEK293 cells to 70% confluence in supplemented DMEM medium (10% bovine fetal serum, 1x glutamate supplement, 1x antibiotic/antimycotic) in 15 cm culture dishes at 37 ºC.~~.~~ 1.1.1.

Commented [A1]: What are the growth conditions? 37 C?

1.1.2. Change the medium to transfection buffer.

1.1.3. Prepare the PEI-DNA mixture for transfection. Use two different 15 cm conical tubes to dilute DNA and PEI 25 K, respectively. Dilute 20 µg of plasmid DNA in a final volume of 500 µL in one tube. Dilute 60 µg of linear PEI 25K in a final volume of 500 µL in the other tube. Incubate at room temperature for 5 min.

1.1.4. Carefully pipette the DNA solution into the PEI tube, mixing once by up-down motion. Incubate at room temperature for 25 min.

~~1.1.5.~~ Drip 1 mL of the PEI-DNA mixture throughout each 15 cm dish. Incubate the cells with the PEI-DNA mixture for 3 h at 37 ºC.~~.~~ 1.1.5.

Commented [A2]: At RT again?

1.1.6. Change the medium to fresh incubation buffer.

1.2. Media collection and storage

1.2.1. Collect the medium from all the dishes 48 h after the transfection of HEK293 cells. Prepare concentrated stocks of the solutions described in the “supernatant modification buffer” section of **Supplemental File 1** and add them to the HEK293 supernatant to achieve the listed final concentrations.

NOTE: Cells can be discarded or recovered for further analysis.

1.2.2. Incubate the medium in ice for 15 min.

1.2.3. Aliquot the medium into centrifuge tubes.

1.2.4. Centrifuge the medium at 10,000 x *g* for 45 min in a 4 °C centrifuge. This step allows the elimination of cell debris and dead cells suspended in the media.

1.2.5. Collect the supernatants, add BSA at a final concentration of 0.1%. and then store at -20 °C. The media can be aliquoted before freezing for faster thawing during the purification step.

NOTE: Storage times of frozen conditioned media of up to 2 months have yielded positive results, longer storage times have not been evaluated.

1.3. Media concentration and purification

1.3.1. Thaw the media in a 37 °C thermoregulated bath.

1.3.2. Aliquot the media into centrifuge tubes.

1.3.3. Centrifuge the medium for 1 h at 3,500 x *g* in a 4 °C cooled centrifuge. This step allows the elimination of remaining cell debris to ensure adequate flow through the chromatography column.

1.3.4. Use the protein concentrators with a 10 kDa cutoff to reduce the media from 500 mL to 100 mL. Follow the manufacturer's recommended centrifugation parameters for optimal concentration.

1.3.5. Add 500 µL of Ni-NTA agarose beads to the concentrated media and incubate overnight at 4 °C in a rocker.

1.3.6. Assemble the chromatography apparatus and pour the media into it. Let it rest for 5 min and then open the 2-way stopcock to let the medium flow through.

1.3.7. Wash the beads with 5 mL of wash buffer for 5 min. Make sure to resuspend the beads in the column. Drain the wash buffer by opening the 2-way stopcock. Repeat 3 times.

1.3.8. Add 1 mL of elution buffer to the column. Make sure to resuspend the beads in the column. Incubate for 15 min, and then collect the eluate in a 1.5 mL microcentrifuge tube. Repeat this step 3 times for complete elution of BDNFAvi.

1.3.9. Load 5 µL of each eluate and different concentrations of commercially available BDNF (40-160 ng) in a 15% polyacrylamide gel. Detect the purified protein by western blotting using an anti-BDNF antibody.

1.3.10. Determine the concentration of the purified BDNFAvi in each eluate using the concentration curve prepared with the commercially available BDNF.

1.3.11. Aliquot and store the purified BDNFAvi at -80 °C.

2. In vitro mono-biotynilation of BDNFAvi using the BirA enzyme

2.1. In vitro mono-biotinylation reaction

2.1.1. Prepare concentrated stock solutions of the biotinylation buffer reagents. The use of concentrated stocks will minimize the dilution of the recombinant protein.

2.1.2. Take an aliquot of 800 ng of BDNFAvi and add the biotinylation buffer reagents and the enzyme BirA in a 1:1 molar relation to BDNF. For example, for a 200 µL final reaction volume add; 100 µL of solution containing 800 ng of BDNFAvi, 20 µL Bicine 0.5 M pH 8.3, 20 µL ATP 100 mM, 20 µL MgOAc 100 mM, 20 µL d-biotin 500 µM, 0.8-1 µg to 1 µL of BirA-GST, and complete to 200 µL with ultrapure water.

NOTE: Successful biotinylation reactions have been performed with aliquots of 400 µL containing a concentration of about 30 ng/ µL BDNFAvi, resulting in a homogeneously biotinylated BDNFAvi to a final concentration of ~20 ng/ µL in the final reaction.

2.1.3. Incubate the mixture at 30 °C in a hybridization oven for 1 h. Mix the content by tube inversion every 15 min.

2.1.4. Add the same volume of ATP and BirA as in step 2.1.2~~1~~ and repeat step 2.1.3.

2.1.5. Store at -80 °C for future analyses or keep on ice for immediate use (e.g., biotinylation quality control).

2.2. Biotinylation analysis

2.2.1. Block 30 µL of streptavidin magnetic beads per BDNF sample in 1 mL of blocking buffer. Incubate at room temperature for 1 h in a microcentrifuge tube rotator.

2.2.2. Precipitate the magnetic beads using a magnetic separation rack for 3 to 5 minutes or until the buffer appears completely cleared of the beads and discard the blocking buffer.

2.2.3. Add 50 µL of fresh blocking buffer and 80 ng of mono-biotinylated BDNFAvi (mbtBDNF) sample to the beads, making sure to resuspend them completely by pippeting.

2.2.4. Incubate at 4 °C for 1 h in a microcentrifuge tube rotator spinning at approximately 20 RPM.~~.~~

2.2.5. Collect the beads using the magnetic separation rack for 3 to 5 minutes, and collect the supernatant, keeping a 30 µL aliquot for analysis.

2.2.6. Wash the beads one time with 500 µL of PBS, and then collect them using the magnetic separation rack for 3 to 5 minutes. Recover the supernatant and keep a 30 µL aliquot for analysis.

2.2.7. Add 10 µL of 4x loading buffer to the beads.

2.2.8. Heat the samples to 97 °C for 7 min to eluate the mbtBDNF.

2.2.9. Detect mbtBDNF using an anti-BDNF specific antibody[19].

Commented [A3]: What is added here and how much? What steps are being referred to?

Commented [A4]: For how long?

Commented [A5]: By pipetting?

Commented [A6]: Is the rotator on? If so, at what speed? Rpm?

Commented [A7]: How long are the beads on the rack?

Commented [A8]: How long are the beads on the rack? 5 min?

Commented [A9]: Please include a citation here.

3. Verification of mbtBDNF biological activity

3.1. Detection of pTrkB and pERK by western blot.

3.1.1. Seed 2 million rat cortical neurons in 60 mm culture dishes.

3.1.2. Culture the neurons for 7 days (DIV7). Then, change the medium to non-supplemented neurobasal mediun when starting the experiment.

3.1.3. One hour after medium change, add mbtBDNF to a final concentration of 50 ng/mL. Incubate for 30 min at 37 ºC. Keep a negative control dish (non-stimulated with BDNF) and a positive control dish (treated with 50 ng/mL of commercially available BDNF).

Commented [A10]: At what temperature?

3.1.4. Collect the medium and gently wash every dish with 1x PBS. Collect and discard the 1x PBS.

3.1.5. Place the dishes on ice and add 50-80 µL of lysis buffer to each dish. Use a cell scraper to lyse the cells.

NOTE: The lysis step should be performed as quickly as possible to avoid protein dephosphorylation and degradation. 1-2 minutes of vigorous scraping are enough to visualize the proteins of interest by western blotting.

3.1.6. Collect the lysis buffer and stir in a vortex mixer at highest speed for 5 s.

3.1.7. Centrifuge the lysis buffer at 14,000 x *g* (4 °C) for 10 min. Collect the supernatant.

3.1.8. Quantify the protein content of the supernatant by BCA protein quantification protocol[20].

Commented [A11]: How is this done? Please specify the method and provide a citation.

3.1.9. Add loading buffer to an aliquot containing 30-50 µg of protein per condition and load it in a 12% polyacrylamide gel for western blotting. Detect pTrkB and pERK using specific phosphor-antibodies to verify BDNFAvi biological activity.

3.2. Verification of BDNF-QD biological activity by pCREB immunofluorescence.

~~3.2.1.~~ Seed 40,000 rat cortical neurons in 10 mm coverslips, previously autoclaved and treated with poly-L-lysine as described previously[21].

Commented [A12]: How was this done? Citation?

3.2.1.

3.2.2. Culture the neurons for 7-8 days in neuronal maintenance buffer (see supplemental materials) at 37 ºC. ~~. Then, change the medium to non-supplemented neurobasal medium when you decide to start the experiment for 1 h.~~

Commented [A13]: At what conditions?

Formatted: No bullets or numbering

~~3.2.2.~~3.2.3. To start the experiment, change the medium to unsupplemented neurobasal medium and incubate at 37 ºC for 1 h.

~~3.2.3.~~3.2.4. Prepare mbtBDNF conjugated to quantum dots (BDNF-QD) by adding to a mbtBDNF aliquot, the necessary volume of quantum dot streptavidin conjugate (streptoavidein-QD) to achieve a 1:1 BDNF-QD molar ratio. Then, dilute to 20 µL with neurobasal medium. Wrap the tube in aluminum foil to protect it from the light.

NOTE: Prepare another tube with the same volume of quantum dot streptavidin conjugate and dilute it to 20 µL with neurobasal medium as a negative control.

~~3.2.4.~~3.2.5. Incubate the mbtBDNF/ streptavidin-QD mixture for 30 min at room temperature in a rocker.

~~3.2.5.~~3.2.6. Dilute the BDNF-QD to the desired final concentration (200 pM and 2 nM) in neurobasal medium.

~~3.2.6.~~3.2.7. After 1 h of incubation with non-supplemented neurobasal medium, stimulate the neurons with BDNF-QD or streptavidin-QD (control) to a final concentration of 200 pM and 2 nM of BDNF for 30 min at 37 °C.

~~3.2.7.~~3.2.8. Wash the coverslips 3 times with 1x PBS (37 °C) and fix the cells for 15 min by treating the coverslip with 4% paraformaldehyde solution containing phosphatase inhibitors.

~~3.2.8.~~3.2.9. Wash the cells 3 times with PBS, and then incubate with blocking/permeabilization buffer (BSA 5%, Triton X-100 0.5%, 1x phosphatase inhibitor) for 1 h.

~~3.2.9.~~3.2.10. Incubate with anti-pCREB antibody 1:500 (in 3% BSA, 0.1% Triton X-100) overnight at 4 °C.

~~3.2.10.~~3.2.11. The following day, wash 3 times with 1x PBS, and incubate for 1 h with the secondary antibody 1:500 (3% BSA, 0.1% Triton X-100).

~~3.2.11.~~3.2.12. Wash 3 times with 1x PBS. Add Hoechst nuclear stain solution (5 µg/mL) for 7 min.

Commented [A14]: Dilution or concentration used?

~~3.2.12.~~3.2.13. Wash 3 times with 1x PBS and mount.

3.3. Visualization of retrograde axonal transport of BDNF-QD in live neurons

3.3.1. Prepare microfluidic chambers and seed neurons as described previously[16].

3.3.2. After 7-8 days in culture, change the medium to non-supplemented neurobasal medium.

3.3.3. Prepare mbtBDNF conjugated to quantum dots (BDNF-QD) by adding to a mbtBDNF aliquot, the necessary volume of quantum dot streptavidin conjugate (streptavidin-QD) to achieve a 1:1 BDNF-QD molar ratio. Then, dilute to 20 µL with neurobasal medium. Wrap the tube in aluminum foil to protect it from the light.

NOTE: Prepare another tube with the same volume of quantum dot streptavidin conjugate and dilute it to 20 µL with neurobasal medium as a control.

3.3.4. Incubate the mbtBDNF/ streptavidin-QD mixture for 30 min at room temperature in a rocker.

3.3.5. Dilute the BDNF-QD to the desired final concentration (2 nM).

3.3.6. After 1 h of incubation with non-supplemented neurobasal medium add the BDNF-QD or the control mixture to the axonal compartments of the microfluidic chamber. Incubate for 210 min at 37 ˚C to ensure a net retrograde transport of BDNF-QD.

3.3.7. For live-cell imaging, visualize axonal retrograde transport in the segment of the microgrooves that is proximal to the cell body compartment using a 100x objective using a microscope suitable for these purpose (37 °C and 5% CO_2). Acquire images at 1 frame/s.

REPRESENTATIVE RESULTS:

The use of a chromatographic column-based protocol allows the processing of significant volumes of HEK293 conditioned media. In **Figure 1**, the results of the purification of BDNFAvi from 500 mL of conditioned media are shown. Consecutive elutions of BDNFAvi from the Ni-NTA agarose beads yield decreasing concentrations of BDNFAvi (**Figure 1A**). After four consecutive elutions (each lasting 15 min), the majority of the BDNF captured by the beads is recovered. The concentrations of the eluates range from 6 to 28 ng/µL, and the total yield amounted to approximately 60 µg of BDNFAvi (**Table 1**). The produced BDNFAvi was then efficiently biotinylated by an in vitro reaction mediated by BirA-GST, as demonstrated by the lack of non-biotinylated BDNFAvi in the supernatant (**Figure 1C**~~)~~**B**. Please note that the biotinylation presented in **Figure 1B**~~C~~ corresponds to an aliquot of the total BDNF produced, but the reaction can be scaled up for bigger volumes.

Then, the biological activity of mbtBDNF was evaluated using 2 different experimental approaches. First, cortical neurons seeded in 60 mm plates (2 million neurons, DIV7) were stimulated with 50 ng/mL of mbtBDNF for 30 min, and then proteins were prepared for western blot analysis. The biological activity of the mbtBDNF was quantified by detecting pTrkB (Y515) and pERK (T202/Y204). Binding of BDNF to TrkB triggers the activation of the receptor through an autophosphorylation reaction in its intracellular domain, and ERK is a known target of the BDNF signaling pathway[22~~19~~]. The bands for both phosphorylated proteins had a similar intensity in neurons treated with commercial BDNF and mbtBDNF, and both showed a stronger signal than control condition (**Figure 2A**). Then, the biological activity of mbtBDNF coupled to streptavidin-QD was evaluated to demonstrate that they can be used in live imaging experiments. Cortical

Commented [A15]: Where is this table?

Commented [A16R15]: This table was submitted in a separate .xlsx file

Commented [A17]: There is no Figure 1C. Please revise.

neurons were seeded in 10 mm covers (40,000 cells per cover, DIV7) and treated with a final concentration of 200 pM or 2 nM BDNF-QD for 30 min before fixing and staining for pCREB. CREB is a transcription factor which is targeted by activated ERK1/2 in cortical neurons[22,29,23,30]. Stimulating neurons with increasing concentrations of BDNF-QD resulted in a dose-dependent increase of phosphorylation of CREB and presence of QD particles surrounding the nucleus (**Figure 2B**), indicating that the BDNF-QD particles were endocytosed and triggered the activation of signaling pathways associated with BDNF-mediated TrkB activation. A twofold increase in pCREB signal was detected when stimulating neurons with a low concentration of BDNF-QD (200 pM), whereas stimulating with 2 nM resulted in a 3.5-fold increase in the pCREB signal (**Figure 2C**). These results demonstrate that the biotinylated BDNFAvi is biologically active, and that it does not lose its activity when coupled to streptavidin-QD, making it suitable for immunofluorescence and live cell imaging.

Finally, the imaging potential of BDNF-QD was evaluated in compartmentalized cultures using microfluidic chambers. Cortical neurons were seeded in microfluidic chambers (15 mm covers, 50,000 neurons per microfluidic chamber, DIV7) to separate the axonal and somatodendritic compartments and were stimulated with 2 nM BDNF-QD for 3.5 h. Live cell microscopy was performed, and the resulting kymographs were used to quantify the speed of BDNF-QD containing organelles (**Figure 3A**). An average moving speed of 0.91 µm/s was detected (**Figure 3B**), which is in line with previous analyses of cytoplasmic dynein-mediated transport[7,16]. Microfluidic chambers treated with 2 nM streptavidin-QD did not show moving QDs in the microgrooves, as shown by the kymograph (**Figure 3A**). Cells grown under the same conditions were stimulated with 500 pM or 2 nM BDNF-QD for 210 min, and then fixed and labelled with a nuclear staining. As shown in **Figure 3C**, neurons show a dose-dependent accumulation of BDNF-QD in all the analyzed sub-compartments, including the proximal and distal portions of the microgroove and the somatodendritic compartment. In contrast, control neurons showed almost no QD signal throughout the chamber. Therefore, the BDNF-QD can be detected in live and fixed cells in microfluidic chambers.

FIGURE AND TABLE LEGENDS:

Figure 1: Production and mono-biotinylation of BDNFAvi in HEK293 cells. HEK293 cells were transfected using the PEI reagent and a BDNFAvi encoding plasmid and the conditioned media was collected after 48 h. BDNFAvi contains a 6x Histidine tag allowing purification using nickel-nitrilotriacetic acid (Ni-NTA) chromatography. Commercially available recombinant human BDNF has an expected molecular weight of ~13 kDa, whereas BDNFAvi displays a molecular weight of ~18 kDa. BDNFAvi bound to the resin was fully eluted with four consecutive elution steps. (**A**) Western blot using anti-BDNF antibodies to detect in house prepared recombinant BDNF and commercial BDNF. Aliquots containing known amounts of commercially available human BDNF and 5 µL of each eluate were loaded into an SDS-PAGE gel for detection of BDNFAvi using an antibody against BDNF. **Table 1** indicates the concentrations of BDNFAvi present in each eluate. The amount and concentration of BDNF in each eluate was obtained by densitometric analysis and interpolation from the concentration curve of commercially available BDNF. (**B**) Verification of BDNFAvi biotinylation. Eighty nanograms of biotinylated BDNFAvi (mbtBDNF) were incubated with 30 µL of streptavidin coupled to magnetic beads (20% slurry) for 1 hr at 4 °C. Then, magnetic

beads were isolated using a magnetic separator. The streptavidin beads were heated with loading buffer to elute the biotinylated BDNFAvi (beads lane). The supernatant (SN lane) was also treated with loading buffer, heated and loaded in the gel (SN lane).

Figure 2: Verification of mbtBDNF biological activity. (**A**) DIV7 cortical neurons were serum starved for 1 h, and then stimulated with 50 ng/mL of commercially-available BDNF or mbtBDNF for 30 min. Proteins were extracted and loaded in an SDS-PAGE gel for analysis of TrkB and ERK1/2 phosphorylation using phospho-specific antibodies and compared to the total levels of the protein using antibodies against total TrkB and ERK1/2. (**B**) DIV7 cortical neurons were serum starved for 1 h, and then stimulated with a final concentration of 200 pM or 2 nM of mbtBDNF coupled to streptavidin-QD (BDNF-QD) for 30 min. Then, cells were fixed and pCREB was labelled for fluorescence microscopy analysis. (**C**) Quantification of nuclear pCREB fluorescence intensity. The results correspond to 90 neurons pooled together from 3 independent experiments, shown as mean ± SEM. The statistical analysis corresponds to a one-way ANOVA with Tukey's multiple comparisons test (****$p < 0.0001$).

Figure 3: Visualization of BDNF-QD in live and fixed cells. (**A**) DIV7 cortical neurons grown in microfluidic chambers were stimulated in the axonal compartment with a final concentration of 2 nM BDNF-QD for 3.5 hrs, and then the proximal portion of the microgrooves was imaged using a live cell microscopy setting. Representative kymographs for control condition (treated with streptavidin-QD) and upon treatment with BDNF-QD are shown. (**B**) Quantification of the speed of moving BDNF-QD. Mobile puncta were defined as those that moved more than 10 µm in the 120 s of recording. (C) DIV7 cortical neurons grown in microfluidic chambers were stimulated in the axonal compartment with a final concentration of BDNF-QD of 500 pM or 2 nM for 3.5 hrs, and then fixed and labelled with Hoechst to visualize the nuclei. Representative images of the somatodendritic compartment and the distal and proximal portions of the microgrooves are shown.

Table 1: Quantification of BDNFAvi purification yield (related to Fig. 1A). HEK293 cells were transfected with a plasmid driving BDNFAvi expression, and the protein was purified by Ni-NTA affinity chromatography. Protein concentration and final yield was calculated by densitometric analysis and interpolation in the known concentration curve of commercially available recombinant human BDNF.

Supplemental File 1: Culture media and buffer components

DISCUSSION:

In this article, an optimized methodology for the production and purification of mbtBDNF in an affinity chromatography-based procedure is described, based on the work of Sung and collaborators[17]. The optimizations include the use of a cost-effective transfection reagent (PEI) while maintaining the efficiency of more expensive transfection methods such as lipofectamine. This optimization translates into a significant cost reduction in the protocol, allowing for scalability while maintaining high cost-effectiveness. The protocol also includes ease of use considerations, including the freezing of conditioned media for up to 2 months. These

optimizations make the procedure adaptable to each laboratory's needs, improve cost-effectiveness, and yield homogeneous and biologically active recombinant BDNF. The protocol can also be adapted to smaller scale productions by replacing the use of the chromatography apparatus with gravitational precipitation of the beads in conical tubes. This constitutes a viable methodology, but its less time-efficient and has resulted in lower yields in our experience. The biotin-labeled BDNF can then be coupled to different streptavidin-bound probes, including fluorophores and paramagnetic nanoparticles, making it a valuable tool to perform diverse types of experiments for the analysis of BDNF post-endocytic trafficking. Therefore, an optimized and simple production protocol for this protein is highly useful to laboratories working in this field.

Production of recombinant proteins with complex post-translational modifications, such as BDNF[24,41], in prokaryotic systems often results in proteins that are not correctly folded and thus have poor biological activity[25,52]. Therefore, expression in mammalian cells is necessary to obtain a bioactive protein. The use of PEI has been described previously as a viable alternative for large-scale production of recombinant proteins in transfected mammalian cells[25,52,26,63], and its efficiency in the transfection of the HEK293 cells in the context of academic laboratories has been highlighted[27,74]. Therefore, the use of this cell line represents a valid option to produce BDNFAvi on a scale that can be managed by an academic laboratory. The proposed protocol could be optimized further by the generation of a HEK293 cell line stably transfected with BDNFAvi, which would eliminate the transient transfection step, thus saving time and resources. Another potential source of optimization is the use of cells in suspension instead of adherent cells. HEK293 cells can be maintained in suspension, generating significant amounts of recombinant protein in the range of grams per liter[28,85].

Another improvement in the protocol is the biotinylation of the BDNFAvi protein using an in vitro strategy, replacing the previous in vivo co-transfection protocol. Transient co-transfection can have unexpected results in terms of the expression of the constructs, as has been demonstrated in multiple cell lines and with several transfection reagents[29,96]. Various factors can affect the expression of transfected proteins in a co-transfection context, including vectors, cell types and plasmid concentration. This multiplicity of factors makes optimization and reproducibility a complex task. On the other hand, an in vitro methodology allows for better control over the conditions in which the biotinylation reaction takes place. This methodology results in reproducible and homogeneous labeling of recombinant BDNF.

As demonstrated by the biological activity verification experiments, the mbtBDNF produced using this protocol is comparable to commercially-available recombinant human BDNF in terms of BDNF-TrkB signaling pathway activation. The data also shows that coupling BDNF to streptavidin-QD does not interfere with BDNF-TrkB signaling. In addition, we showed that BDNF-QD can be detected by epifluorescence microscopy in live and fixed cells. Therefore, mbtBDNF represents a valuable tool for studying retrograde axonal trafficking and it presents significant advantages over alternative probes, such as BDNF-GFP[16].The protocol described in this article provides a reliable and consistent methodology for the production of mbtBDNF, which can then be used in post-endocytic dynamics studies in different neuronal models expressing TrkB or p75. BDNF signaling has potent effects on neuronal morphology and function[3,4,21,17], and has been recently

proposed as a potential therapeutic tool to enhance neuronal regeneration[30,31], making its study relevant in the fields of cellular biology and biomedicine. The study of the effects of BDNF signaling and trafficking will further advance our understanding of neuronal cell biology and may allow for the harnessing of its regenerative potential in clinical settings.

ACKNOWLEDGMENTS:

The authors gratefully acknowledge financial support from Fondecyt (1171137) (FCB), the Basal Center of Excellence in Science and Technology (AFB 170005) (FCB), Millenium-Nucleus (P07/011-F) (FCB), the Wellcome Trust Senior Investigator Award (107116/Z/15/Z) (GS) and a UK Dementia Research Institute Foundation award (GS). This work was supported by the Unidad de Microscopía Avanzada UC (UMA UC).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Huang E, Reichardt L. Neurotrophins: Roles in Neuronal Development and Function. *Annual Review of Neuroscience*, 24: 677-736, 10.1146/annurev.neuro.24.1.677 (2001)

2. Skaper SD. The neurotrophin family of neurotrophic factors: an overview. *Methods in Mollecular Biology*. 846: 1-12, 10.1007/978-1-61779-536-7_1. (2012)

3. Gonzalez A, Moya-Alvarado G, Gonzalez-Billault C, Bronfman FC. Cellular and molecular mechanism regulating neuronal growth by brain-derived neurotrophic factor. *Cytoskeleton*. 73 (10): 612-628, 10.1002/cm.21312. (2016)

4. Cunha C, Brambilla R, Thomas K. A simple role for BDNF in learning and memory? *Frontiers in Mollecular Neuroscience*. 3: 1, 10.3389/neuro.02.001.2010 (2010)

5. Bronfman FC, Lazo OM, Flores C, Escudero CA. Spatiotemporal intracelular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. In: Lewin G, Carter B (eds) Neurotrophic Factor. Handbook of Experimental Pharmacology, vol 220. Springer, Berlin, Heidelberg (2014).

6. Ascano M, Bodmer D, Kuruvilla R. Endocytic trafficking of neurotrophins in neural development. *Trends in Cell Biology*. **22** (5): 266-273, 10.1016/j.tcb.2012.02.005 (2012)

7. Deinhardt K, Salinas S, Verastegui C, Watson R, Worth D, Hanrahan S, Bucci C, Schiavo G. Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. *Neuron*. **52** (2): 293-395, 10.1016/j.neuron.2006.08.018 (2006)

8. Escudero CA et al. c-Jun N-terminal kinase (JNK)-dependent internalization and Rab5-dependent endocytic sorting medaited long-distance retrograde neuronal death induced by axonal BDNF-p75 signaling. *Scientific Reports*. **9**: 6070, 10.1038/s41598-019-42420-6 (2019)

9. Vrabec JP, Levin LA. The neurobiology of cell death in glaucoma. *Eye*. **21 Suppl 1**: S11-4, 10.1038/sj.eye.6702880 (2007)

10. Liot G, Zala D, Pla P, Mottet G, Piel M, Saudou F. Mutant huntingtin alters retrograde transport of TrkB receptors in striatal dendrites. *Journal of Neuroscience*. **33** (15): 6298-6309, 10.1523/JNEUROSCI.2033-12.2013 (2013)

11. Zhou B, Cai Q, Xie Y, Sheng ZH. Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. *Cell Reports*. **2** (1): 42-51, 10.1016/j.celrep.2012.06.010 (2012)

12. Haubensak W, Narz F, Heumann R, Lessmann V. BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. *Journal of Cell Science*. **111** (11): 1483-1493 (1998)

13. Adachi N et al. Glucocorticoid affects dendritic transport of BDNF-containing vesicles. *Scientific Reports*. **5:** 12684, 10.1038/srep12684 (2015)

14. Biocompare: The Buyer's Guide for Life Scientists [Internet]. Mirus Bio. Cellular Toxicity Caused by Transfection: Why is it important?. 2012 Sep 17 [cited 2018 Dec 5]; [about 4 screens]. Available from: https://www.biocompare.com/Bench-Tips/121111-Cellular-Toxicity-Caused-by-Transfection-Why-is-it-important/ (2012)

15. Zhao L et al. Mechanism underlying activity-dependent insertion of TrkB into the neuronal surface. *Journal of Cell Science*. **122** (17): 3123-3136, 10.1242/jcs.047712 (2009)

16. Zhao X, Zhou Y, Weissmiller A, Pearn M, Mobley W, Wu C. Real-time imaging of axonal transport of quantum dot-labeled BDNF in primary neurons. *Journal of Visualized Experiments*. **91**: 51899, 10.3791/51899 (2014)

17. Sung K, Maloney M, Yang J, Wu C. A novel method for producing mono-biotinylated, biologically active neurotrophic factors: an essential reagent for single molecule study of axonal transport. *Journal of Neuroscience Methods.* **200** (2): 121-128, 10.1016/j.jneumeth.2011.06.020 (2011)

18. Deerinck T. The application of fluorescent quantum dots to confocal, multiphoton and electron microscopic imaging. *Toxicologic Pathology*. **36** (1): 112-116, 10.1177/0192623307310950 (2008)

19. Unsain N, Nuñez N, Anastasia A, Mascó DH. Status epilepticus induces a TrkB to p75 neurotrophin receptor switch and increases brain-derived neurotrophic factor interaction with p75 neurotrophon receptor: an initial event in neuronal injury induction. *Neuroscience*. **154** (3): 978-993, 10.1016/j.neuroscience.2008.04.038. (2008)

20. Walker JM. The bicinchoninic acid (BCA) assay for protein quantitation. *Methods Mol Biol.* **32**: 5-8 (1994)

Formatted: Font: (Default) +Body (Calibri), Font color: Auto

Formatted: Indent: Left: 0.5", No bullets or numbering, No widow/orphan control, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

Formatted: Spanish (Spain)

Formatted: English (United States)

Formatted: Font: (Default) +Body (Calibri), Font color: Auto

Formatted: Indent: Left: 0.5", No bullets or numbering, No widow/orphan control, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

Formatted: Font: (Default) +Body (Calibri), Font color: Auto

Formatted: Indent: Left: 0.5", No bullets or numbering, No widow/orphan control, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

21. Moya-Alvarado G, Gonzalez A, Stuardo N, Bronfman FC. Brain-derived neurotrophic factor (BDNF) regulates Rab5-positive early endosomes in hippocampal neurons to induce dendritic branching. *Frontiers in Cellular Neuroscience*. **12**: 493, 10.3389/fncel.2018.00493 (2018)

~~18.~~

~~19.~~22. Sasi M, Vignoli B, Canossa M, Blum R. Neurobiology of local and intercellular BDNF signaling. *Pflugers Archiv European Journal of Physiology*. **469** (5): 593-610, 10.1007/s00424-017-1964-4 (2017)

~~20.~~23. Gonzalez A, Lazo OM, Bronfman FC. The Rab5-Rab11 endosomal pathway is required for BDNF-induced CREB transcriptional regulation in neurons. Pre-print at https://www.biorxiv.org/content/10.1101/844720v1 (2019)

~~21.~~24. Mowla et al. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. *Journal of Biological Chemistry*. **276** (16): 12660-12666, 10.1074/jbc.M008104200 (2001)

~~22.~~25. Longo P, Kavran J, Kim MS, Leahy D. Transient Mammalian Cell Transfection with Polyethyleneimine (PEI). *Methods in Enzymology*. **529**: 227-240, 10.1016/B978-0-12-418687-3.00018-5 (2013)

~~23.~~26. Raymond C, Tom R, Perret S, Moussouami P, L'Abbé D, St-Laurent G, Durocher Y. A simplified polyethyleneimine-mediated transfection process for large-scale and high-throughput applications. *Methods.* **55** (1): 44-51, 0.1016/j.ymeth.2011.04.002 (2011)

~~24.~~27. Dalton A, Barton W. Over-expression of secreted proteins from mammalian cell lines. *Protein Science*. **23** (5): 517-525, 10.1002/pro.2439 (2014)

~~25.~~28. Hunter M, Yuan P, Vavilala D, Fox M. Optimization of protein expression in mammalian cells. *Current Protocols in Protein Science*. **95** (1): e77, 10.1002/cpps.77 (2019)

~~26.~~29. Stepanenko AA, Heng HH. Transient and stable vector transfection: Pitfalls, off-target effects, artifacts. *Mutation Research*. **773**: 91-103, 10.1016/j.mrrev.2017.05.002. (2017)

~~27. Moya-Alvarado G, Gonzalez A, Stuardo N, Bronfman FC. Brain-derived neurotrophic factor (BDNF) regulates Rab5-positive early endosomes in hippocampal neurons to induce dendritic branching. *Frontiers in Cellular Neuroscience*. **12**: 493, 10.3389/fncel.2018.00493 (2018)~~

~~28.~~30. Guerzoni LP, Nicolas V, Angelova A. In vitro modulation of TrkB receptor signaling upon sequential delivery of curcumin-DHA loaded carriers towards promoting neuronal survival. *Pharmaceutical Research.* **34** (2): 492-505, 10.1007/s11095-016-2080-4 (2017)

Formatted: No bullets or numbering

~~29.~~31. Angelova A, Angelov B. Dual and multi-drug delivery nanoparticles towards neuronal survival and synaptic repair. *Neural Regeneration Research*. **12** (6): 886-889, 10.4103/1673-5374.208546. (2017)

Transfection and post transfection incubation buffer
Base Dulbecco's modified Eagle medium (DMEM)
Antibiotic/Antimycotic 1x
Glutamate supplement 1x

Supernatant modifier solutions
To every 20 mL of transfected HEK293 supernatant add:
0.6 g NaCl (Final concentration considering DMEM NaCl content: 0.5 M)
0.5 mL of Imidazole 1 M (Final concentration: 20 mM)
1.7 mL of Na_2HPO_4 0.4 M (Final concentration: 27.12 mM)
0.25 mL of NaH_2PO_4 0.2 M (Final concentration: 1.98 mM)
0.25 mL of 100 X Protease Inhibitor cocktail (Final concentration: 1x)
2.3 mL of distilled H2O
If prepared correctly, pH = 8 will be achieved

Wash Buffer
Na_2HPO_4 20 mM
NaH_2PO_4 20 mM
NaCl 500 mM
Imidazole 20 mM
Protease Inhibitor Cocktail 1x
Adjust to pH 8

Elution Buffer
Na_2HPO_4 20 mM
NaH_2PO_4 20 mM
NaCl 500 mM
Imidazole 300 mM
Glycerol 15%
2-mercaptoethanol 2 mM
Protease Inhibitor Cocktail 1x
Adjust to pH 8

Biotinylation Buffer
Purified BDNF AviTag (800 ng aliquot)
Bicine 50 mM
ATP 10 mM
MgAcO 10 mM

D-biotin 50 µM
BirA-GST in a 1:1 molar relation to BDNF AviTag

Blocking Buffer **(To block streptavidin magnetic beads in the biotinylation verification step)**
3% Bovine Serum Albumin (BSA)
0.1% Triton X100
Dilute in PBS 1x

Lysis Buffer **(For cell lysis in the biological activity verification step)**
Tris 2 mM
NaCl 13.7 mM
EDTA 0.2 mM
8.7% glycerol
0.01% Triton X100
Protease and Phosphatase Inhibitor Cocktails 1x

Unsupplemented neurobasal medium **(for serum deprivation of cortical neuron)**
Base Neurobasal medium
Glutamate supplement 1x
Penicillin-Streptomycin 1x

Neuronal maintenance medium (for neuronal culture maintenance)
Base Neurobasal medium
Glutamate supplement 1x
Penicillin-Streptomycin 1x
2% B-27 supplement