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TESIS DOCTORAL

Mechanisms of long-distance control of dendritic growth by Brain-Derived Neurotrophic Factor (BDNF) in central neurons

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Deja La Vida Volar.

Victor Jara (1966)

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ABBREVIATIONS

4E-BP1/2: Eukaryotic translation initiation factor 4E-binding protein 1

AAV: Adeno-associated viruses

AC: Axonal compartment

Act: Actinomycin D

Akt: Protein Kinase B (PKB)

APPL1: Phosphotyrosine interacting with PH domain and leucine zipper 1

AraC: Cytosine β-D-arabinofuranoside

Arc: Activity-regulated cytoskeleton-associated gen

ARMS: Ankyrin Repeat-rich Membrane Spanning

Bcl-2: B-cell lymphoma 2 protein

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin (BSA)

CaMKII: Ca2+ /calmodulin-dependent protein kinase II

CA: Constitutively active

CB: Cell body compartment

CHX: Cycloheximide

CilioD: Ciliobrevin D

CNS: Central Nervous System

CONICYT: Chilean National Commission for Scientific and Technological Research

CREB: Cyclic AMP-response element binding protein

Ctb: Cholera toxin B subunit

DIV: Days in vitro

DN: Dominant negative

DNA: Deoxyribonucleic acid

DMEM: Dulbecco's Modified Eagle's Medium

DTT: Dithiothreitol

EEA1: Early Endosome Antigen 1

EDTA: Ethylenediaminetetraacetic acid

EGFP: Enhanced green fluorescence protein

ERK: Extracellular signal–regulated kinase

FRAP: Fluorescence recovery after photobleaching

GAP: GTPase-activating protein

GEF: Guanine exchanger factor

GluR1: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

GST: Glutathione S transferase

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HS: Horse serum

IPTG: Isopropyl β-D- thiogalactoside

IRS-1: Insulin receptor substrate 1

JIP1: JNK-interacting protein 1

JNK: c-Jun NH2-terminal kinases

KIF: Kinesin super family

LY: LY294002 (general PI3 kinase inhibitor)

MAP2: Microtubule-associated protein 2

MAPK: Mitogen activated protein kinase

MEM: Minimum essential medium

MG: Microgroove

M-MLV: Maloney Murine Leukemia Virus Reverse Transcriptase

mTORC: Mammalian target of rapamycin complex

mRNA: Messenger ribonucleic acid

NF-kB: Nuclear factor-kB

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartate receptor

NT3: Neurotrophin-3

NT4: Neurotrophin-4

p75: Neurotrophin receptor p75

PBS: Phosphate-buffered saline

pCREB: Phosphorylated cyclic AMP-response element binding protein

PDMS: Polydimethylsiloxane

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC: Protein kinase C

PLCγ: Phospholipase C, gamma

PNS: Peripheral Nervous System

PTB: Phosphotyrosine binding

qRT-PCR: Real-time reverse transcription-PCR

Rab5BD: Rab5 binding domain

RILP: Rab interacting lysosomal protein

RT-PCR: Reverse transcription polymerase chain reaction

S6K1/2: Ribosomal S6 protein kinase 1

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH2: Scr homology 2 domain

SNARES: Soluble NSF attachment protein

Tbp: Tata binding protein

Trk: Tropomyosin-related kinase receptor

TrkB Fc: Recombinant Human TrkB Fc chimera protein

RESUMEN

El factor neurotrófico derivado del cerebro (BDNF) se expresa ampliamente en muchos circuitos del sistema nervioso central (SNC) y se une a sus receptores TrkB y p75 para desencadenar diferentes vías de señalización, tales como las quinasas ERK1/2, PI3K-mTOR y la vía de la PLCγ-Ca^{+2}. De esta manera favorece el crecimiento dendrítico y la plasticidad sináptica. Una vez que BDNF une a sus receptores de membrana TrkB y p75, estos se endocitan formando endosomas de señalización. En el sistema nervioso periférico (SNP) está descrito que las neurotrofinas unen a sus receptores en el axón y transmiten señales al cuerpo celular mediante el transporte axonal de endosomas de señalización favoreciendo la sobrevida neuronal. Sin embargo, el rol funcional de los endosomas de señalización BDNF/TrkB en las neuronas centrales, se desconoce. El propósito general de esta tesis fue estudiar el papel de la ruta endosomal en la señalización a larga distancia de BDNF/TrkB en las neuronas corticales y su regulación por las vías clásicas de regulación como PI3K y PLCγ.

El primer objetivo de nuestro trabajo fue estudiar si BDNF aumenta la actividad de la GTPasa Rab5 de manera temporal y espacial y si esto se traduce en una modificación de la dinámica de estos endosomas en dendritas y cuerpos celulares. Las proteínas Rabs son GTPasas monoméricas que actúan como interruptores moleculares para regular el tráfico de membranas al unirse a una amplia gama de efectores. Entre las Rab GTPasas, Rab5 es la GTPasa clave que regula los endosomas tempranos y es el primer organelo en la ruta endocítica de receptores de membrana. Para estudiar el rol de Rab5 sobre la señalización neuronal de BDNF, nosotros hicimos experimentos de microscopía de fluorescencia en células fijadas y células vivas, además evaluamos, mediante el uso de adenovirus que sobreexpresan un dominante negativo de Rab5, si la actividad de la GTPasa era requerida para la arborización inducida por BDNF. Nuestros estudios mostraron que tiempos breves de tratamiento con BDNF aumentó la colocalización de TrkB con Rab5 en dendritas, aumentando el número y la movilidad de los endosomas positivos para Rab5. Estos hallazgos se complementan con estudios que mostraron que la actividad de Rab5 es requerida para la ramificación dendrítica inducida por BDNF. Estos datos muestran que BDNF regula la dinámica de los endosomas tempranos mediante el aumento de la actividad y número de endosomas Rab5 y sugieren que estos procesos son requeridos para la ramificación dendrítica inducida por BDNF.

Posteriormente, nos preguntamos por el rol funcional de la señalización axonal de BDNF en las neuronas corticales y el posible papel de los endosomas de señalización. Para esto utilizamos cultivos de neuronas corticales sembradas en cámaras de microfluidos, modelo que nos permitió aislar el componente axonal del somatodendrítico y así estudiar mediante microscopía de fluorescencia el efecto de la estimulación axonal de BDNF sobre la ruta de señalización de TrkB en los cuerpos celulares y axones. Nosotros encontramos que la incubación con BDNF en axones aumenta la ramificación dendrítica en los cuerpos celulares. Usando distintos modelos de animales transgénicos, encontramos que este proceso es mayormente mediado por los receptores TrkB y no p75. Además, encontramos que la arborización dependía de la activación del factor de transcripción CREB en el núcleo y la vía PI3K-mTOR en los cuerpos celulares aumentando la síntesis de proteínas somatodendríticas. Por otra parte, la actividad de PI3K en

axones no fue necesaria para el transporte de BDNF, ni para el efecto en la arborización en el cuerpo celular. Mediante el uso de neuronas corticales derivadas de ratones knock-in $TrkB^{F616A}$ y el uso de cultivos compartimentalizados, pudimos mostrar que se requiere de la actividad del receptor TrkB activo en el cuerpo celular inducido por BDNF en el axón, para los efectos dendríticos observados en el cuerpo celular. Por otro lado, las actividades de Rab5 y dineína fueron requeridas para estos efectos. Todos estos resultados en su conjunto sugieren la generación y transporte de endosomas de señalización para la señalización a larga distancia de BDNF.

A continuación, debido a la poca información disponible sobre cómo las rutas rio abajo de TrkB regulan la señalización de larga distancia, estudiamos el papel de la señalización de la PLCγ en la señalización axonal mediada por BDNF. Utilizando cultivos compartimentalizados, demostramos que la actividad axonal de PLCγ es necesaria para la arborización dendrítica y la fosforilación de CREB. Estos resultados se correlacionaron con el aumento de los niveles axonales de Ca^{+2} inducido por BDNF en los axones de una manera dependiente del PLCγ. Adicionalmente, encontramos que la ruta PLCγ/Ca^{+2} es necesaria para la endocitosis de TrkB en el axón.

En resumen, estos resultados sugieren que la señalización axonal a larga distancia de BDNF posee un rol funcional en neuronas corticales, regulando la activación de proteínas claves para la traducción de proteínas y la arborización dendrítica.

ABSTRACT

Brain Derived Neurotrophic Factor (BDNF) is broadly express in many circuits of the central nervous system (CNS) and binds its receptors TrkB and p75. BDNF triggers different signaling pathways, including the kinases ERK1/2, PI3K-mTOR and the PLCγ-Ca^{+2} and inducing dendritic growth and synaptic plasticity. When binding to BDNF, TrkB and p75 are endocytosed to signaling endosomes allowing long-distance signaling by neurotrophin receptors. The long-distance signaling has been well-described in the peripheral nervous system for NGF/TrkA to mediate survival. However, whether there is a functional role for BDNF/TrkB signaling endosomes in central neurons is unknown. The general aim of this thesis was to study the role of signaling endosomes in the process of BDNF long-distance signaling in cortical neurons.

The first objective of our work was to study the regulation of Rab5-positive endosomes by BDNF including activity and dynamics in neurons. Rabs are monomeric GTPases that act as molecular switches to regulate membrane trafficking by binding a wide range of effectors. Among the Rab GTPases, Rab5 is the key GTPase regulating early endosomes and is the first sorting organelle of endocytosed receptors. To study the role of Rab5 on the neuronal signaling of BDNF, we have done experiments with fluorescence microscopy in fixed cells and live imaging. In addition, we have evaluated whether the activity of the GTPase was required for the dendritic arborization induced by BDNF, by transducing the neurons with a dominant negative of Rab5. Here, we showed that short-term treatment with BDNF increased the colocalization of TrkB with Rab5 in dendrites, increasing the number and mobility of Rab5 endosomes. Additionally, we observed that BDNF upregulates the activation of Rab5. These results are complementary to parallel findings indicating that Rab5 activity was required for dendritic branching induced by BDNF. These findings suggest that BDNF regulates the dynamics of Rab5-positive early endosomes by increasing the activity of Rab5, processes required for BDNF-induced dendritic branching.

Afterwards, we asked about the functional role of axonal BDNF signaling in cortical neurons and the possible role of signaling endosomes in this process. We found, that BDNF in axons increases dendritic branching in cell bodies. Using different models of transgenic animals and compartmentalized cultures, we found that this process is mostly mediated by TrkB receptors and not p75 NTR. In addition, we found that arborization depended on the activation of the CREB transcription factor in the nucleus and the PI3K-mTOR pathway in cell bodies increasing the synthesis of somatodendritic proteins. In contrast, the kinase activity of PI3K in axons was neither required for the transport of BDNF nor for the effect on arborization in the cell body. By using cortical neurons derived from TrkBF616A knock-in mice and using compartmentalized cultures, we showed that the activity of the TrkB receptor, activated in the axon by BDNF, is required for the dendritic effects observed in the cell body. On the other hand, the activities of Rab5 and dynein were required for these effects. All these results together, suggest the generation and transport of signaling endosomes for long-distance BDNF signaling in axons.

Next, due to the little information available on how TrkB downstream routes regulate long-

distance signaling, we studied the role of PLCγ-Ca^{+2} in BDNF-mediated axonal signaling. Using compartmentalized cultures, we demonstrate that the axonal activity of PLCγ is necessary for dendritic arborization and phosphorylation of CREB. These results were correlated with the increase in axonal levels of Ca^{+2} induced by BDNF in axons in a PLCγ-dependent manner. Interestingly, we found that PLCγ/Ca^{+2} pathway is necessary for the endocytosis of TrkB in axons.

In summary, these results suggest that the axonal BDNF long-distance signaling has a functional role in cortical neurons, regulating the activation of key proteins to regulate the translation of protein and dendritic branching.

INTRODUCTION

1. Role of neurotrophins in the nervous system

Neurotrophins are a family of growth factors involved in survival, development neurobiology, neurodegenerative diseases and psychiatric disorders (Chao, 2003; Levi-Montalcini, 1987). In mammals are known four neurotrophins; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). Neurotrophins have been shown to activate two classes of receptors; a high-affinity receptor, the tropomyosin-related tyrosine kinase receptors (Trks) (Huang and Reichardt, 2003), and a low-affinity receptor, the p75 neurotrophic receptor (p75) (Ibanez and Simi, 2012). Neurotrophins are homodimeric proteins that are synthesized as precursors (proneurotrophins) and secreted to the extracellular space as mature proteins in both a constitutive and regulated manner. Mature form of NGF is specific for TrkA, BDNF and NT-4 are specific for TrkB and NT-3 activates TrkC and also TrkA with less affinity (Huang and Reichardt, 2003). Neurotrophins act as homodimers and directly bind and dimerize Trks receptors, which results in activation and transphosphorylation of the tyrosine kinase residues present in their cytoplasmic domains. Phosphorylation of tyrosine residues creates specific binding sites for intracellular targets proteins containing phosphotyrosine binding (PTB) and Src homology 2 (SH2) binding domains, which subsequent activates a number of signaling cascades, including the Ras/Raf/MAPK (mitogen-activated protein kinase), phosphoinositide 3-kinase (PI3K), and phospholipase C- γ1 (PLCγ) pathways (Gonzalez et al., 2016; Reichardt, 2006). On the other hand, p75 is member of the tumor necrosis

factor receptor superfamily. In addition to bind with similar affinity all neurotrophins, p75 binds the immature form of neurotrophins (Roux and Barker, 2002). Although p75 does not contain a catalytic motif, p75 interacts with several proteins in its intracellular domain promoting the activation of the nuclear factor-kB (NF-kB) and the c-Jun kinase (JNK) as well as other signaling pathways, regulating both the survival and growing of neurites, as well as cell death and inhibition of axonal growth (Ibanez and Simi, 2012; Kraemer et al., 2014).

2. Brain-derived neurotrophic factor in the central nervous system

BDNF and its receptor TrkB, are widely expressed in peripheral and central neurons through the embryonic, postnatal and adult stages (Park and Poo, 2013). BDNF has been described as a regulator of synaptic transmission (Kang and Schuman, 1995), learning and memory (Alonso et al., 2002; Egan et al., 2003), neurogenesis (Benraiss et al., 2001), survival, dendritic and axonal growth on cortical and hippocampal neurons (Gonzalez et al., 2016; Huang and Reichardt, 2003). In addition, defects in BDNF/TrkB signaling has been described in different neurodegenerative and neuropsychiatric disorders (Hashimoto et al., 2002; Zuccato and Cattaneo, 2009). The density of dendritic arborization determines the number of synaptic inputs, therefore it is critical for assembling the central nervous system (CNS) (Jan and Jan, 2010). Deletion of TrkB in the adult CNS induces dendritic retraction and neurodegeneration of cortical neurons (Xu et al., 2000). In addition, conditional deletion of BDNF from neocortex in embryonic stages using CRE-mediated recombination showed that cortical neurons from layer 2/3 developed normally with normal size and dendrites, but they later shrank in size and exhibited a reduction of dendritic complexity at 3 weeks after birth (Gorski et al., 2003). The signaling pathways that regulate BDNF-induced dendritic branching include the activation of

PI3K/AKT (Protein Kinase B) and ERK1/2 signaling pathways. On the other hand, PLCγ activity appears to be dispensable for increasing dendritic branching, but it is required to induce hippocampal long-term potentiation (Dijkhuizen and Ghosh, 2005; Minichiello et al., 2002).

While the mechanisms underlying changes in gene expression induced by BDNF/TrkB signaling have not been completely established, it has been described that one of the transcription factors mediating BDNF transcriptional regulation is cAMP response element-binding protein (CREB) (Finkbeiner et al., 1997; Xing et al., 1998). In cortical neurons, BDNF induces CREB phosphorylation in the serine 133 by at least two pathways: one that depends on the release of intracellular Ca^{+2} and the activation of CAMKIV and another that depends on the activation of the RAS/ERK/RSK pathway (Finkbeiner et al., 1997). CREB is a major mediator of neurotrophin–mediated transcriptional responses, such as early gene c-fos and Arc, in cortical neurons. In addition, CREB is required for BDNF-induced dendritic branching of hippocampal neurons (Kwon et al., 2011; Gonzales et al, 2019). However, how the activation of this transcription factor is connected with changes in dendritic branching is poorly understood. In that context, Kwon and cols have shown that BDNF increases the expression of the guanine deaminase Cypin in a CREB-dependent manner, and hence Cypin may regulate dendritic branching by direct binding to tubulin heterodimers and promoting microtubule polymerization (Akum et al., 2004; Kwon et al., 2011). Furthermore, CREB regulates both TrkB and BDNF expression by binding to CRE-binding sites in their promotors (Deogracias et al., 2004; Tao et al., 1998), suggesting that the activation of CREB by BDNF can induce a positive feedback loop to increase the transcription of genes related to dendritic arborization.

To coordinate neuronal growth, in addition to the transcription of specific mRNA the concomitant translation of key proteins should be regulated. Local translation is a pivotal mechanism to control protein expression in dendrites and axons. In neurons, BDNF, insulin and IGF-1 have been shown to enhance translation by activating the mammalian target of rapamycin (mTOR) signaling pathway (Garza-Lombo and Gonsebatt, 2016; Kumar et al., 2005; Wang and Proud, 2006). mTOR is a serine/threonine kinase that regulates protein translation, in part, by phosphorylation of p70 ribosomal S6 protein kinase 1 (S6K1/2) and the inhibitor of translation 4E-BP1, which increase the translation of a subset of mRNAs containing a 5'CAP in the 5' untranslated region of the mRNA (Lipton and Sahin, 2014; Panja et al., 2009). mTOR signaling control dendrite size and shape and is involved in the regulation of spine morphology in a BDNF-dependent manner via PI3K/Akt pathway (Kumar et al., 2005). Several lines of evidence suggest that BDNF increases the local translation of a subset of mRNAs in dendrites, including proteins related to CNS plasticity (Leal et al., 2014; Ravindran et al., 2019; Schratt et al., 2004; Takei et al., 2004). Indeed, BDNF induces novel synthesis of Arc and CaMKII proteins in dendrites and synaptic boutons by a mechanism that involve the activation of mTOR and 4E-BP1 phosphorylation (Takei et al., 2004). Additionally, using genome-wide screen and quantitative RT-PCR validation, it was demonstrated that translation of proteins involved in synapsis such as CaMKII, GluR1, NR1/3 and Homer 2 are also regulated by a rapamycin-sensitive mechanism in a BDNF dependent manner (Schratt et al., 2004).

On the other hand, the coordinate action of actin filaments and microtubules and the upstream signaling that regulates their dynamics is critical to promotes neuronal growth (Kalil and Dent, 2014; Van Aelst and Cline, 2004). In this context, it has been shown that TrkB phosphorylates key proteins that regulate the actin dynamic, such as Tiam1, the GEF of the small GTPase Rac1,

which promotes actin polymerization, and the Rho GTPase Cdc42. In addition, BDNF/TrkB also modulates microtubule dynamics, by phosphorylation of different proteins that regulates microtubule polymerization, like the transmembrane scaffold protein Ankyrin Repeat-rich Membrane Spanning (ARMS) or Kidins220 via PI3K/Akt, which interact with MAP1A, MAP1B and MAP2 increasing the microtubule stabilization (Chen et al., 2012; Wu et al., 2009). Thus, BDNF may exert its regulatory effect locally in dendrites through the activation of intracellular signaling that includes component of actin and microtubule cytoskeleton associated proteins, regulating directly the morphological changes in neurons.

The early effects of BDNF, such as filopodia dynamic or synaptic plasticity, results from the post-translation modification such as protein phosphorylation or cytoskeleton arrangement, while the long-term effects, such us dendrite growth or long-term potentiation, arise from modification of transcription and translation. Despite of the effort done to understand the mechanisms of BDNF-mediated dendritic growth, very little information is available about how BDNF regulates this process.

3. Neurotrophin signaling via the endosomal pathway

After secretion, neurotrophins bind their cognate receptors, this complex rapidly activates signaling pathways in the plasma membrane and undergoes internalization. For quite some time, endocytosis of the neurotrophin/receptor complex was considered to be a mechanism solely involved in the downregulation of signal transduction. However, it is now well established that the internalization and post-endocytic trafficking of receptors are essential for signaling and neuronal function. The internalization of receptors would determine the availability of the

receptor in the cellular surface, the location and therefore, the duration of the neurotrophic signal (Bronfman et al., 2007; Cosker and Segal, 2014).

The endocytic process for growth factor receptors can be broadly classifies as being clathrin dependent or clathrin independent. In clathrin dependent endocytosis, receptors and their ligand are recruited to clathrin-coated pits via adaptor proteins. Among them, the BAR domain proteins are critical for early membrane deformation, and the sorting adaptor AP2 that recognizes and sorts the diverse variety of cargoes for endocytosis (Rao et al., 2012). Furthermore, the GTPase dynamin is required for fission of the clathrin-coated vesicles from the plasma membrane to the formed endosome. On the other hand, there are at least eight clathrin-independent mechanisms (Doherty and McMahon, 2009). Among them, micropinocytosis for internalization of larger areas of membrane, a process that involves ruffling of the plasma membrane and requires Rac1, pincher and actin cytoskeleton, and it has been described to be required for Trk signaling (Philippidou et al., 2011; Valdez et al., 2007).

All clathrin-dependent and clathrin-independent pathways of internalization are thought to converge on peripheral early endosomes (Doherty and McMahon, 2009). From there, some components are either rapidly recycled back to the plasma membrane, transported to lysosomes or more slowly recycled through the recycling endosome. Among the proteins that coordinates the dynamics of intracellular trafficking, we found the large Rab family of small GTPases. Rabs control the progression of growth factor receptor endocytosis, beginning with initial internalization at the plasma membrane, and continuing through successive steps of membrane

maturation and cargo transport in the cell (Stenmark, 2009). To date, eleven Rabs have been identified in yeast and over seventy in mammal cells.

Rab GTPases function as molecular switches, cycling between GTP-bound and GDP-bound state. This switch is controlled by guanine nucleotide exchange factors (GEFs), which trigger the binding of GTP, and GTPase-activating proteins (GAPs), which accelerate hydrolysis of the bound GTP to GDP. Rab proteins are localized on specific intracellular compartments and are thus often used as markers for organelles. Particularly for neurotrophic signaling, three Rabs have important roles: Rab5 which regulates the fusion, dynamic and sorting of early endosomes, Rab7 which regulates the late endosomal trafficking to the degradative route and Rab11, which regulates the recycling of receptor and molecules to the plasma membrane (Bucci et al., 2014; Deinhardt et al., 2006; Escudero et al., 2019; Lazo et al., 2013; Moya-Alvarado et al., 2018; Park et al., 2004). For example, the early endosome positive for Rab5, frequently contain the adaptor proteins, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) or Early Endosome Antigen 1 (EEA1) (Kalaidzidis et al., 2015; Wandinger-Ness and Zerial, 2014). APPL1, in particular, has been implicated in both TrkA and TrkB signaling, because it serves as platform for the assembly of signaling complexes regulating the MAPK and Akt pathways (Fu et al., 2011; Lin et al., 2006). The early endosome can subsequently sort the cargo into the late endosome/lysosome pathway or the recycling pathway. Rab7 is the marker of late endosome and constitutes the intermediate compartment between the early endosome and lysosome (Huotari and Helenius, 2011). In the last years, Rab7 has been in the spotlight by its role in the retrograde transport of endosomes containing Trk receptors (Deinhardt et al., 2006; Saxena et al., 2005; Zhou et al., 2012). The active form of Rab7 bind it effector Rab Interacting Lysosomal

Protein (RILP), to engage in retrograde transport by binding to dynein (Johansson et al., 2007). Furthermore, these organelles are positive for Lamp1, a lysosomal marker, and CD63, a multivesicular marker. This last one was recently reported by the Ginty's group as the carrier for TrkA retrograde transport in axons of sympathetic neurons containing not only phosphorylated TrkA receptor, but also other signaling molecules (Ye et al., 2018). On the other hand, after internalization, Trk receptors are able to recycle to the plasma membrane, process regulated by Rab11. In hippocampal neurons, TrkB phosphorylation promotes an increase of Rab11-GTP levels in dendrites and an accumulation of TrkB in this compartment. As a consequence, TrkB levels in dendrites are increased, sensitizing neurons to BDNF and promoting dendritic branching (Lazo et al., 2013). In addition, in mature neurons, Rab11 facilitate the translocation of TrkB in postsynaptic density favoring long-term potentiation (Huang et al., 2013; Song et al., 2015).

4. Long-distance neurotrophic signaling in the nervous system

Due to the polarized morphology of neurons distal dendrites and axons must be communicated to the cell body for proper neuronal homeostasis. This is achieved by bidirectional transport of organelles such as mitochondria, lysosomes and endosomes, among others organelles (Bronfman et al., 2014). There are two main classes of molecular motors that coordinate the transport of cargoes to the minus and plus ends of microtubules. The kinesins are a large family of proteins that coordinates the transport to the plus end of microtubules, thereby moving materials in an anterograde manner to the distal process of axons in neurons (Hirokawa et al., 2010), whereas cytoplasmic dynein is a protein complex that moves cargoes to the minus end

of microtubules, thus moving materials in a retrograde fashion from neuronal distal process to the cell body (Kardon and Vale, 2009). Ligand binding of neurotrophins, promotes Trk internalization of ligand-receptor complexes into signaling endosomes, carrying activated Trk receptor and signaling molecules (Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018). Several studies have shown that endocytosis of TrkB is required for survival, dendritic arborization and cell migration (Zheng et al., 2008; Zhou et al., 2007), suggesting that endosomal signaling has a role in the physiological response induced by TrkB activation.

TrkB receptors are located in both the somatodendritic compartment as well as in neuronal axons, and neurotrophins are released in dendrites and axons (Matsuda et al., 2009) as well as by the target tissues in the peripheral nervous system (Kuruvilla et al., 2004). Therefore, over the years several groups have investigated the cellular and molecular mechanism that allow the intracellular communication of active neurotrophin receptors in distal processes of the neurons with the nucleus.

The signaling endosomes are originated from the plasma membrane when neurotrophin bind their receptor and trigger their endocytosis. Internalization of receptors is a key process not only for long distance propagation of neurotrophic signal, but also for engaging intracellular interactor enabling the activation of distinct signaling pathways (Villarroel-Campos et al., 2018). Long-distance signaling from the target tissues to induce physiological effect in the soma has been well described in the peripheral nervous system for NGF/TrkA signaling (Harrington and Ginty, 2013; Ye et al., 2003). At the cellular level, internalization and retrograde transport of NGF/TrkA complex are required for the promotion of neuronal survival (Ye et al., 2003).

Distal NGF stimulation of dorsal root ganglia neurons (DRG) or sympathetic neurons in compartmentalized cultures trigger the phosphorylation and activation of TrkA. Then, the endocytosed NGF/TrkA complex is retrograde transported by the molecular motor dynein (Heerssen et al., 2004) in a Rab7 positive endosome (BasuRay et al., 2010; Ye et al., 2018). After activation and internalization, the NGF/TrkA complex in signaling endosomes reaches the cell body promoting the phosphorylation of the transcription factor CREB (Riccio et al., 1997). Phosphorylation of CREB upregulates the B-Cell lymphoma 2 protein (Bcl-2) expression promoting neuronal survival (Riccio et al., 1999). During development, upon target innervation NGF enhances the expression of TrkA in sympathetic neurons, establishing a positive feedback loop that enhances prosurvival signals. Besides, the release BDNF and NT4 act as paracrine apoptotic cues that induce death of neighboring cells through p75 (Deppmann et al., 2008; Escudero et al., 2019). In addition to the well stablish role of the retrograde signaling in the survival of peripheral nervous system, NGF signaling also modulates synapse formation and maintenance, directly promoting aggregation of postsynaptic protein complex in dendrites (Lehigh et al., 2017; Sharma et al., 2010), dendritic arborization (Bodmer et al., 2011; Ruit and Snider, 1991; Voyvodic, 1989), and local axon growth (Bodmer et al., 2011).

Compared to the knowledge gathered through the years for the role of NGF/TrkA-long distance signaling. The long-distance effects of BDNF, in the CNS are far less studied and the contribution of BDNF-mediated retrograde signaling to the transcriptional and morphological changes induced in central neurons have not been investigated in vertebrates (Gonzalez et al., 2016). For instance, *in vivo* experiments in retinal ganglion cells (RCG) of *Xenopus laevis* optic tectum, have shown that tectal stimulation with BDNF promotes a potentiation of retinotectal

synapses in a retrograde dependent manner. Moreover, it was observed that this process is dependent on the activity of TrkB and PLCγ. Suggesting that there is a synaptic modification that may be causally linked to structural modification of RGC dendrites after hours of BDNF stimulation (Du and Poo, 2004; Lom et al., 2002). On the other hand, in mammals Jaffrey's lab using compartmentalized cultures in microfluidic chambers of central neurons showed that the long distance signaling of BDNF, from dendrites, promotes transcriptional activation, increasing the synthesis of the activity-regulated cytoskeletal protein (Arc) (Cohen et al., 2011). *Arc* is an immediate-early gene, whose protein is required for synaptic plasticity induced by BDNF (Ying et al., 2002) and its synthesis is regulated by CREB and mTOR (Takei et al., 2004; Ying et al., 2002). Furthermore, Zu-Hang group had shown that snapin mediates the recruitment of dynein to TrkB positive endosomes regulating the activation of CREB and dendritic arborization, supporting the role of TrkB signaling endosomes (Zhou et al., 2012). In addition, they showed that there is a correlation between axonal retrograde transport of TrkB-mCherry and dendritic branching, suggesting that axonal signaling of BDNF and TrkB can promotes dendritic arborization in cortical neurons.

Despite the knowledge we have gathered about how the activities of endosomal GTPases regulates BDNF signaling to increase dendritic branching in CNS neurons. It is unknown whether BDNF can regulate the early sorting endosome activity and if the activity of these endosomes are required for proper long-distance signaling mediated by TrkB. Furthermore, due to the relevance of the BDNF signaling on the synaptic potentiation and dendritic growth, it is key to elucidate the physiological role of the long-distance signaling of BDNF from axons and

to evaluate the contribution of the endosomal pathway and the TrkB downstream signaling pathways in this process.

HYPOTHESIS

In this thesis, we evaluated two different but related hypothesis;

Hypothesis 1. BDNF signaling up-regulates the activity and mobility of Rab5-positive endosomes in neuronal cell bodies and dendrites.

Hypothesis 2. The axonal retrograde transport of BDNF/TrkB signaling endosomes depends on Rab5 activity and increases CREB-dependent dendritic branching requiring the TrkB downstream signaling of PI3K and PLCγ.

GENERAL AIMS

General aim 1. To study whether BDNF signaling regulates the activity and mobility of Rab5-positive endosomes.

General aim 2. To evaluates whether BDNF/TrkB signaling endosomes increases CREB-dependent dendritic branching and whether this process requires Rab5, PI3K and PLCγ activity.

SPECIFIC AIMS

General aim 1: To study whether BDNF signaling regulates the activity and mobility of Rab5-positive endosomes.

1.1 To study the effect of BDNF on Rab5 and Rab11 mobility and transport in dendrites and cell body.

1.2 To evaluate whether BDNF increase Rab5 activity in dendrites and somas

1.3 To evaluate whether BDNF increase Rab5 and Rab11 mRNA levels.

General aim 2. To evaluates whether BDNF/TrkB signaling endosomes increases CREB-dependent dendritic branching and whether this process requires Rab5, PI3K and PLCγ activity.

2.1 To evaluate whether BDNF signaling in axons increases dendritic arborization in cell bodies in a TrkB-dependent manner.

2.2. To evaluate whether BDNF signaling in axons increases dendritic arborization in a CREB-dependent manner.

2.3 To study the role of signaling endosome in BDNF long-distance signaling.

2.4 To study the role of PI3K-mTOR pathway in BDNF long-distance signaling.

2.5 To study the role PLCγ in BDNF long-distance signaling.

CHAPTER I

In this chapter, we approached General aim 1 and the following specific aims:

1.1 To study the effect of BDNF on Rab5 and Rab11 mobility and transport in dendrites and cell body.

1.2 To evaluate whether BDNF increase Rab5 activity in dendrites and somas

1.3 To evaluate whether BDNF increase Rab5 and Rab11 mRNA levels.

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Brain-derived neurotrophic factor (BDNF) regulates Rab5-positive early endosomes in hippocampal neurons to induce dendritic branching

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ABSTRACT

Neurotrophin receptors use endosomal pathways for signaling in neurons. However, how neurotrophins regulate the endosomal system for proper signaling is unknown. Rabs are monomeric GTPases that act as molecular switches to regulate membrane trafficking by binding a wide range of effectors. Among the Rab GTPases, Rab5 is the key GTPase regulating early endosomes and is the first sorting organelle of endocytosed receptors. The objective of our work was to study the regulation of Rab5-positive endosomes by BDNF at different levels, including dynamic, activity and protein levels in hippocampal neurons. Short-term treatment with BDNF increased the colocalization of TrkB in dendrites and cell bodies, increasing the vesiculation of Rab5-positive endosomes. Consistently, BDNF increased the number and mobility of Rab5 endosomes in dendrites. Cell body fluorescence recovery after photobleaching of Rab-EGFP-expressing neurons suggested increased movement of Rab5 endosomes from dendrites to cell bodies. These results correlated with the BDNF-induced activation of Rab5 in dendrites, followed by increased activation of Rab5 in cell bodies. Long-term treatment of hippocampal neurons with BDNF increased the protein levels of Rab5 and Rab11 in an mTOR-dependent manner. While BDNF regulation of Rab5a levels occurred at both the transcriptional and translational levels, Rab11a levels were regulated at the translational level at the time points analyzed. Finally, expression of a dominant-negative mutant of Rab5 reduced the basal arborization of nontreated neurons, and although BDNF was partially able to rescue the effect of Rab5DN at the level of primary dendrites, BDNF-induced dendritic branching was largely reduced. Our findings indicate that BDNF regulates the Rab5-Rab11 endosomal system at different levels and that these processes are likely required for BDNF-induced dendritic branching.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a well-known neurotrophin that belongs to a small family of secreted proteins that includes nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4) (Park and Poo, 2013). BDNF regulates many facets of the central neurons, including neuronal survival and differentiation, neuronal growth, synaptogenesis and plasticity and maintenance of neuronal circuits. BDNF is the most widely expressed neurotrophic factor in the brain and exerts its function by binding to the tropomyosin-related kinase receptor TrkB and the p75 neurotrophin receptor (p75). In addition, BDNF is secreted in an activity-dependent manner by autocrine and paracrine mechanisms (Bronfman et al., 2014).

The neuronal growth effects mediated by BDNF are mainly mediated by its tyrosine kinase receptor TrkB (Gonzalez et al., 2016). For example, BDNF binding to TrkB increases the branching of cortical and hippocampal neurons in dissociated cultures and organotypic slices (Horch and Katz, 2002). In addition, BDNF regulates the survival and migration of cortical neurons (Zheng et al., 2008; Zhou et al., 2007). These effects are induced by the activation of downstream signaling pathways after BDNF/TrkB receptor interaction. After binding BDNF, TrkB dimerizes and undergoes autophosphorylation at specific tyrosine residues of the intracellular domain. These phosphotyrosines are docking sites for adaptor proteins that lead to the activation of several signaling cascades including the mitogen-activated protein kinases (MAPKs), such as ERK1/2, ERK5 and p38, in addition to the phosphatidylinositol-3-kinase (PI3K)-Akt-mTOR pathway, phospholipase-C (PLC)-γ-Ca^{+2} and the small GTPases of the Rho family Cdc42/Rac/RhoA (Huang and Reichardt, 2003; Minichiello, 2009).

Different lines of investigation have shown that internalization and postendocytic trafficking of Trk receptors determine their signaling properties and thus functional outcomes in neurons (Bronfman et al., 2014; Cosker and Segal, 2014). For example, Trk receptors ensure localized signaling responses to extracellular cues in axons (Ascano et al., 2012) and enhance downstream signaling to regulate neuronal differentiation (Zhang et al., 2000) and dendritic arborization (Lazo et al., 2013). Additionally, BDNF signals are retrogradely transported from dendrites to the soma to regulate gene expression (Cohen et al., 2011). Internalization of BDNF/TrkB is required for the sustained activation of PI3K and ERK signaling pathways and neurite outgrowth (Kumar et al., 2005; Zheng et al., 2008). Additionally, after internalization, endocytosed TrkB recruits microtubule-associated molecular motors such as dynein and neuronal kinesin KIF21B, which have both been described to contribute to the directionality of BDNF/TrkB endosomes in dendrites (Ayloo et al., 2017; Ghiretti et al., 2016).

The Rab monomeric GTPases are the main regulators of postendocytic trafficking of endocytic receptors. Rabs act as key regulators of vesicular trafficking by controlling the transport, anchoring and coupling of vesicles through effector binding. Among these effectors are the molecular motors and the SNARES, which generally join the Rabs in their GTP-bound state (Grosshans et al., 2006; Stenmark, 2009). In fact, Rabs are mediators of TrkB endosomal signaling (Lazo et al., 2013; Sui et al., 2015; Zhou et al., 2012). In the literature, more than 60 members of the GTPase Rab family have been described (Stenmark, 2009). Rab5, Rab7 and Rab11 are among the key GTPases known to be involved in BDNF/TrkB signaling (Lazo et al., 2013; Zhou et al., 2012).

After internalization, tyrosine kinase receptors enter the early or sorting endosomes, whose

biology is regulated by Rab5 (Goh and Sorkin, 2013). Independent of the internalization mechanism of receptors, Rab5 tightly regulates the homotypic fusion of endosomes, forming the early or sorting endosome (Stenmark, 2009). There, receptors are sorted to the recycling pathway, which is regulated by Rab11, or to the late endocytic pathway regulated by Rab7 (Bronfman et al., 2014).

Studies from our laboratory and others have established that BDNF/TrkB regulates the activity and dynamics of Rab11-positive endosomes; in turn, Rab11 is required for BDNF-induced dendritic branching and local signaling in dendrites and synapses (Huang et al., 2013; Lazo et al., 2013; Song et al., 2015; Sui et al., 2015). Thus, transit through the early recycling pathway of TrkB receptors is a key step in BDNF signaling in neurons. However, whether BDNF/TrkB regulates Rab5 activity and dynamics in dendrites is unknown. Several lines of evidence indicate that Rab5-positive endosomes are required for proper neuronal morphology. Genetic experiments in Drosophila have shown that dynein and Rab5 are required for dendritic arborization in larvae (Satoh et al., 2008). On the other hand, Rab5 activity is regulated by TrkA in PC12 differentiation assays (Liu et al., 2007). Here, we first studied the short-term effects of BDNF treatment (5-30 min) on Rab5 dynamics and activity and then the long-term effects of BDNF treatment (4-24 h) on Rab5 and Rab11 protein and mRNA levels. We found that BDNF increases the number and dynamics of Rab5-positive endosomes in dendrites. Indeed, fluorescence recovery after photobleaching (FRAP) experiments showed that BDNF increases the recovery of Rab5-positive vesicles in the soma, which correlates with the increased activity of somatic Rab5, suggesting that BDNF increases the activation and movement of dendritic endosomes to cell bodies. Long-term treatment of hippocampal neurons with BDNF increased the protein levels of both Rab5 and Rab11 in an mTOR-dependent manner. In addition, BDNF

also regulated mRNA levels of *rab5* (but not the mRNA levels of *rab11*). Both, Rab5 and Rab11 activity was required for proper morphological changes induced by long-term BDNF (48 h) treatment of neurons. Of note, in contrast to Rab11, reduced Rab5 activity impacted the basal levels of primary dendrites. BDNF was partially able to rescue this effect, but reduced Rab5 activity halted the full dendritic arborization induced by BDNF. Altogether, these results suggest that BDNF regulates the early recycling pathway at different levels to induce dendritic branching.

METHODOLOGY

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.

Materials. Minimum essential medium (MEM, 11700-077), Dulbecco's Modified Eagle's Medium (DMEM, 12800-017), Hank's balanced salt solution (HBSS, 14065-056), neurobasal medium (21103-049), OptiMEM (11058-021), Lipofectamine 2000 (11668-027), glutamine, B27 (17504-044), horse serum (HS, 16050-122), penicillin/streptomycin (15140-148), and trypsin (15090-046) were obtained from Invitrogen (Life Technologies, CA, US). Fetal bovine serum (FBS) HyClone (SH30071.03) was from GE Healthcare Life Science. Poly-L-lysine (P2636), AraC, Glutathione-Sepharose 4B (GE17-0756-01) and isopropyl β-D-thiogalactoside (IPTG, I6758**)** were from Sigma (MO, US). BDNF was purchased from Alomone Labs (Jerusalem, Israel). TrkB-Fc was acquired from R&D Systems (688TK, MN, US). Anti-βIII tubulin antibody, mouse anti-Flag (F3165**)**, Mowiol 4-88 and the inhibitor actinomycin D (A1410) were purchased from Sigma (St. Louis, MO, US). Protease-free bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch (West Grove, PA, US). A MAP2 antibody was purchased from Upstate-Millipore (Billerica, MA). Protein-phosphatase inhibitors were from Thermo Fisher Scientific. The inhibitors cycloheximide (239763) and rapamycin (553210) were purchased from Calbiochem (Darmstadt, Germany). Mouse anti-glutathione-S-

transferase (GST) (Ab92) and mouse anti-Rab5 (ab18211) were purchased from Abcam. Rabbit anti-Rab11 (715300) was purchased from Invitrogen. The Flag-TrkB plasmid was a gift of Dr. Francis Lee (Weill Cornell University, NY, US), EGFP-Rab5 and EGFP-Rab5DN were gifts of Dr. Victor Faundez (Emory University, GA, US), EGFP- Rab11DN was a gift of Dr. Rejji Kuruvilla (John Hopkins University, MD, US), and pGEX-GST-Rabaptin5 was donated by Dr. Vicente Torres (University of Chile, Chile).

Hippocampal neuron primary culture. Embryonic hippocampal neurons from rats of either sex (embryonic days 17-19) were dissected as described previously (Shimada et al., 1998; Fan et al., 2004) in HBSS. After disaggregation, neurons were resuspended in MEM supplemented with 10% HS, 20% D-glucose, and 0.5 mM glutamine and were seeded on coverslips or plastic plates coated with poly-L-lysine (1 mg/ml). For morphological experiments, 7000 cells/cm^2 were seeded on coverslips. For protein or mRNA experiments, 15000 cells/cm^2 were seeded on plastic plates. After 4 h, the culture medium was replaced with neurobasal medium supplemented with 2% B27 and 1X glutamax. Proliferation of nonneuronal cells was limited using cytosine arabinoside at 3 days *in vitro* (DIV). The animals were obtained from the animal facilities of Pontificia Universidad Católica de Chile and euthanatized under deep anesthesia according to the bioethical protocols of our institution.

Analysis of the levels of messenger RNA (mRNA) in hippocampal neurons after BDNF stimulation. Hippocampal neurons at 9 DIV were incubated for 90 min in neurobasal media for depletion of endogenous trophic factors and then were treated with 50 ng/mL BDNF for 4 or 12 h. Total RNA was extracted from primary neurons by using TRIzol and purified using the

RNeasy kit (Qiagen, Hilden, Alemania) according to the manufacturer's instructions. cDNA was prepared by reverse transcription of 1 µg of total RNA with random primers using Maloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). The resulting cDNAs were amplified by using Brilliant II SYBR Green qPCR (Stratagene) with an Mx3000P thermocycler (Stratagene). All mRNA expression data were normalized to *β-actin*, *tbp* and *pjk-1* expression in the corresponding sample (Santos and Duarte, 2008). Finally, $2^{-\Delta\Delta Ct}$ analysis was performed. Oligonucleotide sequences for the primers used are shown in Table 1.

Gene	Primer (5' to 3')
rab5a	F: GGCTAATCGAGGAGCAACAA R: ACAAAGCGAAGCACCAGACT
arc	F: GGAGGGAGGTCTTCTACCGT R: CTACAGAGACAGTGTGGCGG
β-actin	F: CCCGCGAGTACAACCTTCT R: CGTCATCCATGGCGAACT
tbp	F: CTGTTTCATGGTGCGTGACGAT R:AAGCCCTGAGCATAAGGTGGAA
pgk-1	F: TGCTGGGCAAGGATGTTCTGTT R:ACATGAAAGCGGAGGTTCTCCA
rab11a	F: AAAGTTACCCTGCTGCCTGG R: CTGCCAGGAAAGGAGACTGG

Table 1. Primers used to evaluate the mRNA levels of Rab5a, Arc, β-actin, TBP and PGK-1. F and R correspond to Forwards and Reverse primers, respectively

Western blot analyses. To study Rab5a and Rab11a protein levels, neurons were depleted with neurobasal media in the presence or absence of 5 µM actinomycin D (for Rab5a) or 25 µM cycloheximide (for Rab5a and Rab11a) with 50 ng/mL BDNF for 24 h or were treated in the presence or absence of 200 nM rapamycin for 60 min and then stimulated for 4 and 12 h with 50 ng/mL BDNF in the presence or absence of the drug. Next, cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% SDS and 1% Triton X-100)

containing protease and phosphatase inhibitors. Standard SDS gel electrophoresis and Western blotting procedures were used to analyze the cell extracts using anti-Rab5a (1:1000), anti-Rab11a (1:1000) and anti-β-III tubulin (1:1000) antibodies.

Immunoendocytosis of Flag-TrkB and colocalization. Neurons were transfected with Flag-TrkB and EGFP-Rab5 using Lipofectamine 2000 and the manufacturer's instructions when cultures were at 7 DIV. Forty-eight hours later, neurons were incubated for 90 min in neurobasal media for depletion of endogenous trophic factors and treated with mouse anti-Flag antibodies conjugated to an Alexa Fluor 555 fluorochrome (20 μg/mL). After 30 min, the cells were washed with PBS at 37°C and stimulated with 50 ng/mL BDNF for 5 or 15 min, fixed and compared with noninternalized controls (cells not treated with BDNF). Images of neurons were acquired using confocal microscopy, processed with deconvolution algorithms, and then colocalization of Flag-TrkB with EGFP-Rab5 was analyzed by calculating Manders correlation index (M1) (Bolte and Cordelieres, 2006).

Live-cell imaging of EGFP-Rab5. Neurons were transfected with EGFP-Rab5 as described above. After 24 h, the cells were depleted with neurobasal media during 180 min. Then, the cells were transferred to a Tyrode media (124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM D-glucose and 25 mM HEPES, pH 7.4). Live-cell imaging was performed on a Nikon Eclipse C2 confocal microscope equipped with a live-cell temperature controller (LCI cu-501) and digital camera connected to a computer with Software NIS-Elements C. Images of a single neuron transfected with EGFP-Rab5 were acquired using a 60X objective at intervals of 7.3 s for 5 min to establish the basal level of distribution and dynamic. After 5 min, neurons were stimulated with 50 ng/mL BDNF, allowing 3 min for diffusion of the ligand, and we started an

additional 30 min of capture.

Quantification of the number of endosomes-like vesicles containing EGFP-Rab5 in dendrites was performed by comparing the fraction of total dendritic Rab5 that was found in structures larger than 0.2 μm^2. Images of the video were segmented with ImageJ, and the number of endosome-like vesicles was quantitated in 30-μm segments of primary dendrites.

Analysis of the mobility of Rab5-positive endosomes was performed by comparing the distribution of fluorescence in the same dendrite at different time points (0, 5, 15, 30 min). We quantified the number of particles moving more than 5 μm as a mobile fraction in nonstimulated neurons (control) and in neurons treated with BDNF for 5-30 min.

Live-cell imaging and fluorescence recovery after photobleaching (FRAP) of EGFP-Rab5. The neurons were transfected with EGFP-Rab5 at 8 DIV. After 24 h, the cells were depleted from B27 for 180 min in neurobasal media. Then, the cells were transferred to a Tyrode media supplemented with TrkB/Fc (200 nM), and live-cell imaging was performed on a Nikon Eclipse C2 confocal microscope equipped with a live-cell temperature controller (LCI cu-501). Images of a single neuron transfected with EGFP-Rab5 were acquired using a 60X objective at intervals of 4 s for 5 min each at 5, 15 and 30 min to establish the basal level. After a brief wash with Tyrode media, neurons were stimulated with 50 ng/mL BDNF, allowing 3 min for diffusion of the ligand, and we started an additional 30 min of capture for intervals of 4 s for 300 s each at 5, 15 and 30 min. For the FRAP assay, a prebleach image was acquired at 2% laser power, after which a selected area was bleached at 100% laser power with 10 successive bleach scans separated by 1 s, assisted by the microscope software. Postbleach recovery images were

acquired every 7.3 s for 300 s. Postacquisition image processing was performed using ImageJ. Adjustment and analyses were performed on the videos as brightness/contrast adjustments to all pixels in the images and as manual tracking of objects across multiples frames, respectively (Snapp et al., 2003). To quantify the percentage of endosome-like vesicles in the cell bodies of neurons transfected with EGFP-Rab5, first a threshold of the photobleached zone was applied. Prior to bleaching, a quantification was performed using the same selected region of interest (ROI). Then, the number of vesicles that recovered fluorescence associated with EGFP-Rab5 was quantified at 0, 5, 15 and 30 min after photobleaching.

Microscopy detection and quantification of active Rab5 in dendrites and cell bodies. The fusion protein Rab5BD-GST was produced in BL21 E. coli, transformed with a pGEX-GST-Rabaptin5 plasmid and stimulated for 4 h with IPTG. The Rab5BD-GST protein was purified from bacteria lysate using glutathione-Sepharose beads. For use as a probe, the protein was eluted in a solution of reduced glutathione. Similar methods have been described previously for other GTPases such as GST-FIP3 (Lazo et al., 2013). To test the protein as a probe, hippocampal neurons at 8 DIV were transfected with EGFP-Rab5DN, EGFP-Rab5CA or EGFP; in addition, nontransfected neurons were stimulated with 50 ng/mL BDNF for 5 or 30 min, fixed with paraformaldehyde (PFA), permeabilized and blocked in 3% fish gelatin in incubation buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA, 0.25 M sucrose and 0.2% Triton X-100, pH 7.2) for 45 min. The neurons were then incubated overnight with ~10 μg/mL Rab5BD-GST in incubation buffer at 4°C. After 2 brief washes in HBSS, the neurons were fixed again in PFA, washed in PBS and then a standard immunofluorescence assay with rabbit anti-GST (1:500) and mouse anti-MAP2 (1:1000) was performed.

The neurons to be quantified were selected based on the MAP2 labeling to avoid the specific selection of a neuron with high or low levels of Rab5BD-GST. Three primary dendrites and the cell body were identified, and the integrated intensity was measured (intensity of the signal standardized by the area) per cell body and associated dendrites. The background was calculated from images of neurons treated with GST, and this baseline was calculated for and subtracted from each dataset.

Stimulation and measurement of dendritic arborization induced by BDNF. Hippocampal neurons (7 DIV) were transduced with EGFP, EGFP-Rab5DN or EGFP-Rab11DN adenoviruses and stimulated with 50 ng/mL BDNF in culture medium. After 48 h, dendritic arborization was analyzed by Sholl analysis (Sholl, 1953) and by counting the number of branching points as described previously (Lazo et al., 2013). For analysis of dendritic branching, the neurons were immunostained with anti-MAP2. Dendrites were visualized by confocal microscopy using a Zeiss Axiovert 2000 inverted microscope equipped with a laser scanning module and Pascal 5 software (Carl Zeiss). Images were acquired using a 63X objective at 1024 X 1024-pixel resolution along the z-axis of whole cells. Z-stacks were integrated, and the images were segmented to obtain binary images. Ten concentric circles with increasing diameters (10 μm each step) were traced around the cell body, and the number of intersections between dendrites and circles was counted and plotted for each diameter. The adenovirus vector work was performed under biosafety level 2 conditions using a Labculture Class II, Type A2 cabinet (ESCO, Singapore). Analysis was performed using the ImageJ program.

Statistics. For statistical analysis, the GraphPad Prism 7 program was used. Multiple

comparisons were performed with ANOVA with Bonferroni's posttest. To determine if two sets of data were significantly different from each other the Student's t-test was applied.

RESULTS

BDNF increases the colocalization of TrkB with Rab5-positive endosomes, increasing its vesiculation and mobility in dendrites.

Neurotrophins use the early endosomal route, regulated by the Rab5 monomeric GTPase, to signal and regulate different physiological processes (Ascano et al., 2012; Deinhardt et al., 2006; Lazo et al., 2013). However, to date, there are no studies addressing the functional relationship of Rab5-positive endosomes with BDNF signaling. To address this issue, we analyzed the dynamics and activity of Rab5 endosomes upon short-term administration (5-30 min) of BDNF. First, we studied whether TrkB and Rab5 colocalize after BDNF treatment by cotransfecting hippocampal neurons with EGFP-Rab5 and Flag-TrkB tagged on its NH2 domain. As reported previously for the colocalization of Rab11 and TrkB, we performed immunoendocytosis by labeling the surface expression of TrkB in the absence or presence of BDNF (Lazo et al., 2013). We observed that on neurons that were not stimulated with BDNF, the TrkB receptors were dispersed to the periphery of the cell bodies and dendrites in large patches (Fig 1A). In addition, EGFP-Rab5 was concentrated in the cell body, although it was possible to identify some Rab5-positive endosomes in the dendrites (Fig 1A). After stimulation with BDNF, the EGFP-Rab5 distribution was more vesiculated, and there was an apparent increase in the presence of EGFP-Rab5 in dendrites. TrkB distribution also appeared more vesiculated after 15 min of BDNF treatment (Fig 1A). In addition, BDNF increased the colocalization of TrkB-positive endosomes with EGFP-Rab5 endosomes in cell bodies in a time-dependent manner (Fig 1B), as well as in dendrites (Fig 1C). Interestingly, after 5 min of BDNF treatment, the colocalization of TrkB and Rab5 in dendrites was already the same as at 15 min of stimulation. However, in the soma, the

colocalization was increased after 15 min of treatment compared to that at 5 min (Fig 1B and C). These results suggest that BDNF increases TrkB and Rab5 colocalization and changes the dynamics of Rab5 in the dendrites and somas of hippocampal neurons.

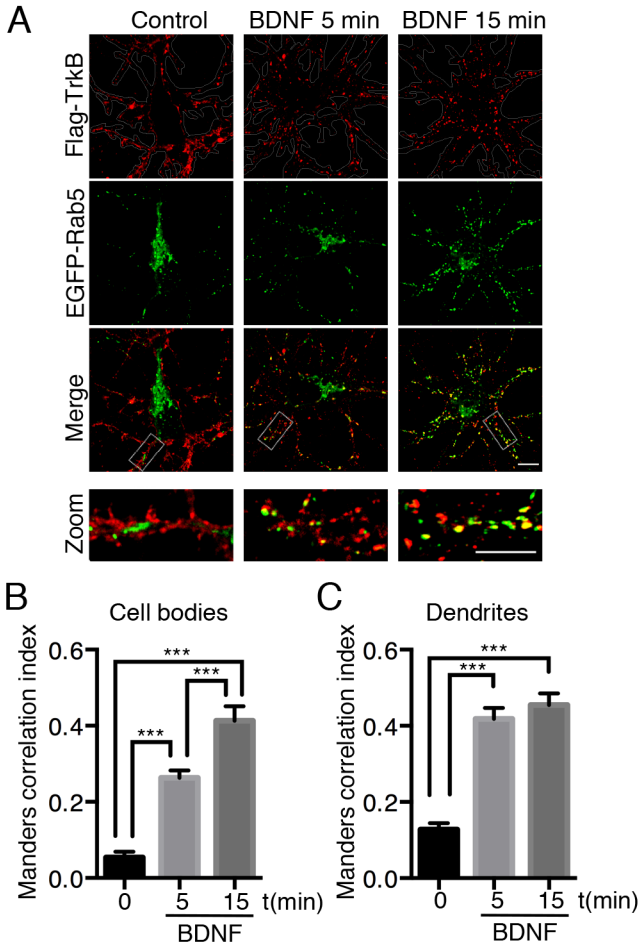
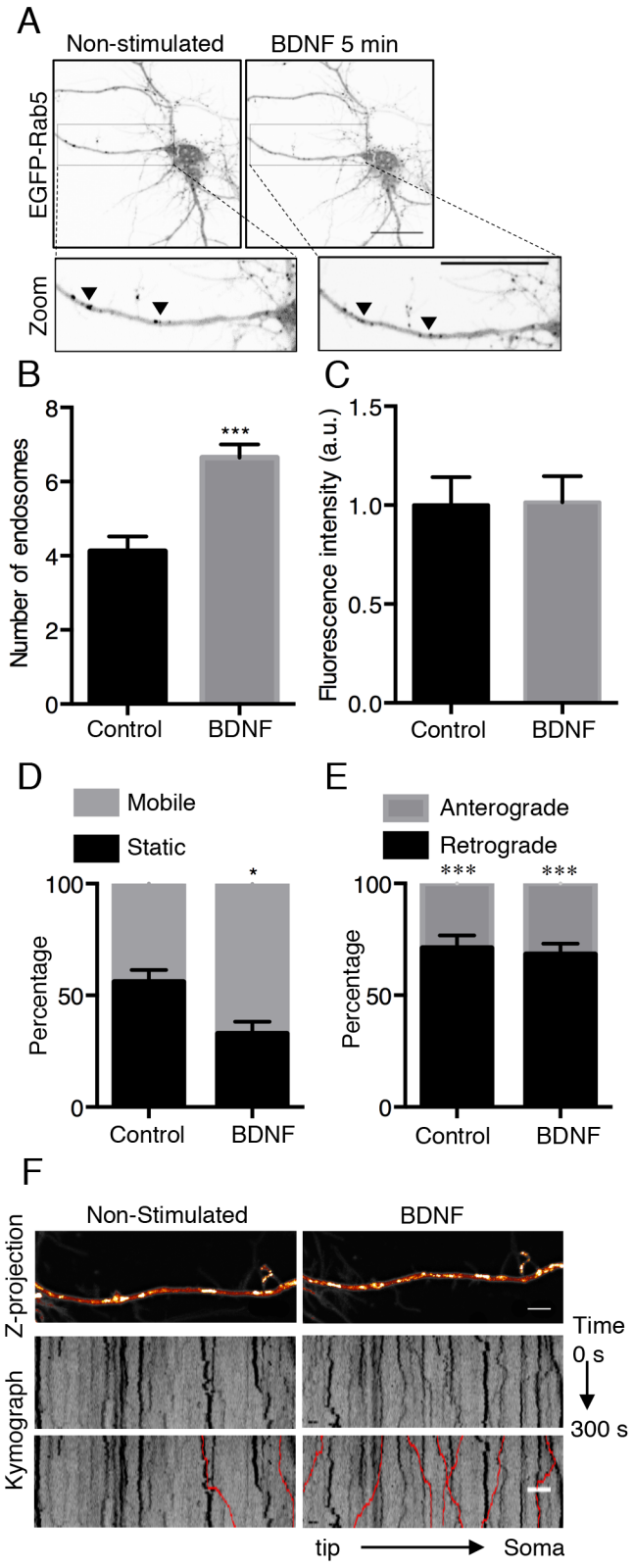


Figure 1. Endocytosed TrkB receptors colocalize with Rab5 after BDNF stimulation in dendrites and cell bodies of hippocampal neurons. Hippocampal neurons (7 DIV) were cotransfected with Flag-TrkB and EGFP-Rab5 (green). After 48 h, neurons were incubated with anti-Flag antibodies (red) at 4°C for 30 min. TrkB internalization was stimulated with BDNF at 37°C for 5 or 15 min and then the neurons were fixed and observed by confocal microscopy. (A) In nonstimulated neurons, TrkB receptors are localized in the plasma membrane, and Rab5 is located within the neurons (time 0). After BDNF stimulation, TrkB receptors show an intracellular distribution and colocalize with Rab5. (B) Quantification of colocalization of TrkB-Flag with EGFP-Rab5 in cell bodies using Manders correlation index. (C) Quantification of colocalization of Flag-TrkB with EGFP-Rab5 in primary dendrites using Manders correlation index. N = 30 neurons from 3 different experiments. The results are expressed as the mean ± SEM. *** $p < 0.001$ by ANOVA with Bonferroni's post-test.

To further study whether BDNF regulates Rab5-positive endosomes, we studied the dynamics of EGFP-Rab5 in transfected hippocampal neurons by time-lapse microscopy of living cells before and after 5 min of BDNF stimulation. We found an increase in the number of Rab5-positive endosomes (Fig 2A and B), defined as dark vesicles using a threshold analysis in ImageJ, without changing the total EGFP-Rab5-associated fluorescence (Fig 2C). Additionally, the mobility of Rab5 after BDNF treatment was increased, measured as endosomes that moved more than 5 μM in a time lapse of 300 s, shown as red lines in the kymograph (Fig 2D and F). Interestingly, the movement of EGFP-Rab5 is biased to the retrograde direction, as reported before in the literature (Ayloo et al., 2017; Kollins et al., 2009), a process that was not changed with the addition of BDNF (Fig 2E).


A
Non-stimulated
BDNF 5 min
EGFP-Rab5
Zoom
B
Number of endosomes
***
Control
BDNF
C
Fluorescence intensity (a.u.)
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D
Mobile
Static
Percentage
*
Control
BDNF
E
Anterograde
Retrograde
Percentage
***
***
Control
BDNF
F
Non-Stimulated
BDNF
Z-projection
Kymograph
Time
0 s
300 s
tip
Soma

Figure 2. BDNF increases the number and mobility of Rab5-positive endosomes in dendrites. (A) Representative image illustrating changes in the number of Rab5-positive endosomes in nonstimulated conditions (control) and after 5 min of BDNF treatment (BDNF) in the same neuron. EGFP-Rab5 endosomes are observed as dark spots within dendrites. A zoomed-in image of the dendrite is shown in the lower part, indicating that BDNF increases the number of Rab5-positive endosomes in dendrites (arrowheads). Scale bar, 10 μm. (B) Quantification of the number of endosomes in 30 μm^2 of dendrites in nonstimulated neurons (control) and after BDNF treatment (5 min). Endosomes were segmented by a fluorescence threshold and then quantified using ImageJ software. A total of 40 dendrites from 6 neurons were included in the study from 3 independent experiments. (C) Quantification of the fluorescence intensity of EGFP-Rab5 in the dendrites of neurons in the nonstimulated and BDNF conditions. (D) Quantification of mobile and static particles in dendrites expressed as a percentage based on the total number of particles per condition. Endosomes that traveled 5 μm or more after 300 s of recording were considered mobile endosomes. (E) Quantification of anterograde and retrograde mobile particles in dendrites expressed as a percentage of the total number of particles in each condition. (F) Representative image of a Z-projection of dendrites in the nonstimulated and BDNF conditions, showing the change in the mobile fraction of EGFP-Rab5. In the lower part is the kymograph of each neurite during the 300 s recording. In red are the endosomes considered to be mobile vesicles. Scale bar, 5 μm. The results are expressed as the mean ± SEM. *$p < 0.5$; or ***$p < 0.001$ by Student's t-test.

BDNF increases the recovery of vesicular Rab5 after photobleaching in the cell body, a process that correlates with increased Rab5 activity.

To better understand the effect of BDNF on the mobility of EGFP-Rab5 endosomes in dendrites, we performed FRAP assays of hippocampal neurons stimulated with BDNF for 5 min. When a dendrite was photobleached, two populations of endosomes where observed: static (white and cyan arrowheads) and mobile (yellow arrowheads) (Fig 3A, B and Supplementary Video 1). Immediately after photobleaching, there was a recovery of cytoplasm-associated fluorescence, as shown with the blue arrow (Fig 3A). Then, an endosome derived from the static endosome, shown as the cyan arrowhead (blue arrow), recovered the fluorescence of the static photobleached endosome (yellow and white arrowheads in Fig 3A).

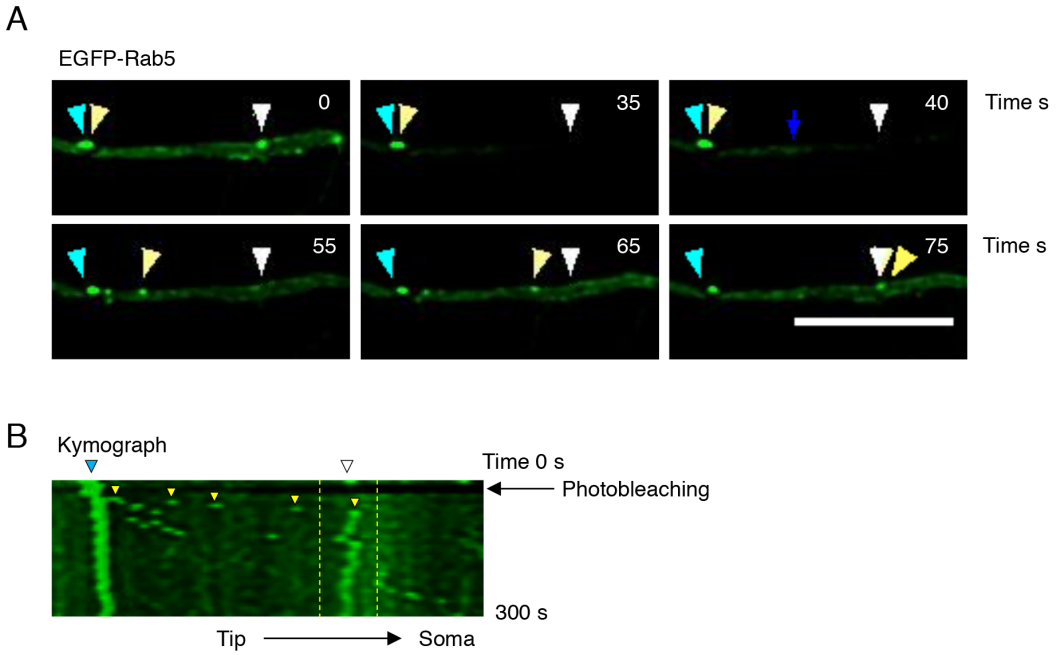

Figure 3. Evaluation of EGFP-Rab5 movement by time-lapse microscopy after photobleaching in hippocampal neurons treated with BDNF. (A) Representative image of FRAP performed in primary dendrites of hippocampal neurons treated with BDNF. The recovery was evaluated during 300 s. Images were taken every 7.3 s. Representative individual frame of time-lapse performed in a neuron expressing EGFP-Rab5. The image shows both cytosolic-associated EGFP-Rab5 and vesicular-associated EGFP-Rab5 fluorescence (green) in dendrites prior to FRAP (0 s), during FRAP (35 s) and after photobleaching (until 75 s). The blue arrow shows nonvesicular (or soluble) EGFP-Rab5 fluorescence recovery. The white arrowhead indicates a static endosome that is photobleached (35 s), which is recovered after 40 s by recruiting a mobile endosome, which is indicated with the yellow arrowhead. The cyan arrowhead shows a static endosome that generates the endosome labeled with a yellow arrowhead. Scale bar, 10 μm. (B) Kymograph of the endosome movement event shown in A. In between the yellow lines is located the endosome recovered (indicated by the white arrowhead in A) by a retrograde-transported endosome, which is indicated by the yellow arrowhead in A. The black arrow indicates the moment in which the photobleaching was performed (10 s).

Consistently, when the soma-associated fluorescence was bleached, it was possible to observe vesicles moving retrogradely towards the soma, as shown in the panels of Fig 4 and Supplementary Videos 2 and 3. Altogether, these results suggest that by increasing the number and mobility of Rab5-positive endosomes, as shown in Fig 2, BDNF increases the retrograde transport of Rab5-positive endosomes to the soma.

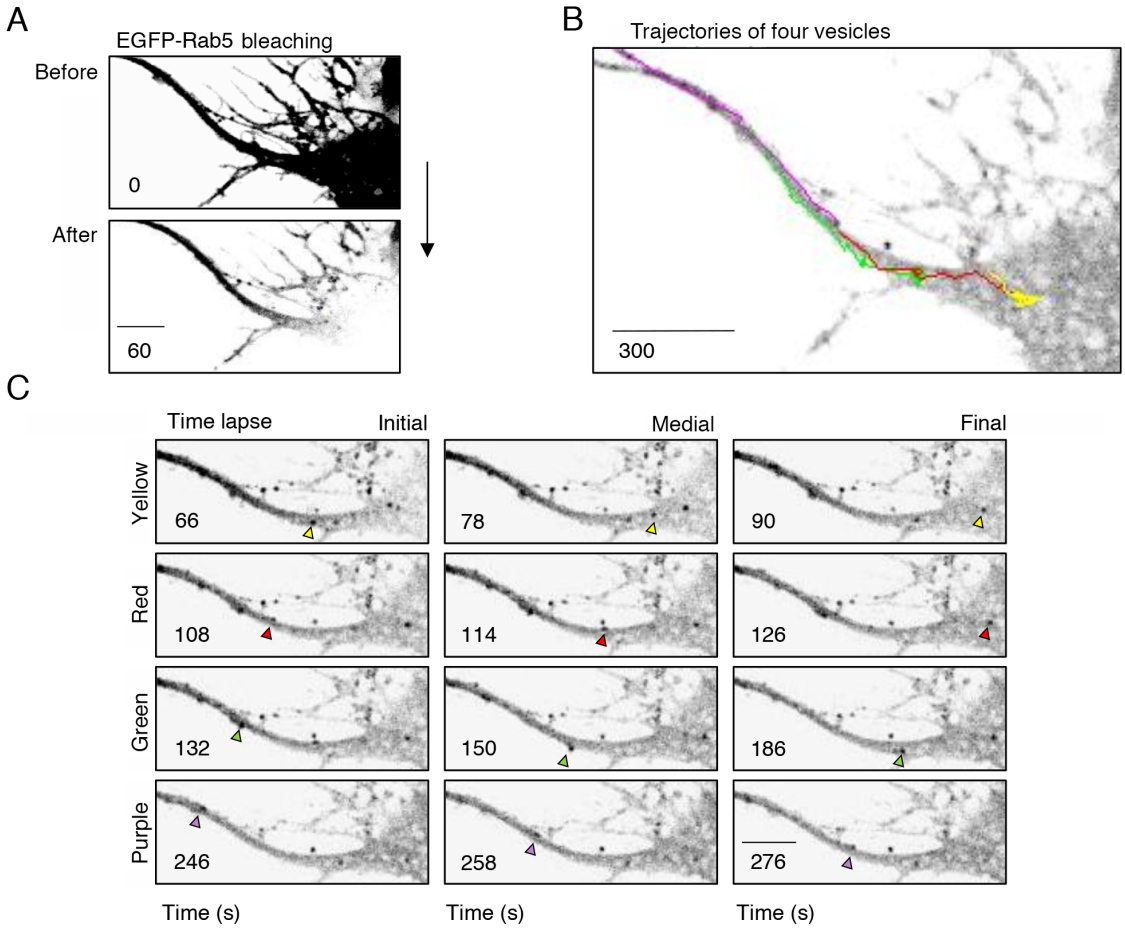

Figure 4. Retrograde transport of EGFP-Rab5 endosomes from dendrites to the cell body. (A) Cell body and primary dendrite of a hippocampal neuron before (upper panel) and after photobleaching (lower panel). (B) Representative image of a time-lapse recording of EGFP-Rab5-associated fluorescence after photobleaching indicating the trajectories of four vesicles positive for EGFP-Rab5 performed in a primary dendrite and soma of a hippocampal neuron stimulated with BDNF. (C) Representative image of the initial, medial and final point of the trajectories shown in B. The vesicles whose trajectories were labeled in B are indicated with arrowheads of the same color of the trajectory. The numbers inside the panels indicate the seconds after photobleaching. Scale bar, 10 µm.

To study this possibility, we utilized FRAP assays of the complete cell bodies, including the initial segments of the dendrites, and studied the recovery of EGFP-Rab5 fluorescence in the soma of the cell bodies of cells treated with or without BDNF for 30 min. We noticed that there were two components in the EGFP-Rab5-associated fluorescence that were recovered after photobleaching. One accounted for the fluorescence associated with cytoplasmic EGFP-Rab5, and the other accounted for the fluorescence of EGFP-Rab5 associated with vesicles (Fig 3A, blue arrow and yellow arrowhead). When we quantified the FRAP in the cell body of EGFP-Rab5-transfected neurons, we did not observe changes in the kinetics of fluorescence recovery in neurons treated with BDNF compared to control neurons (Fig 5B). However, we observed that after BDNF stimulation, the recovery of Rab5-positive endosomes was faster than that in the control condition (Supplementary Videos 4 and 5). Therefore, we applied a threshold to each image obtained after photobleaching, as indicated in Fig 5A, and quantified the fluorescence associated with EGFP-Rab5-positive vesicles. For these experiments, we considered the initial number of vesicles before the photobleaching as 100% of particles and then quantified the number of visible vesicles in the cell body after 5, 15 and 30 min of BDNF stimulation. We found that BDNF increased the number of vesicles in a time-dependent manner compared to the number observed in nontreated neurons (Fig 5A, yellow box in zoom, and Fig 5C). In addition, we repeated this protocol in neurons expressing EGFP-Rab11, and we observed that BDNF did not increase the recovery of EGFP-Rab11 fluorescence in the cell bodies (Fig 5D and Supplementary Fig 1). Because EGFP-Rab11 fluorescence in the cell bodies appeared to be less vesicular and less defined than EGFP-Rab5 fluorescence, we were unable to quantify discrete Rab11 endosomes (Supplementary Fig 1). Our experiments suggest that BDNF increases the transport of Rab5 endosomes towards the cell body.

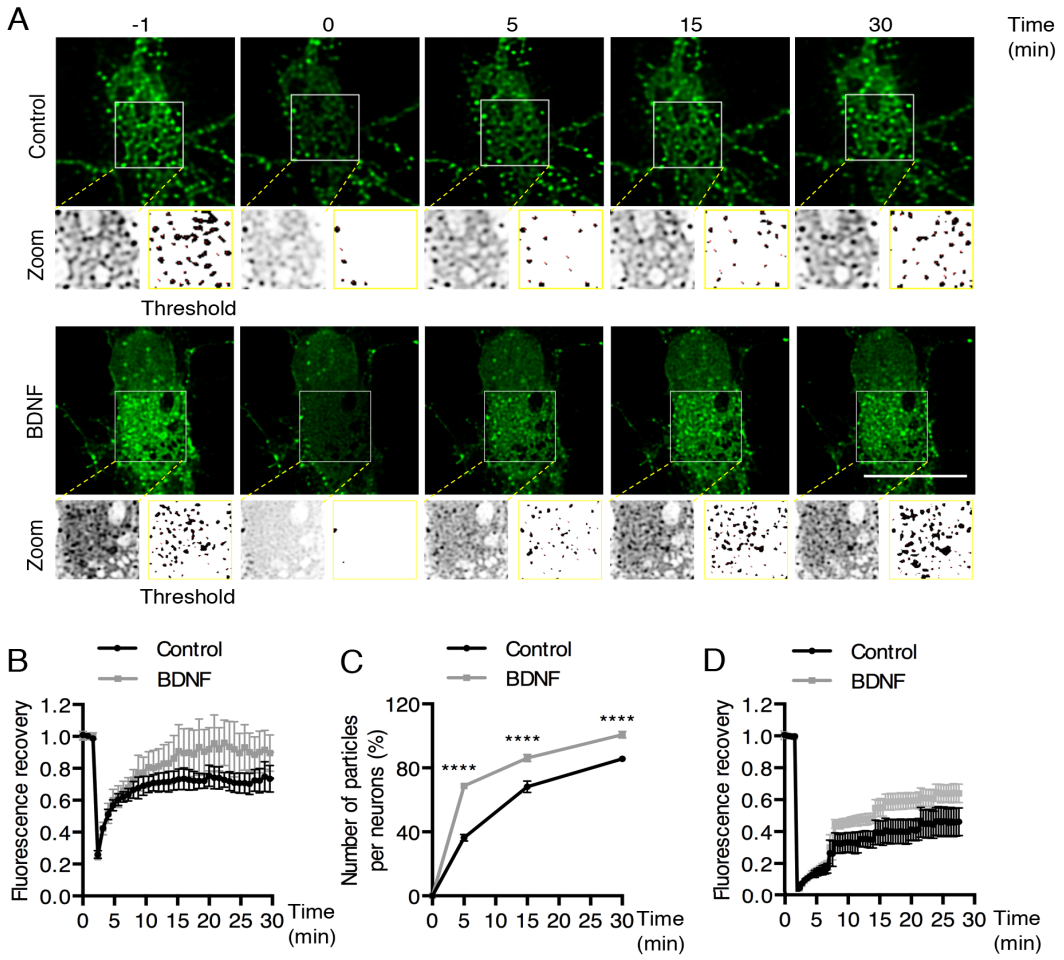

Figure 5. BDNF increases the recovery of Rab5-positive endosomes after photobleaching. (A) FRAP was performed in cell bodies of hippocampal neurons (time 0), and recovery was evaluated after 5, 15 and 30 min of photobleaching. Representative individual frame of a recording performed in a neuron expressing EGFP-Rab5. Recording was performed under control conditions or after BDNF treatment. The image shows endosome-like Rab5 particles in the cell body prior to FRAP (-1 min), during FRAP (0 min) and after FRAP (5, 15, 30 min). The white square indicated in the picture shows a zoomed-in image. On the right, a threshold image of the zoomed image (left) shows the endosomes quantified in C. (B) Time course of fluorescence recovery after FRAP of EGFP-Rab5-associated fluorescence in the photobleached soma. Black squares indicate the control neurons (n = 7) and gray squares the BDNF-stimulated neurons (n = 7). (C) The graph indicates the percentage (100% fluorescence was established at 1 min before FRAP) of endosome-like particles quantified (at t = 0, 5, 15 and 30 min) after FRAP. The black squares indicate the values obtained for the control neurons (n = 7), and gray squares indicate stimulated BDNF neurons (n = 7). (D) Time course of fluorescence recovery after FRAP of EGFP-Rab11-associated fluorescence in the photobleached soma. Black squares indicate the control neurons (n = 5) and gray squares the BDNF-stimulated neurons (n = 6). Four independent experiments were performed. Scale bar, 10 µm. The results are expressed as the mean ± SEM. ****$p < 0.0001$; by two-way ANOVA with Bonferroni's post-test.

To assess whether the increased TrkB/Rab5 colocalization and Rab5 mobility in dendrites and somas correlate with increased Rab5 activity after BDNF stimulation, we studied the distribution of active Rab5 *in situ* using the GST-fused with the Rab5 binding domain of Rabaptin5 (Rab5BD-GST), which specifically recognizes the GTP-bound active form of Rab5 (Wu et al., 2014). Using Rab5BD-GST as a probe of active-endogenous Rab5 (Rab5-GTP), followed by staining with an antibody against GST, we found that the treatment of neurons with BDNF for 5 and 30 min increased the amount of Rab5-GTP in the cell bodies and dendrites of hippocampal neurons in a time-dependent manner, with no changes in the levels of endogenous Rab5 measured by Western blotting (Fig 6A, B, C and E). In cell bodies, there was a significant increase in Rab5 activity after 30 min of BDNF stimulation that was not due to increased levels of Rab5 protein by BDNF treatment (Fig 6B and E). However, in dendrites, we observed increased levels of Rab5-GTP after only 5 min of BDNF stimulation (Fig 6D), similar to the results for TrkB and Rab5 colocalization in dendrites and cell bodies (Fig 1). As negative and positive controls for this experiment, we used hippocampal neurons transfected with a Rab5 dominant-negative (Rab5DN) or a Rab5 constitutively active (Rab5CA) mutant, respectively. Neurons expressing Rab5DN displayed significantly lower Rab5BD-GST labeling than neurons expressing Rab5CA (Fig 6D). Altogether, these results suggest that BDNF increases the activity of Rab5 in the soma and dendrites of hippocampal neurons in a spatial- and time-dependent manner.

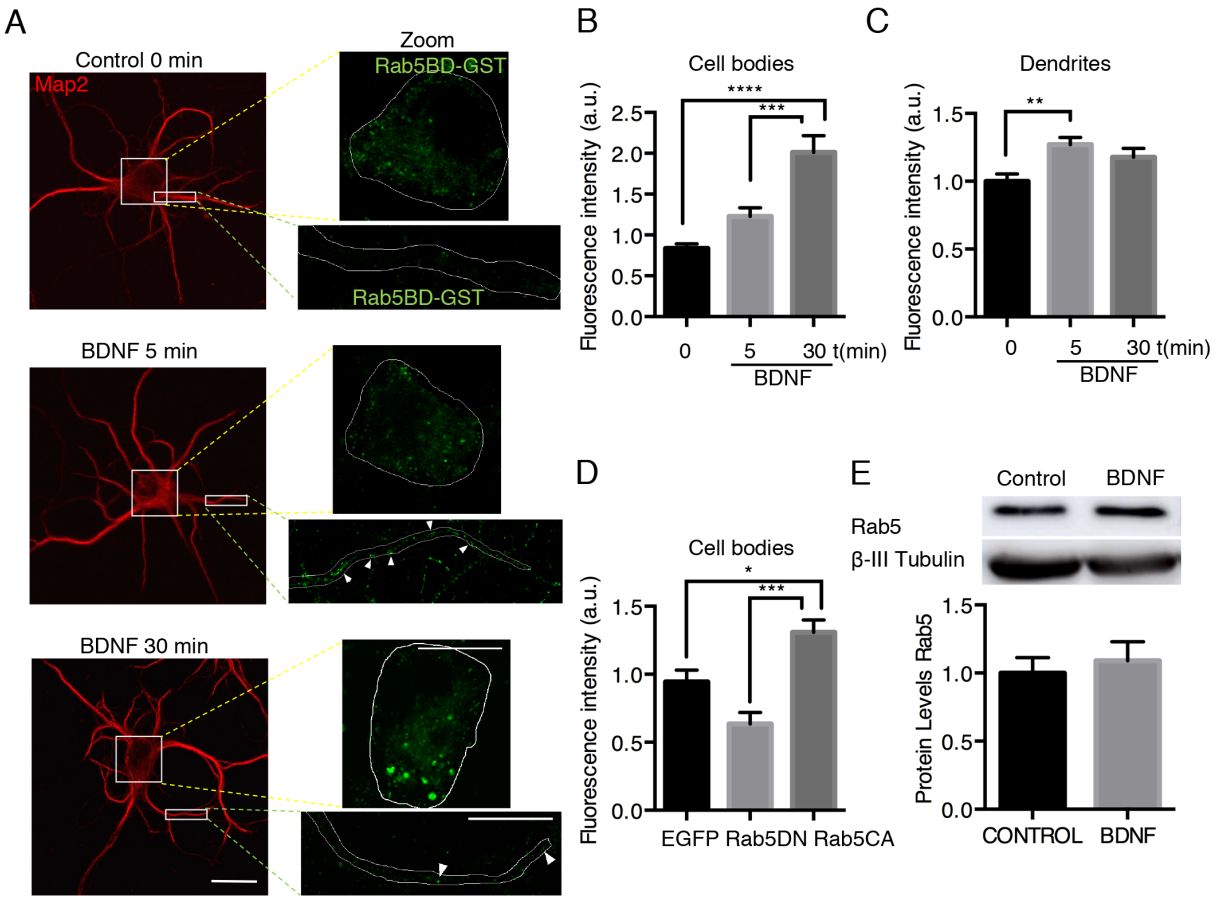

Figure 6. BDNF increases the activity of Rab5. (A) Neurons were nonstimulated (control time 0 min) or stimulated with BDNF for 5 min (BDNF 5 min) and 30 min (BDNF 30 min). Representative image of MAP2 immunostaining (red) and Rab5BD-GST (green). The white box indicates the region shown in the amplified photos of the cell body in the left panel and the dendrite shown in the right panel. Scale bar, 10 µm. (B) Quantification of fluorescence intensity in the cell body. (C) Quantification of fluorescence intensity in primary dendrites of control neurons and neurons treated for 5 min and 30 min with BDNF. The results for the soma and dendrites were normalized to the values of control neurons (time 0 min). A total of 28-29 cell bodies and 66-87 dendrites were included from 4 independent experiments. (D) Quantification of fluorescence intensity in the cell bodies of 7 DIV neurons transfected with EGFP, EGFP-Rab5DN or EGFP-Rab5CA. The results are expressed as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ by ANOVA with Bonferroni's posttest. (E) The Rab5a and ß-III tubulin levels of hippocampal neurons (9 DIV) stimulated with BDNF for 30 min. Lower part, quantification of the Rab5 levels of three independent experiments. The results are expressed as the mean ± SEM by Student's t-test.

Long-term treatment of hippocampal neurons with BDNF results in increased protein levels of Rab5 and Rab11.

BDNF signaling increases protein levels by increasing transcription in a CREB-dependent manner downstream of PLC-gamma and ERK1/2 or translation in a mTOR-dependent manner downstream of PI3K and ERK1/2 signaling (Gonzalez et al., 2016). Therefore, we studied the effect of long-term administration of BDNF (4-24 h) on Rab5 and Rab11 protein levels. First, we studied whether the administration of BDNF for 4 or 12 h regulated the levels of the *rab5a* and *rab11a* genes. We found that BDNF increased the levels of *rab5a* in a time-dependent manner; an approximately 4-fold increase in *rab5a* was observed after 4 h of BDNF treatment (Supplementary Fig 2A), whereas after 12 h of BDNF stimulation, the levels of *rab5a* decreased to approximately 0.5-fold over the levels of the control (Supplementary Fig 2B). Conversely, the level of *rab11a* was unchanged by BDNF treatment at any of the time points studied (Supplementary Fig 2A and 2B). In this context, we first evaluated whether BDNF increases the protein levels of Rab5 after 24 h of BDNF treatment. We found that BDNF increased the level of Rab5a in approximately 20% of hippocampal neurons (Fig 7A) in a transcription- and translation-dependent manner, which is consistent with the results presented in Fig 7B and C, showing that actinomycin D and cycloheximide reduced the levels of Rab5 after BDNF treatment. Since BDNF increases protein translation in an mTOR-dependent manner (Takei et al., 2004), we evaluated whether the increase in Rab5a protein levels was sensitive to rapamycin, an mTOR pathway inhibitor (Schratt et al., 2004). We observed that 4 h of BDNF treatment did not affect the protein levels of Rab5; however, the presence of rapamycin decreased the increase in Rab5 protein levels caused by 12 h of BDNF treatment to the basal level (Fig 7D and E). These results indicate that BDNF regulates Rab5 protein levels by increasing or stabilizing its

mRNA and by increasing its translation in an mTOR-dependent manner.

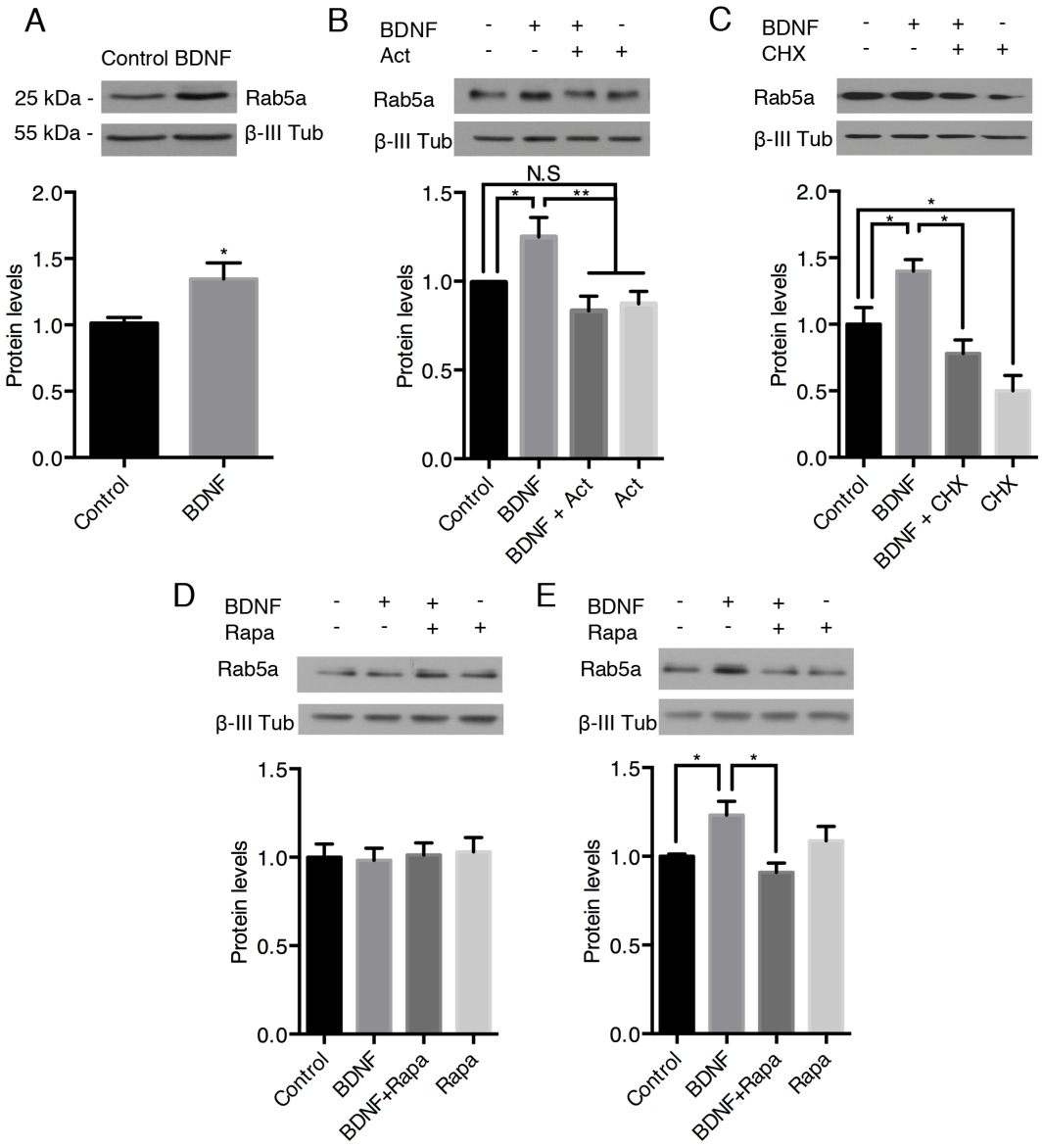

Figure 7. BDNF increases the protein levels of Rab5a. The Rab5a and ß-III tubulin levels in hippocampal neurons (9 DIV) stimulated with BDNF for 24 h (A) in the presence or absence of Actinomycin D (B) or cycloheximide (CHX) (C). Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent 5 independent experiments. The results are expressed as the mean ± SEM. *p < 0.05; **p < 0.01 by ANOVA with Bonferroni's posttest. (D-E) Ra5a levels after 4 h (D) or 12 h (E) of treatment with rapamycin (Rapa) in the presence or absence of BDNF. Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent 4 independent experiments. The results are expressed as the mean ± SEM. *p < 0.05; **p < 0.01 by ANOVA with Bonferroni's post-test.

We also analyzed the protein levels of Rab11 upon BDNF treatment. Similar, to the findings for Rab5, BDNF increased the level of Rab11 after 24 h of treatment; this effect was abolished by cycloheximide (Fig 8A and B). In contrast, 4 h of BDNF treatment did not affect the protein level of Rab11 (Fig 8C), while 12 h of BDNF treatment increased the level of Rab11 to values similar to 24 h of treatment (Fig 8D). In addition, the BDNF effect on the Rab11 protein levels (12 h treatment) was diminished by rapamycin (Fig 8D). All together, these results indicate that BDNF regulated the levels of both the Rab5 and Rab11 GTPases at the translational level in an mTOR-dependent manner.

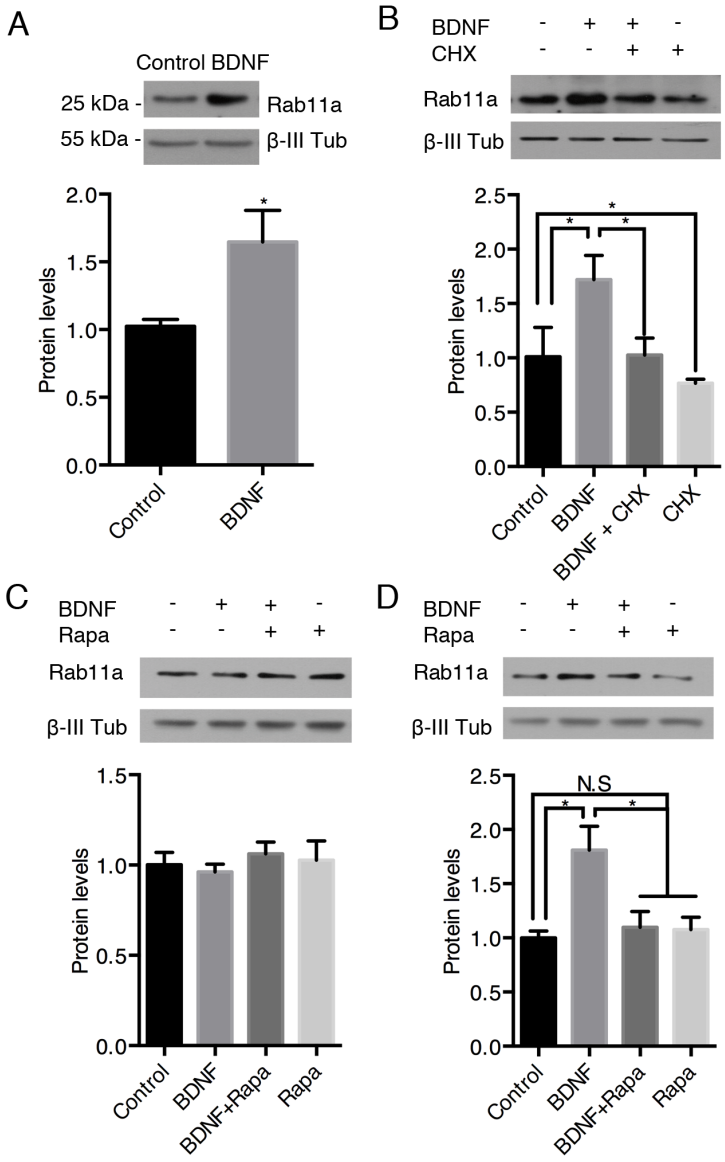

Figure 8. BDNF increases the protein levels of Rab11a. The Rab11a and ß-III tubulin levels in hippocampal neurons (9 DIV) stimulated with BDNF for 24 h (A) in the presence or absence of cycloheximide (CHX) (B). Bottom panel, densitometric quantification of the Ra5a levels normalized to the ß-III tubulin levels. The data represent 5 independent experiments. The results are expressed as the mean ± SEM. *$p < 0.05$; **$p < 0.01$ by ANOVA with Bonferroni's posttest. (C-D) Ra11a levels after 4 h (C) or 12 h (D) of treatment with rapamycin (Rapa) in the presence or absence of BDNF. Bottom panel, densitometric quantification of the Ra11a levels normalized to the ß-III tubulin levels. The data represent 4 independent experiments. The results are expressed as the mean ± SEM. *$p < 0.05$; by ANOVA with Bonferroni's post-test.

Long-term treatment of hippocampal neurons with BDNF results in increased dendritic branching that is impaired by reducing the activity of the Rab5 and Rab11 proteins.

It is well known that BDNF induces an increase in dendritic branching both *in vivo* and *in vitro* (Gonzalez et al., 2016). To evaluate whether Rab5 activity is required for BDNF-induced dendritic branching in hippocampal neurons, we stimulated neurons expressing EGFP or the dominant-negative mutant of Rab5 (EGFP-Rab5DN) with BDNF for 48 h. We found that the expression of EGFP-Rab5DN produced a change in the morphology of the somato-dendritic arbor in comparison with neurons that only expressed EGFP (Fig 9A). Using Sholl analysis and the quantification of branching points, we found that the expression of Rab5DN reduces the branching points compared to the control condition (Fig 9A-C). Although neurons expressing Rab5DN responded to BDNF by increasing the number of primary dendrites, they were not able to respond to the same extent as neurons expressing EGFP and treated with BDNF, which showed an increase in branching points in addition to an increase in the number of primary dendrites (Fig 9 C). These results are somehow different from those observed when neurons express a dominant-negative mutant for Rab11 (Rab11DN). Similar to our previous observations (Lazo et al., 2013), neurons expressing Rab11DN have a similar number of dendrites to neurons expressing EGFP. However, they did not respond to BDNF (Fig 9 D-F). These results indicate that Rab5 activity is required for the maintenance of dendritic arbors *in vitro,* and although EGFP-Rab5-expressing neurons responded to BDNF by increasing the number of primary dendrites, Rab5 activity is required to observe the effect of BDNF on the branching of higher level dendrites.

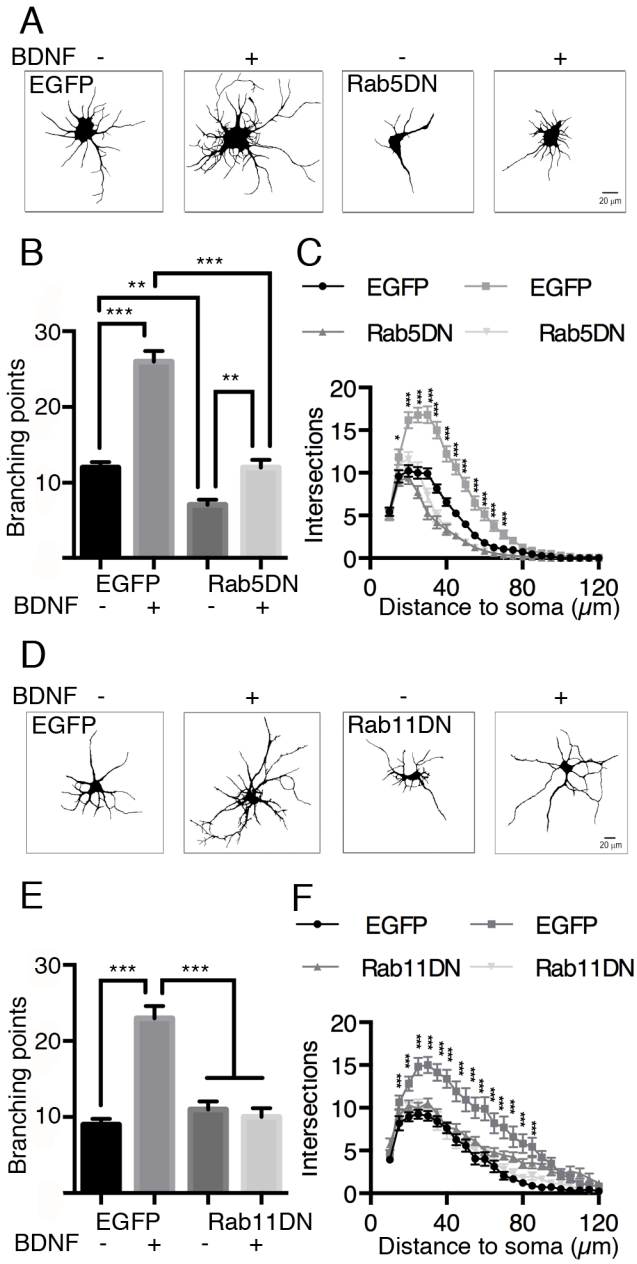
A
BDNF - + - +
EGFP
Rab5DN
B
Branching points
EGFP
Rab5DN
BDNF - + - +
C
EGFP
EGFP
Rab5DN
Rab5DN
Intersections
Distance to soma (μm)
D
BDNF - + - +
EGFP
Rab11DN
E
Branching points
EGFP
Rab11DN
BDNF - + - +
F
EGFP
EGFP
Rab11DN
Rab11DN
Intersections
Distance to soma (μm)

Figure 9. The expression of dominant-negative mutants of Rab5 (Rab5DN) decreases BDNF-induced dendritic branching in hippocampal neurons. (A) Representative image of hippocampal neurons (7 DIV) transduced with adenovirus EGFP (in A at the left) or with a dominant-negative mutant of Rab5 fused to EGFP (Rab5DN in A at the right) and stimulated with BDNF for 48 h. After fixation, the neurons were labeled with anti-MAP2, observed by fluorescence microscopy and subjected to morphometric analysis. (B) Sholl analysis of the arborization profiles of neurons expressing EGFP and Rab5DN-EGFP in the presence or absence of BDNF. (C) Quantification of the branching points of neurons that overexpressed Rab5DN or EGFP and exposed to BDNF. N = 27-34 neurons from 3 different experiments. (D) Representative image of hippocampal neurons (7 DIV) that were transduced with adenovirus EGFP (in D at the left) or a dominant-negative mutant of Rab11 fused to EGFP (Rab11DN in A at the right) and stimulated with BDNF for 48 h. After fixation, the neurons were labeled with anti-MAP2, observed by fluorescence microscopy and subjected to morphometric analysis. (E) Sholl analysis of the arborization profiles of neurons expressing EGFP and Rab11DN-EGFP in the presence or absence of BDNF. (F) Quantification of the branching points of neurons that overexpressed Rab11DN or EGFP exposed to BDNF. N = 16-19 neurons from 3 different experiments. The results are expressed as the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by two-way ANOVA with Bonferroni's post-test.

DISCUSSION

Several lines of evidence have consistently shown that the internalization and transit of Trk receptors through the endocytic pathway are required for proper signaling and neuronal function (Bronfman et al., 2014; Cosker and Segal, 2014). The mechanism by which neurotrophin receptors use the endosomal pathway for signaling in neurons is well documented. For example, "signaling endosomes" containing ligand-bound neurotrophin receptors have been extensively described for axon-to-nucleus communication in peripheral neurons (Bronfman et al., 2003; Delcroix et al., 2003; Harrington et al., 2011). Additionally, BDNF signaling endosomes have been described to have a role in dendrite-to-nucleus communication in central neurons (Cohen et al., 2011). However, how neurotrophins regulate the endosomal system for proper signaling is just beginning to be understood (Cosker and Segal, 2014). Rabs are monomeric GTPases that act as molecular switches to regulate membrane trafficking. They achieve this function by binding a wide range of effectors that include SNAREs, signaling molecules and molecular motors. Among the Rab GTPases, Rab5 is the key GTPase regulating early endosomes and the first endocytic station of endocytosed receptors (Stenmark, 2009). Of note, several lines of evidence have shown that there is crosstalk between Rab5 activity and tyrosine kinase signaling receptors (Chiariello et al., 1999; Jozic et al., 2012; Ong et al., 2014). Our aim was to study the regulation of the Rab5-positive endosomes in relation to BDNF and at different levels, including dynamics, activity and protein levels in hippocampal neurons. We found that BDNF increased the colocalization of TrkB in dendrites and cell bodies, increasing the vesiculation of Rab5-positive endosomes in the somatodendritic compartment. These findings correlated with the increased mobility of Rab5 endosomes in dendrites and increased the movement of Rab5 endosomes from dendrites to the cell body. Consistently, BDNF induced an early activation of

Rab5 in dendrites (5 min) followed by increased activation of Rab5 in cell bodies (30 min). Long-term treatment of hippocampal neurons with BDNF (12-24 h) increased the protein levels of Rab5 and Rab11 in an mTOR-dependent manner. Finally, expression of a dominant-negative mutant of Rab5 reduced the basal arborization of nontreated neurons and BDNF-induced arborization. We propose that BDNF increases the activity of Rab5 in dendrites to foster local dendritic growth and to increase BDNF signaling propagation to the cell soma.

We have previously shown that BDNF/TrkB increases the activity of Rab11 in dendrites of hippocampal neurons by increasing local recycling and thus signaling of BDNF (Lazo et al., 2013). Rab5 regulates the fusion of endocytosed vesicles to form early endosomes where receptors are sorted to the recycling pathway that is regulated by Rab11. Here, we show that BDNF signaling also regulates the activity of Rab5, suggesting that BDNF in dendrites increases the activity of both GTPases to increase the early recycling pathways for local signaling. One intriguing aspect of our research, however, is that the mobility of both endosomes was oppositely regulated by BDNF. While BDNF decreases the mobility of Rab11 to allow local recycling (Lazo et al., 2013), it increases both the number and mobility of Rab5-positive vesicles in dendrites (current study). The movement of Rab5 vesicles increased in both the anterograde and retrograde directions. However, Rab5 movements were biased to the retrograde direction, consistent with a study indicating that 60% of microtubules are oriented with the minus end towards the soma in mammalian cells (Ayloo et al., 2017). It is possible that while anterograde movement of Rab5-positive vesicles is required for dendritic growth, retrograde movement of Rab5 resulted in increased levels of Rab5-positive vesicles in the cell body. Consistently, we showed by live-cell microscopy that Rab5-positive endosomes moved from primary dendrites to the cell body. While performing live-cell microscopy of dendritic EGFP-Rab5 transfected

neurons after photobleaching, we observed that Rab5-associated fluorescence recovered in the same place, in addition to the observed mobile vesicles (Fig 3), suggesting that we monitored both stationary and mobile early endosomes. Altogether, our research suggests that BDNF defines a different population of Rab5 early endosomes that sort components to the recycling pathway for local recycling, and another population engages in long-distance trafficking to the soma or to distal dendrites. It is possible that a coordinated action of actin-based motors regulates local trafficking of signaling receptors since both Rab5 and Rab11 interact with myosin proteins to coordinate local membrane trafficking (Masters et al., 2017; Schafer et al., 2014; Sui et al., 2015).

Different lines of evidence have shown that both dynein and neuronal kinesin KIF21B engage TrkB-BDNF for long-distance trafficking in dendrites (Ayloo et al., 2017; Ghiretti et al., 2016). On the other hand, active Rab5 has been described to bind the Hook-interacting protein complex, which interacts with dynein and dynactin to regulate the retrograde transport of axonal proteins in neurons (Guo et al., 2016). Additionally, there is evidence that dynein and dynactin contribute to 85-98% of long-inward translocation of Rab5 early endosomes in HeLa cells (Flores-Rodriguez et al., 2011). Of note, dynein-mediated transport of Rab5-positive early endosomes is required for dendritic branching in *Drosophila melanogaster* dopamine neurons (Satoh et al., 2008). These results are consistent with our findings showing that Rab5 activity is required for the stability of dendrites and BDNF-mediated dendritic branching in hippocampal neurons (Fig 7). Altogether, these results suggest that microtubule-associated molecular motors, most likely dynein, drive the long-distance movement of Rab5 endosomes from dendrites to the soma in response to BDNF, which is a process required for dendritic branching. This process might be important in dendrite-to-nucleus communication as suggested by the results showing that

dendritic BDNF increases expression of the immediate early genes c-fos and Arc in the cell bodies of both hippocampal and striatal neurons (Cohen et al., 2011; Liot et al., 2013).

We also observed that BDNF increases Rab5 vesiculation and the number of Rab5-positive endosomes in dendrites, a process that correlates with increased vesicles containing active Rab5. Fusion and fission events are required for proper early endosome function and sorting of endocytosed receptors and ligands (Skjeldal et al., 2012). Rab5 regulates these process by regulating fusion of newly endocytosed receptors to form the early or sorting endosomes; from there, fission events allow sorting into the endocytic pathways (Driskell et al., 2007). It is possible that BDNF increases fusion and fission events to increase the vesiculation of Rab5 in dendrites, or increases the recruitment of cytosolic inactive Rab5 to newly formed or preexisting endosomes (Fig 10). However, direct evidence of these phenomena remain to be analyzed by a more refined technique such as fluorescence resonance energy transfer as performed by Verboogen to visualize SNARE trafficking and fusion (Verboogen et al., 2017).

Like other Rab proteins, Rab5 activity and localization is regulated by GEFs, GAPs and different effectors (Stenmark, 2009; Zerial and McBride, 2001). Different extracellular cues have been described to regulate Rab5 activity. For instance, in PC12 cells, NGF activation of TrkA recruits RabGAP5, which inactivates Rab5, producing a delay in the maturation of signaling endosomes and prolonging signaling and neurite outgrowth in PC12 cells (Liu et al., 2007). Consistently, expression of a dominant-negative Rab7 in PC12 cells enhances NGF-mediated signaling (Saxena et al., 2005) while it abolishes axonal retrograde transport of TrkB-positive endosomes in motor neurons (Deinhardt et al., 2006). On the other hand, in cortical neurons, semaphorin 3A increases the activity of Rab5 in axons to promote growth cone collapse (Wu et al., 2014), suggesting that the activation of Rab5 might induce different outcomes

depending on the extracellular cues, the signaling pathways activated and the neuronal processes that are regulated. We observed that BDNF increases Rab5 activity and that these processes are required for BDNF-dependent dendritic arborization, suggesting that in hippocampal neurons, BDNF-mediated activation of Rab5 is required for proper signaling, contrary to the results observed in PC12 cells (Liu et al., 2007). One question that arises is how BDNF regulates the activity of Rab5. There are no antecedents that could lead us to hypothesize a direct effect of TrkB signaling on Rab5 activity. However, we could speculate that by phosphorylating Rab5, BDNF modulates its interaction with GEFs, thus increasing its activation. In support of this speculation is the fact that different kinases including ERK1, a BDNF/TrkB downstream kinase, phosphorylate Rab5 (Chiariello et al., 1999), and Rin1, a GEF for Rab5, has been associated with other RTKs to increase Rab5 activity (Hunker et al., 2006). The activation of Rab5 should be a tightly regulated process, and we observed that the activation of Rab5 induced by BDNF is time dependent without promoting sustained activation. Sustained activation of Rab5 in axons disrupts retrograde axonal trafficking of NGF signals in basal forebrain cholinergic neurons, suggesting that Rab5 activity must be tightly regulated for proper neuronal function (Xu, et al., 2016). Overactivation of Rab5 could be deleterious for neurons. Indeed, we have observed that expression of Rab5CA induces neurodegeneration in hippocampal cultures (data not shown), a phenomenon that it is not observed when hippocampal neurons are transduced with Rab11CA (Lazo et al., 2013).

Our results indicate that both Rab5 and Rab11 activity are required for BDNF-induced dendritic branching, indicating that the transit and correct endosomal sorting of BDNF receptors are required for proper signaling. For example, retrolinkin, a receptor that tethers vesicles, interacts with endophilin A1, a protein involved in generating endocytic necks, which is recruited to the

early endosomal compartment in response to BDNF (Burk et al., 2017). Both proteins are required for BDNF early endocytic trafficking and spatiotemporal regulation of BDNF-induced ERK activation (Fu et al., 2011).

Finally, we found that BDNF increases both the mRNA and proteins levels of Rab5a in an mTOR-dependent manner. The activation of mTOR kinase has been described as a key signaling pathway regulating the translation of proteins mediated by BDNF (Leal et al., 2014). Specificity is achieved because BDNF, in addition to regulating translation, induces a specific miRNA-dependent repression (specific miRNA downregulation) and stabilizes the Dicer-TRBP complex, increasing global maturation of miRNA (Ruiz et al., 2014). The fact that the protein levels of Rab5 and Rab11 are upregulated by BDNF in an mTOR-dependent manner suggests that the specific growth program initiated by BDNF acts as a positive feedback loop to increase BDNF- and Rab5-Rab11-dependent dendritic growth. Indeed, mTOR activation is required for dendritic arborization of central neurons (Jaworski et al., 2005) and, consistently, both Rab5 and Rab11 activity is required for BDNF-induced neuronal growth.

Our results and those of others allow us to propose a model (Fig 10) addressing the functional role between the early recycling pathway regulated by Rab5-Rab11 and BDNF/TrkB signaling in neurons. We propose that BDNF is able to regulate the endosomal system by regulating the activity of Rab5 and Rab11 in a time- and space-dependent manner. This process allows both increased local signaling in dendrites and increased signaling in cell bodies. While this model predicts two different populations of recycling endosomes (dendritic Rab11 endosomes versus the perinuclear cell body Rab11 recycling endosome), the early endosomal pathway might be coordinating dendritic and cell body signaling.

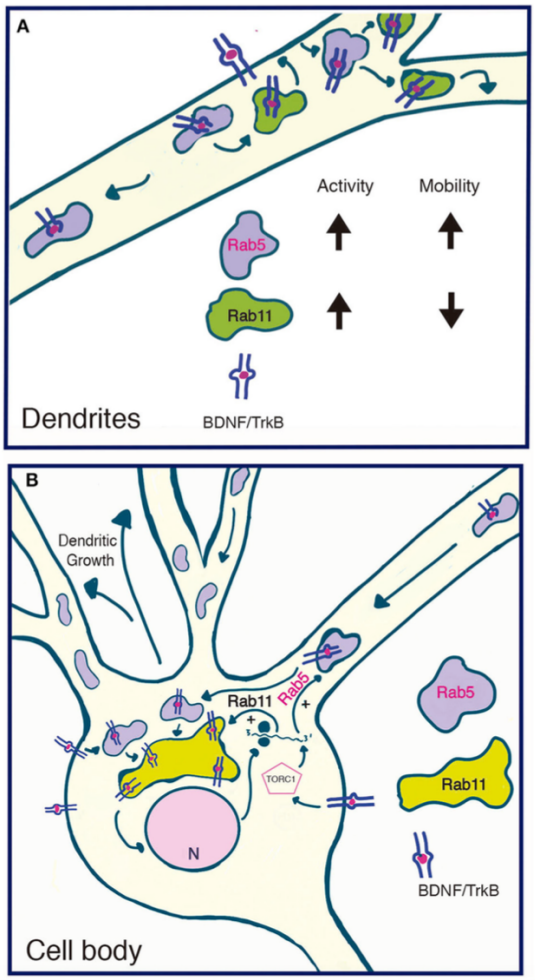

Figure 10. Model summarizing the functional relationship between the early recycling pathway and BDNF/TrkB signaling. (A) In dendrites, BDNF increases the activity and reduces the mobility of Rab11-endosomes, fostering the local recycling of TrkB in dendrites and increasing local BDNF signaling (Lazo OM, et al 2013). On the other hand, BDNF transiently increases the activity of Rab5, a process that increases the number and mobility of Rab5 endosomes in dendrites. Since Rab5 early endosomes are upstream of Rab11 recycling endosomes, increased activity of Rab5 may, on the one hand, foster the Rab11-dependent recycling of TrkB in dendrites and, on the other hand, increase the transport of Rab5 endosomes to the soma. (B) In the soma, increased activity of both Rab5 (this paper) and Rab11 (Lazo OM, et al 2013) by BDNF may increase the cell body recycling of TrkB, increasing the long-lasting signaling of BDNF required for dendritic growth. Increased activity of the TORC1 complex by BDNF increased the protein levels of Rab5 and Rab11 acting as a positive feedback loop that contributes to BDNF-induced dendritic branching.

Altogether, our results suggest that Rabs are key proteins that regulate BDNF signaling, and further research is required to better understand the mechanism that leads to BDNF-mediated activation of Rab5 and Rab11 and how this process is coordinated with molecular motors for both local and long-distance signaling of BDNF.

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REFERENCES

Ascano, M., Bodmer, D., and Kurovilla, R. (2012). Endocytic trafficking of neurotrophins in neural development. Trends in Cell Biology *22*, 266-273.

Ayloo, S., Guedes-Dias, P., Ghiretti, A.E., and Holzbaur, E.L.F. (2017). Dynein efficiently navigates the dendritic cytoskeleton to drive the retrograde trafficking of BDNF/TrkB signaling endosomes. Mol Biol Cell *28*, 2543-2554.

Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. J Microsc *224*, 213-232.

Bronfman, F.C., Lazo, O.M., Flores, C., and Escudero, C.A. (2014). Spatiotemporal intracellular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. Handb Exp Pharmacol *220*, 33-65.

Bronfman, F.C., Tcherpakov, M., Jovin, T.M., and Fainzilber, M. (2003). Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. J Neurosci *23*, 3209-3220.

Burk, K., Murdoch, J.D., Freytag, S., Koenig, M., Bharat, V., Markworth, R., Burkhardt, S., Fischer, A., and Dean, C. (2017). EndophilinAs regulate endosomal sorting of BDNF-TrkB to mediate survival signaling in hippocampal neurons. Sci Rep *7*, 2149.

Chiariello, M., Bruni, C.B., and Bucci, C. (1999). The small GTPases Rab5a, Rab5b and Rab5c are differentially phosphorylated *in vitro*. FEBS Lett *453*, 20-24.

Cohen, M.S., Bas Orth, C., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2011). Neurotrophin-mediated dendrite-to-nucleus signaling revealed by microfluidic compartmentalization of dendrites. Proc Natl Acad Sci U S A *108*, 11246-11251.

Cosker, K.E., and Segal, R.A. (2014). Neuronal signaling through endocytosis. Cold Spring Harb Perspect Biol *6*.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., and Schiavo, G. (2006). Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron *52*, 293-305.

Delcroix, J.D., Valletta, J.S., Wu, C., Hunt, S.J., Kowal, A.S., and Mobley, W.C. (2003). NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron *39*, 69-84.

Driskell, O.J., Mironov, A., Allan, V.J., and Woodman, P.G. (2007). Dynein is required for receptor sorting and the morphogenesis of early endosomes. Nat Cell Biol *9*, 113-120.

Farnsworth, C.C., Seabra, M.C., Ericsson, L.H., Gelb, M.H., and Glomset, J.A. (1994). Rab geranylgeranyl transferase catalyzes the geranylgeranylation of adjacent cysteines in the small GTPases Rab1A, Rab3A, and Rab5A. Proc Natl Acad Sci U S A *91*, 11963-11967.

Flores-Rodriguez, N., Rogers, S.S., Kenwright, D.A., Waigh, T.A., Woodman, P.G., and Allan, V.J. (2011). Roles of dynein and dynactin in early endosome dynamics revealed using automated tracking and global analysis. PLoS One *6*, e24479.

Fu, X., Yang, Y., Xu, C., Niu, Y., Chen, T., Zhou, Q., and Liu, J.J. (2011). Retrolinkin cooperates with endophilin A1 to mediate BDNF-TrkB early endocytic trafficking and signaling from early endosomes. Mol Biol Cell *22*, 3684-3698.

Ghiretti, A.E., Thies, E., Tokito, M.K., Lin, T., Ostap, E.M., Kneussel, M., and Holzbaur, E.L.F. (2016). Activity-Dependent Regulation of Distinct Transport and Cytoskeletal Remodeling Functions of the Dendritic Kinesin KIF21B. Neuron *92*, 857-872.

Fan GH, Lapierre LA, Goldenring JR, Sai J, Richmond A (2004) Rab11- family interacting protein 2 and myosin Vb are required for CXCR2 re- cycling and receptor-mediated chemotaxis. Mol Biol Cell 15:2456–2469.

Goh, L.K., and Sorkin, A. (2013). Endocytosis of receptor tyrosine kinases. Cold Spring Harb Perspect Biol *5*, a017459.

Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billaut, C., and Bronfman, F.C. (2016). Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor. Cytoskeleton *73*, 612-628.

Grosshans, B.L., Ortiz, D., and Novick, P. (2006). Rabs and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci U S A *103*, 11821-11827.

Guo, X., Farias, G.G., Mattera, R., and Bonifacino, J.S. (2016). Rab5 and its effector FHF contribute to neuronal polarity through dynein-dependent retrieval of somatodendritic proteins from the axon. Proc Natl Acad Sci U S A *113*, E5318-5327.

Harrington, A.W., St Hillaire, C., Zweifel, L.S., Glebova, N.O., Philippidou, P., Halegoua, S., and Ginty, D.D. (2011). Recruitment of actin modifiers to TrkA endosomes governs retrograde NGF signaling and survival. Cell *146*, 421-434.

Horch, H.W., and Katz, L.C. (2002). BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat Neurosci *5*, 1177-1184.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem *72*, 609-642.

Huang, S.H., Wang, J., Sui, W.H., Chen, B., Zhang, X.Y., Yan, J., Geng, Z., and Chen, Z.Y. (2013). BDNF-dependent recycling facilitates TrkB translocation to postsynaptic density during LTP via a Rab11-dependent pathway. J Neurosci *33*, 9214-9230.

Hunker, C.M., Giambini, H., Galvis, A., Hall, J., Kruk, I., Veisaga, M.L., and Barbieri, M.A. (2006). Rin1 regulates insulin receptor signal transduction pathways. Exp Cell Res *312*, 1106-1118.

Jaworski, J., Spangler, S., Seeburg, D.P., Hoogenraad, C.C., and Sheng, M. (2005). Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway. J Neurosci *25*, 11300-11312.

Jozic, I., Saliba, S.C., and Barbieri, M.A. (2012). Effect of EGF-receptor tyrosine kinase inhibitor on Rab5 function during endocytosis. Arch Biochem Biophys *525*, 16-24.

Kollins, K.M., Bell, R.L., Butts, M., and Withers, G.S. (2009). Dendrites differ from axons in patterns of microtubule stability and polymerization during development. Neural Dev *4*, 26.

Kumar, V., Zhang, M.X., Swank, M.W., Kunz, J., and Wu, G.Y. (2005). Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci *25*, 11288-11299.

Lazo, O.M., Gonzalez, A., Ascano, M., Kuruvilla, R., Couve, A., and Bronfman, F.C. (2013). BDNF regulates Rab11-mediated recycling endosome dynamics to induce dendritic branching. J Neurosci *33*, 6112-6122.

Leal, G., Comprido, D., and Duarte, C.B. (2014). BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology *76 Pt C*, 639-656.

Liot, G., Zala, D., Pla, P., Mottet, G., Piel, M., and Saudou, F. (2013). Mutant Huntingtin alters retrograde transport of TrkB receptors in striatal dendrites. J Neurosci *33*, 6298-6309.

Liu, J., Lamb, D., Chou, M.M., Liu, Y.J., and Li, G. (2007). Nerve growth factor-mediated neurite outgrowth via regulation of Rab5. Mol Biol Cell *18*, 1375-1384.

Masters, T.A., Tumbarello, D.A., Chibalina, M.V., and Buss, F. (2017). MYO6 Regulates Spatial Organization of Signaling Endosomes Driving AKT Activation and Actin Dynamics. Cell Rep *19*, 2088-2101.

Minichiello, L. (2009). TrkB signalling pathways in LTP and learning. Nat Rev Neurosci *10*, 850-860.

Ong, S.T., Freeley, M., Skubis-Zegadlo, J., Fazil, M.H., Kelleher, D., Fresser, F., Baier, G., Verma, N.K., and Long, A. (2014). Phosphorylation of Rab5a protein by protein kinase C is crucial for T-cell migration. J Biol Chem *289*, 19420-19434.

Park, H., and Poo, M.M. (2013). Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci *14*, 7-23.

Ruiz, C.R., Shi, J., and Meffert, M.K. (2014). Transcript specificity in BDNF-regulated protein synthesis. Neuropharmacology *76 Pt C*, 657-663.

Santos, A.R., and Duarte, C.B. (2008). Validation of internal control genes for expression studies: effects of the neurotrophin BDNF on hippocampal neurons. J Neurosci Res *86*, 3684-3692.

Satoh, D., Sato, D., Tsuyama, T., Saito, M., Ohkura, H., Rolls, M.M., Ishikawa, F., and Uemura, T. (2008). Spatial control of branching within dendritic arbors by dynein-dependent transport of Rab5-endosomes. Nat Cell Biol *10*, 1164-1171.

Saxena, S., Bucci, C., Weis, J., and Kruttgen, A. (2005). The small GTPase Rab7 controls the endosomal trafficking and neuritogenic signaling of the nerve growth factor receptor TrkA. J Neurosci *25*, 10930-10940.

Schafer, J.C., Baetz, N.W., Lapierre, L.A., McRae, R.E., Roland, J.T., and Goldenring, J.R. (2014). Rab11-FIP2 interaction with MYO5B regulates movement of Rab11a-containing recycling vesicles. Traffic *15*, 292-308.

Schratt, G.M., Nigh, E.A., Chen, W.G., Hu, L., and Greenberg, M.E. (2004). BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. J Neurosci *24*, 7366-7377.

Shimada A, Mason CA, Morrison ME (1998) TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. J Neurosci 18:8559–8570.

Sholl, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. J Anat *87*, 387-406.

Skjeldal, F.M., Strunze, S., Bergeland, T., Walseng, E., Gregers, T.F., and Bakke, O. (2012). The fusion of early endosomes induces molecular-motor-driven tubule formation and fission. J Cell Sci *125*, 1910-1919.

Snapp, E.L., Altan, N., and Lippincott-Schwartz, J. (2003). Measuring protein mobility by photobleaching GFP chimeras in living cells. Curr Protoc Cell Biol *Chapter 21*, Unit 21 21.

Song, M., Giza, J., Proenca, C.C., Jing, D., Elliott, M., Dincheva, I., Shmelkov, S.V., Kim, J., Schreiner, R., Huang, S.H., et al. (2015). Slitrk5 Mediates BDNF-Dependent TrkB Receptor Trafficking and Signaling. Dev Cell *33*, 690-702.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol *10*, 513-525.

Sui, W.H., Huang, S.H., Wang, J., Chen, Q., Liu, T., and Chen, Z.Y. (2015). Myosin Va mediates BDNF-induced postendocytic recycling of full-length TrkB and its translocation into dendritic spines. J Cell Sci *128*, 1108-1122.

Takei, N., Inamura, N., Kawamura, M., Namba, H., Hara, K., Yonezawa, K., and Nawa, H. (2004). Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci *24*, 9760-9769.

Verboogen, D.R.J., Baranov, M.V., Ter Beest, M., and van den Bogaart, G. (2017). Visualizing Intracellular SNARE Trafficking by Fluorescence Lifetime Imaging Microscopy. J Vis Exp.

Wu, K.Y., He, M., Hou, Q.Q., Sheng, A.L., Yuan, L., Liu, F., Liu, W.W., Li, G., Jiang, X.Y., and Luo, Z.G. (2014). Semaphorin 3A activates the guanosine triphosphatase Rab5 to promote growth cone collapse and organize callosal axon projections. Sci Signal *7*, ra81.

Xu, W., Weissmiller, A.M., White, J.A., Fang, F., Wang, X., Wu, Y., Pearn, M.L., Zhao, X., Sawa, M., Chen, S., Gunawardena, S., Ding, J., Mobley, W.C., Wu, C. (2016). Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. J Clin Invest 126, 1815-1833.

Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. Nat Rev Mol Cell Biol *2*, 107-117.

Zhang, Y., Moheban, D.B., Conway, B.R., Bhattacharyya, A., and Segal, R.A. (2000). Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. J Neurosci *20*, 5671-5678.

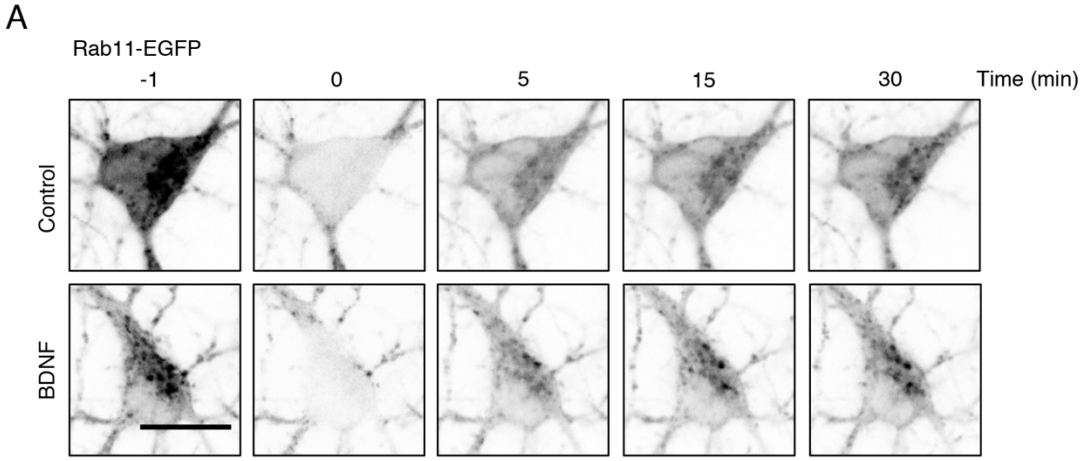
Zheng, J., Shen, W.H., Lu, T.J., Zhou, Y., Chen, Q., Wang, Z., Xiang, T., Zhu, Y.C., Zhang, C., Duan, S., et al. (2008). Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth. J Biol Chem *283*, 13280-13288.

Zhou, B., Cai, Q., Xie, Y., and Sheng, Z.H. (2012). Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Rep *2*, 42-51.

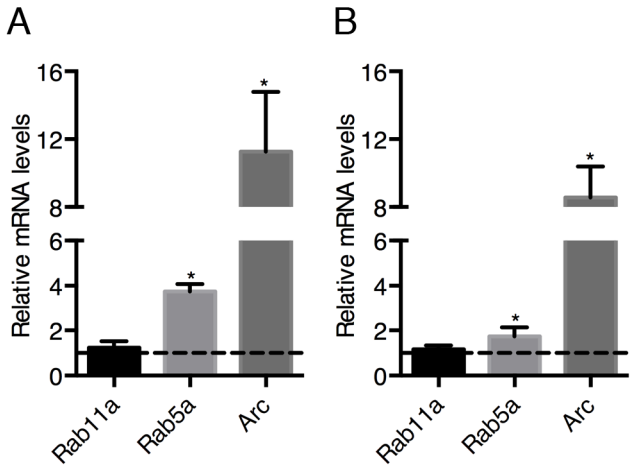
Zhou, P., Porcionatto, M., Pilapil, M., Chen, Y., Choi, Y., Tolias, K.F., Bikoff, J.B., Hong, E.J., Greenberg, M.E., and Segal, R.A. (2007). Polarized signaling endosomes coordinate BDNF-induced chemotaxis of cerebellar precursors. Neuron *55*, 53-68.

Zhou, X.P., Wu, K.Y., Liang, B., Fu, X.Q., and Luo, Z.G. (2008). TrkB-mediated activation of geranylgeranyltransferase I promotes dendritic morphogenesis. Proc Natl Acad Sci U S A *105*, 17181-17186.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. FRAP in the cell body of EGFP-Rab11. FRAP was performed in cell bodies of hippocampal neurons (time 0), and recovery was evaluated after 5, 15 and 30 min. Representative individual frames of a recording performed in a neuron expressing EGFP-Rab11. Recording was performed in control conditions or after BDNF treatment. The panel shows EGFP- Rab11 endosomes in the cell body before FRAP (-1 min), just after FRAP (0 min) and after 5, 15, 30 min of FRAP.



Supplementary Figure 2. BDNF stimulation increases rab5a but not rab11a mRNA levels. A total of 8-9 DIV hippocampal neurons were stimulated with BDNF (50 ng/mL) for 4 h (A) or 12 h (B). mRNA levels of rab5a, rab11a and arc were analyzed by qPCR. Expression levels were normalized by β-actin, Tbp and Pgk-1 mRNA levels. Arc mRNA levels were evaluated as a positive control of BDNF stimulation. Quantification was done of relative levels of mRNA with 2^{-Ct}, showed as a fold changes of nonstimulated neurons (black dashed line). The results are expressed as the mean ± SEM, from 3 independent experiments. *<0.5; by Student's T-test.

CHAPTER II

In this chapter, we approached General aim 2 and the following specific aims:

2.1 To evaluate whether BDNF signaling in axons increases dendritic arborization in cell bodies in a TrkB-dependent manner.

2.2. To evaluate whether BDNF signaling in axons increases dendritic arborization in a CREB-dependent manner.

2.3 To study the role of signaling endosome in BDNF long-distance signaling.

2.4 To study the role of PI3K-mTOR pathway in BDNF long-distance signaling.

BDNF axonal signaling endosomes mediates long-distance dendritic growth by activating CREB/PI3K-mTOR-dependent translation in neuronal cell bodies

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ABSTRACT

Brain Derived Neurotrophic Factor (BDNF) is broadly express in many circuits of the central nervous system (CNS) and binds TrkB and p75 to trigger different signaling pathways, including ERK1/2 and PI3K-mTOR, to induce dendritic growth and synaptic plasticity. When binding to BDNF, TrkB and p75 are endocytosed to signaling endosomes to continue signaling inside the cell. Whether BDNF/TrkB-p75 signaling endosomes in axons are regulating long-distance signaling to cell bodies to modify neuronal morphology is unknown. Here, we studied the functional role of BDNF/TrkB-p75 signaling endosomes and BDNF signaling pathways in long-distance regulation of dendritic growth using compartmentalized cultures of rat and mouse cortical neurons derived from $p75^{exonIII}$ knock-out or $TrkBF^{616A}$ knock-in mice. By applying BDNF to distal axons we showed the capacity of axonal BDNF to increase dendritic arborization in cell bodies. This process depended on TrkB, but not p75 activity. Dendritic arborization induced by axonal BDNF signaling required both nuclear CREB activation and translation of newly synthesized proteins in a CREB and PI3K-mTOR dependent-manner. Indicating that PI3K-mTOR pathway in cell bodies promotes the translation of newly synthesized transcripts. Interestingly, PI3K activity was not required in the axons for long-distance responses. Although, BDNF/TrkB increases Rab5 activity and the activity of Rab5 and dynein were required for BDNF-long distance signaling. All together, these results show that BDNF/TrkB in axons regulates the endosomal pathway allowing long-distance control of dendritic growth. Our results suggest a role of BDNF-TrkB signaling endosomes wiring circuits in the CNS.

INTRODUCTION

During development, neurons grow specific dendritic threes which is a key process for the establishment of proper central nervous system (CNS) connectivity (Jan and Jan, 2010). After birth, dendrites and synapsis undergo plastic changes that are required for physiological processes such as learning and memory. The morphology of the dendritic arbor is maintained and/or undergoes plastic changes in a process that requires activity-dependent transcription factors (TFs), protein translation and extracellular signaling molecules (Sutton and Schuman, 2006; Wong and Ghosh, 2002; Yap and Greenberg, 2018). A well-known activity regulated gene is the member of the neurotrophin family of growth factors, brain-derived neurotrophic factor (BDNF), whose expression is required for the maintenance of dendrites of adult striatal and cortical neurons (layer II and III) (Gorski et al, 2003; Horch et al, 1999). Furthermore, BDNF increases dendritic branching of cortical and hippocampal neurons in dissociated cultures and in organotypic slices (Cheung et al., 2007; Horch and Katz, 2002). BDNF binds to both receptors the tyrosine kinase receptor B (TrkB) and the pan neurotrophin receptor p75 (Huang and Reichardt, 2003; Yan et al., 1997). However, the growth promoting action of BDNF is specifically associated to TrkB; consistently, a mutant mouse with inducible deletion of TrkB receptors exhibit a significant reduction of dendritic arbor and neurodegeneration in cortical neurons (Xu et al., 2000).

The neuronal growth mediated by TrkB activation in neurons is triggered by signaling pathways such as ERK1/2 and PLCγ that activate the TF, cAMP response element CRE-binding protein

(CREB) (Finkbeiner et al., 1997; Xing et al., 1998, Gonzalez et al 2019), mediating activity-dependent transcription (Flavell and Greenberg, 2008; Yap and Greenberg, 2018). Also, the activation of the class 1 phosphoinositide 3-kinase (PI3K)/Akt/ and the mammalian target of rapamycin (mTOR) (Dijkhuizen and Ghosh, 2005; Minichiello et al., 2002) contribute to this process. Several lines of evidence suggest that BDNF increases the local translation of a subset of mRNAs in dendrites in a mTOR dependent manner, including proteins related to CNS plasticity (Leal et al., 2014; Ravindran et al., 2019; Schratt et al., 2004; Takei et al., 2004). Thus, mTOR controls dendrite size and shape in a BDNF-dependent manner via PI3K/Akt pathway (Kumar et al., 2005).

Ligand binding of BDNF promotes TrkB internalization of ligand-receptor in endocytic organelles. A wealth amount of evidences has shown that a complex of neurotrophins and neurotrophin receptors continue signaling inside the cell in a specialized organelle named signaling endosomes, where it encounters specific signaling adaptors (Grimes et al, 1996; Bronfman et al, 2014; Debaisieux et al, 2016). Consistently, several studies have shown that endocytosis of TrkB is required for survival, dendritic arborization and cell migration (Zheng et al., 2008; Zhou et al., 2007), suggesting that endosomal signaling has a role in the physiological responses to TrkB. In line with these results, we have shown that BDNF/TrkB signaling increases the activity and mobility of key Rab GTPases regulating endosomal trafficking, particularly Rab5 (early-endosomes) and Rab11 (recycling endosomes) and in turns the activity of these GTPases are required for BDNF-induced dendritic branching in a CREB-dependent manner (Lazo et al 2013; Moya-Alvaro et al 2018; Gonzalez et al 2019).

Due to the polarized morphology of neurons, distal dendrites and axons must be communicated to the cell body for proper neuronal homeostasis. This is achieved by bidirectional transport of organelles in a process that requires microtubule associated molecular motors (Bronfman et al., 2014; Hirokawa et al, 2010; Terenzio et al, 2017). It has been shown, that BDNF is retrograde transported in axons of cortical neurons (Olenick et al., 2019; Zhou et al., 2012). Furthermore, endosomes containing activated TrkB are associated with the dynein adaptor snapin and, the disruption of snapin/dynein interaction decreases dendritic arborization (Zhou et al., 2012). However, few studies have addressed the long-distance effects of BDNF in the CNS (Cohen et al., 2011; Lom et al., 2002)). On the other hand, long-distance communication of neurotrophins from target tissue to cell bodies has been described in the peripheral nervous system (Harrington and Ginty, 2013; Ye et al., 2003; Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018)). Mechanistically speaking, the favorite hypothesis, due to the large body of evidences, is that signaling endosome originating in synaptic terminals propagate distal neurotrophin signals to the nucleus for transcriptional regulation, process mediated by TFs such as CREB (Riccio et al, 1997; Watson et al, 1999; Haihong et al, 2003). In that context, our lab and others have described that CREB is phosphorylated in the nucleus of cortical neurons when BDNF is added exclusively to the axons of compartmentalized neuronal cultures (Bronfman et al., 2014; Deinhardt et al., 2006; Zhou et al., 2012), suggesting that CREB may mediate long-distance signaling by BDNF.

Here, we used compartmentalized cultures of rat and mouse cortical neurons using microfluidic chambers to study the mechanism of long-distance BDNF signaling in CNS neurons. We show

that axonal stimulation with BDNF promotes dendritic growth in neuronal cell bodies. This effect was mainly dependent on TrkB, and although p75 down-regulation affected the basal morphology of neurons it did not affect long-distance BDNF responses. As previously reported, axonal BDNF increases activation of CREB and CREB activity was required for long-distance BDNF dendritic growth. Both process dependent on dynein mediated transport of activated TrkB and Rab5 activity, suggesting that the process requires TrkB signaling endosomes transport. Interestingly, PI3K activity was required only in the cell body compartment to promote dendritic arborization and mTOR activation induced by axonal BDNF signaling. Consistently, the increase in the activity of CREB and mTOR induced by BDNF stimulation in axons promotes somatodendritic protein synthesis. Our finding suggests that there is a functional role of BDNF/TrkB signaling endosomes in regulating long-distance dendritic growth in central neurons.

METHODOLOGY

Primary culture of cortical neurons. Embryonic cortical neurons from rats (embryonic days 17–19) were obtained from the animal facilities of the Pontificia Universidad Catolica de Chile and euthanatized under deep anesthesia according to the bioethical protocols of our institution. The rat or mouse brain cortex were dissected and dissociated into single cells in HBSS (Hank´s Balance Salt Solution; Thermo-Fisher, cat# 14025134). After disaggregation, neurons were resuspended in MEM/Horse Serum (Thermo-Fisher, cat# 16050122) and were seeded on microfluidic chambers at a low density (40-50 x 10^3 cells/chamber) or in mass culture with a density of 25 x 10^3 cells/well in 12 mm coverslips or 1.8 x 10^6 cell/ 60 mm plate. After 4 h, the culture medium was replaced with neurobasal medium (Thermo-Fisher, cat# 21103049) supplemented with 2% B27 (Life Technologies, cat# 17504044), 1x glutamax (Thermo-Fisher, cat# 35-050-061) and 1x pen/strep (Thermo-Fisher, cat# 15140-122). Proliferation of non-neuronal cells was limited by the use of cytosine arabinoside (0.25 µg/mL AraC; Sigma-Aldrich, cat# C1768) when MEM/HS was replaced to neurobasal (Gonzalez et al, 2019; Taylor et al., 2003).

Microfluidic devices. The molds to prepare the compartmentalized chambers was provided by Prof Eran Perlson from Tel Aviv University (Gluska et al, 2016). The microfluidic chambers were prepared with syldgard^{TM} 184 silicone elastomer base (Poirot, cat# 4019862), according to the manufacturer's instructions. Two days before performing primary cultures, glass coverslips (25 mm) were incubated with poly-D-lysine (0.1 mg/mL, Corning, cat# 354210). The

next day, poly-D-lysine was washed and a microfluidic chamber with 400 µm microgrooves was place on the coverslip. Then, inside of the chamber a solution of laminin (2 µg/mL in water, Invitrogen, cat# 23017015) was added. The same day that the primary culture was performed, the laminin solution was changed to DMEM/HS media (Dulbecco Minimum Essential Medium supplemented with 10% horse serum, 1x glutamax and 1x antibiotic/antimycotic, Thermo-Fisher, cat# 15240062).

Quantification of dendritic arborization induced by BDNF. Cortical neurons (6 DIV) were transfected with 0.5 ug of the plasmid driving the expression of EGFP (CA, USA) using 0.8 uL of lipofectamin 2000 (Invitrogen, cat#11668-019) in 30 uL of optimem (Thermo-Fisher, cat# 11058021). After 2 hours, the optimem with the plasmids was replaced for neurobasal media supplemented with 2% B27, 1x Glutamax and 1x pen/Strep for 1 hour. The cell body compartment was incubated with neurobasal medium supplemented with TrkB-Fc (100 ng/mL, B&D system, 688TK) for all treatments. The drugs were incubated in the cell body compartment or axonal compartment as follow. In the cell body compartment; 1NM-PP1 (1 µM, Cayman, cat# 221244-14-0), KG501 (10 µM, Sigma-Aldrich, cat# 70485), LY294002 (10 µM, Calbiochem, cat# 440202). In the axonal compartment: K252a (0.2 µM, Tocris, cat# 1683), 1NM-PP1 (1 µM), LY294002 (10 µM), Ciliobrevin D (20 µM, Calbiochem, cat# 250401). After 1 hour, BDNF (50 ng/mL, Alomone, cat# B-250) was added to the axonal compartment together with the subunit B of the choleratoxin fluorescently labelled (Ctb, 1 µg/mL, Thermo-Fisher, cat# C34777). After 48 h, neurons were washed with PBS 37 ºC and then fixed with fresh 4 % paraformaldehyde (Sigma-Aldrich, cat# 158127) in PBS (PFA-PBS) at 37ºC for 15 minutes, then the chamber is removed and the neurons were permeabilized and blocked with 5% BSA

(Jackson, cat# 001-000-162) and 0.5% Triton X-100 (Sigma-Aldrich, cat# 234729) in PBS and then incubated with anti-MAP2 ((1:500), Merk-Millipore, cat# AB5622) in incubation solution (3% BSA with 0.1% Triton X-100 in PBS), after washes (3x buffer) neurons were treated with a donkey anti-mouse Alexa 647 (Molecular probes, cat# A-31571), 1:500, in incubation solution and mounted for fluorescence microscopy visualization using Mowiol 4-88 (Calbiochem, cat# 475904).

Dendritic arborization was analyzed in cortical neurons labelled with Ctb and transfected with EGFP and labeled with MAP2. Primary dendrites, branching points and Sholl's analysis (Sholl, 1953) were quantified (see below). For visualization, confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C was used. Images were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis each 0,5 µm of whole cells, 5-7 optical slices. Z-stacks were integrated, and the images were segmented to obtain binary images. Ten concentric circles with increasing diameters (5 µm each step) were traced around the cell body, and the number of intersections between dendrites and circles was counted and plotted for each diameter. Analysis was performed using the ImageJ plugin developed by the Anirvan Gosh Laboratory (http://biology.ucsd. edu/labs/ghosh/software). The number of total primary dendrites and branching points of all dendrites was manually counted from the segmented images.

Evaluation of protein phosphorylation by immunofluorescence. Cortical neurons (5-6 DIV) were incubated with Ctb555 (Ctb, 1 μg/mL) overnight. For all treatments, 6-7 DIV cortical neurons were incubated with neurobasal medium supplemented with TrkB-Fc (100 ng/mL) in the cell body compartment. The drugs were incubated in the cell body compartment or axonal compartment as follow. In the cell body compartment; LY294002 (10 μM), Torin 1 (0.25 μM, Tocris, cat# 4247). In the axonal compartment: Ciliobrevin D (20 μM). After 1 hour, BDNF (50 ng/mL) was added to the axonal compartment together with Ctb647 (1 μg/mL, Thermo-Fisher, cat# C34778). After the time point indicated in the figure, samples were fixed with 4 % PFA containing phosphatase inhibitor (1x, Thermo-Fisher, cat# A32957) in PBS during 15 min. Blockage and permeabilization was done with blocking solution (5% BSA, 0.3% Triton X-100, 1x phosphatase inhibitor in PBS) for 1 hour. The antibodies were incubated overnight (4ºC) in incubation buffer (BSA, 3%, Triton X100, 0.1%, in PBS). The antibodies used were: anti-phospho CREB, 1:500, (S133, Cell Signaling cat# 9198), anti-phospho S6 ribosomal protein 1:100 (S235/236, Cell Signaling, cat# 2211S), anti-phospho 4E-BP1, 1:500 (S65, Cell Signaling, cat# 9456S). The secondary antibody used was a donkey anti rabbit Alexa488 ((1:500), Molecular Probes, cat# A-21206) and was incubated for 90 minutes in BSA (3%), Triton X100 (0.1%) in PBS. The samples were incubated with Hoechst 33342 (5 μg/mL, Invitrogen, cat# 62249) for nuclei visualization. We visualized the entire neuron in the cell body compartment with confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Images were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis. Five to seven optical section of 0,5 μm thick were considered from a whole cell.

Neuron cell viability evaluation through TUNEL assay. For detection of fragmented DNA, 6 DIV cortical neurons were exposed to KG501 (10 μM) in the cell body compartment and Ctb555 (1 μg/mL) in the axonal compartment, during 48 hrs in neurobasal supplemented with B27. As a positive control Oligomycin A (10 μM, Sigma-Aldrich, cat# 75351) was incubated in the cell body compartment during 5 min. Neurons were washed with PBS and fixed in 4% PFA in PBS for 15 min at room temperature. After that, neuron-attached coverslips were washed in PBS three times for 5 min each. For permeabilization, neurons were placed onto ice-cold citrate/triton solution (0.1% citrate; 0.1% triton X-100) and kept for 2 min on ice, then coverslip were washed twice to remove permeabilization solution and incubated with labeling TUNEL mix (Kit for In Situ Cell Death Detection Kit, Fluorescein, Roche cat# 11684795910) for 1h at 37ºC. The samples were incubated with Hoechst (5 μg/mL). TUNEL assay was analyzed by fluorescence and/or confocal microscopy and total Hoechst nuclear staining as well as TUNEL positive apoptotic nuclei were counted by using the Image J.

Immunoendocytosis of Flag-TrkB in microfluidic chambers to evaluate retrograde transport of TrkB from axons to dendrites. Cortical neurons (5 DIV) were transfected with 0.5 ug of the plasmid Flag-TrkB (gift from Prof. Francis Lee, NYU, USA) using 0.8 uL of lipofectamin 2000 in 30 uL optimen per chamber. After 48 hours, cortical neurons (7 DIV) were incubated at 4ºC for 10 minutes and then treated with an anti-Flag antibody, 1:750, (Sigma-Aldrich, cat#F3040) for 45 minutes only in the axonal compartment at 4 ºC. After, cortical neurons were washed briefly with warm neurobasal and were incubated with BDNF (50 ng/mL) in the axonal compartment for 180 minutes. Finally, samples were fixed with 4 % PFA with phosphatase inhibitor in PBS during 15 min. Blockage and permeabilization was done with

blocking solution (5% BSA, 0.3% Triton X-100, 1x phosphatase inhibitor in PBS) for 1 hour. The antibodies anti phosphorylated TrkB Y816 ,1:200, (Sigma-Aldrich, cat# ABN1381) or anti-Rab ,1:250, (Abcam, cat# AB18211) were incubated overnight (4 ºC) in incubation buffer (BSA, 3%, Triton X100, 0.1%, in PBS). The secondary antibody used was a donkey anti rabbit Alexa488, 1:500, and donkey anti-mouse Alexa555, 1:500, (Invitrogen, cat# A-31570) was incubated for 90 minutes in incubation buffer. We visualized entire neuron in the cell body compartment and the microgrooves compartment with confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Images were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis each 0,5 µm of whole cells, 5-7 optical slices.

Microscopy detection and quantification of active Rab5 in dendrites and cell bodies. The fusion protein Rab5BD-GST (gift from Prof. Vicente Torres, University of Chile, Chile) was produced in BL21 E. coli, transformed with a pGEX-GST-Rabaptin5 plasmid and stimulated for 4 hours with IPTG (Promega, cat# V3955). The Rab5BD-GST protein was purified from bacteria lysate using glutathione-Sepharose beads (Sigma-Aldrich, cat# **GE17-0756-01)**. For use as a probe, the protein was eluted in a solution of reduced glutathione (Sigma-Aldrich, cat# G4251) (Moya-Alvarado 2018). Seven DIV Neurons were preincubated with K252a (0.2 µM) during 1 hour in the axonal compartment, then, axonal compartment was stimulated with BDNF (50 ng/mL) or Ctb555 (1 µg/mL) for 30 minutes, fixed with 4 % PFA in PBS for 15 minutes, permeabilized and blocked in 3% fish gelatin (Sigma-Aldrich, cat# G704) in incubation buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA, 0.25 M sucrose and 0.2% Triton X-100, pH 7.2) for 45 min. Then, neurons were incubated overnight with ~10

µg/mL Rab5BD-GST in incubation buffer at 4°C. After 2 brief washes in HBSS, neurons were fixed again with 4% PFA in PBS, then samples were permeabilized and blocked with 3% fish gelatin (Jackson, cat# 001-000-162) and 0.5% Triton X-100 (Sigma-Aldrich, cat# 234729) in PBS and then incubated with anti-GST, 1:500, (Thermo-Fisher, cat# 8-326) and anti- β-III Tubulin, 1:750, (Sigma-Aldrich, Cat T8578) in incubation solution (3% fish gelatin with 0.1% Triton X-100 in PBS), after washes (3x buffer) neurons were treated with donkey anti-rabbit Alexa488, 1:500, and donkey anti-mouse Alexa647, 1:500, in incubation solution and mounted for fluorescence microscopy visualization.

To avoid biases in the selection of a neuron with high or low levels of Rab5BD-GST, neurons were selected according to the β-III tubulin labelling for quantification. We visualized the proximal part to the microgrooves of axonal compartment with confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Images were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis. Three to five optical sections of 0,25 µm thick were considered per axons and 30 µm length of axons were quantified for anti-GST fluorescence. The background was calculated from images of neurons treated with GST, and this baseline was calculated for and subtracted from each dataset.

Evaluation of the somatodendritic TrkB activity in compartmentalized cultures using cortical neurons derived from the TrkB^{F616A} mice. Cortical neurons derived from TrkB^{F616A} (6 DIV) mice were incubated in the axonal compartment with Ctb555 (1 µg/mL) overnight. Then, neurons were washed with neurobasal. In the control and BDNF treatments, cultures were

depleted of B27 (neurobasal, with 1x glutamax) during 1 hour and next maintained with more volume of media in the CB in the presence of TrkB-Fc (100 ng/mL). The axonal compartment (AC) was treated with BDNF (50 ng/mL) for 3 hrs. For inhibition of TrkB activity in the cell bodies compartment (CB), the cultures were depleted from media during one hour in the presence of 1NM-PP1 (1 μM) in the CB, but with more media in the AC to avoid the leak of 1NM-PP1 to the AC. Next, neurons were incubated in the presence or absence of BDNF (50 ng/mL) in the AC with more volume in the AC and the CB compartment continued with 1NM-PP1 (1 μM) and TrkB-Fc (100 ng/mL) for 3 hours. When the activity of TrkB was inhibited in the AC, neurons were depleted from media during one hour in the presence of 1NM-PP1 (1 μM) in the AC, then cells were incubated with BDNF (50 ng/mL) in the AC in the presence of 1NM-PP1 and TrkB-Fc during 3 hours with more volume in the CB. For all conditions, the CB was incubated during the experiment with TrkB-Fc (100 ng/mL) in neurobasal without B27. After 3 hours of BDNF treatment, neurons were washed with PBS 1X and cell were fixed with 4 % PFA in PBS during 15 minutes at room temperature. Finally, an immunofluorescence against phospho proteins was performed as described above. Phospho TrkB (Y816; 1:200) and phospho CREB (S133, 1:500) was evaluated. To evaluate phospho TrkB and phospho CREB levels, we visualized entire neuron in the cell body compartment with confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Images were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis of whole cells. Dots associated to Phospho-TrkB levels in the cell body were quantified from Ctb positive cells and normalized to the size of cell body compartment. Phospho CREB levels were evaluated as described above.

Evaluation of the TrkB activity and downstream signaling in non-compartmentalized cultures using cortical neurons derived from the TrkB^{F616A} mice. Cortical neurons derived from TrkB^{F616A} **(**7 DIV**)** mice were depleted from media during 1 hour in the presence or absence of 1NM-PP1 (1 μM). Then neurons were treated as follow: control: vehicle 1 hour. BDNF: incubation of BDNF (50 ng/mL) during 30 minutes, next were rinse twice with PBS 1x and incubated with vehicle for 30 minutes. 1NM-PP1 (Pre): neurons were incubated with BDNF in the presence of 1NM-PP1 during 1 hour. 1NM-PP1 (Post): neurons were stimulated with BDNF during 30 minutes, next were rinse twice with PBS 1x and incubated with 1NM-PP1 during 30 minutes. Next, TrkB and Akt activity were evaluated by westernblot and CREB, 4E-BP1 and S6 ribosomal protein (S6r) were evaluated by immunofluorescence. For westernblot, cells were lysed with RIPA buffer (0.1 % SDS, 0.5 % NP40, 10 mM Tris-HCl (pH7.5), 1 mM EDTA, 150 mM NaCl, 0.5 % deoxicolic acid) containing protease and phosphatase inhibitors. Standard SDS gel electrophoresis and Western blotting procedures were used to analyze the cell extracts using anti-phosphorylates pTrkB (Y816, 1:1000), TrkB (1:1000, BD bioscience cat#610101). pAkt (S437, 1:1000, Cell signaling cat#9271), Akt (1:1000, Cell signaling cat#9272) and GAPDH (6C5, 1:1000, Santa Cruz cat#sc-32233) antibodies. For immunofluorescence, neurons were washed with PBS 1X and cell were fixed with 4 % PFA in PBS during 15 minutes at room temperature. Finally, an immunofluorescence against phospho proteins was performed as described above. Anti-phospho CREB (S133; 1:500) Anti-phospho S6 ribosomal protein (S235/236, 1:100), Anti-phospho 4E-BP1 (S65, 1:500) and anti- β-III tubulin (1:750). we visualized entire neuron in the cell body compartment with confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Images were acquired using a 20x objective for pCREB and 60x

objective for 4E-BP1 and S6r at 1024x1024 pixel resolution along the z-axis of whole cells.

Evaluation of protein synthesis by Click-iT chemistry and Arc immunofluorescence. Compartmentalized cortical neurons (6 DIV) were incubated in the AC with Ctb488 overnight. At 7 DIV neurons were washed with warm PBS 1x, then incubated with DMEM without methionine (Life Technologies, cat# 21013024), supplemented with glutamax 1x and L-cystine (0.201 mM, Sigma-Aldrich, cat#C7602) for 60 minutes. The media was changed to DMEM supplemented with Click-iT AHA (0.1 mM, Thermo-Fisher, Click-iT cat# C10269; AHA cat#C10102) (standardization was done with AHA 0.2 mM, 0.1 mM, 0.05 mM and 0.02 mM), for 5 hours. In addition, the AC of neurons was treated with BDNF (50 ng/mL) and the cell body compartment was incubated with TrkB-Fc (100 ng/mL) and Torin 1 (0.25 μM) or KG501 (10 μM). Neurons were fixed with 4 % PFA in PBS for 15 minutes. Then, the protocol was completed according to the manufacturer's instructions using alkyne alexa fluor 555 (2.5 μM, Invitrogen, cat# A20013) to conjugate with AHA. Finally, immunofluorescence against Arc (1:300, Abcam, cat# AC118929) was performed as described above for MAP2 immunostaining.

BDNF-avi production and purification. HEK293FT cells were cultured in DMEM, 4.5 g/l glucose, 10% FBS, 1% penicillin/streptomycin. For transfection, HEK293FT cells were grown in 20 plates of 15 cm to ~70% confluency. Cells were changed to 25 ml of DMEM-high glucose without serum. For transfection, 30 μg of pcDNA3.1-BDNFavi plasmids DNA (gift from Prof. Chengbiao Wu, UCSD, USA) was added to one ml of DMEM-high glucose media and then 90 μg PEI (45 μL, Polysciences Inc, cat# 23966-1) were added. The mixture was incubated at room temperature for 25 min and was added into the media by dropwise method. Transfected

HEK293FT cells were incubated at 37 ºC, 5% CO_2 for 48 hour post transfection and then, the media was collected for protein purification.

Media was harvested and adjusted to 30 mM phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole (Sigma-Aldrich, cat# I5513) and a cocktail of protease inhibitors (Sigma-Aldrich, cat# P8340). After incubation on ice for 15 min, media was cleared by centrifugation at 9500 rpm for 45 min using a Hettich 46R centrifuge. Ni-NTA resins (Qiagen) were rinsed twice with the washing buffer (30 mM phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole and a cocktail of protease inhibitors). Ni-NTA resins (Qiagen, cat# 30250) were added to the media at a concentration of 0.3 ml Ni-NTA/100 ml media and incubated for 180 minutes at 4 ºC. The media/Ni-NTA slurry were loaded onto a column and the captured Ni-NTA resins were washed with 10 ml wash buffer and eluted with 1 ml elution buffer (30 mM phosphate buffer, pH8.0, 500 mM NaCl, 300 mM imidazole, protease inhibitors). The purity and concentration of BDNF-avi was assessed by Western blot using a standards curve of BDNF (Alomone, cat# B250), and anti-BDNF antibody (1:500, Alomone, cat# ANT-010).

BDNF-avi biotinylation. Purified BDNF-avi was dialyzed with Sanderson water for 15 minutes. To biotinylate the BDNF-avi protein, an enzymatic reaction was performed. BDNF-avi (600-800 ng) were incubated with biotinylation buffer (50 mM Bicine, 10 mM MGAcO, 50 µM D-Biotin) in the presence of 10 mM ATP and BirA-GST (BirA 1:1 BDNF-avi) during 1 hour at 30ºC with 600 RPM agitation. Then, 10 mM ATP and BirA-GST were added again and the incubation was allowed for one additional hour at 30ºC with 600 RPM agitation.

BDNF-bt coupling to streptavidin quantum dots and visualization in microfluidic devices. To bind BDNF-bt to streptavidin quantum dots -655, (Thermo-Fisher, cat# Q10121MP)

(BDNF-QD), 1 µL of QD-655 was added to 10 µL of BDNF-bt (approx. 3 ng/ µL) and taken to a final volume of 20 µL with neurobasal media. Then, the mixture was incubated at room temperature in an orbital rocker for 30 minutes.

To evaluate the spatial location of BDNF-QD within cells, cortical neurons were depleted with neurobasal media during 1 hour in the presence or absence of LY294002 (10 µM) or Ciliobrevin D (20 µM). Then, the AC was incubated with BDNF-QD (2 nM) in the presence or absence of LY294002 or Ciliobrevin D for 4 hours. Neurons were fixed with 3 % PFA in PBS for 15 minutes at room temperature, washed with PBS and mounted in mowiol. We visualized the proximal part of the microgrooves to the cell bodies compartment with epifluorescence microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Three to five optical sections (3-5) of 0,25 µm thick were considered per axons. Images were acquired using a 100x objective at 1024x1024 pixel resolution along the z-axis. The number of BDNF-QD or QD alone were quantified in 90 μm^2 region of axons located in the microgrooves.

Statistical analysis. The results are expressed as the average ± standard error of the mean (SEM). Sholl's analysis curves were compared with two-way repeated measures ANOVA, followed by Bonferroni's multiple comparisons. Student's t-test or one-way ANOVA followed by appropriated multiple comparisons test was performed depending on the number of groups used in each experiment. Details about specific test used, level of significance and number of replicates are indicated in each figure legends. Statistical analyses were performed using GraphPad Prism 7 (Scientific Software).

RESULTS

BDNF axonal signaling promotes dendritic branching in a TrkB-dependent manner.

Using an *in-vitro* model of microfluidic chambers we evaluated whether the axonal stimulation of BDNF promotes dendritic arborization using three different approaches; rat cortical neurons incubated with the tyrosine kinase inhibitor K252a (Tapley et al., 1992), cortical neurons derived from $TrkB^{F616A}$ knockin mice (Chen et al., 2005), and cortical neurons derived from p75 $KO^{exonIII}$ mice (Lee et al., 1992). To evaluate morphological changes in neurons, we transfected compartmentalized cultures of cortical neurons (6 DIV) with plasmids driving the expression of EGFP and then we performed MAP2 immunostaining after neuronal fixation (Fig.1A). To identify the neurons that projected their axons to the axonal compartment (AC), we used the subunit B of the cholera toxin fluorescently labelled (Ctb). Ctb, is internalized and retrogradely transported in neuronal axons and accumulates in the Golgi apparatus in neuronal cell bodies (Escudero et al, 2019) (Fig. 1A and Supplementary Fig. 1A). The axonal treatment of Ctb allows us to control the true compartmentalization of the microfluidics chambers, just cultures that show Ctb accumulation in the Golgi apparatus and not all over the neurons were considered for analysis (Supplementary Fig. 1B). Also, quantifications were performed just in neurons that were labelled by Ctb. Since BDNF is mainly released from dendrites (Matsuda et al., 2009), the selectivity of BDNF axonal stimulation was achieved by incubating with the TrkB-Fc chimera the neuronal somas in the cell body compartment (CB) (Shelton et al., 1995). TrkB-Fc neutralizes the activity of endogenous BDNF released by neurons, indeed when TrkB-Fc was

not included in the CB compartment there was an increase in the basal level of dendritic arborization (Fig 1B, Supplementary Fig.1C).

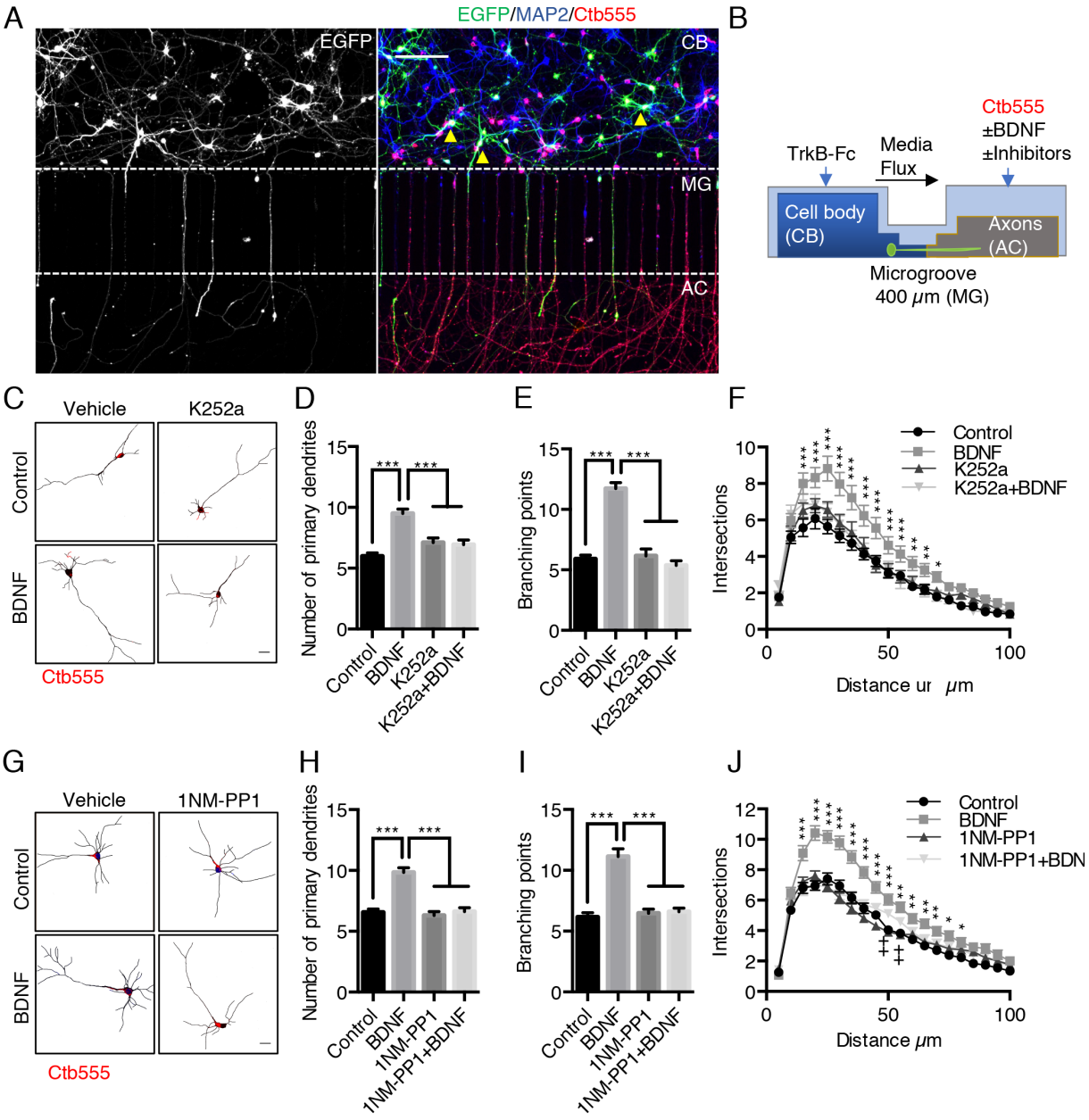


Figure 1. BDNF added to axons promotes dendritic branching in a TrkB-dependent manner. (A) Representative images of two compartmentalized microfluidic chambers after BDNF/Ctb555 incubation in the axonal compartment. Cortical neurons were seeded in microfluidic chambers of 400 μm long and 15 μm width microgrooves. Left panel, EGFP immunofluorescence showed in white. Right panel, EGFP in green, MAP2 in blue and Ctb555 in red. Ctb555 in the cell body and co-staining with MAP2 is shown in magenta, in light blue are labelled neurons co-stained with EGFP/MAP2/Ctb555 indicates neurons projecting their axons through the microgrooves (yellow arrow). **(B)** Experimental design to study BDNF retrograde signaling. At Six DIV neurons were transfected with a plasmid driving the expression of EGFP. The cell bodies compartment (CB) was incubated with TrkB-Fc (100 ng/mL). The axonal compartment (AC) was stimulated with BDNF (50 ng/mL) in addition to the fluorescently-labelled B subunit of choleratoxin (Ctb555) in presence or absence of different inhibitors. Treatments with BDNF and TrkB-Fc were performed for 48 hours to evaluate dendritic arborization. Finally, neurons were fixed, and immunofluorescence was performed against MAP2. **(C)** Representative images of rat cortical neurons in the CB (red indicated Ctb555) from compartmentalized cultures treated in the axons with DMSO (control), K252a (200 nM), BDNF (50 ng/mL) or BDNF and preincubated with K252a **(D-F).** Quantification of primary dendrites **(D)**, branching points **(E)** and Sholl analysis **(F)**, from neurons labelled with EGFP/MAP2/Ctb555 for each experimental condition. n= 40-45 neurons from 3 independent experiment. **(G)** Representative images of mouse $TrkB^{F616A}$ cortical neurons in the CB (red indicated Ctb555) from compartmentalized cultures treated in the axons with DMSO (control), 1NM-PP1 (1 μm), BDNF (50 ng/mL), or BDNF pretreated with 1NM-PP1. **(H-J)** Quantification of primary dendrites **(H)**, branching points **(I)** and Sholl analysis **(J)**, from neurons labelled with EGFP/MAP2/Ctb555 in the four different experimental conditions as described in G. Scale bar 20 μm. n= 40-45 neurons from 3 independent experiment. *$p<0.05$, **$p< 0.01$, ***$p< 0.001$, ++$p<0.01$ NM-PP1 vs 1NM-PP1+BDNF. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons (D, E, H and I). Two-way ANOVA with Bonferroni post-test for multiple comparisons (F and J). Results are expressed as ± SEM.

Following the above-mentioned experimental design, we found that BDNF added to axons was able to increase primary dendrites, branching points and the overall dendritic arbor in the cell body of rat (Fig. 1C-F) or mice (Fig. 1G-J) cortical neurons. Also, we found that this effect required axonal TrkB signaling. The treatment of rat cortical cultures with K252A in axons abolished axonal BDNF effect (Fig. 1C-F). Consistently, the treatment of axons with 1NM-PP1 of compartmentalized neuronal cortical cultures, derived from $TrkB^{F616A}$ knockin mice

abolished axonal BDNF effect in neuronal morphology (Fig. 1G-J) in the same line with the results observed with K252A in rat-derived cortical cultures.

To evaluate the contribution of p75 to the dendritic arborization induced by BDNF, we used cortical neurons derived from p75 $KO^{exonIII}$ mice. Using the same experimental design described for Figure 1A we prepared cultures derived from p75 $KO^{exonIII}$ ($p75^{+/+}$), heterozygous ($p75^{+/-}$) and homozygous p75 $KO^{exonIII}$ ($p75^{-/-}$) mice. We observed that the number of branching points induced by axonal stimulation with BDNF was unchanged by the p75 genotype (Fig. 2A-C). However, we observed that the absent of p75 changes the basal morphology of the dendritic arbor. While neurons possessed a smaller number of primary dendrites, there was an increased number of distal dendrites (Fig. 2D). Consistently, with the result presented in Fig 2A-C, axonal BDNF was able to increase the number of primary dendrites in p75 $KO^{exonIII}$ to similar levels compared to neurons derived from $p75^{+/+}$ littermates derived from p75 $KO^{exonIII}$ heterozygotes (Fig.2E). All together, these results indicate that BDNF in axons of cortical neurons increases the dendritic arbor in a TrkB activity dependent-manner.

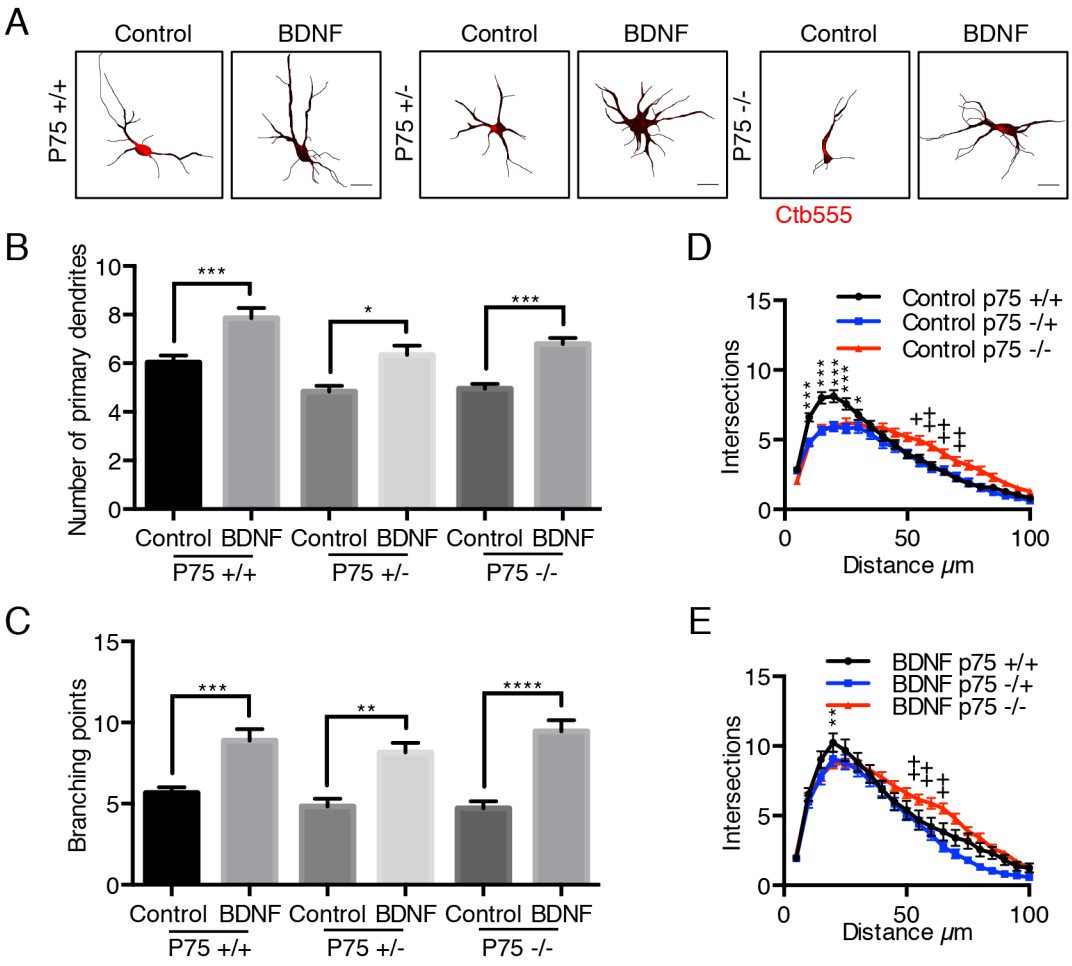


Figure 2. BDNF increases dendritic arborization in a p75-independent manner. (A) Representative images of p75 KO^{exonIII} cortical neurons in the CB (red indicated Ctb555) from compartmentalized cultures of p75$^{+/+}$, p75$^{+/-}$ or p75$^{-/-}$ treated with BDNF (50 ng/mL) in axons. Quantification of primary dendrites **(B)** and branching points **(C)** from neurons labelled with MAP2/Ctb555 in the different mouse genotypes as described in A. **(D)** Sholl analysis of cultures treated with TrkB-Fc in the CB compartment (non-stimulated) condition from each genotype. **(E)** Sholl analysis of cultures treated with TrkB-Fc in the CB compartment and BDNF in the AC from each mouse genotype. n= 40-50 neurons from 3 independent mice, in 2 independent experiments. *p<0.05, **p< 0.01, ***p< 0.001, ++p<0.01 p75$^{-/-}$ vs p75$^{+/+}$. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons (B and C). Two-way ANOVA with Bonferroni post-test for multiple comparisons (D and E). Results are expressed as ± SEM.

CREB activity is required for BDNF long-distance signaling

Several groups have shown that axonal BDNF signaling promotes nuclear CREB phosphorylation in different neuronal models (Deinhardt et al., 2006; Watson et al., 1999; Zhou et al., 2012); however, the physiological relevance of CREB activation in this process has not been evaluated. In order to evaluate CREB activation, we used a well-described polyclonal antibody against phosphorylated CREB at serine 133 (Lonze et al, 2002; Gonzales et al, 2019) and we performed a time course of CREB phosphorylation induced by axonal BDNF treatment. With this aim, at 5 DIV we incubated Ctb555 in the axonal compartment overnight, to identify all the neurons that projected their axons. At 6 DIV, we added BDNF together with Ctb647 at different time points to parallel bulk retrograde transport with the visualization of CREB phosphorylation (Fig. 3A). BDNF induced an increase of nuclear CREB phosphorylation as soon as 30 minutes, with two peaks one at 30 and a second peak at 180 minutes. Although, Ctb647 accumulation was observed at early times points of BDNF treatment, we observed robust Ctb647 accumulation mainly at 180 minutes (Fig. 3B).

To corroborate that CREB activity was required for dendritic arborization, we stimulated axons with BDNF of cortical neurons incubated with KG501 in the cell body compartment for 48 hours. KG501 is a small molecule that disrupt the interaction between CREB and CREB Binding Protein (CBP) (Best et al., 2004), (Fig. 3D). Interestingly, the presence of KG501 produces a small decrease, but not statistically significant, of the basal dendritic arborization. However, KG501 abolished the increase of dendritic arborization induced by the treatment of axons with BDNF (Fig. 3F, G and H). In addition, we evaluated whether p75 was required for

phosphorylation of CREB upon BDNF stimulation. Consistently with the results observed in Fig. 2, the absence of p75 did not impacted in BDNF-induced CREB phosphorylation (Fig. Supplementary 2 A and B). Indicating that, in our model, p75 is not required for BDNF-induced CREB phosphorylation.

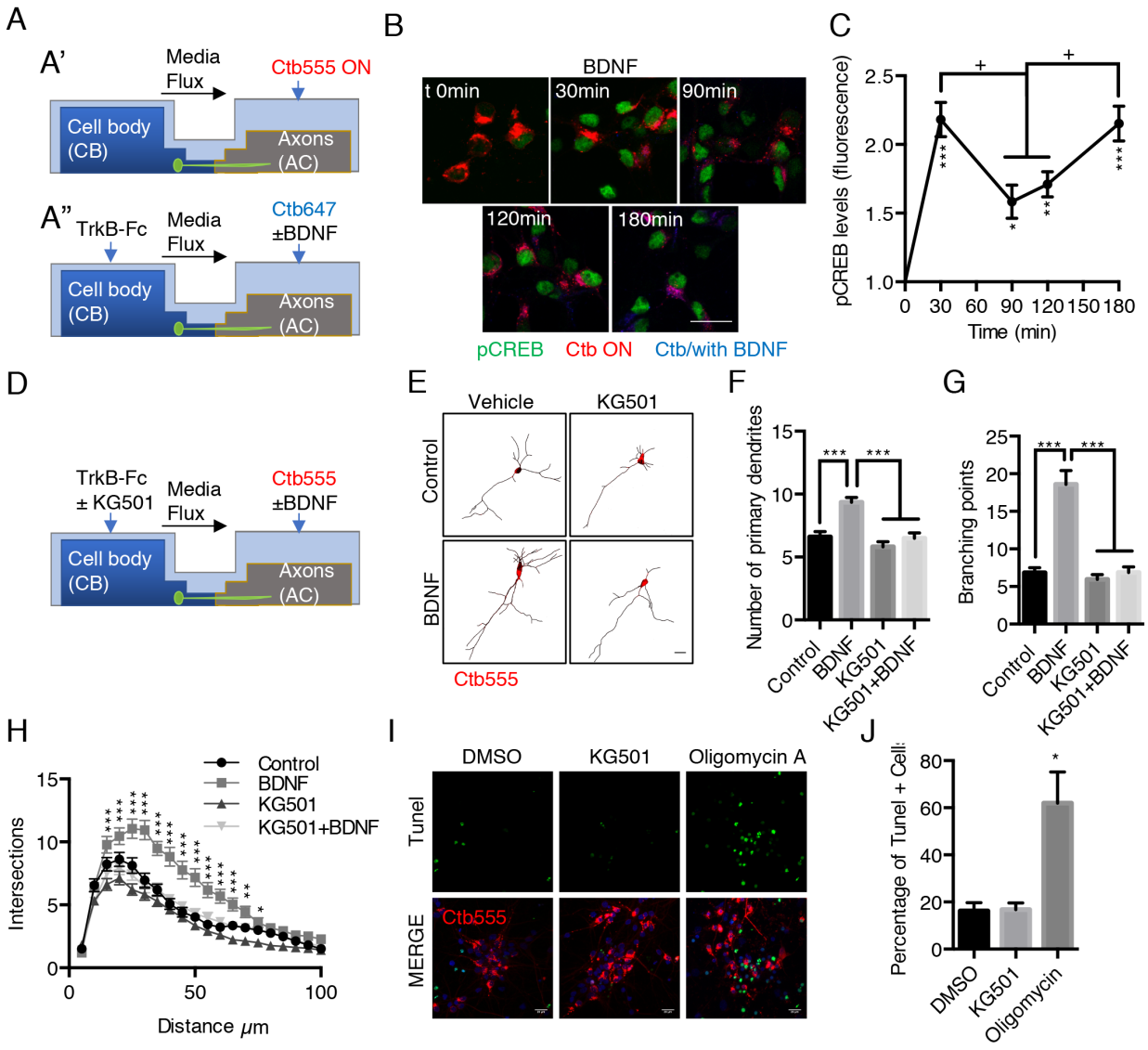


Figure 3. CREB activity is required for axonal BDNF induced long-distance dendritic arborization. (A) Schematic representation of the protocol to evaluate CREB phosphorylation induced by axonal BDNF. Five DIV cortical neurons were retrograde labeled with Ctb555 (in red) overnight (A'). Next, the culture medium was changed to serum-free medium for 90 minutes and stimulated with BDNF (50 ng/mL) for 5, 30, 90, 120 and 180 minutes along with Ctb647 (in blue) in the axons (A"). Finally, the cultures were fixed and activated CREB was visualized by phospho CREB (pCREB, S133) immunofluorescence performed in CB. **(B)** Representative figures of nuclear pCREB immunostaining in control neurons and stimulated with BDNF in axons for different times points as indicated in A. Scale bar 10 μm. **(C)** Quantification of fluorescence (arbitrary units, A.U) of pCREB in the nucleus in the presence or absence of BDNF. Just neurons positive for Ctb555 were included in the analysis. n= 30-40 neurons from 2 independent experiment. *p<0.05, **p< 0.01, ***p< 0.01 vs control; + p<0.05 vs BDNF 90 and 120 min. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons. **(D)** Experimental design to study the requirement of CREB activation for axonal BDNF-induced dendritic arborization. The CB was incubated with TrkB-Fc (100 ng/mL) and KG501 (10 μM) for 1 hour. Then, the AC was stimulated with BDNF (50 ng/mL) for 48 hours in the presence of Ctb555 in the axons in the presence or absence of KG501 in the CB. Finally, neurons were fixed and immunofluorescence was performed against MAP2. **(E)** Representative images of neurons in the CB (red indicates Ctb555) from compartmentalized cultures treated with DMSO (control), KG501, BDNF or BDNF in the presence of KG501 CB. Scale bar 20 μm. **(E-H)** Quantification of primary dendrites **(F)**, branching points **(G),** Sholl analysis **(H)**, from neurons labelled with EGFP/MAP2/Ctb555 in each experimental condition. n= 30-35 neurons from 3 independent experiment. *p<0.05, **p< 0.01, ***p< 0.001. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons (F and G). Two-way ANOVA with Bonferroni post-test for multiple comparisons was used for Sholl analysis. Results are expressed as ± SEM.**(I)** To control for cell death induced by the KG501 drug, we performed experiments as indicated in D by after fixation cell were staining for DNA fragmentation using Tunel (in green). Just neurons labelled with Ctb555 were considered. As a positive control Oligomycin (10 μM) treatments as indicated in methods was used. **(J)** Quantification of Tunel positive cells. Scale bar 20 μm. n=2 chambers per condition from 3 independent experiments. *p=0.05. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons.

Since CREB has been widely described as mayor regulator of neurotrophins-induced survival in PNS neurons (Bonni et al., 1999; Lonze and Ginty, 2002), we assessed whether, in our conditions, KG501 had any effect on neuronal survival using the Tunel assay. We found that after 48 hours of treatment, KG501 did not affected neuronal survival. As a positive control, we used oligomycin A, an inhibitor of oxidative phosphorylation in the mitochondria, that induces apoptotic cell death (Fig. 3I and J). Taken together, our result suggests that axonal BDNF promotes CREB phosphorylation and that CREB-dependent transcription is required for long-distance dendritic branching induced by BDNF.

The transport of endocytic organelles by the molecular motor dynein is required for BDNF long-distance signaling.

In axons, long-range retrograde transport of organelles and signaling molecules is performed by the minus-end microtubule molecular motor dynein (Hirokawa et al., 2010). Several groups have shown that in central neurons, BDNF and TrkB are efficiently retrogradely transported (Olenick et al., 2019; Zhou et al., 2012). To further confirm that this process is dynein dependent, we monobiotinylated BDNF *in vitro* and we labelled it with streptavidin quantum-dots (QD) for *in vivo* imaging (Stuardo et al, 2019). We found that BDNF-QD was efficiently targeted to the retrograde transport pathway (Supplementary Figure 3) and that this process was reduced in about 80% by inhibition of dynein with Ciliobrevin D (CilioD), a specific inhibitor of dynein ATP activity (Sainath and Gallo, 2015) (Fig. 4A and B). To evaluate whether axonal dynein inhibition reduced the transport of axonal TrkB signaling, we evaluated nuclear phosphorylation of CREB and dendritic arborization induced by axonal BDNF stimulation in

the presence or absence of CilioD in axons (Fig 4C). First, we evaluated the extend of CREB phosphorylation in the nucleus upon axonal BDNF stimulation. With this aim, we incubated the axonal compartment overnight with Ctb555 to identify all the neurons that projected their axons, then at the next day we incubated the axonal compartment with BDNF and Ctb647 in the presence or absence of CilioD (Fig. 4C and D). The presence of CilioD in the axons decreased CREB phosphorylation at 30 minutes in almost a 40% (Fig. 4D and E) and at 180 minutes completely abolish CREB phosphorylation (Fig. 4D and F) induced by BDNF axonal stimulation in addition to the accumulation of Ctb647 (in magenta) (Fig. 4D). Consistently with the result presented in Figure 3, reduced CREB phosphorylation induced by dynein inhibition (48 hrs treatment) negatively regulated dendritic arborization induced by axonal BDNF (Fig. 4G - J).

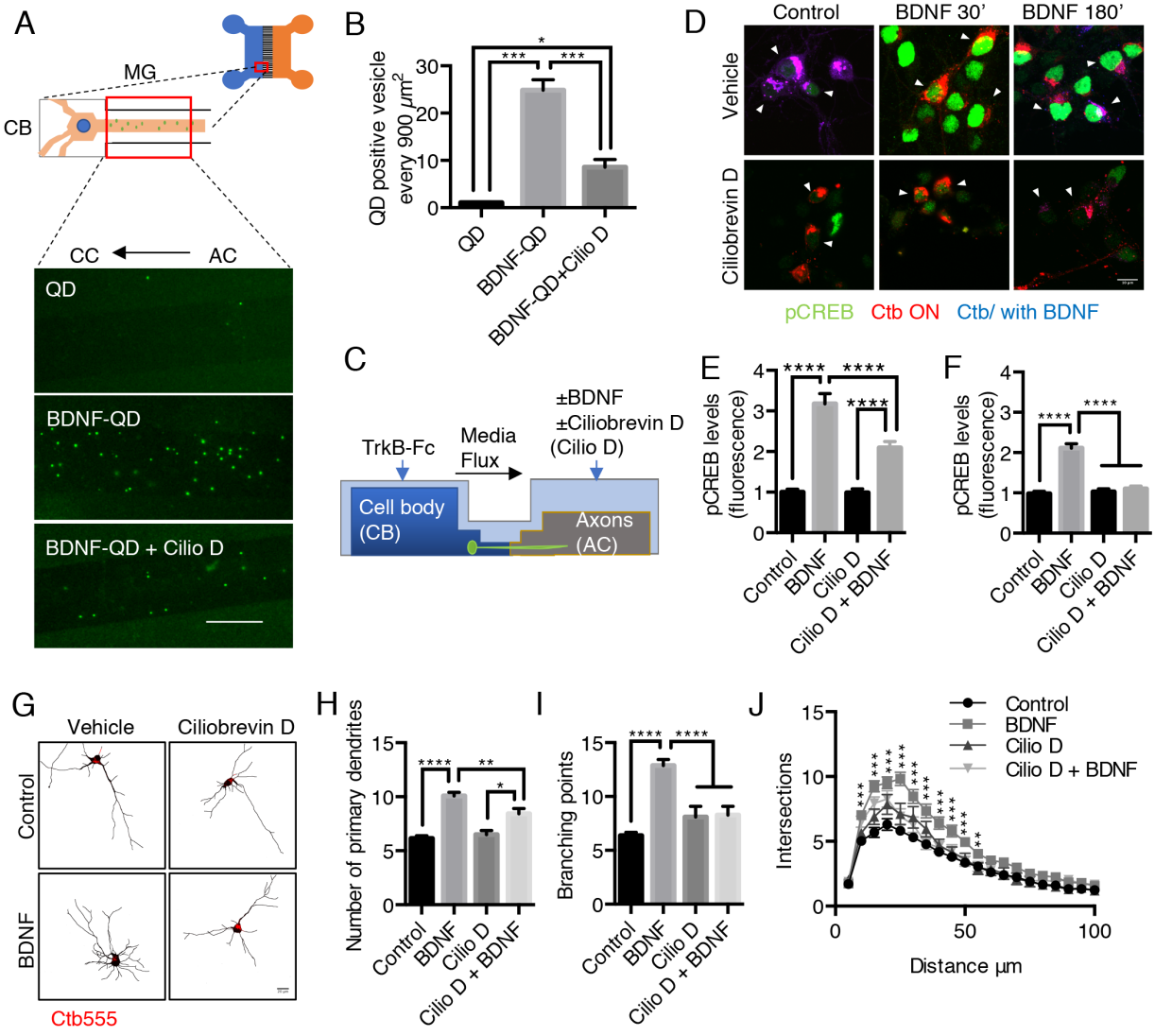

A
MG
CB
CC
AC
QD
BDNF-QD
BDNF-QD + Cilio D
B
QD positive vesicle every 900 μm²
QD
BDNF-QD
BDNF-QD+Cilio D
C
TrkB-Fc
Media Flux
±BDNF
±Ciliobrevin D (Cilio D)
Cell body (CB)
Axons (AC)
D
Control
BDNF 30'
BDNF 180'
Vehicle
Ciliobrevin D
pCREB
Ctb ON
Ctb/ with BDNF
E
pCREB levels (fluorescence)
Control
BDNF
Cilio D
Cilio D + BDNF
F
pCREB levels (fluorescence)
G
Vehicle
Ciliobrevin D
Control
BDNF
Ctb555
H
Number of primary dendrites
I
Branching points
J
Intersections
Distance μm
Control
BDNF
Cilio D
Cilio D + BDNF

Figure 4. Dynein activity is required for phosphorylation of CREB and dendritic arborization induced by BDNF. (A) Schematic representation of protocol to evaluate retrograde transport of BDNF. Biotinylated BDNF conjugated to Streptavidin quantum dot 655 (QD) (2 nM) was incubated in the AC for 4 hrs in the presence or absence of Ciliobrevin D (20 μM). Then, after washed, neurons were fixed and the accumulation of BDNF-QD was evaluated in the proximal compartment of the microgrooves (MG). As a control, non-conjugated QDs were used. Representative images of BDNF-QD (dots in green) of the proximal region of MG in each condition. **(B)** Quantification of QD positive vesicles in the proximal region of MG every 900 μm per each condition. Scale bar 10 μm. N= 36 microgrooves from 3 independent experiment were analyzed. **(C)** Experimental design to study dynein dependent axonal BDNF signaling in compartmentalized cortical neurons. Five DIV cortical neurons were retrograde labeled with Ctb555 (in red) over-night. The next day, the culture medium was changed to serum-free medium for 90 minutes in the presence or absence of Ciliobrevin D in the AC. Then, AC was stimulated with BDNF (50 ng/mL) for 30 and 180 minutes along with Ctb647 (in blue) in the axon in the presence or absence of Ciliobrevin D. Finally, the cultures were fixed and phosphoCREB (pCREB, S133) was visualized by immunofluorescence. **(D)** Representative images of nuclear pCREB in neurons stimulated or non-stimulated with BDNF, Ctb555 (in red) and Ctb647(in blue) added to axons in the presence or absence of axonal Ciliobrevin D. Scale bar 10 μm. **(E-F)** Quantification of the fluorescence (Arbitrary Units, A.U.) associated with pCREB immunostaining in the nucleus of neurons labeled with Ctb555 and stimulated with BDNF during 30 minutes (E) or 180 min (F). n= 83-111 neurons from 3 independent experiment. **(G)** Six DIV cortical neurons were transfected with EGFP, then the CB was treated with TrkB-Fc (100 ng/mL) and the AC was treated with Ctb555 and BDNF (50 ng/mL) with or without Ciliobrevin D (20 μM) during 48 hours. Finally, neurons were fixed and immunofluorescence was performed against MAP2. Representative images of the neuronal morphology of neurons labelled with Ctb555 (in red) and treated in the AC during 48 hours from compartmentalized cultures with DMSO (control), Ciliobrevin D, BDNF or BDNF and pretreated with Ciliobrevin D. **(H-J)** Quantification of primary dendrites **(H)**, branching points **(I),** Sholl analysis **(J)**, from neurons labelled with EGFP/MAP2/Ctb555 in each experimental condition as indicated in G. Scale bar 20 μm. n= 34-65 neurons from 3 independent experiment. *p<0.05, **p< 0.01, ***p< 0.001, ****p<0.0001. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons (E, F, H and I). Statistical comparison of the Sholl analysis was done by two-way ANOVA with Bonferroni post-hoc test for multiple comparisons.

Several lines of research have suggested that signaling endosomes requires a Rab5-dependent sorting step for retrograde transport of neurotrophin receptors (Deinhardt et al, 2006; Escudero et al, 2019; Goto-Silva et al, 2019). To study whether BDNF/TrkB long-distance signaling requires Rab5 activity, we first studied whether endocytosed TrkB co-localized with

endogenous Rab5. Cortical neurons in microfluidic devices were transfected with a plasmid driving the expression of TrkB NH2-tagged with the Flag epitope (Flag-TrkB) and we incubated distal axons with a Flag-specific monoclonal antibody in the presence of BDNF for 30 or 180 minutes. Then, we evaluated if the Flag staining colocalized with endogenous Rab5 endosomes using a specific antibody against Rab5. We observed that 30 minutes of BDNF treatment was not enough to observe an extensive accumulation of Flag-TrkB in the microgrooves, required to analyzed co-localization (Supplementary figure 4A). Therefore, we performed longer time exposure with BDNF (180 min) and we analyzed the TrkB and Rab5 co-localization in axons in the axonal compartment as well as in the microgrooves, were TrkB staining corresponded to retrogradely transported TrkB receptor (Supplementary fig. 4A and Fig.5A). Interestingly, we observed that in the axonal compartment 60% of Flag-TrkB dots colocalized with Rab5 positive vesicles. This colocalization decrease to a 40% in the microgrooves (Fig. 5B), suggesting that during its transport TrkB-positive endosomes matures to another type of endosomes.

The active form of Rab5 recruits different adaptors allowing early endosomes to interact with molecular motors such as dynein and myosins, or SNAREs proteins allowing the fusion of vesicles and endosomal kinases allowing the synthesis of phosphatidylinositols (Christoforidis et al., 1999; Olenick et al., 2019; Wandinger-Ness and Zerial, 2014; Satoh et al, 2008). Previously, we have shown that BDNF promotes Rab5 activation in dendrites and cell bodies of hippocampal neurons (Moya-Alvarado et al., 2018). To assesses whether in axons, BDNF also promotes the activation of Rab5 in compartmentalized cortical neurons, we used the glutathione-S-transferase (GST)-fused Rab5 binding domain of Rabaptin5 (Rab5BD-GST). This protein specifically recognizes the GTP-bound active form of Rab5 (Wu et al., 2014) and

by using an antibody against GST we have measured the Rab5-GTP levels *in situ* by immunofluorescence (Moya-Alvarado et al, 2018). With the same methodology, we found that the treatment of axonal compartment of cortical neurons with BDNF for 30 minutes increased the amount of Rab5-GTP in the axons (Fig. 5 C and D). To corroborate that the activation of Rab5 is TrkB specific, we incubated the axonal compartment with K252a or Ctb. We observe that K252a abolished BDNF-induced Rab5 activation and the addition of Ctb did not increased Rab5 activity (Fig. 5D and supplementary fig. 4B), both results suggest that BDNF specifically increases the Rab5 activity in a TrkB-dependent manner in axons. Finally, to evaluate whether there is a functional role of Rab5 in long-distance signaling mediated by BDNF, we evaluated nuclear CREB phosphorylation induced by axonal BDNF in compartmentalized neurons transduced with Rab5DN adenoviruses. We observed that the presence of a dominant negative form of Rab5 prevented axonal BDNF-CREB induced phosphorylation (Fig. 5E and F). Altogether, our results indicate that BDNF long-distance signaling requires TrkB internalization to a Rab5-early positive endosome for dynein-dependent retrograde transport.

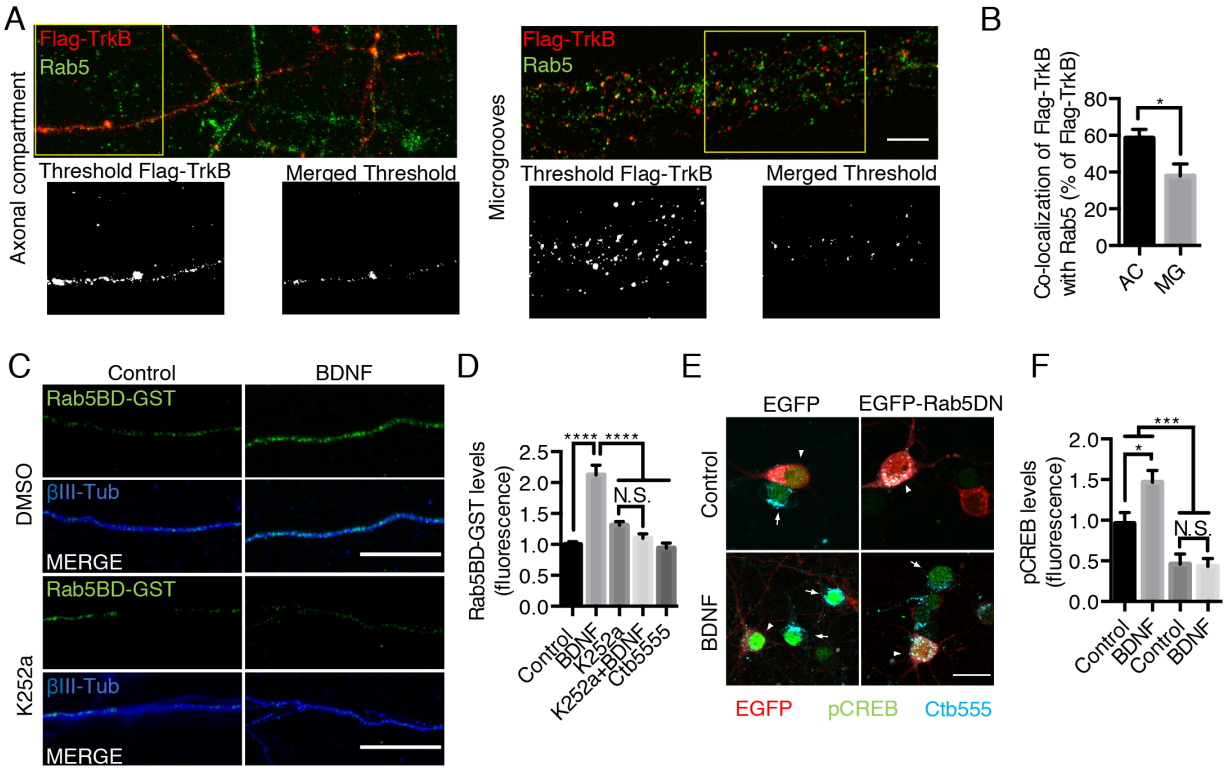


Figure 5. Rab5 activity is required for CREB phosphorylation induced by BDNF in axons. **(A)** Representative image of Flag-TrkB and Rab5 co-localization in the axonal compartment and microgroove compartment of neurons stimulated with BDNF (50 ng/mL) during 180 minutes. In the lower part, there is a zoom of the yellow square images shown in the upper part of the panel. In the right panel, in white the fluorescence associated with Flag-TrkB and in the left panel, the fluorescence associated with the co-localization of TrkB with Rab5 immunostaining in the AC or MG. Scale bar 10 µm. **(B)** Quantification of Flag-TrkB and Rab5 co-localization expressed as percentage. Results are expressed as ± SEM. *p<0.05. Statistics was done by student's t-test **(C)** Representative image of Rab5BD-GST (green) and βIII-Tubulin (blue) of the axonal compartment of cortical neurons non-stimulated or stimulated with BDNF (50 ng/mL, 30 minutes) in the presence or absence of K252a (200 nM). Scale bar 20 µm. **(D)** Quantification of the fluorescence intensity (relative to the control) in the axonal compartment in each condition. n=24-53 axons from 4 independent experiments. **(E)** Cortical neurons infected with EGFP or EGFP-Rab5DN for 48 hours and then stimulated with BDNF (50 ng/mL) in the axonal compartment. Representative image of phosphorylated CREB (pCREB) in the cell body of neurons in the control or treated with BDNF. EGFP (red), pCREB (green) and Ctb555 (cyan). Scale bar 20 µm. **(F)** Quantification of pCREB in the nucleus of neurons in the different experimental conditions. n=24-34 neurons from 2 independent experiment. Results are expressed as ± SEM. *p<0.05, ****p< 0.0001. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons

Somatodendritic activity of axonal TrkB is required for long-distance signaling triggered by BDNF in axons.

Several articles have shown that BDNF in axons is processively transported in the retrograde direction accumulating in cell bodies (Olenick et al., 2019; Zhou et al., 2012; Xie et al, 2012).

To our knowledge, there are no evidence that active TrkB reaches the cell body and continues signaling allowing CREB activation. We and others have shown that CREB activation requires Erk1/2 signaling (Finkbeiner et al, 1997; Gonzalez et al, 2019); therefore, it is possible that active Erk in the axons is transported in a complex with dynein and not in signaling endosomes together with active TrkB. Such a mechanism has been described before in the PNS for axonal-injury signals (Perlson et al, 2005). To evaluate whether axonal-derived active TrkB is required for BDNF axonal signaling, we performed two different approaches. First, a pulse and chase experiment to visualize active TrkB in endosomes and then we used compartmentalized cultures derived from the $TrkB^{F616A}$ knockin mice to inhibit TrkB activity using the inhibitor 1NM-PP1 in cell bodies. First, we transfected cortical neurons in microfluidic devices with the Flag-TrkB plasmid and then we treated distal axons with Flag-specific antibody at 4ºC (40 min), washed and treated the axon with or without BDNF for 3 hours at 37ºC. After fixation, we evaluated the co-localization of Flag immunostaining with an antibody against phosphorylated TrkB (pTrkB) in the microgrooves in the proximity to the axonal compartment (distal microgrooves), in the microgrooves in the proximity with the cell bodies compartment (proximal microgrooves) and in the cell body compartment (CB) (Supplementary Fig. 5A). In the distal microgrooves, we

observed that the Flag-TrkB protein is retrogradely transported in absent of stimulation, but BDNF increases its retrograde transport (Supplementary Fig. 5B). In the proximal microgrooves, little Flag labeling is observed in the absence of axonal BDNF, but there is a large labelling for Flag-TrkB in the presence of axonal BDNF. There, some vesicle co-localized with both pTrkB and Flag-TrkB (Supplementary Fig. 5C). Colocalization of pTrkB with Flag-TrkB was even more obvious in the close proximity to the somas (Supplementary Fig. 5 D and E). These results suggest that axonal BDNF induces the retrograde transport of TrkB and that retrogradely transported TrkB is active in neuronal cell bodies.

To further corroborate that active TrkB in axonal-derived endosomes is required for the propagation of axonal BDNF signaling into the cell body, we used neuronal cortical cultures derived from the $TrkB^{F616A}$ mice. We first sought to characterize whether 1NM-PP1 turned off the activity of TrkB and BDNF-dependent CREB phosphorylation and Akt/mTORC1 activation by Western blot and immunofluorescence in non-compartmentalized neurons. We stimulated the neurons with BDNF for 30 minutes and then, we incubated with 1NM-PP1 or DMSO for additional 30 minutes. The purpose was to corroborate that the 1NM-PP1 is able to reduce the activity of TrkB and its downstream signaling pathways after the receptor has been activated. As a control, we preincubated the neurons with 1NM-PP1 for 1 hour to prevent the activation of TrkB (Supplementary Fig.6A) and as we expected, the preincubation of neurons with 1NM-PP1 prevented the activation of TrkB and Akt. Finally, we observed that the treatment with 1NM-PP1 after BDNF stimulation decreased the phosphorylation of TrkB and Akt by Western blot (Supplementary Fig.6B and C). Consistently, by immunofluorescence, we observed that this treatment decreases the nuclear phosphorylation of CREB and the phosphorylation of 4E-

BP1 and 6S ribosomal protein (S6r), two proteins downstream of TORC1 activation induced by BDNF (Supplementary Fig 6D and E). These results indicate that 1NM-PP1 is able to turn off the TrkB receptor after its activation and that the TrkB receptor activity is required to sustain TrkB-signaling pathways.

Subsequently, we directly tested whether we could reduce the TrkB activity in the cell body after axonal stimulation of BDNF in $TrkB^{F616A}$ mice-derived neurons. Somatodendritic TrkB activity was tested after treating with 1NM-PP1 in the cell body compartment or in the axonal compartment. BDNF applied in the axonal compartment increases the immunostaining for endogenous phosphorylated TrkB in the cell bodies compartment (Fig. 6A). Consistently with the observations in non-compartmentalized neuronal cultures, the presence of 1NM-PP1 in the axonal compartment completely abolished the accumulation of phosphorylated TrkB in cell bodies (Fig. 6B and C). On the other hand, the presence of 1NM-PP1 in the cell body decreased the amount of active TrkB in cell bodies after axonal stimulation with BDNF (Fig. 6B and C). These results suggest that 1NM-PP1 turned off the phosphorylation of endosomal TrkB after arriving to the cell body. To further corroborate this possibility, we studied the phosphorylation of CREB after the stimulation of axons with BDNF in the presence or absence of 1NM-PP1 in cell bodies of compartmentalized cultures derived from $TrkB^{F616A}$ mice. We found that somatodendritic TrkB activity was required for CREB phosphorylation induced by axonal BDNF stimulation (Fig. 6D and E). These results together with the observations presented in this research suggested that BDNF is transported together with TrkB in signaling endosomes to the cell body where the complex continues signaling.

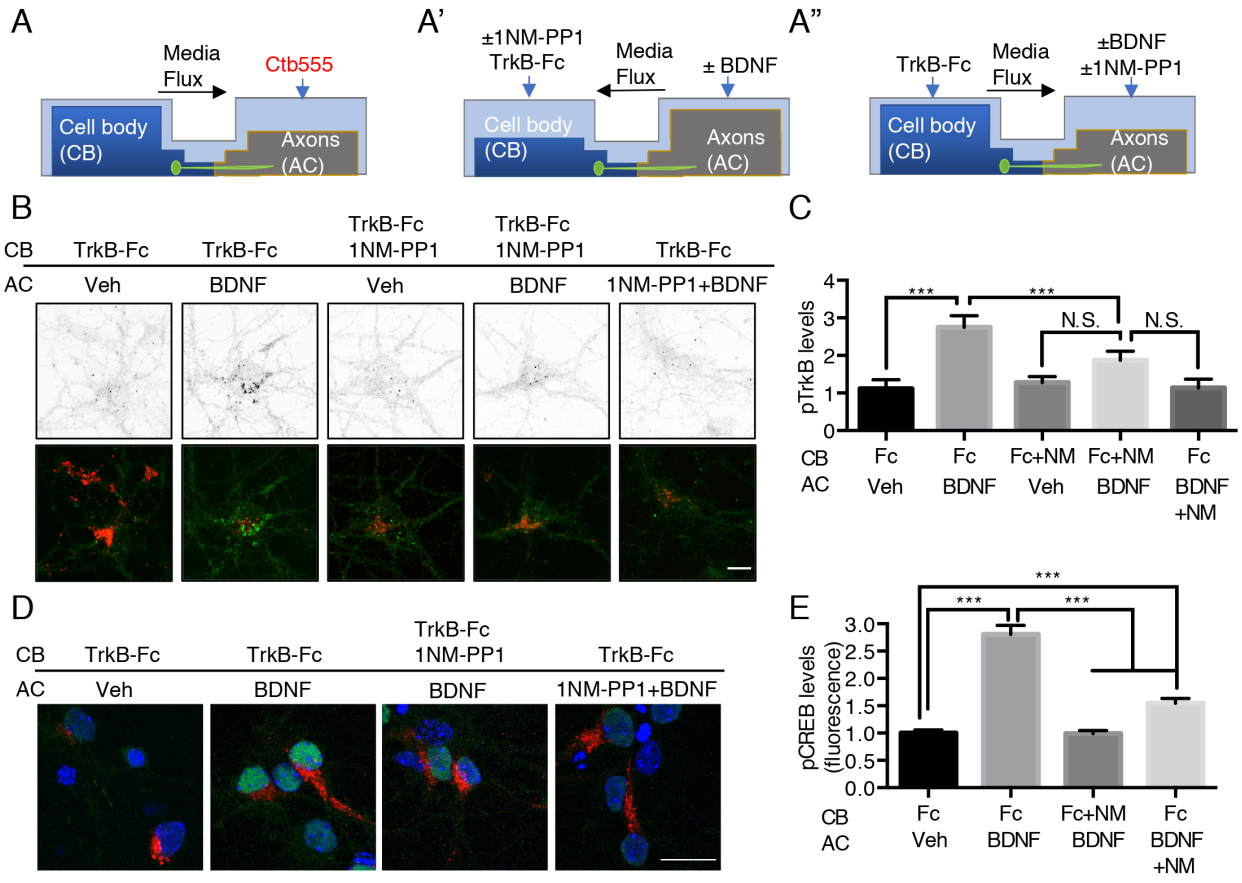


Figure 6. Somatodendritic activity of axonal TrkB is required for BDNF long-distance signaling of BDNF. (A-A'') Schematic representation of the protocol used for stimulating neurons. **(A)** At 6 DIV AC of cortical neurons from TrkB^{F616A} was incubated with Ctb555 overnight. Next day (7 DIV) there was two types of treatments: (A') Neurons were depleted of B27 supplement during 1 hour in the presence of 1NM-PP1 (1 μM) in the CB, with the flux toward the CB. Then, the CB was treated with TrkB-Fc (100 ng/mL) with 1NM-PP1 and the AC was treated with or without BDNF (50 ng/mL) during 3 hours. (A'') Neurons were depleted of B27 supplement during 1 hour in the presence or absence of 1NM-PP1 (1 μM) in the AC, with the flux toward the AC. Then the CB was treated with TrkB-Fc, and the AC was incubated with or without BDNF in the presence or absence of 1NM-PP1 during 3 hours. Finally, neurons were fixed and an immunofluorescence against phosphoTrkB (pTrkB, Y816) or phosphoCREB (pCREB, S133) was performed. **(B)** Representative images of pTrkB in the CB compartment of Ctb positive neurons treated as described in A. **(C)** Quantification of pTrkB levels in the cell body of each treatment as indicated in B. n=3 independent experiment. **(D)** Representative image of cortical neurons immunostained for pCREB stimulated with BDNF in the axonal compartment for 3 hrs in the presence or absence of 1NM-PP1 in the CB (1NM- PP1/BDNF) or AC (1NM-PP1 + 1NM-PP1) respectively. **(E)** Quantification of pCREB levels in Ctb positive neurons of each condition. n= 78-86 neurons from 3 independent experiment. **p< 0.01, ***p<

0.001. vs control. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons.

Somatodendritic PI3K/Akt/mTOR activity is required for BDNF long-distance signaling.

BDNF-mediated dendritic arborization is achieved by activating the PI3K/Akt/mTOR signaling pathway increasing local translation (Dijkhuizen and Ghosh, 2005; Kumar et al., 2005). In addition, PI3K has been shown to mediated retrograde NGF-signaling in sympathetic neurons (Kuruvilla et al., 2000). On the other hand, it was reported that Rab5/APPL1 endosomes were positive for TrkB and Akt (Goto-Silva et al., 2019), suggesting that axonal PI3K/Akt signaling may contribute to long-distance dendritic arborization induced by BDNF. With this aim, we incubated the AC of compartmentalized cortical neurons with BDNF in the presence or absence of LY294002, a potent general PI3K inhibitor (Fig. 7A). Surprisingly, the presence of LY294002 in axons did not affect the dendritic arborization induced by axonal BDNF (Fig. 7B-E). Confirming previous results (Finsterwald et al; 2010), LY294002 was able to reduce BDNF-induce dendritic arborization in non-compartmentalized cortical cultures, indicating that LY294002 was active in cortical neurons (Supplementary Fig. 7). Also, inhibition of PI3K activity in the axons did not reduced the transport of BDNF-QD (Fig. 7F and G). Indicating that PI3K activity is not required for retrograde transport of BDNF axonal signaling. To this end, we investigated whether PI3K signaling in the somatodendritic compartment was required for axonal BDNF-induced dendritic arborization by treating the CB compartment of compartmentalized cultures with LY294002 (Fig. 7H). We observed that the presence of LY294002 in the CB inhibited the dendritic arborization induced by BDNF (Fig. 7I-L), suggesting that PI3K activation in cell bodies is required for long-distance signaling induced by BDNF in axons.

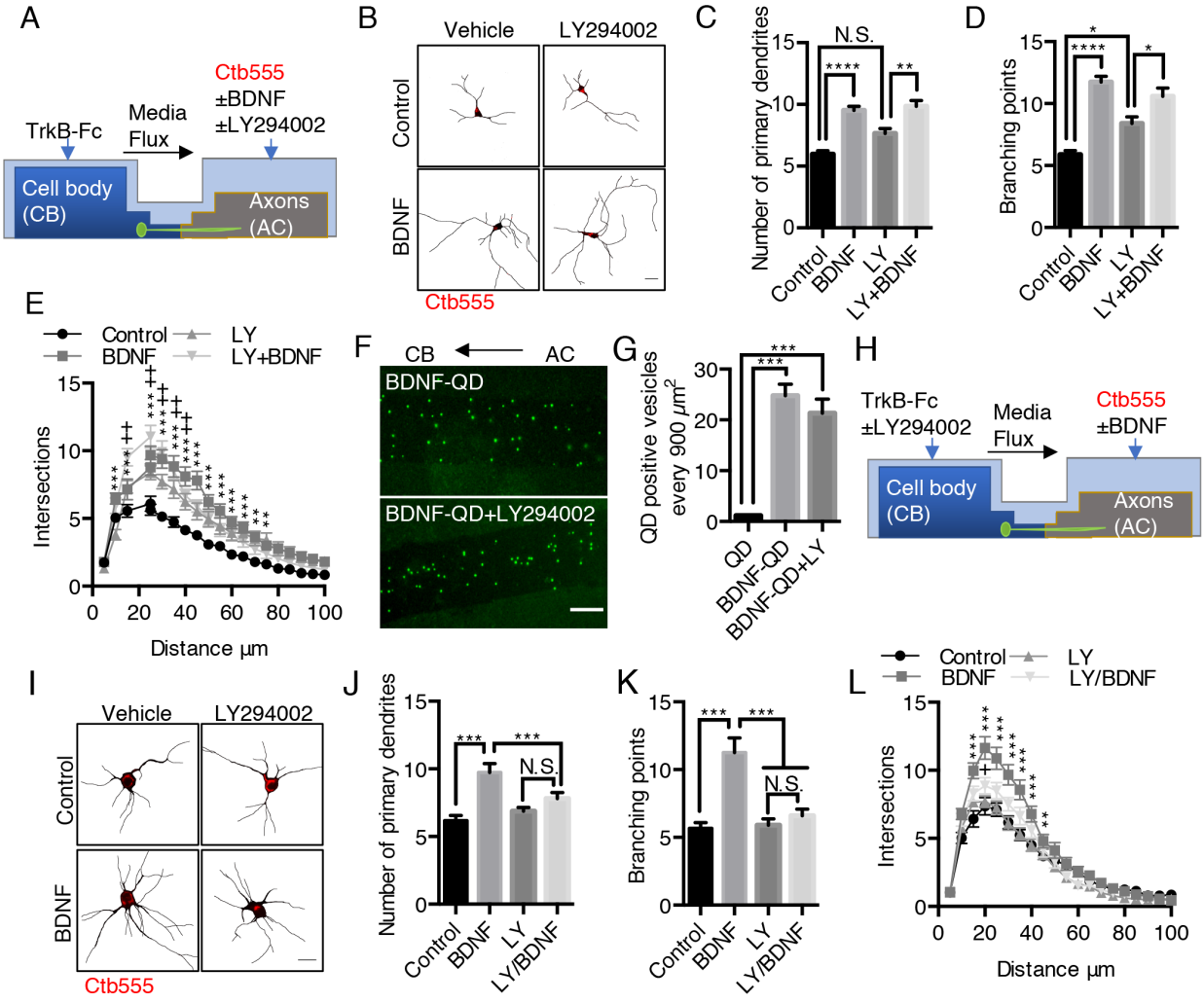

A
TrkB-Fc
Media Flux
Ctb555 ±BDNF ±LY294002
Cell body (CB)
Axons (AC)
B
Vehicle
LY294002
Control
BDNF
Ctb555
C
Number of primary dendrites
N.S.
Control
BDNF
LY
LY+BDNF
D
Branching points
E
Control
BDNF
LY
LY+BDNF
Intersections
Distance μm
F
CB
AC
BDNF-QD
BDNF-QD+LY294002
G
QD positive vesicles every 900 μm²
QD
BDNF-QD
BDNF-QD+LY
H
TrkB-Fc ±LY294002
Media Flux
Ctb555 ±BDNF
Cell body (CB)
Axons (AC)
I
Vehicle
LY294002
Control
BDNF
Ctb555
J
Number of primary dendrites
Control
BDNF
LY
LY/BDNF
K
Branching points
L
Control
BDNF
LY
LY/BDNF
Intersections
Distance μm

Figure 7. Activation of PI3K activity in the cell bodies and not in the axons (AC) was required for dendritic arborization induced by axonal BDNF. (A) Schematic representation of the protocol to stimulate compartmentalized cortical neurons. Six DIV cortical neurons were transfected with EGFP, then the CB was treated with TrkB-Fc (100 ng/mL) and the AC was treated with Ctb555 and BDNF (50 ng/mL) with or without LY294002 (10 μM) during 48 hours. Finally, neurons were fixed and immunofluorescence was performed against MAP2. **(B)** Representative images of neurons in the CB (red indicated Ctb555) from compartmentalized cultures treated with DMSO in the presence or absence of BDNF and LY294002 in AC. **(C-E)** Quantification of primary dendrites **(C)**, branching points **(D),** and Sholl analysis **(E)**, from neurons labelled with EGFP/MAP2/Ctb555 in the different experimental conditions as described in B. Scale bar 20 μm. n= 29-48 neurons from 3 independent experiment. **(F)** The Ac was treated with 2 nM of BDNF-QD for 4 hours in the presence or absence of LY294002 (10 μM) to promote the retrograde transport of BDNF. Then, neurons were fixed and the accumulation of BDNF-QD was evaluated in the proximal compartment of the microgrooves (MG). Representative image of BDNF-QD (in green dots) accumulation in the proximal part of MG in each treatment. Scale bar 10 μm. **(G)** Quantification of QD positive vesicles in the proximal region of MG every 900 μm per each condition. Scale bar 10 μm. N= 36 microgrooves from 3 independent experiment were analyzed. **(H)** Schematic representation of the protocol to stimulate compartmentalized cortical neurons. Six DIV cortical neurons were transfected with EGFP, then the CB was treated with TrkB-Fc (100 ng/mL) in the presence or absence of LY294002 (10 μM) and the AC was treated with Ctb555 and BDNF (50 ng/mL) during 48 hours. Finally, neurons were fixed and immunofluorescence was performed against MAP2. **(I)** Representative images of neurons in the CB (red indicated Ctb555) from compartmentalized cultures treated in the CB with DMSO (control) or LY294002 and in the AC with or without BDNF. Scale bar 20 μm. **(C-E)** Quantification of primary dendrites **(C)**, branching points **(D),** Sholl analysis **(E)**, from neurons labelled with EGFP/MAP2/Ctb555. n= 25-30 neurons from 3 independent experiment. *p<0.05, **p< 0.01, ***p< 0.001, +p<0.05 LY294002 vs LY294022/BDNF. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons (C, D, G, J, and K). Statistical comparison of the Sholls analysis were done by two-way ANOVA with Bonferroni post-hoc test for multiple comparisons.

Once PI3K is activated, it phosphorylates PIP2 two PIP3 in membranes leading to the recruitment of Akt and its phosphorylation by PKD. In turn, Akt activates the mTOR kinase pathway by reducing the activity of the Rheb GAP leading to the activation of mTOR (Garza-Lombo and Gonsebatt, 2016). First, to evaluate the activation of the mTOR pathway by BDNF

in cortical neurons we stimulated non-compartmentalized cultures with BDNF during 1 hour in the presence or absence of LY294002 (PI3K inihibitor), and Torin1, a specific inhibitor of mTOR that will reduce the activity of both the TORC1 and TORC2 complex (Liu et al., 2010). We observed by Western blot that BDNF promotes the phosphorylation of TrkB, Akt, S6r, and 4E-BP1 in cortical neurons and that the presence of LY294002 and Torin1 fully inhibit the phosphorylation of Akt, S6r and 4E-BP1 but not TrkB (Supplementary figure 8A) as expected. Then, we evaluated whether the mTOR pathway is involved in increasing local translation of protein upon arrival of pTrkB signaling endosomes to cell bodies. With this aim, using rat compartmentalized cortical neurons, we evaluated in the neuronal cell bodies the time course of 4E-BP1 phosphorylation induced by axonal BDNF stimulation. We observed that BDNF increases the phosphorylation of 4E-BP1 at 90 minutes with the higher level at 180 minutes (Fig. 8A and B), and similar results were obtained with the phosphorylation of the S6r (Supplementary Fig. 8B). To evaluate whether the activation of 4E-BP1 and S6r by axonal BDNF was dependent on the somatodendritic activity of PI3K and mTOR, we treated compartmentalized neurons with the LY294002 or Torin1 inhibitors in the CB (Fig. 8C). The somatodendritic 4E-BP1 phosphorylation induced by axonal BDNF was significantly reduced by the presence of these inhibitors (Fig. 8D and E). Also, the treatment of axons with the dynein inhibitor CilioD reduced the somatodendritic 4E-BP1 phosphorylation induced by axonal BDNF (Fig. 8D and E). Similar results were obtained with phospho S6r (Supplementary Fig 8C). All together, these results indicate that BDNF/TrkB signaling endosome activates PI3K/Akt/mTOR pathway in cell bodies suggesting that upon arrival to cell bodies, signaling endosomes turns on protein translation.

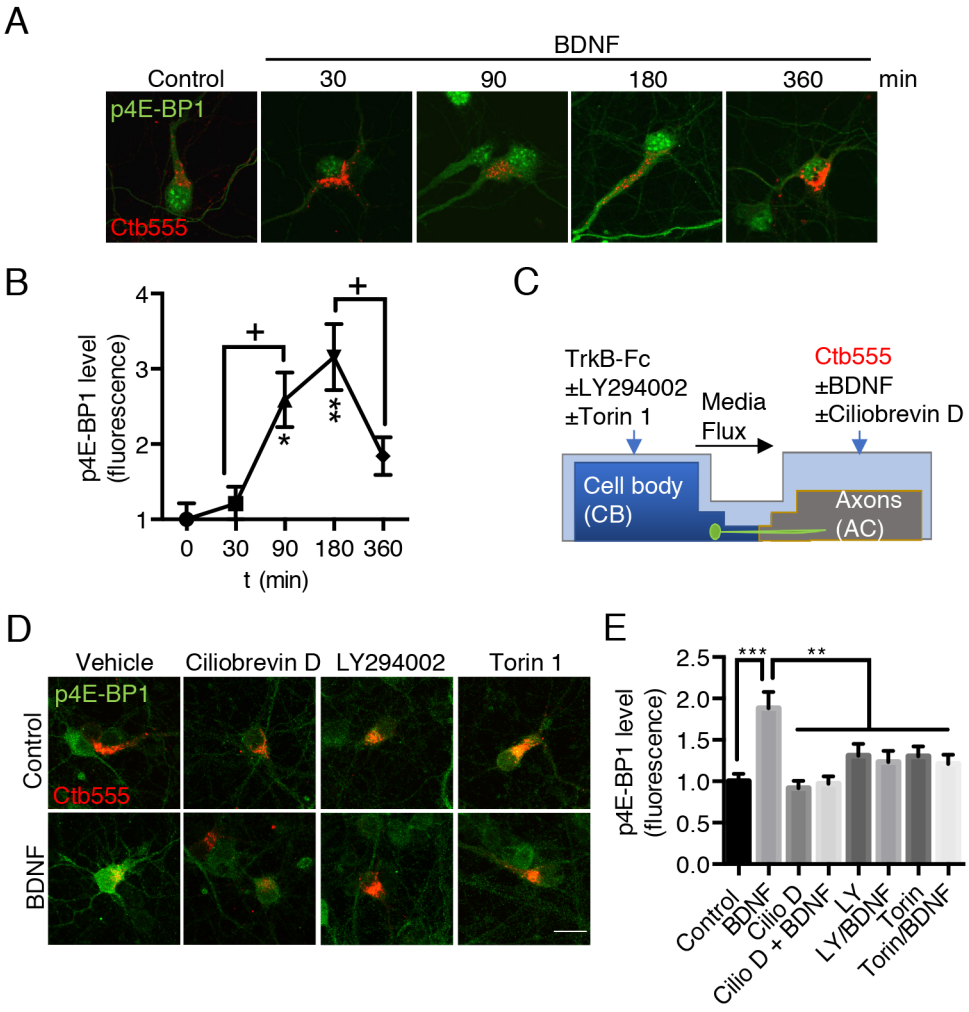


Figure 8. Axonal BDNF promotes mTOR activation in the cell body of compartmentalized cortical neurons in a PI3K-dependent manner. (A) Representative images of phosphorylated 4E-BP1 (p4E-BP1) immunostaining (in green) and Ctb555 (in red). Six DIV compartmentalized cortical neurons were incubated with Ctb555 overnight. At 7 DIV, neurons were treated in the axonal compartment with BDNF (50 ng/mL) during 30, 60, 180 or 360 minutes. **(B)** Time-course quantification of somatodendritic p4E-BP1 associated immunofluorescence in primary dendrites. **(C)** Schematic representation of the protocol used to evaluate the effect of different pharmacological inhibitors on the axonal BDNF-induced phosphorylation of 4E-BP1 in cell bodies. Six DIV cortical neurons were pretreated with DMSO (control), LY294002 (10 µm; LY), Torin 1 (0.25 µm; Torin) in the CB compartment or Ciliobrevin D (20 µm; Cilio D) in the AC for 1 hour. Then, BDNF was added during 180 minutes in the AC in the presence or absence of these inhibitors. **(D)** Representative images of p4E-BP1 (in green) in somatodendritic compartment of neurons stimulated with BDNF, in the presence or absence of different inhibitors. **(E)** Quantification of somatodendritic p4E-BP1 in each treatment of neurons labeled with Ctb555 (in red). Scale bar 20 µm. n= 31-36 neurons from 3 independent experiment. *p<0.05, **p< 0.01, ***p< 0.001. vs control; +p<0.05 vs BDNF 90 and 360 min in B. Results are expressed as ± SEM. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons.

CREB and mTOR activities are required for protein synthesis in the somatodendritic compartment.

The results presented above, indicating that BDNF in axons activates the phosphorylation of CREB (Fig. 3) and the activation of mTOR (Fig. 8) suggested that BDNF/TrkB signaling endosome activates the translation of newly synthesized transcripts. To test this hypothesis, we used the metabolic labeling of newly synthesized protein using the click-IT chemistry that uses the incorporation of AHA, a modified amino acid that resembles methionine (Dieterich et al., 2010). In addition, to evaluate by immunofluorescence the level of *de novo* protein synthesis, we evaluated the levels of Arc, an immediate-early gene, whose protein is required for synaptic plasticity induced by BDNF (Ying et al., 2002) and it synthesis is regulated by CREB and mTOR (Takei et al., 2004; Ying et al., 2002). First, we standardized the clickt-IT AHA methodology, in non-compartmentalized neurons. With that aim, we stimulated the neurons with BDNF during 5 hours in the presence or absence of cycloheximide. As we expected, BDNF increased the AHA fluorescence and the presence of cycloheximide completely reduced the fluorescence of AHA (Supplementary Figure 9)(Dieterich et al., 2010). Using compartmentalized cultures, we depleted the neurons media of methionine and B27 for 1 hour, next, we added AHA reagent in all the chamber and BDNF exclusively in the AC during 5 hours in the presence or absence of KG501 or Torin1 in the CB (Fig. 9A). As shown in Figure 9, BDNF causes a significant increase of both AHA incorporation in newly synthesized proteins and Arc levels in cell body and dendrites (Fig. 9B-D). Remarkably, inhibition of CREB and mTOR, resulted in a significant reduction in the AHA incorporation in newly synthesized proteins and Arc protein levels in the

cell body induced by BDNF (Fig. 9B-D). All together, these results indicate that BDNF/TrkB long-distance signaling turns on transcription and translation of specific mRNAs.

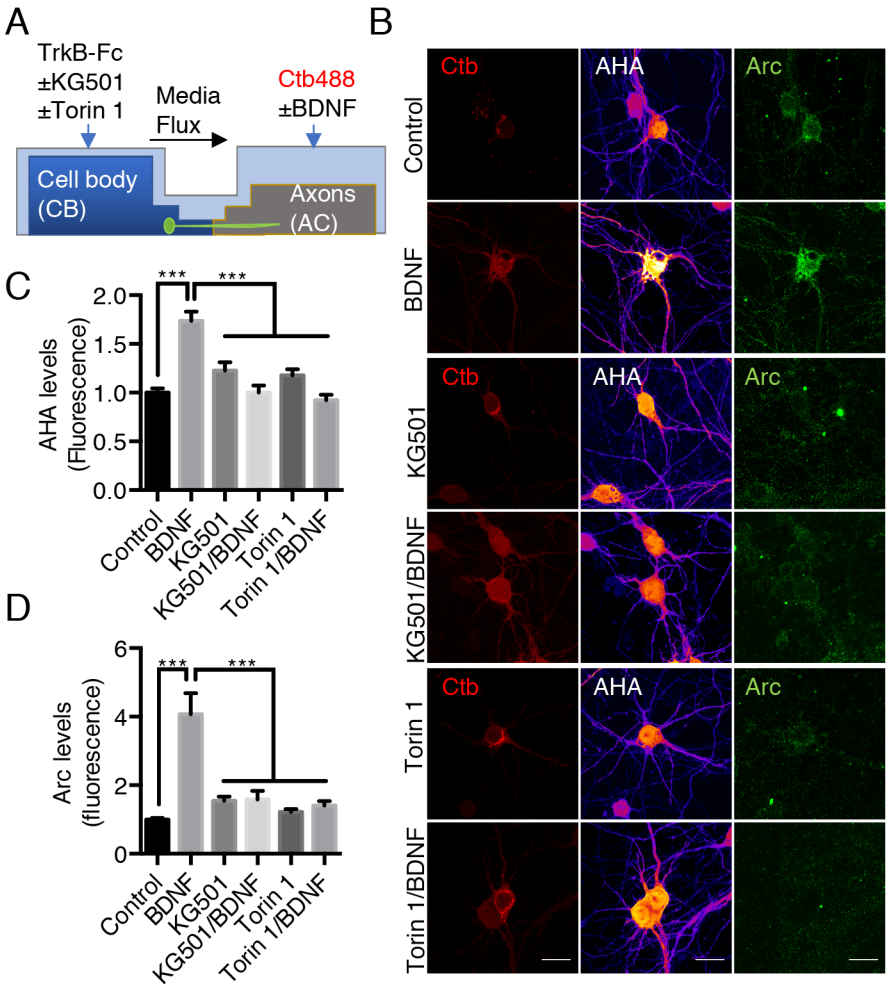


Figure 9. Axonal BDNF promotes somatodendritic protein synthesis in a mTOR and CREB dependent manner. **(A)** Schematic representation of the protocol to evaluate protein synthesis. Six-7 DIV cortical neurons were incubated in free methionine media during 1 hour in the presence or absence of KG501 (10 µm) or Torin 1 (0.25 µm) in the CB. Then, both the CB and AC were incubated with AHA, but just the axonal compartment was stimulated with BDNF (50 ng/mL) in the presence of Ctb488 during 5 hours. **(B)** Representative images of neurons positive for Ctb488, AHA staining and Arc immunofluorescence of control condition and stimulated with BDNF, in the presence or absence of KG501 and Torin 1. **(C)** Quantification of AHA fluorescence in the primary dendrite of neurons labeled with Ctb488 in each different treatment. **(D)** Quantification of Arc fluorescence in the somas of neurons labelled with Ctb488 in each different treatment. Scale bar 20 µm. n= 30-48 neurons from 3 independent experiment. ***p< 0.001. Statistics was done by one-way ANOVA test and Bonferroni post-test for multiple comparisons.

DISCUSSION

Long-distance communication of neurotrophins is well-described in the PNS, where targeted derived-neurotrophins control survival and axonal growth of sympathetic neurons (Harrington and Ginty, 2013; Ye et al., 2003; Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018; Escudero et al, 2019). Whether long-distance signaling have a role in the physiology of the CNS in mammals is not known. Using cultures of compartmentalized cortical neurons, we showed that BDNF in axons is able to increase dendritic arborization in cell bodies in a TrkB-dependent manner. Axonal BDNF increased nuclear CREB activation and translation of newly synthesized proteins in a CREB and PI3K-mTOR dependent-manner. Interestingly, PI3 kinase activity was not required in the axons for long-distance responses, contrary to the observation in sympathetic axons about NGF transport and signaling (Kuruvilla et al., 2000). We also provide several lines of evidences indicating that active TrkB, in signaling endosomes, is responsible for axon to cell body responses. The transport of BDNF/TrkB axonal signaling required Rab5 and dynein activity, suggesting that internalization through the early endosomal pathway is a required sorting step for retrograde transport.

Neurotrophin signal transduction in neurons is a complex process because the polarized morphology of neurons. Most neurons have long axons that can extend centimeters in the brain and spinal cord or even meters in the peripheral nervous system. TrkB receptors are localized in dendrites and axons in the plasma membrane and in intracellular vesicles throughout development (Gomes et al., 2006). On the other hand, BDNF is secreted in an autocrine and paracrine manner by the regulated-secretory pathway in the somatodendritic compartment and

in dense core vesicles in axons (Matsuda et al., 2009; Lo et al, 2011). Thus, neurons have to integrate both the local and long-distance neurotrophin signals. Examples of local responses were described by Horch and Kats, where they showed that BDNF released from single cell promotes dendritic growth in neighboring neurons (Horch and Katz, 2002). On the other hand, BDNF long-distance effects were described for the retinal ganglion cells (RGC) of *Xenopus* tadpoles, where long-distance BDNF stimulation enhances RGC dendritic arborization, while local BDNF reduces it (Du and Poo, 2004; Lom et al., 2002). Furthermore, previous work in mammal cells have shown that endosomal signaling of BDNF/TrkB is required for dendritic branching in mammal neurons (Zheng et al., 2008, Zhou et al, 2012). All these results, support the idea that long-distance signaling mediated by signaling endosomes regulates dendritic arborization in mammalian cells.

As mentioned above BDNF is secreted in an autocrine manner and TrkB is located in axons and dendrites; therefore, the experimental design performed in our study allowed to distinguish between axonal BDNF signaling and somatodendritic signaling. The microfluidic devices used to prepare compartmentalized cultures of cortical neurons possesses two chambers, separated by 400 long microgrooves to allow fluidic isolation (Gluska et al, 2016). The cell bodies compartment (CB) was kept with a larger volume compared to the axonal compartment (AC) in most of the experiments. In addition, we incubated the CB with TrkB-Fc to capture all the somatodendritic released of BDNF. Moreover, only the dendritic morphology of neurons that projected their axons in the AC were included in the analysis, allowing us to study selectively BDNF axonal signaling.

Since BDNF can bind p75 and TrkB, we ought to analyze whether long-distance signaling was dependent on TrkB signaling and the requirement for p75 expression in this process. We observed that inhibition of TrkB signaling, using the 1NM-PP1 in compartmentalized cultures derived from $TrkB^{F616A}$ mice showed a reduction in dendritic arborization. Similar results were obtained in compartmentalized cultures of rat cortical neurons treated with the drug K252a in the axons, a drug that reduces tyrosine kinase activity. Suggesting that the activity of the TrkB receptor is required for the long-distance signaling from axons in cortical neurons. Similarly, long-distance signaling by BDNF and NGF also requires axonal signaling of Trks in sensory and sympathetic neurons respectively. On the other hand, p75 signaling has been reported as having opposing roles compare to Trks signaling since it can induce apoptosis or reduce axonal branching when binding different ligands and receptors (Ibanez and Simi, 2012; Kraemer et al., 2014). Besides, p75 negatively regulates dendrite complexity and spine density in hippocampal neurons (Zagrebelsky et al., 2005). We observed that the absence of p75 produced a decrease of basal dendritic arborization of cortical neurons in microfluidic devices, but the presence of BDNF recover the dendritic complexity, and even increase the distal dendritic arbor, over 50 μm from soma, suggesting that p75 participates in the regulation of dendritic arborization, but is not required for BDNF axonal signaling.

CREB is a TF that is endogenously expressed in neurons and it has a major role in activity-dependent gene expression (Flavell and Greenberg, 2008; Yap and Greenberg, 2018) and plays a central role in physiological brain processes such as learning and memory (Finkbeiner et al., 1997; Xing et al., 1998; Lonze and Ginty, 2002). Indeed, mutations in several co-regulators or

downstream TFs factors are associated with genetic diseases leading to autism and cognitive disabilities (Lyu et al, 2016; Zhou et al, 2016; McGirr et al, 2016; Wang et al, 2018). CREB-dependent gene expression is regulated by two different signaling pathways. The first one by increasing the intracellular Ca^{+2} and the activation of CaMKIV and the second depends on activation of Ras/ERK pathway (Finkbeiner et al., 1997; Minichiello et al., 2002; Redmond et al., 2002). Whether BDNF in axons is able to activate CREB in cell bodies was suggested before in several studies (Deinhardt et al., 2006; Watson et al., 1999; Zhou et al., 2012); however, no studies have addressed the physiological role of CREB activation by BDNF-long distance signaling. In this work, we showed that CREB was required for BDNF-induced long-distance dendritic arborization. Also, CREB activation was required to increase general protein synthesis and the protein expression of Arc.

We performed a time course study of CREB activation after BDNF addition to axons and we studied its dependence on dynein activity using the inhibitor Ciliobrevin D. We found a sustained activation of CREB after 3 hours of BDNF addition to axons, results that are different of what we have observed in non-compartmentalized cultures of hippocampal neurons. In hippocampal neurons, addition of BDNF increases CREB phosphorylation after 15 minutes and by 1 hour of stimulation, CREB phosphorylation is significantly downregulated (Gonzalez et al 2019). Of note, in compartmentalized cortical cultures, we found that CREB activation, after 30 minutes BDNF addition to axons was reduced by a 40% by axonal treatment with Ciliobrevin D. On the other hand, 3 hours of BDNF stimulation, was reduced by an 80%, to a similar extend to what BDNF-QD retrograde transport was reduced. It is possible to speculated that there is and additional component that contributes to the early activation of CREB by axonal BDNF.

Unpublished results of our lab have shown that BDNF in axons is able to increase intracellular calcium levels (Chapter III, this thesis). Long-distance calcium waves have been described after axonal injury in the PNS (Cho et al., 2013). It is possible that there are two components that allow CREB activation by axonal BDNF, fast propagating calcium waves followed by axonal retrograde transport of signaling endosomes. Interestingly, it has been described that both rapid and sustained CREB phosphorylation are required for proper transcription regulation (Dolmetsch et al., 2001) suggesting that in neurons, retrograde BDNF-signaling may contribute to sustained CREB phosphorylation for CREB-dependent maintenance of dendritic arborization. However, more studies are required to support this hypothesis.

Upon ligand binding, TrkB receptors are internalized into endosomes that are retrograde transported to neuronal cell bodies (Xie et al, 2012). Here, we showed that TrkB is active in the somatodendritic compartment and the activity of the receptor is required in the cell body for CREB phosphorylation. We used the drug 1NM-PP1 in the cell body to specifically decrease TrkB activity. The fact that 1NM-PP1 reduced the activation of an already active TrkB suggested that 1NM-PP1 binds the ATP binding site of receptor, reducing the tyrosine activity of TrkB without affecting neurotrophin binding (Chen et al, 2005). There are two possibilities that can explain our results. The first one is that TrkB is being phosphorylated and desphosphorylated during its trajectory along the axon. Such a process, will allow 1NM-PP1 to bind the ATP binding cassette of the receptor and prevents its phosphorylation. In the second possibility, BDNF retrograde signaling promotes the phosphorylation of somatodendritic TrkB receptors and this process is required to maintain CREB phosphorylation. In agreement with this last idea, other groups have shown that retrogradely transported TrkA in sympathetic

neurons accumulate in the somatodendritic compartment locally increasing the phosphorylation of other TrkA receptors in dendrites and cell body (Lehigh et al., 2017; Yamashita et al., 2017). The Rab monomeric GTPases are the main regulators of postendocytic trafficking of endocytic receptors. After internalization, tyrosine kinase receptors enter to the early or sorting endosome, whose biology is regulated by Rab5 (Goh and Sorkin, 2013). Independent of the internalization mechanism of receptors, Rab5 tightly regulates the homotypic fusion of endosomes, forming the sorting endosome (Stenmark, 2009). In the early endosome, the receptor is sorted to the recycling pathway, which is regulated by Rab11 or to the late endocytic pathway regulated by Rab7 (Bronfman et al., 2014). We observed that in the microgrooves almost 40% of TrkB immunostaining colocalize with Rab5 immunostaining, suggesting that TrkB is internalized in Rab5 positive endosomes and then sorted to endosomes negative for Rab5. These results indicate that endosomal sorting and transport of signaling endosomes is an active process requiring the active modulation of the endosomal and transport machinery, consistently, Erk1/2 signaling has been shown to be required for dynein-dependent transport of BDNF/TrkB positive endosomes in axons (Mitchell et al, 2012). Moreover, we have shown that BDNF/TrkB signaling is able to increase the activity of the Rab11 and Rab5 GTPases in the dendrites of central neurons (Lazo et al 2013; Moya-Alvarado et al 2018). In this work, we showed that BDNF increases the activity of Rab5 in the axon in a TrkB-dependent manner, and consistent with the idea that a Rab5-dependent sorting step is required for retrograde transport of TrkB signaling endosomes, we found reduced phosphorylation of CREB when we reduced the activity of Rab5 using a dominant negative mutant. Previously, in motoneurons it was shown that neurotrophins receptors are retrogradely transported in endocytic organelle together with a fragment of tetanus neurotoxin TeNT H(C). Rab5 is essential for an early step in sorting of

TeNT H(C) but was absent from axonally transported vesicles that were mainly Rab7 positive (Deinhardt et al, 2006). Other authors, have found that retrograde transported TrkB receptors co-localize with AP2-LC3 organelle, these organelles are not enriched for Rab7 (Kononenko et al, 2017). Suggesting that authophagosomes may have a role for retrograde transport of neurotrophin receptor (Kononenko et al, 2017). Also, in sympathetic neurons retrograde transport of TrkA was associated with both Rab7-positive multivesicular bodies and Rab11 positive endosomes (Bardford et al, 2018; Mengchen et al, 2018). Suggesting that there is a diversity of Trks signaling organelles originating in the axons (Villarroel-Campos et al, 2018).

Endosome identity and function are specified not only for Rab proteins but also by the presence of distinct phosphoinositide species (Schink et al., 2016). We observed that BDNF increases the Rab5-GTP form in axons. When Rab5 is activated, it recruits effector proteins such as EEA1, Rabex5 (Christoforidis et al, 1999) and phosphoinositide kinase class I PI3K (Goto-Silva et al., 2019). Next, SAND-1/Mon1 acts as a disruptor of the loop of activation induced by Rabex5 and promotes the recruitment of Rab7 to the endosome (Poteryaev et al, 2010). Then, WDR91, a Rab7 effector, inhibits PI3K activity on early-to-late endosomes (Liu et al., 2017), allowing the maturation and progression of signaling endosome pathway. All together, these data support a role of TrkB signaling in supporting the transition from early to late endosome for retrograde transport.

It is interesting that PI3K activity in axons does not play a role on retrograde transport of signaling endosomes. As we discussed above, a role of PI3K was anticipated due to the role of phosphoinositide accumulation in early endosomes regulating Rab5-Rab7 transition. This is

different to the findings in sympathetic neurons for TrkA signaling, where PI3K activity was required (Kuruvilla et al., 2000). Although, we found that LY294002 axonal treatment did not reduced BDNF retrograde transport, still is possible that PI3K is required for signaling endosome function since TrkB promotes Rab5 activation in axons and Rab5-GTP recruits the class I PI3K complex to early endosomes. Owing to, PI3K has a scaffolding protein activity, that it is not inhibited by LY294002 or wortmannin (Bilanges et al, 2019). For example, p110β is a subunit of class I PI3K scaffolding proteins (Hirsch et al, 2009) and its reduction impairs internalization of transferrin receptor and the epidermal growth factor receptor (EGFR). This phenotype is rescue by the overexpression of the kinase dead p110β subunit (Ciriaolo et al, 2008; Jia et al, 2008). Moreover, the inhibition of PI3K with wortmannin or LY294002 did not affect the proper retrograde transport of EGF receptor to the lysosomal compartment (Chen and Wang, 2000), suggesting that the regulation of TrkB trafficking could be more similar to EGFR retrograde transport than TrkA.

On the other hand, the increase on PI3K activity in the cell bodies was required for BDNF-long distance signaling. Indeed, when PI3K activity was reduced in the cell bodies, the increase in dendritic branching and activation of mTOR downstream pathways was completely abolished, suggesting that kinase activity of PI3K in the cell body is required for BDNF/TrkB signaling (Luikart et al, 2008). On the other hand, PI3K and Akt kinase activation promotes mTOR phosphorylation induced by BDNF. It has been shown, that TrkB endocytosis is required for PI3K/Akt signaling in neuronal cells (Zheng et al., 2008). In addition, in other cell model TORC1 is located in late endosomes where it activity is regulated by insulin signaling through PI3K activity (Flinn et al., 2010; Sancak et al., 2008; Marat et al, 2017). Here, we found that

BDNF axonal signaling promotes somatodendritic phosphorylation of 4E-BP1 and S6 ribosomal protein in a dynein dependent manner, suggesting that mTOR in cell bodies is activated by signaling endosomes.

Using Click-iT AHA chemistry, we showed that axonal BDNF promotes protein synthesis in the somatodendritic compartment. AHA incorporation is a method for pulse-labeling of new synthesized proteins, that allow to examine changes in protein distribution and turnover when coupled with proximity ligation assay (PLA) (Dieck et al., 2015). We observed that BDNF in axons was able to turn on protein synthesis in the cell bodies in a CREB and PI3K/mTOR dependent manner. These results were parallel by an increase in the protein levels of Arc. It is known that the *Arc* mRNA is anterograde transported to dendrites to be locally synthetized (Steward and Worley, 2002), suggesting that axonal signaling may increase local translation in dendrites. The fact that these effects were also dependent on CREB suggest that CREB-dependent transcript are specifically translated upon signaling endosome arrival to cell bodies.

Our study demonstrates that BDNF axonal signaling promotes an increase on protein synthesis in the somatodendritic compartment by increasing both, CREB and mTOR activation in an endosomal-dependent manner. Consequently, the arriving of active TrkB to the cell body promotes an increase in the dendritic arborization in a by the upregulation of CREB activity. It is possible that the retrograde transport of long-distance signaling endosome will have an impact of synaptic plasticity, since it has been shown in CNS neurons that BDNF in axons induces a long-distance persistent long-term potentiation on dendrites of retinotectal synapsis in *Xenopus* (Kang and Schuman, 1995; Du and Poo, 2004). Consistently, the activation of CREB has a key

role in the activity-dependent gene expression, playing a central role in learning and memory (Alonso et al., 2002; Egan et al., 2003). Therefore, our results suggest that BDNF signaling has a direct role on the long-distance regulation of synaptic plasticity and neuronal growth.

REFERENCES

Alonso, M., Vianna, M.R., Izquierdo, I., and Medina, J.H. (2002). Signaling mechanisms mediating BDNF modulation of memory formation in vivo in the hippocampus. Cell Mol Neurobiol *22*, 663-674.

Barford, K., Keeler, A., McMahon, L., McDaniel, K., Yap, C. C., Deppmann, C. D., and Winckler, B. (2018). Transcytosis of TrkA leads to diversification of dendritic signaling endosomes. Sci Rep *8*, 4715

Best, J.L., Amezcua, C.A., Mayr, B., Flechner, L., Murawsky, C.M., Emerson, B., Zor, T., Gardner, K.H., and Montminy, M. (2004). Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. Proc Natl Acad Sci U S A *101*, 17622-17627.

Bilanges, B., Posor, Y., and Vanhaesebroeck, B. (2019). PI3K isiforms in cell signalling and vesicle trafficking. Nat Rev Mol Cell Biol. doi: 10.1038/s41580-019-0129-z.

Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A., and Greenberg, M.E. (1999). Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science *286*, 1358-1362.

Bronfman, F.C., Lazo, O.M., Flores, C., and Escudero, C.A. (2014). Spatiotemporal intracellular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. Handb Exp Pharmacol *220*, 33-65.

Ciraolo, E., Iezzi, M., Marone, R., Marengo, S., Curcio, C., Costa, C., Azzolino, O., Gonella, C., Rubinetto, C., Wu, H., Dastrù, W., Martin, E.L., Silengo, L., Altruda, F., Turco, E., Lanzetti, L., Musiani, P., Rückle, T., Rommel, C., Backer, J.M., Forni, G., Wymann, M.P., and Hirsch, E. (2008). Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development. Sci Signal *1*, ra3.

Chen, X., Ye, H., Kuruvilla, R., Ramanan, N., Scangos, K.W., Zhang, C., Johnson, N.M., England, P.M., Shokat, K.M., and Ginty, D.D. (2005). A chemical-genetic approach to studying neurotrophin signaling. Neuron *46*, 13-21.

Chen, X., and Wang, Z. (2000). Regulation of intracellular trafficking of the EGF receptor by Rab5 in the absence of phosphatidylinosito 3-kinase activity. EMBO Rep *2*, 68-74.

Cheung, Z.H., Chin, W.H., Chen, Y., Ng, Y.P., and Ip, N.Y. (2007). Cdk5 is involved in BDNF-stimulated dendritic growth in hippocampal neurons. PLoS Biol *5*, e63.

Cho, Y., Sloutsky, R., Naegle, K., and Cavalli, V. (2013). Injury-induced HDAC5 nuclear export is essential for axon regeneration. Cell *155*, 894-908.

Christoforidis, S., McBride, H.M., Burgoyne, R.D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621-625.

Cohen, M.S., Bas Orth, C., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2011). Neurotrophin-mediated dendrite-to-nucleus signaling revealed by microfluidic compartmentalization of dendrites. Proc Natl Acad Sci U S A *108*, 11246-11251.

Cosker, K.E., and Segal, R.A. (2014). Neuronal signaling through endocytosis. Cold Spring Harb Perspect Biol *6*.

Debaisieux, S., Encheva, V., Chakravarty, P., Snijders, A.P., and Schiavo, G. (2016). Analysis of signaling endosome composition and dynamics using SILAC in embryonic stem cell-derived neurons. Mol Cell Proteomics *15*, 542–557.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., and Schiavo, G. (2006). Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron *52*, 293-305.

Dieterich, D.C., Hodas, J.J., Gouzer, G., Shadrin, I.Y., Ngo, J.T., Triller, A., Tirrell, D.A., and Schuman, E.M. (2010). In situ visualization and dynamics of newly synthesized proteins in rat hippocampal neurons. Nat Neurosci *13*, 897-905.

Dijkhuizen, P.A., and Ghosh, A. (2005). BDNF regulates primary dendrite formation in cortical neurons via the PI3-kinase and MAP kinase signaling pathways. J Neurobiol *62*, 278-288.

Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M., and Greenberg, M.E. (2001). Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science *294*, 333-339.

Du, J.L., and Poo, M.M. (2004). Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature *429*, 878-883

Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M., Lu, B., and Weinberger, D.R. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*, 257-269.

Escudero, C.A., Cabeza, C., Moya-Alvarado, G., Maloney, M.T., Flores, C.M., Wu, C., Court, F.A., Mobley, W.C., and Bronfman, F.C. (2019). c-Jun N-terminal kinase (JNK)-dependent internalization and Rab5-dependent endocytosis sorting mediate long-distance retrograde neuronal death induced by axonal BDNF-p75 signaling. Sci Rep *9*, 6070.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., and Greenberg, M.E. (1997). CREB: a major mediator of neuronal neurotrophin responses. Neuron *19*, 1031-1047.

Finsterwald, C., Fiumelli, H., Cardinaux, J.R., and Martin, J.L. (2010). Regulation of dendritic development by BDNF requires activation of CRTC1 by glutamate. J Biol Chem *285*, 28587-28595.

Flavell, S.W., and Greenberg, M.E. (2008). Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Annu. Rev. Neurosci *31*, 563–590.

Flinn, R.J., Yan, Y., Goswami, S., Parker, P.J., and Backer, J.M. (2010). The late endosome is essential for mTORC1 signaling. Mol Biol Cell *21*, 833-841.

Garza-Lombo, C., and Gonsebatt, M.E. (2016). Mammalian Target of Rapamycin: Its Role in Early Neural Development and in Adult and Aged Brain Function. Front Cell Neurosci *10*, 157.

Gluska, S., Chein, M., Rotem, N., Ionescu, A., and Perlson, E. (2016). Tracking quantum-dot labeled neurotrophic factors transport along primary neuronal axons in compartmental microfluidic chambers. Methods cell biol *131*, 365-387.

Goh, L.K., and Sorkin, A. (2013). Endocytosis of receptor tyrosine kinases. Cold Spring Harb Perspect Biol *5*, a017459.

Gomes, R.A., Hampton, C., El-Sabeawy, F., Sabo, S.L., and McAllister, A.K. (2006). The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. J Neurosci *26*, 11487-11500.

Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billaut, C., and Bronfman, F.C. (2016). Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor. Cytoskeleton (Hoboken) *73*, 612-628.

Gonzalez, A., Lazo, O.M., and Bronfman, F.C. (2019). The Rab5-Rab11 endosomal pathway is required for BDNF-induced CREB transcriptional regulation in neurons. J Neurosci (Submitted).

Gorski, J.A., Zeller, S.R., Tamowski, S., and Jones, K.R., (2003). Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. J Neurosci *23*, 6856-6865.

Goto-Silva, L., McShane, M.P., Salinas, S., Kalaidzidis, Y., Schiavo, G., and Zerial, M. (2019). Retrograde transport of Akt by a neuronal Rab5-APPL1 endosome. Sci Rep *9*, 2433.

Grimes, M.L., Zhou, J., Beattie, E.C., Yuen, E.C., Hall, D.E., Valletta, J.S., Topp, K.S., LaVail, J.H., Bunnett, N.W., and Mobley, W.C. (1996). Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J Neurosci *16*, 7950–7964.

Haihong, Y., Kuruvilla, R., Zweifel, L.S., and Ginty, D.D. (2003). Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron *39*, 57-68.

Harrington, A.W., and Ginty, D.D. (2013). Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci *14*, 177-187.

Hirokawa, N., Niwa, S., and Tanaka, Y. (2010). Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Neuron *68*, 610-638.

Hirsch, E., Braccini, L., Ciraolo, E., Morello, F., and Perino, A. (2009). Twice upon a time: PI3K's secret double life exposed. Trends Biochem. Sci *34*, 244–248.

Horch, H.W., Kruttgen, A., Portbury, S.D., and Katz, L.C. (1999). Destabilization of cortical dendrites and spines by BDNF. Neuron *23*, 353–364.

Horch, H.W., and Katz, L.C. (2002). BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat Neurosci *5*, 1177-1184.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem *72*, 609-642.

Ibanez, C.F., and Simi, A. (2012). p75 neurotrophin receptor signaling in nervous system injury and degeneration: paradox and opportunity. Trends Neurosci *35*, 431-440.

Jan, Y.N., and Jan, L.Y. (2010). Branching out: mechanisms of dendritic arborization. Nat Rev Neurosci *11*, 316-328.

Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S.H., Zhang, J., Signoretti, S., Loda, M., Roberts, T.M., and Zhao, J.J. (2008). Essential roles pf PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature *454*, 776-779.

Kang, H.J., and Schuman, E.M. (1995). Neurotrophin-induced modulation of synaptic transmission in the adult hippocampus. J Physiol Paris *89*, 11-22.

Kononenko, N.L., Classen, G.A., Kuijpers, M., Puchkov, D., Maritzen, T., Tempes, A., Malik, A.R., Skalecka, A., Bera, S., Jaworski, J., and Haucke, V. (2017). Retrograde transport of TrkB-containing autophagosomes via the adaptor AP-2 mediates neuronal complexity and prevents neurodegeneration. Nat Commun *8*, 14819.

Kraemer, B.R., Yoon, S.O., and Carter, B.D. (2014). The biological functions and signaling mechanisms of the p75 neurotrophin receptor. Handb Exp Pharmacol *220*, 121-164.

Kumar, V., Zhang, M.X., Swank, M.W., Kunz, J., and Wu, G.Y. (2005). Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci *25*, 11288-11299.

Kuruvilla, R., Ye, H., and Ginty, D.D. (2000). Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron *27*, 499-512.

Lazo, O.M., Gonzalez, A., Ascano, M., Kuruvilla, R., Couve, A., and Bronfman, F.C. (2013). BDNF regulates Rab11-mediated recycling endosome dynamics to induce dendritic branching. J Neurosci *33*, 6112-6122.

Leal, G., Comprido, D., and Duarte, C.B. (2014). BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology *76 Pt C*, 639-656.

Lee, K.F., Li, E., Huber, L.J., Landis, S.C., Sharpe, A.H., Chao, M.V., and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell *69*, 737-749.

Lehigh, K.M., West, K.M., and Ginty, D.D. (2017). Retrogradely Transported TrkA Endosomes Signal Locally within Dendrites to Maintain Sympathetic Neuron Synapses. Cell Rep *19*, 86-100.

Liu, K., Xing, R., Jian, Y., Gao, Z., Ma, X., Sun, X., Li, Y., Xu, M., Wang, X., Jing, Y., Guo, W., and Yang, C. (2017). WDR91 is a Rab7 effector required for neuronal development. J Cell Biol *216*, 3307-3321.

Liu, Q., Chang, J.W., Wang, J., Kang, S.A., Thoreen, C.C., Markhard, A., Hur, W., Zhang, J., Sim, T., Sabatini, D.M., and Gray, N.S. (2010). Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benz o[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. J Med Chem *53*, 7146-7155.

Lo, K.Y., Kuzmin, A., Unger, S.M., Petersen, J.D., and Silverman, M.A. (2011). KIF1A is the primary anterograde motor protein required for the axonal transport of dense-core vesicles in cultured hippocampal neurons. Neurosci Lett *491*, 168–173.

Lom, B., Cogen, J., Sanchez, A.L., Vu, T., and Cohen-Cory, S. (2002). Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci *22*, 7639-7649.

Lonze, B.E., and Ginty, D.D. (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron *35*, 605-623.

Lonze, B.E., Riccio, A., Cohen, S., and Ginty, D.D. (2002). Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron *34*, 371–385.

Luikart, B.W., Zhang, W., Wayman, G.A., Kwon, C.H., Westbrook, G.L., and Parada, L.F. (2008). Neurotrophin-dependent dendritic filopodial motility:A convergence on PI3K signaling. J Neurosci *28*, 7006-7012.

Lyu, J. W., Yuan, B., Cheng, T. L., Qiu, Z. L., and Zhou, W. H. (2016). Reciprocal regulation of autism-related genes MeCP2 and PTEN via microRNAs. Sci. Rep *6*, 20392

Marat, A., Wallroth, A., Lo, W.T., Müller, R., Norata, G.D., Falasca, M., Shultz, C., and Haucke, V. (2017). mTORC1 activity repression by late endosomal phosphatidylinositol 3,4-bisphosphate. Science *356*, 968-972.

Matsuda, N., Lu, H., Fukata, Y., Noritake, J., Gao, H., Mukherjee, S., Nemoto, T., Fukata, M., and Poo, M.M. (2009). Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J Neurosci *29*, 14185-14198.

Mengchen, Y., Lehigh, K.M., and Ginty, D.D. (2018). Multivesicular bodies mediate long-range retrograde NGF-TrkA signaling. eLife *7*, pii:e33012.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron *36*, 121-137.

Mitchell, D.J., Blasier, K.R., Jeffery, E.D., Ross, M.W., Pullikuth, A.K., Suo, D., Park, J., Smiley, W.R., Lo, K.W., Shabanowitz, J., Deppmann, C.D., Trinidad, J.C., Hunt, D.F., Catling, A.D., and Pfister, K.K. (2012). Trk activation of the ERK1/2 kinase pathway stimulates intermediate chain phosphorylation and recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport. J Neurosci *32*, 15495-15510.

Moya-Alvarado, G., Gonzalez, A., Stuardo, N., and Bronfman, F.C. (2018). Brain-Derived Neurotrophic Factor (BDNF) Regulates Rab5-Positive Early Endosomes in Hippocampal Neurons to Induce Dendritic Branching. Front Cell Neurosci *12*, 493.

Olenick, M.A., Dominguez, R., and Holzbaur, E.L.F. (2019). Dynein activator Hook1 is required for trafficking of BDNF-signaling endosomes in neurons. J Cell Biol *218*, 220-233.

Perlson, E., Hanz, S., Ben-Yaakov, K., Segal-Ruder, Y., Seger, R., and Fainzilber, M. (2005). Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron 45, 715-726

Ravindran, S., Nalavadi, V.C., and Muddashetty, R.S. (2019). BDNF Induced Translation of Limk1 in Developing Neurons Regulates Dendrite Growth by Fine-Tuning Cofilin1 Activity. Front Mol Neurosci *12*, 64.

Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999-1010.

Riccio, A., Pierchala, B.A., Ciarallo, C.L., and Ginty, D.D. (1997). An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science *277*, 1097–1100.

Sainath, R., and Gallo, G. (2015). The dynein inhibitor Ciliobrevin D inhibits the bidirectional transport of organelles along sensory axons and impairs NGF-mediated regulation of growth cones and axon branches. Dev Neurobiol *75*, 757-777.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496-1501.

Satoh, D., Sato, D., Tsuyama, T., Saito, M., Ohkura, H., Rolls, M. M., Ishikawa, F., and Uemura, T. (2008). Spatial control of branching within dendritic arbors by dynein-dependent transport of Rab5-endosomes. Nat Cell Biol *10*, 1164–1171.

Schink, K.O., Tan, K.W., and Stenmark, H. (2016). Phosphoinositides in Control of Membrane Dynamics. Annu Rev Cell Dev Biol *32*, 143-171.

Schratt, G.M., Nigh, E.A., Chen, W.G., Hu, L., and Greenberg, M.E. (2004). BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. J Neurosci *24*, 7366-7377.

Scott-Solomon, E., and Kuruvilla, R. (2018). Mechanisms of neurotrophin trafficking via Trk receptors. Mol Cell Neurosci *91*, 25-33.

Shelton, D.L., Sutherland, J., Gripp, J., Camerato, T., Armanini, M.P., Phillips, H.S., Carroll, K., Spencer, S.D., and Levinson, A.D. (1995). Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci *15*, 477-491.

Shimada, A., Mason, C.A., and Morrison, M.E. (1998). TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. J Neurosci *18*, 8559-8570.

Sholl, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. J Anat *87*, 387-406.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol *10*, 513-525.

Steward, O., and Worley, P. (2002). Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? Neurobiol Learn Mem *78*, 508-527.

Stuardo N., Moya-Alvarado, G., and Bronfman, F.C. (2019), Optimized in vitro mono-biotinylated BDNF production after semiquantitative purification from HEK293 cells supernatant for axonal trafficking studies.In preparation

Sutton, M., and Schuman, E.M., (2006). Dendritic protein synthesis, synaptic plasticity, and memory. Cell *127*, 49-58.

Takei, N., Inamura, N., Kawamura, M., Namba, H., Hara, K., Yonezawa, K., and Nawa, H. (2004). Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent

local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci *24*, 9760-9769.

Tapley, P., Lamballe, F., and Barbacid, M. (1992). K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. Oncogene *7*, 371-381.

Taylor, A.M., Rhee, S.W., Tu, C.H., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2003). Microfluidic Multicompartment Device for Neuroscience Research. Langmuir *19*, 1551-1556.

Terenzio, M., Schiavo, G., and Fainzilber, M. (2017). Compartmentalized signaling in neurons: from cell biology to neuroscience. Neuron *96*, 667–679.

tom Dieck, S., Kochen, L., Hanus, C., Heumuller, M., Bartnik, I., Nassim-Assir, B., Merk, K., Mosler, T., Garg, S., Bunse, S., Tirrel, D.A., and Shuman, E.M. (2015). Direct visualization of newly synthesized target proteins in situ. Nat Methods *12*, 411-414.

Villarroel-Campos, D., Schiavo, G., and Lazo, O. M. (2018). The many disguises of the signalling endosome. FEBS letters *592*, 3615–3632.

Wandinger-Ness, A., and Zerial, M. (2014). Rab proteins and the compartmentalization of the endosomal system. Cold Spring Harb Perspect Biol *6*, a022616.

Wang, H., Xu, J., Lazarovici, P., Quirion, R., and Zheng, W. (2018). cAMP response element-binding protein (CREB): a possible signaling molecule link in the pathophysiology of schizophrenia. Front Mol Neurosci *11*, 255.

Watson, F.L., Heerssen, H.M., Moheban, D.B., Lin, M.Z., Sauvageot, C.M., Bhattacharyya, A., Pomeroy, S.L., and Segal, R.A. (1999). Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. J Neurosci *19*, 7889-7900.

Wong, R.O., and Ghosh, A., (2002). Activity-dependent regulation of dendritic growth and patterning. Nat Rev Neurosci *3*, 803-812.

Wu, K.Y., He, M., Hou, Q.Q., Sheng, A.L., Yuan, L., Liu, F., Liu, W.W., Li, G., Jiang, X.Y., and Luo, Z.G. (2014). Semaphorin 3A activates the guanosine triphosphatase Rab5 to promote growth cone collapse and organize callosal axon projections. Sci Signal *7*, ra81.

Xie, W., Zhang, K., and Cui, B. (2012). Functional characterization and axonal transport of quantum dot labeled BDNF. Integr Biol (Camb) *4*, 953-960.

Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A., and Greenberg, M.E. (1998). Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol *18*, 1946-1955.

Xu, B., Zang, K., Ruff, N.L., Zhang, Y.A., McConnell, S.K., Stryker, M.P., and Reichardt, L.F. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron *26*, 233-245.

Yamashita, N., Joshi, R., Zhang, S., Zhang, Z.Y., and Kuruvilla, R. (2017). Phospho-Regulation of Soma-to-Axon Transcytosis of Neurotrophin Receptors. Dev Cell *42*, 626-639 e625.

Yan, Q., Rosenfeld, R.D., Matheson, C.R., Hawkins, N., Lopez, O.T., Bennett, L., and Welcher, A.A. (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience *78*, 431-448.

Yap, E.L., and Greenberg, M.E. (2018). Activity-regulated transcription: Bridging the gap between neural activity and behavior. Neuron *100*, 330-348.

Ye, H., Kuruvilla, R., Zweifel, L.S., and Ginty, D.D. (2003). Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron *39*, 57-68.

Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., and Bramham, C.R. (2002). Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J Neurosci *22*, 1532-1540.

Zagrebelsky, M., Holz, A., Dechant, G., Barde, Y.A., Bonhoeffer, T., and Korte, M. (2005). The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons. J Neurosci *25*, 9989-9999.

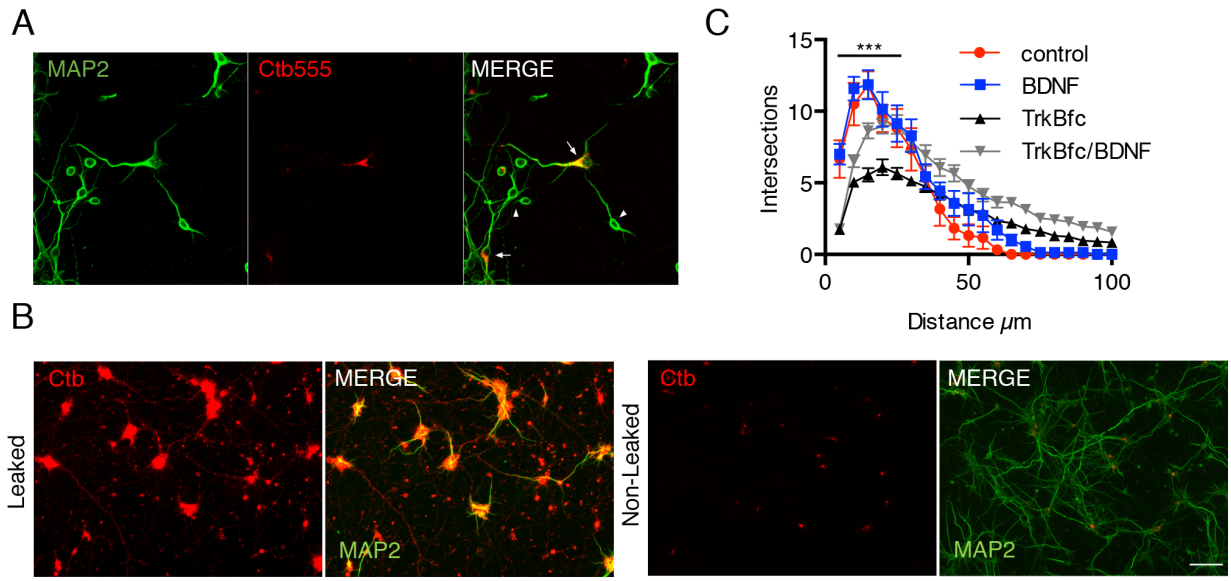
Zheng, J., Shen, W.H., Lu, T.J., Zhou, Y., Chen, Q., Wang, Z., Xiang, T., Zhu, Y.C., Zhang, C., Duan, S., and Xiong, Z.Q. (2008). Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth. J Biol Chem *283*, 13280-13288.

Zhou, B., Cai, Q., Xie, Y., and Sheng, Z.H. (2012). Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Rep *2*, 42-51.

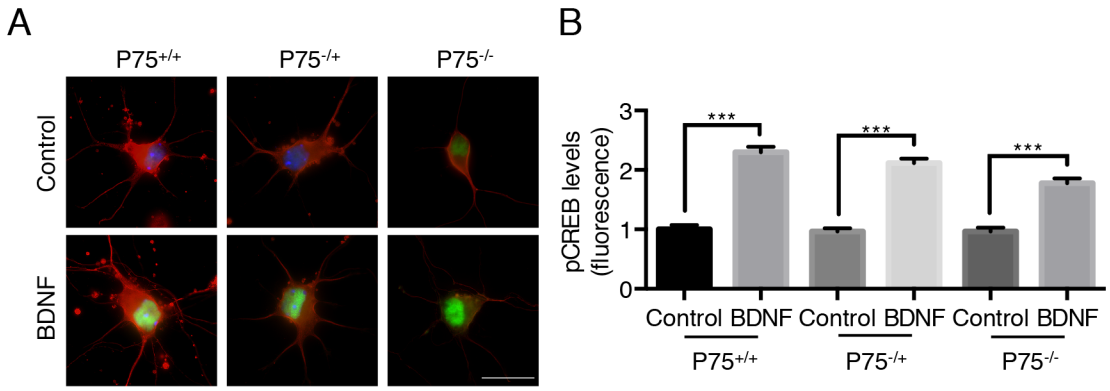
Zhou, P., Porcionatto, M., Pilapil, M., Chen, Y., Choi, Y., Tolias, K.F., Bikoff, J.B., Hong, E.J., Greenberg, M.E., and Segal, R.A. (2007). Polarized signaling endosomes coordinate BDNF-induced chemotaxis of cerebellar precursors. Neuron *55*, 53-68.

Zhou, W. J., Xu, N., Kong, L., Sun, S. C., Xu, X. F., Jia, M. Z., Wang, Y., and Chen, Z.Y. (2016). The antidepressant roles of Wnt2 and Wnt3 in stress-induced depression-like behaviors. Transl. Psychiatry *6*, e892.

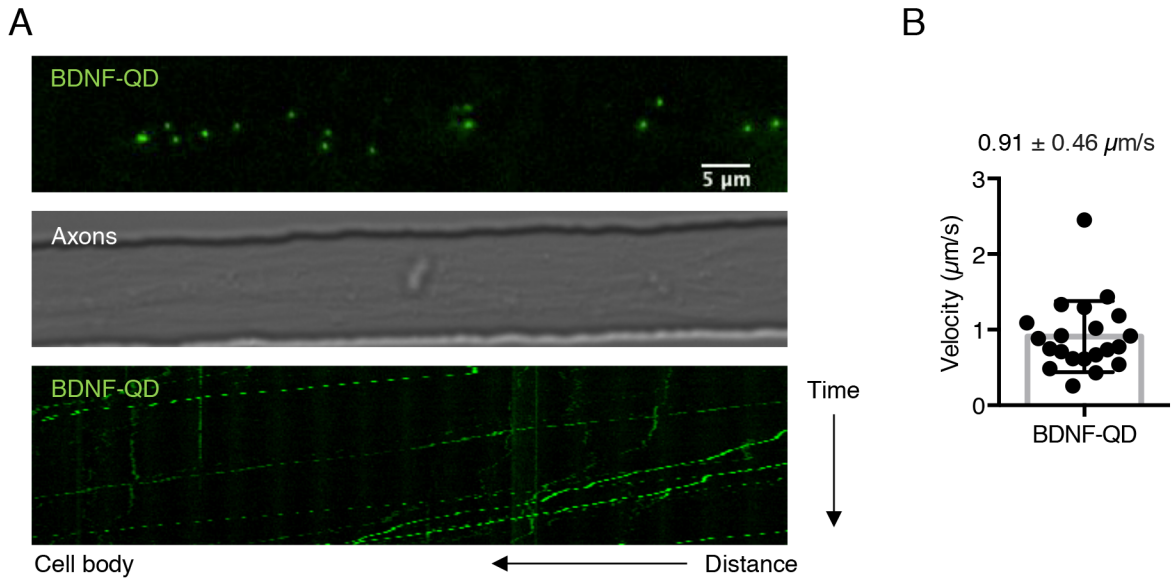
SUPPLEMENTARY FIGURES



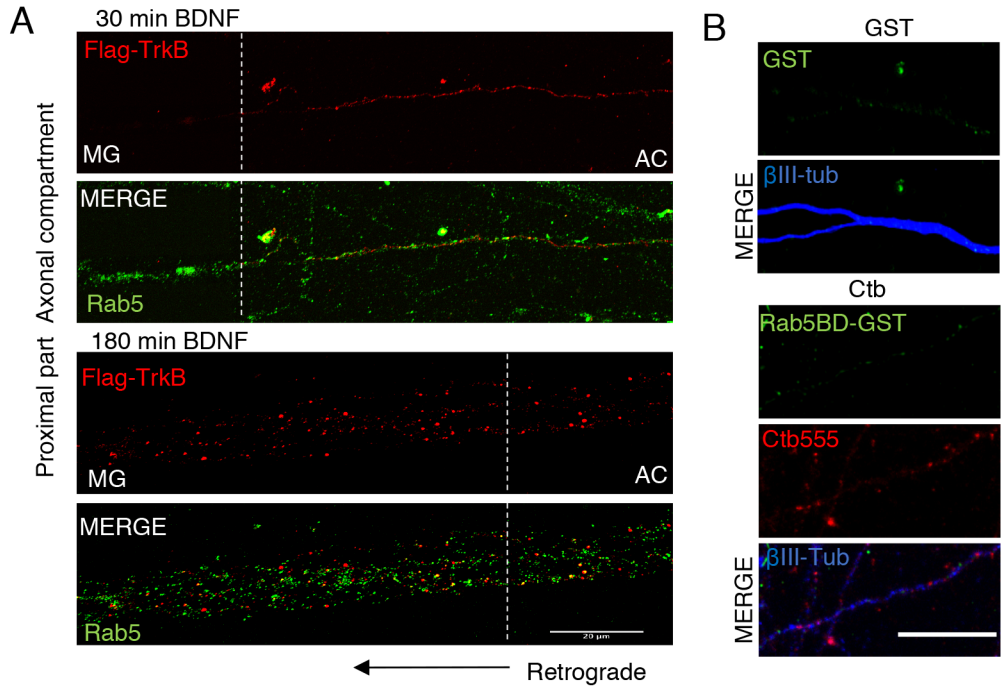
Supplementary Figure 1. Experimental design to study BDNF retrograde signaling in compartmentalized cultures of cortical neurons. (A) Representative images of compartmentalized cultures incubated with Ctb555 in the axonal compartment for 48 hrs. Neurons were immunostained with Map2 (in green). Arrow shows neurons that projected their axons to the AC. Arrowhead shows neurons that their axon is not projecting to the AC and therefore are not labelled with Ctb555. **(B)** Representative image of microfluidic chamber that are not well-compartmentalized (leaked, left panel) or that compartmentalization has been achieve (non-leaked, right panel). In red, Ctb555. In green, Map2. Scale bar 50 µm. **(C)** Sholl analysis of dendritic arborization of rat cortical neurons stained with Ctb555 from compartmentalized. The CB of chambers was incubated with or without TrkB-Fc (100 ng/mL), and the AC was treated with or without BDNF (50 ng/mL) during 48 hours. Results are expressed as ± SEM. $P<0.001$, from 2 independent experiments. Statistical comparison of the Sholls analysis were done by two-way ANOVA with Bonferroni post-hoc test for multiple comparisons.



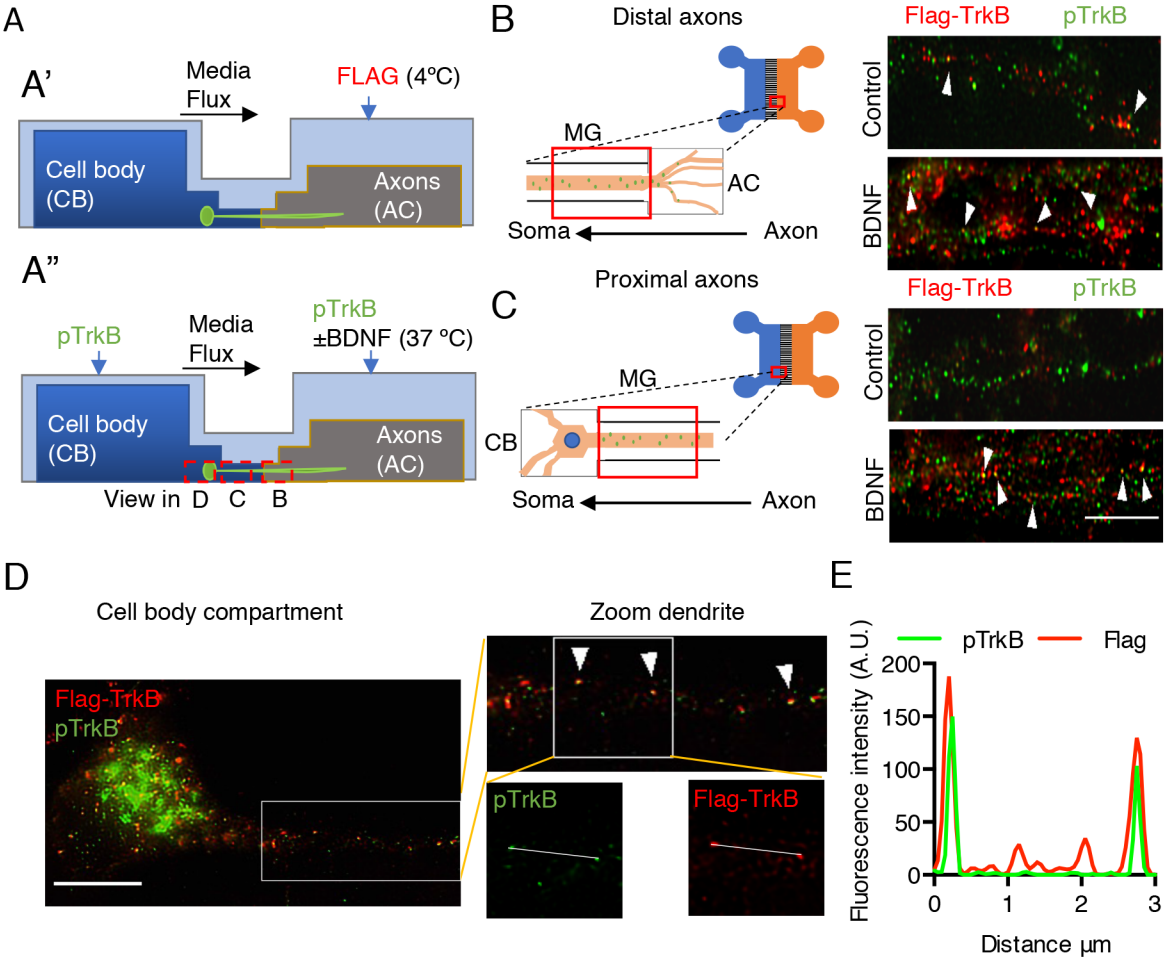
Supplementary Figure 2. p75 is not required for CREB activation induced by BDNF in cortical neurons. Seven DIV cortical neurons of $p75^{+/+}$, $p75^{+/-}$ or $p75^{-/-}$ mice were treated with BDNF during 30 min, washed and fix for immunofluoresce against phosphorylated CREB (pCREB, S133) as indicated in methods. **(A)** Representative image of pCREB (in green) and βIII-Tubulin (in red) of neurons stimulated with BDNF in each genotype. **(B)** Quantification of pCREB fluorescence intensity in the nucleus of neurons derived from the 3 different p75 genotypes and with or without BDNF treatment. n= 30 neurons from 2 independent experiment. Results are expressed as ± SEM. ***p< 0.01. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons.



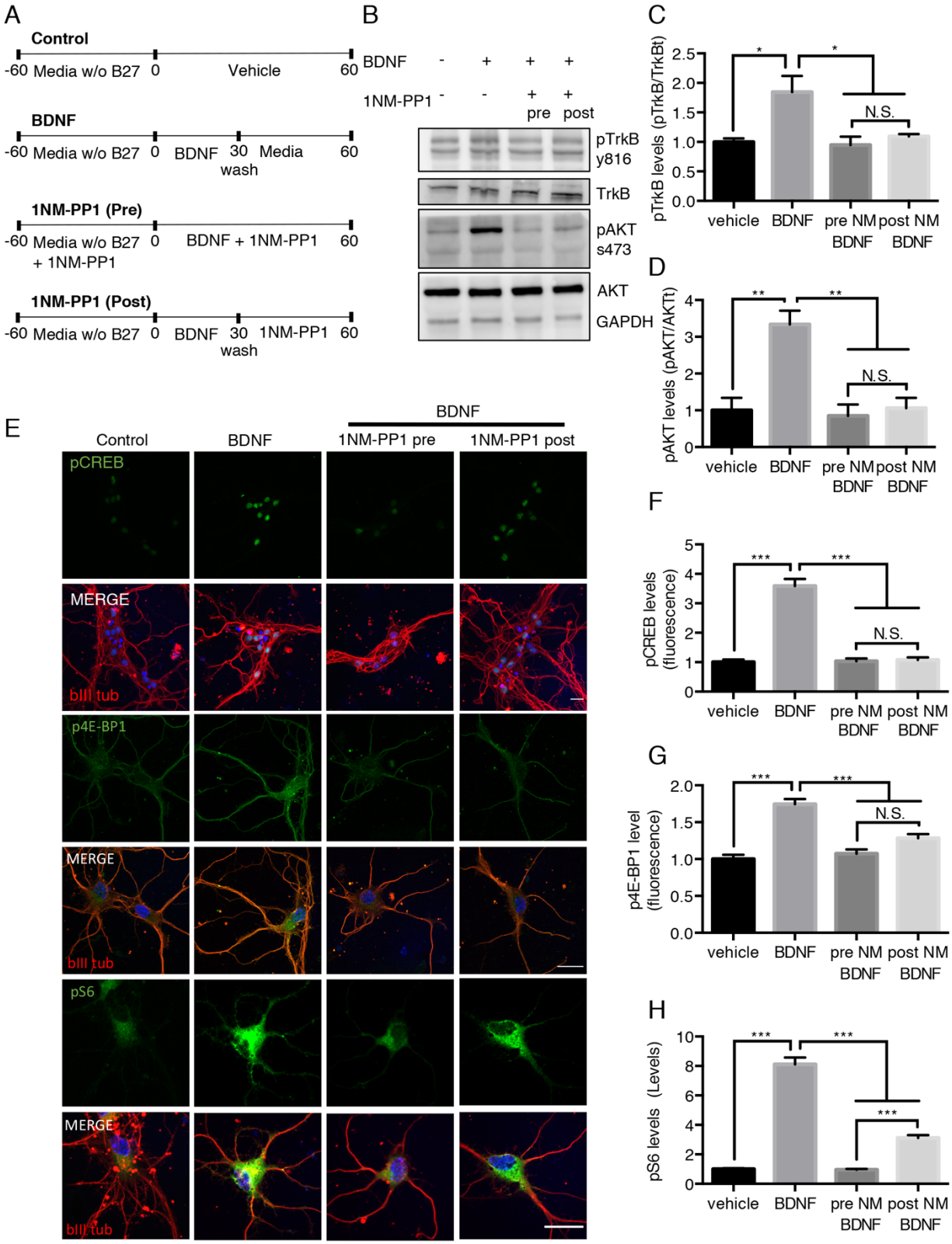
Supplementary Figure 3. BDNF-QD is retrogradely transported in axons of cortical neurons. (A) Cortical neurons in microfluidic chambers were incubated in the axonal compartment with 2 nM of BDNF-QD (Green dots). After 4 hours, the microgrooves proximal compartment was video recorded during 2 minutes. Differential interference contrast image of axons. Representative kymographs of QD-BDNF transport in cortical neurons. **(B)** Quantification of the velocity of BDNF-QD obtained from in vivo recording of microgrooves.



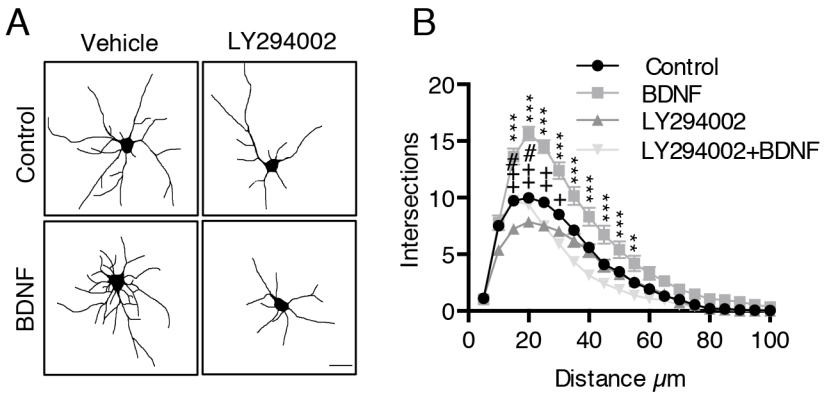
Supplementary Figure 4. Standardization of TrkB labeling with anti-Flag antibodies and control of the methodology to measure *in situ* Rab5-GTP levels using the Rab5BD-GST protein. (A) Representative image of Flag-TrkB (Red) and endogenous Rab5 (Green) co-localization in the axonal compartment and microgroove compartment of neurons stimulated with BDNF (50 ng/mL) during 30 or 180 minutes. Images show both the microgroove compartment (MG) and the axonal compartment (AC). Scale bar 20 µm. **(B)** Representative image of GST staining of cortical neurons in non-stimulated conditions (left panel) and Rab5BD-GST staining of cortical neurons stimulated with Ctb555 in the axonal compartment (right panel).


A
A'
Media Flux
FLAG (4°C)
Cell body (CB)
Axons (AC)
A"
pTrkB
Media Flux
pTrkB ±BDNF (37 °C)
Cell body (CB)
Axons (AC)
View in D C B
B
Distal axons
MG
AC
Soma
Axon
C
Proximal axons
MG
CB
Soma
Axon
Flag-TrkB
pTrkB
Control
BDNF
Flag-TrkB
pTrkB
Control
BDNF
D
Cell body compartment
Flag-TrkB
pTrkB
Zoom dendrite
pTrkB
Flag-TrkB
E
pTrkB
Flag
Fluorescence intensity (A.U.)
200
150
100
50
0
0
1
2
3
Distance µm

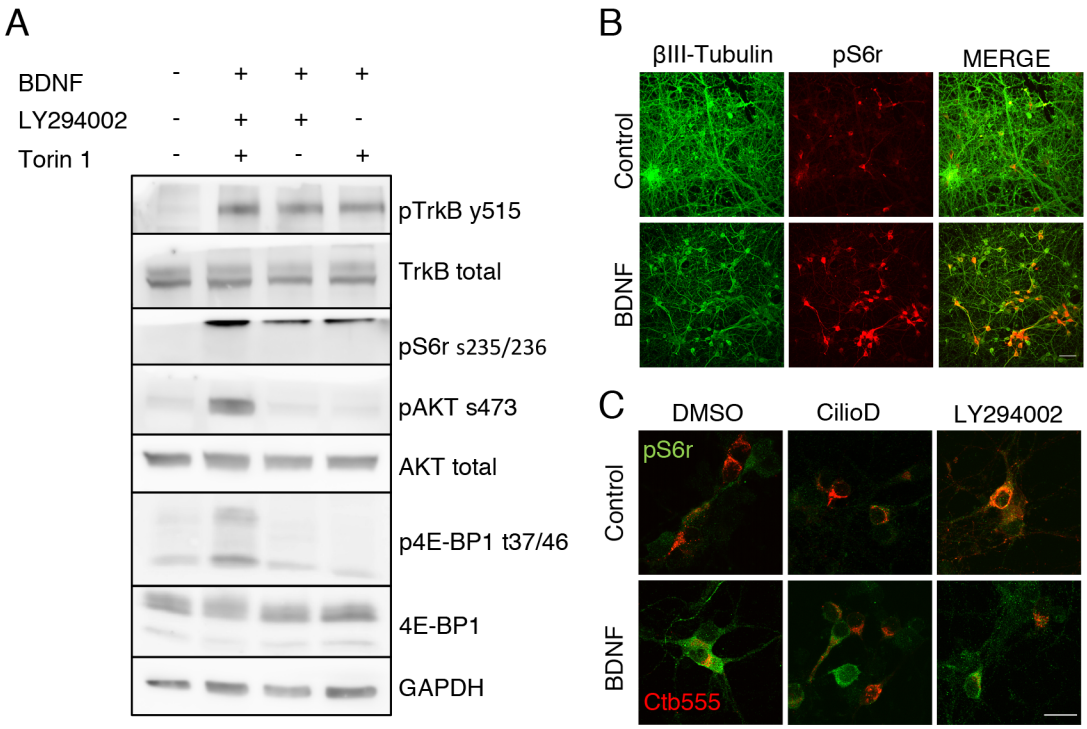
Supplementary Figure 5. Endosomal TrkB is active in the cell body after axonal stimulation with BDNF. (A) Schematic representation of the protocol used to label axonal TrkB. Five DIV compartmentalized cortical neurons were transfected with Flag-TrkB for 48 hrs. (A') At DIV seven, the axonal compartment was incubated with anti-Flag antibodies for 40 min at 4 ºC. (A'') Then, neurons were incubated at 37°C in the presence and absence of BDNF (50 ng/mL). Finally, immunofluorescence was performed against phosphorylated TrkB (Y816, pTrkB) (in green) and anti-Flag (in red). **(B and C)** The schemes showed in the left panels allows the identification of the localization of the axonal immunostainings showed in the right panels. **(B)** Immunofluorescence of pTrkB and internalized TrkB in distal axons in the absence (upper panel) or presence of BDNF (50 ng/mL) (lower panel). **(C)** Immunofluorescence of pTrkB and internalized Flag-TrkB in proximal axons in the absence (upper panel) or presence (lower panel) of BDNF. **(D)** Immunofluorescence of pTrkB and internalized Flag-TrkB in compartmentalized neurons treated with BDNF in the axons as shown in A. Representative images of neuron in BDNF condition in cell body compartment. In the right panel, a zoom of a proximal dendrite of the neurons shown in the left panel. The arrows indicate the co-colocalization of Flag (in red) and pTrkB (in green). Scale bar 5 μm. **(E)** Graphs showing the fluorescence intensity profile pixel by pixel on the white lines shown in the right panels in D. Green line indicates the fluorescent profile of the pTrkB-associated fluorescence and the red lines indicate the fluorescent profile of the Flag-TrkB associated fluorescence.


A
Control
-60 Media w/o B27 0 Vehicle 60
BDNF
-60 Media w/o B27 0 BDNF 30 Media wash 60
1NM-PP1 (Pre)
-60 Media w/o B27 0 BDNF + 1NM-PP1 60
+ 1NM-PP1
1NM-PP1 (Post)
-60 Media w/o B27 0 BDNF 30 1NM-PP1 60
wash
B
BDNF - + + +
1NM-PP1 - - + +
pre post
pTrkB y816
TrkB
pAKT s473
AKT
GAPDH
C
pTrkB levels (pTrkB/TrkBt)
vehicle BDNF pre NM BDNF post NM BDNF
N.S.
D
pAKT levels (pAKT/AKTt)
N.S.
E
Control
BDNF
BDNF
1NM-PP1 pre
1NM-PP1 post
pCREB
MERGE
bIII tub
p4E-BP1
pS6
F
pCREB levels (fluorescence)
G
p4E-BP1 level (fluorescence)
H
pS6 levels (Levels)

Supplementary Figure 6. 1NM-PP1 is able to reduce TrkB activation in TrkB^{F616A} cortical neurons. (A) Diagrams of the experimental designs used for stimulating neurons in non-compartmentalized cultures. 7 DIV cortical neurons were depleted from media during 1 hour in the presence or absence of 1NM-PP1 (1 μM). Then neurons were treated as follow: control: vehicle 1 hour. BDNF: incubation of BDNF (50 ng/mL) during 30 minutes, next were rinse twice and incubated with vehicle. 1NM-PP1 (Pre): incubation with BDNF in the presence of 1NM-PP1 during 1 hour. 1NM-PP1 (Post): incubation with BDNF during 30 minutes, next were rinse twice and incubated with 1NM-PP1 during 30 minutes. **(B)** Immunoblot of pTrkB (Y816), TrkB, pAkt (S437), Akt and GAPDH from cortical neurons stimulated as indicated in A. **(C)** Quantification of pTrkB levels normalizated with total TrkB. **(D)** Quantification of pAkt levels normalizated with total Akt. n=3 independent experiments. **(E)** Representative image of cortical neurons treated as indicated in A. In green is shown pCREB, p4E-BP1 or pS6, in red is shown bIII tubulin. **(F)** Quantification of fluorescence (arbitrary units, A.U) of pCREB in the nucleus of neurons. **(G)** Quantification of fluorescence (arbitrary units, A.U) of p4E-BP1 in the primary dendrites. **(H)** Quantification of fluorescence (arbitrary units, A.U) of pS6 in primary dendrites. n=49-72 neurons from 3 independent experiments. Results are expressed as ± SEM. *p< 0.05; **p<0.01; ***p< 0.001. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons.



Supplementary Figure 7. The PI3K inhibitor LY294002 reduced BDNF-induced dendritic arborization in non-compartmentalized cortical neurons (A) Representative images of rat cortical neurons from non-compartmentalized cultures treated with vehicle, LY294002 (10 μM), BDNF (50 ng/mL) or BDNF and preincubated with LY294002. **(B)** Quantification of total dendritic branching by Sholl analysis. n=25-32 from 2 independent experiment. **p<0.01; ***p<0.001 control vs BDNF; ++p<0.01 control vs LY294002. Results are expressed as ± SEM. Statistical comparison of the Sholl analysis was done by two-way ANOVA with Bonferroni post-hoc test for multiple comparisons.



Supplementary figure 8. Axonal BDNF promotes somatodendritic S6 ribosomal protein phosphorylation in compartmentalized cultures. Western blot of 7 DIV non-compartimentalized neurons treated with BDNF (50 ng/mL) in the presence or absence of LY294002 (10 µM) or Torin 1 (0.25 µM) during 1 hour phospho TrkB (pTrkB, Y515), TrkB, phosphor Akt (pAkt, S473), Akt total, phospho S6 ribosomal protein (pS6r, S235/236), phospho 4E-BP1 (p4E-BP1, T37/46), 4E-BP1 total and GAPDH was evaluated. **(B)** Representative images of β-III-tubulin (in green) and pS6r (in red) of cortical neurons stimulated from the axonal compartment with BDNF (50 ng/mL) during 180 min. Scale bar, 50 µm. **(C)** Evaluation of pS6r (in green) in the somatodendritic compartment of neurons stimulated with BDNF (50 ng/mL) for 180 min, in the presence or absence of Ciliobrevin D (Cilio D, 20 µM) in the AC or LY294002 (10 µM) in the CB. Scale bar 20 µm. Results from 2 independents experiments.

A

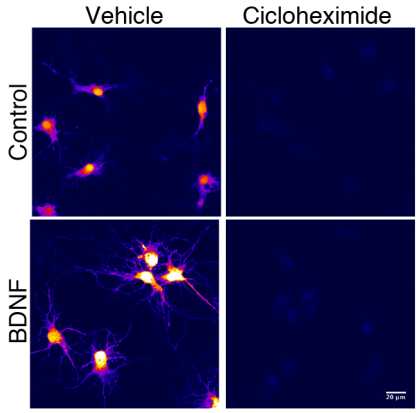


Figure 9. Evaluation of *de novo* protein synthesis by Click-IT AHA of neurons stimulated with BDNF. (A) Representative image 7 DIV cortical neurons in non-compartmentalized culture incubated with DMEM without methionine for 1 hr. Then, media was changed for DMEM with AHA (0.1 mM) in the presence of BDNF (50 ng/mL) and preincubated or not with cicloheximide 10 µM.

CHAPTER III

In this chapter, we approached General aim 2 and the following specific aim:

2.5 To study the role PLCγ in BDNF long-distance signaling.

Axonal PLCγ activity is required for BDNF long-distance signaling

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ABSTRACT

We have shown that long-distance signaling induced by the activation of axonal TrkB by brain-derived neurotrophic factor (BDNF) increases dendritic arborization in a CREB-dependent manner. The binding of BDNF by TrkB triggers the activation of different signaling pathways, including ERK1/2, PLCγ and PI3K/mTOR, to induce dendritic growth and synaptic plasticity. How TrkB down-streams pathways regulates long-distance signaling is unclear. Here, we studied the role of PLCγ-Ca^{+2} in BDNF/TrkB-induced long-distance signaling using compartmentalized cultures. We showed that dendritic branching and CREB phosphorylation, induced by axonal BDNF stimulation, required the activation of the PLCγ in the axons of cortical neurons. Also, BDNF axonal stimulation increased Ca^{+2} waives and both PLCγ and Ca^{+2} were required for TrkB endocytosis, suggesting that axonal PLCγ activation is required for the generation of signaling endosomes.

INTRODUCTION

Neurotrophins are extracellular cues that regulate multiples aspects of neuronal development, such us cell survival, synapse maturation and neuronal growth (Huang and Reichardt, 2003). Brain-derived neurotrophic factor (BDNF) with it receptors TrkB and p75 are mayor regulators of dendritic branching in cortical and hippocampal neurons (Cheung et al., 2007; Horch and Katz, 2002; Xu et al., 2000). Upon binding to TrkB, BDNF activates three main downstream signaling molecules, including phospholipase C gamma (PLCγ) MAP kinase (MAPK) and phosphoinositide 3-kinase (PI3K) (Reichardt, 2006). Neurons have a polarized morphology; thus, dendrites and axons must be properly communicated with the cell body to maintain neuronal homeostasis. TrkB is located both in somatodendritic and axonal compartment of neurons (Arimura et al., 2009) and BDNF is released in dendrites and axons (Matsuda et al., 2009), regulating dendritic and axonal growth (Gonzalez et al., 2016; Huang and Reichardt, 2003), synaptic transmission (Kang and Schuman, 1995; Zhang et al., 2013) and learning and memory (Alonso et al., 2002a; Alonso et al., 2002b; Egan et al., 2003). It has been reported that axonal extracellular application of BDNF triggers the increase of intracellular Ca^{+2} in a PLCγ-dependent manner (Li et al., 2005). BDNF promotes the release of intracellular Ca^{+2} storages by activation of the inositol 3,4,5-triphophate receptor (IP3R) and also the entry of extracellular Ca^{+2} by the activation of the transient receptor-potential cation channel subfamily C (TRPC) (Leal et al., 2015; Li et al., 2005). Also, it has been shown that calcium influx induces dendritic growth via activation of transcriptional program that involves activation of Calmodulin kinase IV (CAMKIV) and the activation of cAMP response element-binding protein (CREB) transcription factor (Redmond et al., 2002).

In cortical and hippocampal neurons, the binding of BDNF to TrkB promotes its dimerization and autophosphorylation, inducing the internalization of the receptor into signaling endosomes (Bronfman et al., 2014; Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018). We previously showed that BDNF axonal signaling promotes dendritic arborization in a TrkB-dependent manner (Moya-Alvarado et al., 2019 in preparation, Chapter II). TrkB is endocytosed in axons and retrogradely transported to the cell body by the molecular motor dynein. In the cell body compartment, activated TrkB upregulates the CREB and PI3K/mTOR activity increasing the transcription and translation of proteins (Moya-Alvarado in preparation 2019, Chapter II). Although, long-distance signaling of BDNF has been described in cortical neurons (Cohen et al., 2011; Zhou et al., 2012), there is little information of the cellular mechanism and the downstream signaling pathways regulating this process. Several lines of evidence support the participation of the PLCγ and increased intracellular Ca^{+2} in long-distance signaling mediated by BDNF. It was reported in hippocampal neurons that the internalization of BDNF/TrkB complex is regulated by an increase in Ca^{+2} influx through N-methyl-d-aspartate receptors (NMDAR) (Du et al., 2003). Furthermore, the axonal stimulations of retinal ganglionic cells (RCG) with BDNF promotes a retrograde potentiation of retinal synapses in a TrkB and PLCγ dependent-manner (Du and Poo, 2004), suggesting that PLCγ can participate in BDNF long-distance signaling regulating TrkB internalization. Interestingly, in the axons of sympathetic neurons, TrkA via PLCγ regulates the activation of calcineurin to dephosphorylate dynamin 1 coordinating the receptor endocytosis and axonal growth (Bodmer et al., 2011). Suggesting that the regulation of Trks endocytosis by PLCγ and calcium is a conserved process among different

type of neurons.

Here, we show that BDNF long-distance signaling depend on the axonal activity of PLCγ. Using compartmentalized cultures of cortical neurons, we show that axonal PLCγ activity in axons is required for dendritic arborization and CREB phosphorylation in cell bodies. Consistently with other works, BDNF increased the axonal Ca^{+2} levels in axons in a PLCγ dependent manner (Du and Poo, 2004). We showed that the PLCγ/Ca^{+2} pathway is required for TrkB endocytosis, suggesting that axonal PLCγ can regulate the generation of signaling endosomes for retrograde BDNF/TrkB signaling.

METHODOLOGY

Primary culture of cortical neurons. Embryonic cortical neurons from rats (embryonic days 17–19) were obtained from the animal facilities of the Pontificia Universidad Catolica de Chile and euthanatized under deep anesthesia according to the bioethical protocols of our institution. Cortex were dissected and dissociated into single cells in HBSS (Hank´s Balance Salt Solution; Thermo-Fisher, cat# 14025134). After disaggregation, neurons were resuspended in MEM/Horse Serum (Thermo-Fisher, cat# 16050122) and were seeded on microfluidic chambers at a low density (40-50 x 10^3 cells/chamber) or in mass culture 25 x 10^3 cells/well in 12 mm coverslips or 1.8 x 10^6 cell/ 60 mm plate. After 4 h, the culture medium was replaced with neurobasal medium (Thermo-Fisher, cat# 21103049) supplemented with 2% B27 (Life Technologies, cat# 17504044), 1x glutamax (Thermo-Fisher, cat# 35-050-061) and 1x pen/strep (Thermo-Fisher, cat# 15140-122). Proliferation of non-neuronal cells was limited by the use of cytosine arabinoside (0.25 µg/mL AraC; Sigma-Aldrich, cat# C1768) when MEM/HS was replaced to neurobasal(Gonzalez et al, 2019; Taylor et al., 2003).

Microfluidic devices. The molds to prepare the compartmentalized chambers was provided by Prof Eran Perlson from Tel Aviv University (Gluska et al, 2016). The microfluidic chambers were prepared with syldgard^{TM} 184 silicone elastomer base (Poirot, cat# 4019862), according to the manufacturer's instructions. Two days before performing primary cultures, glass coverslips (25 mm) were incubated with poly-D-lysine (0.1 mg/mL, Corning, cat# 354210). The next day, poly-D-lysine was washed and a microfluidic chamber with 400 µm microgroove was place on the coverslip. Then, inside of the chamber laminin (2 µg/mL in water, Invitrogen, cat#

23017015) was added. The same day that the primary culture was performed the laminin solution was changed to DMEM/HS media (Dulbecco Minimum Essential Medium supplemented with 10% horse serum, 1x glutamax and 1x antibiotic/antimycotic, Thermo-Fisher, cat# 15240062).

Quantification of dendritic arborization induced by BDNF. Cortical neurons (6 DIV) were transfected with 0.5 ug of the plasmid containing EGFP (CA, USA) using 0.8 uL of lipofectamin 2000 (Invitrogen, cat#11668-019) in 30 uL of optimem (Thermo-Fisher, cat# 11058021). After 2 hours, the optimem media was replaced for neurobasal media supplemented for 1 hour. The cell body compartment was incubated with neurobasal medium supplemented with TrkB-Fc (100 ng/mL, B&D system, 688TK) for all treatments. The drugs were incubated in the cell body compartment or axonal compartment as follow. In the axonal compartment: U73122 (5 μM, Sigma-Aldrich cat# U6756). After 1 hour, BDNF (50 ng/mL, Alomone, cat# B-250) was added to the axonal compartment together with fluorescent subunit B of choleratoxin (Ctb, 1 μg/mL, Thermo-Fisher, cat# C34777). After 48 hours, neurons were washed with PBS 37ºC and then, fixed with fresh 4 % PFA-PBS at 37ºC for 15 minutes (Paraformaldehyde (Sigma-Aldrich, cat# 158127) in PBS). Then the chamber was removed and the neurons were permeabilized and blocked with 5% BSA (Jackson, cat# 001-000-162) and 0.5% Triton X-100 (Sigma-Aldrich, cat# 234729) in PBS and then incubated with anti-MAP2 (1:500, Merk-Millipore, cat# AB5622) in incubation solution (3% BSA with 0.1% Triton X-100 in PBS). After washes (3x buffer), neurons were treated with a donkey anti-mouse Alexa 647 (1:500, Molecular probes, cat# A-31571) in incubation solution and mounted for fluorescence microscopy visualization using Mowiol 4-88 (Calbiochem, cat# 475904).

Dendritic arborization was analyzed in cortical neurons labelled with Ctb and labelled for EGFP and MAP2. Primary dendrites, branching points and Sholl's analysis (Sholl, 1953) were quantified (see below). For visualization, confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C was used. Five to seven optical section of 0,5 µm thick from the whole cells were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis. Z-stacks were integrated, and the images were segmented to obtain binary images. Ten concentric circles with increasing diameters (5 µm each step) were traced around the cell body, and the number of intersections between dendrites and circles was counted and plotted for each diameter. Analysis was performed using the ImageJ plugin developed by the Anirvan Gosh Laboratory (http://biology.ucsd. edu/labs/ghosh/software). The number of total primary dendrites and branching points of all dendrites was manually counted from the segmented images.

Treatments and immunofluorescence for phospho-proteins. To evaluate the accumulation of phosphorylated CREB (pCREB) in the nucleus, and the phosphorylation of PLCγ (pPLCγ) in axons, cortical neurons (5-6DIV) were incubated with Ctb555 (Ctb, 1 µg/mL) overnight. Next, cortical neurons (6-7 DIV) were incubated with neurobasal medium with TrkB-Fc (100 ng/mL) in the cell body compartment. To inhibit the PLCγ we used the drug U73122 (5 µM) in the axons or cell bodies depending on the experimental design. After 1 hour, BDNF (50 ng/ml) was added to the axonal compartment together with Ctb647 (1 µg/mL, Thermo-Fisher, cat# C34778). After 20 min (pPLCγ immunofluorescence) or 180 minutes (pCREB immunofluorescence) samples were fixed with 4 % PFA with phosphatase inhibitor (1x) for 15

min. Permeabilization and blocking was done in the presence of bovine serum albumin (BSA, 5 %) Triton X100 (0.5%) for 1 hour in PBS. The antibodies were incubated overnight at 4 ºC in the presence of BSA (3%), Triton X100 (0.1%) in PBS, the antibodies used were: Anti-phospho CREB (1:500, Cell signaling cat# 9198), anti-phospho PLCγ1 (pY783.28, 1:200, Santa Cruz cat# sc-136186). The secondary antibodies were incubated for 90 minutes in BSA (3%), Triton X100 (0.1%) in PBS. Finally, the samples were incubated with Hoechst 33342 (5 μg/mL, Invitrogen, cat# 62249) and mounted in Mowiol 4-88. Neurons were visualized by confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Five to seven optical section of 0,5 μm thick from the whole cells were acquired using a 60x objective at 1024x1024 pixel resolution along the z-axis.

Immunoendocytosis of Flag-TrkB to evaluate the effect of PLCγ activity in the internalization of TrkB. Cortical neurons (5DIV) were transfected with 0.5 ug of the Flag-TrkB plasmid (gift from Prof. Francis Lee, NYU, USA) using 0.8 uL of lipofectamin 2000 in 30 uL opti-men per chamber. After 48 hours, cortical neurons (7 DIV) were incubated at 4ºC for 10 minutes and then treated with an anti-Flag antibody (1:750, Sigma-Aldrich, cat#F3040) for 45 minutes, in the presence or absence of U73122 (5 μM) or BAPTA-AM (20 μM). Cortical neurons were washed briefly with warm neurobasal and were incubated with BDNF (50 ng/mL) in the presence or absence of U73122 or BAPTA-AM for 20 minutes. Then, neurons were fixed in 4 % PFA and samples were incubated with a donkey anti mouse Alexa488 (1:500, Invitrogen, cat# R-37114) without permeabilization. Finally, samples were blocked and permeabilized as described above and immunostained with donkey anti mouse Alexa555 (1:500, Invitrogen, cat#

A-31570). As a control, 20 min internalization of Ctb555 was evaluated in the presence or absence of U73122 (5 μM).

Measurement of intracelular Ca^{+2}. The changes of intracellular calcium concentration were examined by Fluo4-AM staining. Neurons were incubated with Fluo4-AM (2 μM, Invitrogen cat# F14201) in Neurobasal medium for 30 min at 37 °C, followed by incubation for another 15 min after rinsing with Hanks' balanced saline solution. Ca^{+2} imaging was performed by confocal microscopy using a Nikon Eclipse C2 confocal microscope equipped with a digital camera connected to a computer with Software NIS-Elements C. Cells were excited with a laser at 488 nm, and the intensity of the fluorescence was collected at 525 nm as the Fluo4-AM signal. The images were collected before and after BDNF treatment (30 seconds after initial video-recording), and the fluorescent intensity was analyzed with ImageJ. To measure the calcium in the axonal terminals in the presence of U73122 (5 μM), neurons were preincubated in the axonal terminal with U73122 after Fluo4-AM loading.

Western blot analyses. To study phosphorylated dynamin 1 protein levels, neurons were depleted with neurobasal media in the presence or absence of U73122 (5 μM), then cortical neurons were stimulated with BDNF (50 ng/mL) for 15 minutes. Next, cells were lysed with RIPA buffer (0.1 % SDS, 0.5 % NP40, 10 mM Tris-HCl (pH7.5), 1 mM EDTA, 150 mM NaCl, 0.5 % deoxicolic acid) containing protease and phosphatase inhibitors. Standard SDS gel electrophoresis and Western blotting procedures were used to analyze the cell extracts using anti-phosphorylates dynamin 1 (S795 (F-11; 1:500), Santa Cruz cat#(F-11) sc-377568), anti-dynamin1 (E-11, 1:500, Santa Cruz cat# sc-17807) and anti-phosphorylated PLCγ1 (pY783.27, 1:1000) and GAPDH (6C5, 1:1000, Santa Cruz cat#sc-32233) antibodies.

Statistical analysis. The results are expressed as the average ± standard error of the mean (SEM). Sholl's analysis curves were compared with two-way repeated measures ANOVA, followed by Bonferroni's multiple comparisons. Moreover, Student's t-test or one-way ANOVA followed by appropriated multiple comparisons test was performed depending on the number of groups used in each experiment. Details about specific test used, level of significance and number of replicates are indicated in each figure legends. Statistical analyses were performed using GraphPad Prism 7 (Scientific Software).

RESULTS

Axonal PLCγ regulates BDNF/TrkB long-distance signaling in cortical neurons.

Previously, we have described that axonal stimulation with BDNF promotes dendritic arborization in a CREB-dependent manner. Interestingly, when characterizing the TrkB downstream signaling pathways, we found that PI3K activity is required in the cell body but not in axons to induce dendritic arborization and mTOR activation (Moya-Alvarado in preparation 2019, Chapter II). One downstream pathway involved in TrkB signaling in axons is PLCγ in RCG and sympathetic neurons (Bodmer et al., 2011; Du and Poo, 2004). Moreover, PLCγ is described to have a role on neurite outgrowth in sensory neurons (Sciarretta et al., 2010). Using an *in-vitro* model of compartmentalized cultures of cortical neurons we evaluated the change in the dendritic morphology induced by axonal stimulation of BDNF and we asked whether the presence, in the axonal compartment of one pharmacological inhibitor of PLCγ, U73122 (Bleasdale et al., 1990), affect the retrograde signaling of BDNF, using the protocol described in our previously work (Moya-Alvarado et al., 2019 in preparation, Chapter II). In brief, we expressed EGFP in six DIV cortical neurons seeded in microfluidic devices of 400 μm long-microgrooves. We incubated TrkB-Fc chimera protein (Shelton et al., 1995) in the cell bodies compartment (CB) to neutralize the activity of endogenous BDNF released by neurons. To identify the neurons that projected their axons to the axonal compartment (AC), we have used a fluorescent version of the subunit B of the cholera toxin (Ctb) (Escudero et al, 2019), which it is internalized in axons and retrogradely transported up to the Golgi apparatus of neurons that

have axons in the AC (Fig. 1A). To identify the somatodendritic domain of neurons an MAP2 immunofluorescence was performed. As we observed previously, BDNF, added to axons increased the arborization (Fig. 1B and C), primary dendrites (Fig. 1D) and branching points (Fig. 1E) (Moya-Alvarado et al., 2019 in preparation, Chapter II**)**. However, the presence of U73122 in the axonal compartment prevent the effect induced by BDNF (Fig. 1), suggesting that PLCγ is required for long-distance axonal signaling of BDNF.

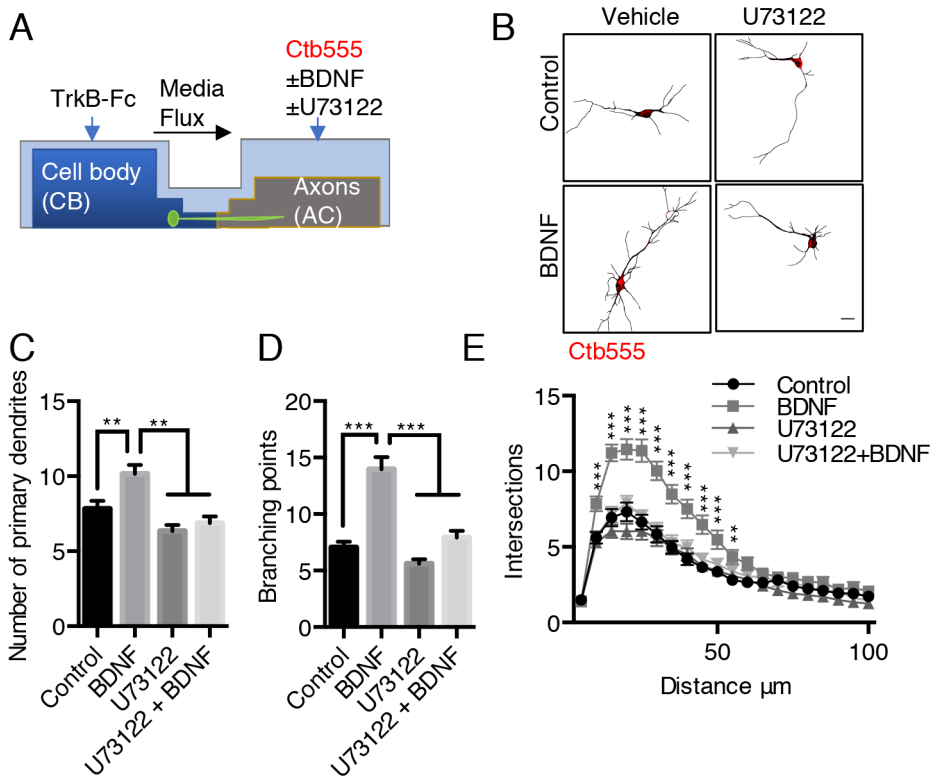

Figure 1. Axonal PLCγ activity is required for dendritic arborization induced by BDNF. **(A)** Six DIV cortical neurons were transfected with a plasmid driving the expression of EGFP. The CB was incubated with TrkB-Fc (100 ng/mL). The AC was stimulated with BDNF (50 ng/mL) in addition to Ctb555 in presence or absence of U73122 (5 µM). Treatments were performed for 48 hours. Finally, neurons were fixed and immunofluorescence was performed against MAP2. **(B)** Examples of neurons in the CB (Ctb555 in red) from compartmentalized cultures treated as follow; control situation, treated only with U73122 in AC, stimulated with BDNF, stimulated with BDNF and treated with U73122 in AC. **(C-E)** Quantification of primary dendrites **(C)**, branching points **(D),** Sholl analysis **(E)**, from neurons labelled with EGFP/MAP2/Ctb555 for each treatment Scale bar 20 µm. n= 27-38 neurons from 3 independent experiment. Results are expressed as ± SEM. **p< 0.01, ***p< 0.01. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons (C-D). Statistics of the Sholl analysis was done by two-way ANOVA followed by Bonferroni post-test for multiple comparisons.

We have described that axonal stimulation with BDNF promotes CREB phosphorylation, and in addition, the activity of CREB is required for dendritic arborization (Moya-Alvarado et al., 2019 in preparation, Chapter II; Bronfman et al., 2014). Furthermore, the mutation in the PLCγ docking site of TrkB impairs the phosphorylation of CREB and CaMKIV (Minichiello et al., 2002), suggesting that the activity of PLCγ is required for CREB phosphorylation. These results were obtained in non-compartmentalized cultures. Therefore, we tested whether the activity of PLCγ was required both in the cell body or axonal compartment in our model. In order to evaluate CREB phosphorylation, at five DIV we incubated Ctb555 in the axonal compartment overnight, to identify all the neurons that projected their axons. At the next day, we added BDNF with Ctb647 in the axonal compartment in the presence or absence of U73122 in the axonal or in the cell body compartment. As we previously reported, axonal signaling of BDNF induced an increase of CREB phosphorylation (Fig. 2) (Moya-Alvarado et al., 2019 in preparation, Chapter II**)**. Interestingly, the presence of PLCγ inhibitor decreases CREB activation only when added to the axonal compartment (Fig. 2A and B), having no effect when added in the cell body compartment (Fig. 2C and D), suggesting that PLCγ has a role mainly in BDNF/TrkB axonal signaling.

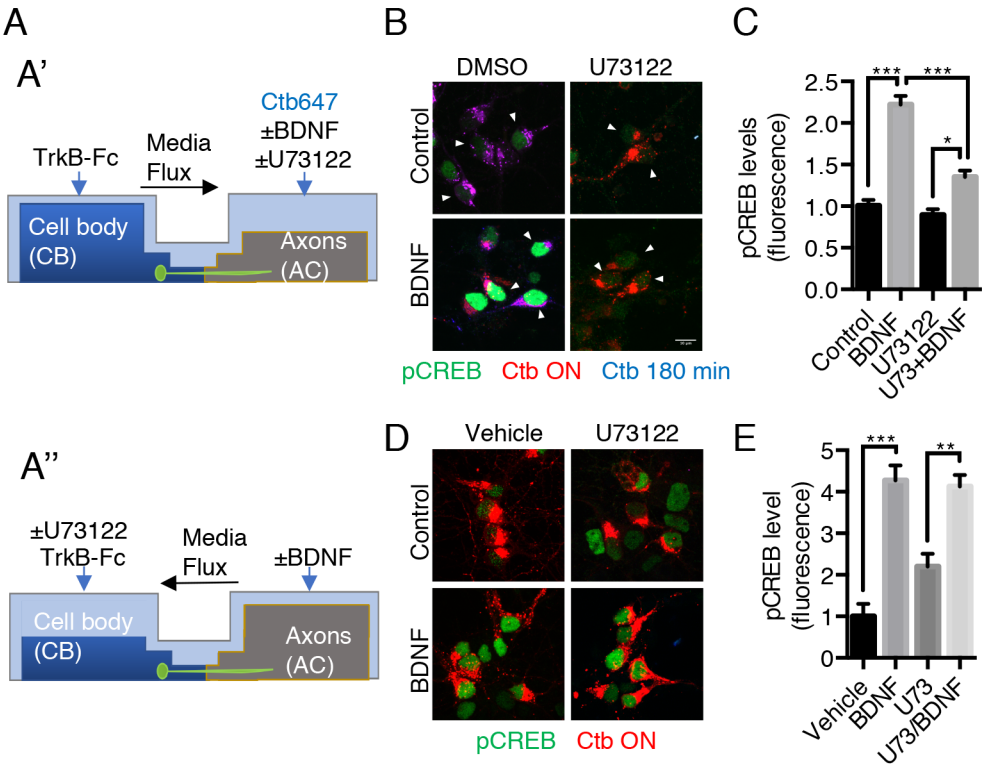

Figure 2. Axonal PLCγ activity is required for somatodendritic CREB phosphorylation. **(A)** Schematic representation of the protocol used for stimulating neurons. For both protocols Five DIV cortical neurons were retrograde labeled with Ctb555 (in red) overnight. (A') At six DIV, the culture medium was changed to serum-free medium for 90 minutes in the presence or absence of U73122 (5 µM) in the AC, and stimulated with BDNF (50 ng/mL) for 180 minutes along with Ctb647 (in blue) in the AC, in the presence or absence of U73122 with the flux toward the AC. (A'') At six DIV, the culture medium was changed to serum-free medium for 90 minutes in the presence or absence of U73122 (5 µM) in the CB with the flux toward the CB, then AC was incubated with BDNF (50 ng/mL) for 180 minutes. Finally, the cultures were fixed and phosphorylated CREB (pCREB, S133) immunofluorescence was performed in cell bodies (B) Representative figures of nuclear pCREB in neurons with or without BDNF stimulation labelled with Ctb647 (in blue) and Ctb555 (in red) added to axons in the presence or absence of axonal U73122. Scale bar 10 µm. **(B)** Quantification of pCREB in the nucleus of neurons labeled with Ctb555 (red) in each condition. n= 90-114 neurons from 3 independent experiment. **(C)** Representative figures of nuclear pCREB (in green) in neurons with or without BDNF stimulation labelled with Ctb555 (in red) added to axons in the presence or absence of U73122 in CB. **(D)** Quantification of pCREB in the nucleus of neurons labeled with Ctb555 in each condition. n= 43-60 neurons from 2 independent experiment. Results are expressed as ± SEM. **p< 0.01, ***p< 0.01. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons.

PLCγ is locally activated in the axonal compartment by BDNF.

To test whether BDNF was activating PLCγ in axons, we treated axons of cortical neurons with BDNF for 15 minutes and assessed the phosphorylation of PLCγ by immunofluorescence. Immunofluorescence analyses with a phospho-specific antibody reveal that BDNF increases the PLCγ phosphorylation in axons of cortical neurons (Fig. 3A and B). It is known that activation of PLCγ by TrkB leads to an increase in intracellular Ca^{+2} (Zirrgiebel et al., 1995). To assess whether BDNF increases Ca^{+2} in axons in a PLCγ dependent manner, we loaded the neurons with Fluo-4 AM, a cell permeant calcium indicator (Cheng et al., 2017) and we video recorded Fluo-4 AM fluorescence in the axons during 1 minute after BDNF addition. The incubation of BDNF to axons produced a single Ca^{+2} release point that generate an extended retrograde signal that cover completely the axons in the recording zone (Fig. 3C). Of note, not all the axons responded at the same time, or with the same intensity. Nevertheless, the presence of U73122 completely abolished the increase in the calcium release induced by BDNF in axons (Fig. 3C and E). We measured the velocity of the retrograde signal considering the initial point of calcium increase until the last point observable in the video recording, the velocity measured was 4.47±0.15 μm/s (Fig. 3D), showing a faster movement compared to dynein-dependent TrkB/BDNF retrograde trafficking (Xie et al., 2012; Goto-Silva et al., 2019; Moya-Alvarado et al., 2019 in preparation, Chapter II)).

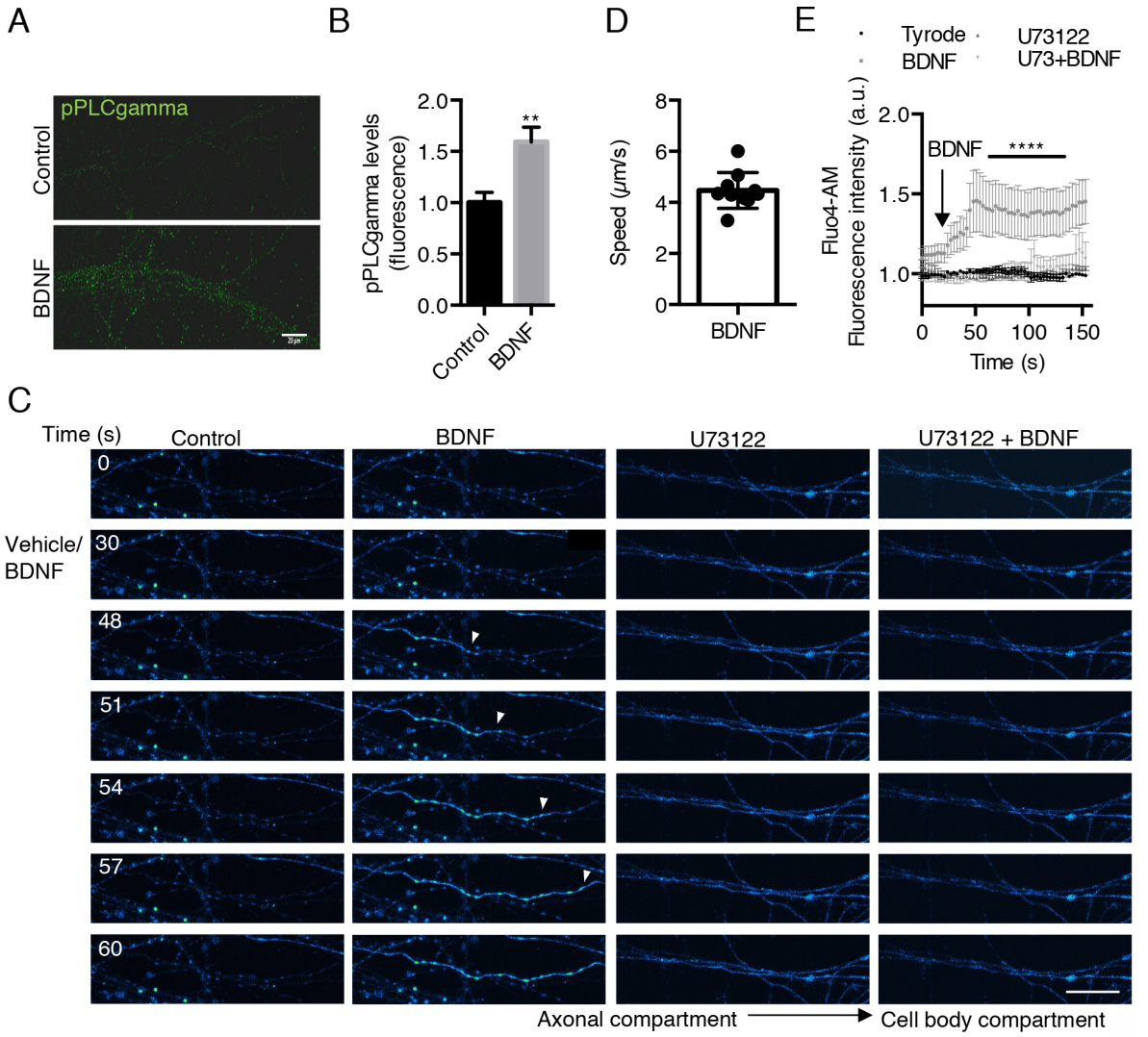
A
pPLCgamma
Control
BDNF
B
pPLCgamma levels (fluorescence)
2.0
1.5
1.0
0.5
0.0
**
Control
BDNF
D
Speed (μm/s)
8
6
4
2
0
BDNF
E
Tyrode
BDNF
U73122
U73+BDNF
Fluo4-AM Fluorescence intensity (a.u.)
2.0
1.5
1.0
BDNF
****
0
50
100
150
Time (s)
C
Time (s)
Control
BDNF
U73122
U73122 + BDNF
0
Vehicle/ BDNF
30
48
51
54
57
60
Axonal compartment
Cell body compartment

Figure 3. Axonal BDNF promotes intracellular calcium increase in a PLCγ-dependent manner. (A) Representative figure of phosphorylated PLCγ (pPLCy, pY783.28) in axonal compartment of neurons stimulated with BDNF (50 ng/mL) for 20 min. **(B)** Quantification of the immunofluorescence associated with pPLCγ each 30 μm in axons of neurons stimulated with BDNF. Results are expressed as ± SEM. **p< 0.01. Statistics was done by Student's *t*-test. **(C)** Evaluation of calcium signaling induced by BDNF. The change of fluorescence intensity associated to Fluo4-AM (2 μM) was used to measure the concentration change of cytosolic calcium. Representative images of compartmentalized cultures loaded with Fluo4-AM in the axonal compartment treated with vehicle or BDNF (50 ng/mL) in the axonal compartment with or without U73122 (5 μM) pre-treatment in the axonal compartment. Live-cell imaging of each axonal field in the axonal compartment recorded before BDNF treatment (0 seconds) and during 60 seconds of BDNF treatment. Scale bar, 10 μm. **(D)** Quantification of the velocity of calcium back propagation of the fluorescence signal associated with Fluo4-AM in BDNF condition. **(E)** Mean Fluo4-AM fluorescence intensity (± SEM) for each treatment at different snap-shot times. Normalization and statistics for each treatment was done using their corresponding mean '0 second' basal fluorescence as a reference. n = 8-12 axons from three independent compartmentalized cultures per each treatment in 2 independent experiments. Statistics was done by two-way ANOVA test followed by Bonferroni's post-test for multiple comparisons. **** P<0.0001.

PLCγ regulates TrkB internalization.

Neurotrophin binding to Trks results in the internalization of the receptor-ligand complex into vesicles, which are transported to neuronal cell bodies to propagate retrograde signals (Beattie et al., 1996; Bronfman et al., 2014). It has been shown that PLCγ regulates both the epidermal growth factor receptor (EGFR) (Delos Santos et al., 2017) and TrkA internalization in a calcium dependent manner (Bodmer et al., 2011). Furthermore, TrkB internalization is regulated by neuronal activity and calcium influx (Du et al., 2003). Suggesting that PLCγ is involved in the endocytosis of TrkB. To test that hypothesis, we transfected cortical neurons with a plasmid that drives the expression of a TrkB receptor amino-tagged with a Flag epitope (Flag-TrkB). Forty-eight hours later, we treated the neurons with a Flag antibody at 4ºC. Next, we stimulated

neurons with BDNF in the presence or absence of PLCγ inhibitor (Fig. 4A). BDNF treatment, leads to a robust internalization of the TrkB receptors (eFlag-TrkB) (Fig. 4B and C) and the levels of the internalized receptor were reduced (53% decrease) when PLCγ activity was inhibited by U73122 (Fig. 4B and C). U73122 did not have an effect on the levels of TrkB on the plasma membrane (mFlag-TrkB) (Fig. 4D), nor in the internalization of Ctb555 (Fig. 4E). We also evaluated whether a Ca^{+2} chelator BAPTA-AM could affect the internalization of TrkB. As we expected the presence of BAPTA-AM, decreases the internalization of TrkB (Fig. 4F). In sympathetic neurons, TrkA internalization is regulated by dynamin-1 dephosphorylation (Bodmer et al., 2011). To evaluate if BDNF regulates dynamin dephosphorylation, we incubated cortical neurons with BDNF during 15 minutes and evaluated by Western blot the phosphorylation of dynamin-1 and PLCγ. Interestingly, we observed that BDNF stimulation did not affected the phosphorylation of dynamin-1 (Fig. 4G), suggesting that PLCγ regulates TrkB internalization in a Ca^{+2} dependent manner but independent on dynamin 1 dephosphorylation.

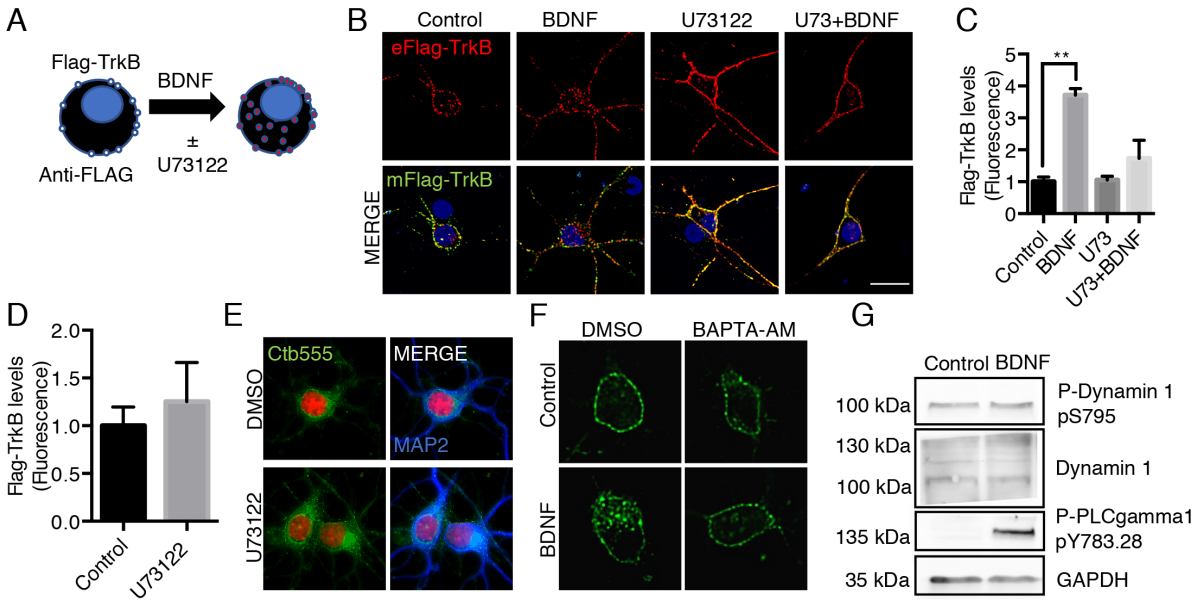

Figure 4. PLCγ activity is required for TrkB internalization. (A) Schematic representation of immunoendocytosis of Flag-TrkB. Five DIV neurons were transfect with a plasmid driving the expression of Flag-TrkB. After 48 hours, neurons were incubated with anti-Flag antibody. Then, neurons were treated with BDNF (50 ng/mL) in the presence or absence of U73122 (5 µM) or BAPTA-AM (20 µM) for 20 min to induce the endocytosis at 37 ºC. Finally, neurons were fixed and the Flag epitope was detected by immunostaining. **(B)** Representative image of the endocytosis of Flag-TrkB in control condition, BDNF, U73122 and BDNF with U73122. Upper figure shows endocytosed Flag-TrkB (eFlag-TrkB, in red), lower images shows merge with membrane Flag-TrkB (mFlag-TrkB, in green). **(C)** Quantification of fluorescence intensity of intracellular Flag-TrkB- in each treatment. Scale bar, 20 µm **(D)** Quantification of the plasma membrane associated fluorescence of TrkB in control condition and in the presence or absence of U73122. n= 5-10 neurons from 2 independent experiment. Results are expressed as ± SEM. **p< 0.01. Statistics was done by one-way ANOVA test followed by Bonferroni post-test for multiple comparisons. **(E)** Representative image of Ctb555 endocytosis in the presence or absence of U73122. In green, Ctb555, in blue, Map2 and in red, Hoechst. **(F)** Representative images of TrkB-Flag endocytosis in control condition, BDNF, BAPTA-AM and BDNF with BAPTA-AM. Image representative from 2 independent experiments. **(G)** Representative image of a Western blot of neurons treated with BDNF during 15 min. Immunobloting of the proteins, phosphorylated dynamin (p-Dynamin 1, S795), dynamin, pPLCy 1 (Y793.28) and GAPDH.

DISCUSSION

We have described that BDNF long-distance signaling from axons increases CREB and mTOR activation inducing dendritic arborization (Moya-Alvarado et al., 2019 in preparation, Chapter II). However, the downstream signaling pathways involved in this process are poorly understood. Here, we show that axonal PLCγ activity is required for dendritic arborization and CREB phosphorylation induced by BDNF axonal signaling. Furthermore, the local axonal activation of PLCγ regulates intracellular Ca^{+2} increase and TrkB endocytosis.

We have previously described that somatodendritic, but not axonal signaling of PI3K is required for BDNF axonal signaling (Moya-Alvarado et al., 2019 in preparation, Chapter II). Here, we showed that axonal activity of PLCγ is required for dendritic arborization induced by BDNF in axons. Interestingly, an *in vivo* experiment with retinal ganglion cells (RCG) of the *Xenopus laevis* optic tectum, showed that tectal stimulation with BDNF promotes a potentiation of retinotectal synapses in a retrograde dependent manner. This process was dependent on the activity of TrkB and PLCγ, suggesting that there are synaptic modification that may be causally linked to structural modification of RGC dendrites after hours of BDNF stimulation in the axons (Du and Poo, 2004; Lom et al., 2002). In our model, due to the days in vitro that cortical neurons were (6-8), they have not developed synaptic contacts; therefore, we did not evaluate whether axonal BDNF increases synapses in dendrites by axonal stimulation. However, in future experiment it will be important to evaluate whether BDNF axonal signaling promotes synaptic strengthening in mature neurons using compartmentalized cultures.

In the same context, we observed that axonal but not cell body activity of PLCγ is required for CREB phosphorylation induced by BDNF axonal signaling, suggesting that PLCγ has a local role in cortical axons regulating the initial steps of signaling endosomes generation. It has been shown that a mutation in the TrkB docking site for PLCγ downregulates BDNF-induced activation of CREB (Minichiello et al., 2002).

In non-compartmentalized cultures, the bath application of BDNF promotes a strong increase in the frequency of global calcium transients (He et al., 2005; Lang et al., 2007), but BDNF local application induces a point spread signal in dendrites (Lang et al., 2007). We observed that BDNF axonal stimulation promoted an increase in intracellular Ca^{+2} and similar to the observation reported in dendrites, we observed a single Ca^{+2} spread point that generate an extended retrograde signal that cover the axons along the recording zone. Since, TrkB is located in all the surface of the axons, it is intriguing that Ca^{+2} waves are generated in apparently random locations along the axons. Of note, we observed that the velocity of the calcium waves was faster than the average velocity of BDNF-QD (1.11±0.05 μm/s) (Xie et al., 2012) and (1,5 ±0.3 μm/s) for GFP-TrkB (Goto-Silva et al., 2019), suggesting that this increase is independent of the retrograde transport of the signaling endosome. However, it remains to be studied whether intracellular calcium is required for the intracellular transport of signaling endosomes. Although very speculative, the increase in calcium could be regulating fusion/fission events in the endosomal pathway (Chen et al., 2002; Hay, 2007; Luzio et al., 2007) required for retrograde transport of signaling endosomes.

In sensory neurons back-propagation of calcium at an injury site has been reported along the axon toward the cell soma (Cho et al., 2013). This process regulates the dual leucine zipper kinase (DLK), which activates c-Jun NH2 terminal kinase (JNK3), which is linked to axonal transport via JNK-interacting protein JIP3, a protein that interact with kinesin and dynactin (Cavalli et al., 2005; Rishal and Fainzilber, 2014). Interestingly, it has been shown that JIP3 is an adaptor for the anterograde transport of TrkB mediated by kinesin 1 (Huang et al., 2011). However, the increase of intracellular calcium in axons increases the affinity of JIP3 for dynactin compared to kinesin (Cavalli et al., 2005; Rishal and Fainzilber, 2014). Therefore, it is possible that the increased in intracellular calcium ions mediated by BDNF/TrkB in axons increases the TrkB/JIP3/dynactin/dynein complex to promotes the retrograde transport of the TrkB receptor. Moreover, it has been reported that the increase in the intracellular concentration of calcium ions regulates Trk receptor internalization (Bodmer et al., 2011; Du et al., 2003). It is possible that the propagation of calcium waves increases the probability of TrkB internalization and retrograde transport along the axon.

PLCγ has been described as a regulator of endocytosis of TrkA (Bodmer et al., 2011) and the EGFR (Delos Santos et al., 2017). Two different mechanism has been reported for the regulation of these two different tyrosine kinase receptors. For TrkA, Ca^{+2} contributes to enhance clathrin-mediated endocytosis in neurons due to calcineurin-dependent dephosphorylation of dynamin 1 (Bodmer et al., 2011). On the other hand, for EGFR, calcineurin and dynamin 1 were dispensable for the internalization of EGFR. In this case, calcium ions recruits synaptojanin 1 (Sjn1) to clathrin-coated pits in a PKC-dependent manner induced by the activation of PLCγ

(Delos Santos et al., 2017). In our study, we observed that PLCγ regulates internalization of TrkB, in a calcium dependent manner, without affecting the phosphorylation of dynamin suggesting that PLCγ regulates the internalization of TrkB in a PKC- dependent manner.

In summary, this study has revealed a specific function for PLCγ signaling in the axonal compartment of cortical axons, regulating the signaling of BDNF/TrkB from distal axons to the cell body to increase CREB-dependent dendritic branching. Several questions remain for future studies, including how PLCγ/Ca^{+2} regulates the internalization of TrkB receptors in the axons, and the contribution of Ca^{+2} to the trafficking of signaling endosomes.

REFERENCES

Alonso, M., Vianna, M.R., Depino, A.M., Mello e Souza, T., Pereira, P., Szapiro, G., Viola, H., Pitossi, F., Izquierdo, I., and Medina, J.H. (2002a). BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. Hippocampus *12*, 551-560.

Alonso, M., Vianna, M.R., Izquierdo, I., and Medina, J.H. (2002b). Signaling mechanisms mediating BDNF modulation of memory formation in vivo in the hippocampus. Cell Mol Neurobiol *22*, 663-674.

Arimura, N., Kimura, T., Nakamuta, S., Taya, S., Funahashi, Y., Hattori, A., Shimada, A., Menager, C., Kawabata, S., Fujii, K.*, et al.* (2009). Anterograde Transport of TrkB in Axons Is Mediated by Direct Interaction with Slp1 and Rab27. Developmental Cell *16*, 675-686.

Beattie, E.C., Zhou, J., Grimes, M.L., Bunnett, N.W., Howe, C.L., and Mobley, W.C. (1996). A signaling endosome hypothesis to explain NGF actions: potential implications for neurodegeneration. Cold Spring Harb Symp Quant Biol *61*, 389-406.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., and Bunting, S. (1990). Selective-Inhibition of Receptor-Coupled Phospholipase-C-Dependent Processes in Human Platelets and Polymorphonuclear Neutrophils. J Pharmacol Exp Ther *255*, 756-768.

Bodmer, D., Ascano, M., and Kuruvilla, R. (2011). Isoform-Specific Dephosphorylation of Dynamin1 by Calcineurin Couples Neurotrophin Receptor Endocytosis to Axonal Growth. Neuron *70*, 1085-1099.

Bronfman, F.C., Lazo, O.M., Flores, C., and Escudero, C.A. (2014). Spatiotemporal intracellular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. Handb Exp Pharmacol *220*, 33-65.

Cavalli, V., Kujala, P., Klumperman, J., and Goldstein, L.S.B. (2005). Sunday Driver links axonal transport to damage signaling. Journal of Cell Biology *168*, 775-787.

Chen, J.L., Ahluwalia, J.P., and Stamnes, M. (2002). Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. Journal of Biological Chemistry *277*, 35682-35687.

Cheng, Q., Song, S.H., and Augustine, G.J. (2017). Calcium-Dependent and Synapsin-Dependent Pathways for the Presynaptic Actions of BDNF. Frontiers in Cellular Neuroscience *11*.

Cheung, Z.H., Chin, W.H., Chen, Y., Ng, Y.P., and Ip, N.Y. (2007). Cdk5 is involved in BDNF-stimulated dendritic growth in hippocampal neurons. PLoS Biol *5*, e63.

Cho, Y., Sloutsky, R., Naegle, K.M., and Cavalli, V. (2013). Injury-Induced HDAC5 Nuclear Export Is Essential for Axon Regeneration. Cell *155*, 894-908.

Cohen, M.S., Bas Orth, C., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2011). Neurotrophin-mediated dendrite-to-nucleus signaling revealed by microfluidic compartmentalization of dendrites. Proc Natl Acad Sci U S A *108*, 11246-11251.

Cosker, K.E., and Segal, R.A. (2014). Neuronal signaling through endocytosis. Cold Spring Harb Perspect Biol *6*.

Delos Santos, R.C., Bautista, S., Lucarelli, S., Bone, L.N., Dayam, R.M., Abousawan, J., Botelho, R.J., and Antonescu, C.N. (2017). Selective regulation of clathrin-mediated epidermal growth factor receptor signaling and endocytosis by phospholipase C and calcium. Molecular Biology of the Cell *28*, 2802-2818.

Du, J., Feng, L.Y., Zaitsev, E., Je, H.S., Liu, X.W., and Lu, B. (2003). Regulation of TrkB receptor tyrosine kinase and its internalization by neuronal activity and Ca2+ influx. Journal of Cell Biology *163*, 385-395.

Du, J.L., and Poo, M.M. (2004). Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature *429*, 878-883.

Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M.*, et al.* (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*, 257-269.

Escudero, C.A., Cabeza, C., Moya-Alvarado, G., Maloney, M.T., Flores, C.M., Wu, C., Court, F.A., Mobley, W.C., and Bronfman, F.C. (2019). c-Jun N-terminal kinase (JNK)-dependent internalization and Rab5-dependent endocytosis sorting mediate long-distance retrograde neuronal death induced by axonal BDNF-p75 signaling. Sci Rep *9*, 6070.

Gluska, S., Chein, M., Rotem, N., Ionescu, A., and Perlson, E. (2016). Tracking quantum-dot labeled neurotrophic factors transport along primary neuronal axons in compartmental microfluidic chambers. Methods cell biol *131*, 365-387.

Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billaut, C., and Bronfman, F.C. (2016). Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor. Cytoskeleton (Hoboken) *73*, 612-628.

Goto-Silva, L., McShane, M.P., Salinas, S., Kalaidzidis, Y., Schiavo, G., and Zerial, M. (2019). Retrograde transport of Akt by a neuronal Rab5-APPL1 endosome. Sci Rep *9*, 2433.

Hay, J.C. (2007). Calcium: a fundamental regulator of intracellular membrane fusion? Embo Rep *8*, 236-240.

He, J., Gong, H., and Luo, Q.M. (2005). BDNF acutely modulates synaptic transmission and calcium signalling in developing cortical neurons. Cell Physiol Biochem *16*, 69-76.

Horch, H.W., and Katz, L.C. (2002). BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat Neurosci *5*, 1177-1184.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem *72*, 609-642.

Huang, S.H., Duan, S., Sun, T., Wang, J.E., Zhao, L., Geng, Z., Yan, J., Sun, H.J., and Chen, Z.Y. (2011). JIP3 Mediates TrkB Axonal Anterograde Transport and Enhances BDNF Signaling by Directly Bridging TrkB with Kinesin-1. Journal of Neuroscience *31*, 10602-10614.

Kang, H., and Schuman, E.M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science *267*, 1658-1662.

Lang, S.B., Stein, V., Bonhoeffer, T., and Lohmann, C. (2007). Endogenous brain-derived neurotrophic factor triggers fast calcium transients at synapses in developing dendrites. Journal of Neuroscience *27*, 1097-1105.

Leal, G., Afonso, P.M., Salazar, I.L., and Duarte, C.B. (2015). Regulation of hippocampal synaptic plasticity by BDNF. Brain Res *1621*, 82-101.

Li, Y., Jia, Y.C., Cui, K., Li, N., Zheng, Z.Y., Wang, Y.Z., and Yuan, X.B. (2005). Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature *434*, 894-898.

Lom, B., Cogen, J., Sanchez, A.L., Vu, T., and Cohen-Cory, S. (2002). Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. Journal of Neuroscience *22*, 7639-7649.

Luzio, J.P., Bright, N.A., and Pryor, P.R. (2007). The role of calcium and other ions in sorting and delivery in the late endocytic pathway. Biochem Soc T *35*, 1088-1091.

MacAskill, A.F., Rinholm, J.E., Twelvetrees, A.E., Arancibia-Carcamo, I.L., Muir, J., Fransson, A., Aspenstrom, P., Attwell, D., and Kittler, J.T. (2009). Miro1 Is a Calcium Sensor for Glutamate Receptor-Dependent Localization of Mitochondria at Synapses. Neuron *61*, 541-555.

Maday, S., Twelvetrees, A.E., Moughamian, A.J., and Holzbaur, E.L.F. (2014). Axonal Transport: Cargo-Specific Mechanisms of Motility and Regulation. Neuron *84*, 292-309.

Matsuda, N., Lu, H., Fukata, Y., Noritake, J., Gao, H., Mukherjee, S., Nemoto, T., Fukata, M., and Poo, M.M. (2009). Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J Neurosci *29*, 14185-14198.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron *36*, 121-137.

Moya-Alvarado, G., Stuardo, N., Chengbiao, W., Mobley, W., Perlson, E., Bronfman, F.C. (2019). BDNF axonal signaling endosomes mediates long-distance dendritic growth by activating CREB/PI3K-mTOR-dependent translation in neuronal cell bodies. Chapter II, In preparation.

Redmond, L., Kashani, A.H., and Ghosh, A. (2002). Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. Neuron *34*, 999-1010.

Reichardt, L.F. (2006). Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci *361*, 1545-1564.

Rishal, I., and Fainzilber, M. (2014). Axon-soma communication in neuronal injury. Nature Reviews Neuroscience *15*, 32-42.

Saotome, M., Safiulina, D., Szabadkai, G., Das, S., Fransson, A., Aspenstrom, P., Rizzuto, R., and Hajnoczky, G. (2008). Bidirectional Ca2+-dependent control of mitochondrial dynamics by the Miro GTPase. P Natl Acad Sci USA *105*, 20728-20733.

Sciarretta, C., Fritzsch, B., Beisel, K., Rocha-Sanchez, S.M., Buniello, A., Horn, J.M., and Minichiello, L. (2010). Bmc Dev Biol *10*.

Scott-Solomon, E., and Kuruvilla, R. (2018). Mechanisms of neurotrophin trafficking via Trk receptors. Mol Cell Neurosci *91*, 25-33.

Shelton, D.L., Sutherland, J., Gripp, J., Camerato, T., Armanini, M.P., Phillips, H.S., Carroll, K., Spencer, S.D., and Levinson, A.D. (1995). Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J Neurosci *15*, 477-491.

Shimada, A., Mason, C.A., and Morrison, M.E. (1998). TrkB signaling modulates spine density and morphology independent of dendrite structure in cultured neonatal Purkinje cells. J Neurosci *18*, 8559-8570.

Sholl, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. J Anat *87*, 387-406.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nature Methods *2*, 599-605.

Taylor, A.M., Rhee, S.W., Tu, C.H., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2003). Microfluidic Multicompartment Device for Neuroscience Research. Langmuir *19*, 1551-1556.

Xie, W., Zhang, K., and Cui, B. (2012). Functional characterization and axonal transport of quantum dot labeled BDNF. Integr Biol (Camb) *4*, 953-960.

Xu, B., Zang, K., Ruff, N.L., Zhang, Y.A., McConnell, S.K., Stryker, M.P., and Reichardt, L.F. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron *26*, 233-245.

Zhang, Z.T., Fan, J., Ren, Y.X., Zhou, W., and Yin, G.Y. (2013). The release of glutamate from cortical neurons regulated by BDNF via the TrkB/Src/PLC-gamma 1 pathway. J Cell Biochem *114*, 144-151.

Zhou, B., Cai, Q., Xie, Y., and Sheng, Z.H. (2012). Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Rep *2*, 42-51.

Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Thoenen, H., and Lindholm, D. (1995). Characterization of Trkb Receptor-Mediated Signaling Pathways in Rat Cerebellar Granule Neurons - Involvement of Protein-Kinase-C in Neuronal Survival. J Neurochem *65*, 2241-2250.

CONCLUSION

Here using an *in vitro* model of central nervous system neurons, we showed the relevance of the endosomal signaling of the neurotrophin BDNF and its receptor TrkB on the long distance signaling.

In non-compartmentalized hippocampal neurons:

BDNF increases the vesiculation of Rab5-positive endosomes in the somatodendritic compartment. BDNF increases the movement of Rab5 endosomes from dendrites to the cell body and BDNF induced an early activation of Rab5 in dendrites (5 min) followed by increased activation of Rab5 in cell bodies (30 min).

By using microfluidic chambers, we provide evidence that:

Axonal stimulation with BDNF promotes an increase of dendritic arborization by the formation of a signaling endosome in a TrkB dependent manner. Rab5 and dynein activity are required for the axonal signaling to arrive to the cell body. PI3K activity is not required in the axonal compartment for the retrograde transport of BDNF signaling endosomes, but it is required in the cell body compartment to induce dendritic arborization and mTOR activation. Somatodendritic CREB and mTOR activation are require to regulate protein synthesis.

To deepen more in the regulation of downstream pathways of TrkB in the long distance signaling we studied the PLCγ signaling.

Axonal PLCγ activity is required for dendritic arborization and CREB phosphorylation induced by BDNF axonal signaling. Local axonal activation of PLCγ regulates intracellular Ca^{+2} increase. PLCγ regulates TrkB endocytosis.

DISCUSSION

During neuronal development, neurons begin to extend processes that culminate in the formation of functional synapses (Jan and Jan, 2010). Neurons are highly polarized cells and are capable of extending long processes to form circuits. This generate a challenge for intracellular signaling and maintenance of neuronal homeostasis. (Fainzilber et al, 2011; Bronfman et al, 2014). In the CNS, BDNF plays an important role in regulating survival, growth and plasticity. (Huang and Reichardt, 2003). In the PNS, the work of different groups has contributed to the evidences that the retrograde transport of signaling endosomes, containing neurotrophins and its receptors, is essential for neuronal survival and neuronal growth (Cosker et al, 2008; Ginty and Segal, 2002; Zhou et al, 2012). However, the physiological role of long-distance signaling in the central nervous system and how neurotrophin signaling regulates endocytic trafficking is just beginning to be understood. The study of how neurotrophic signaling regulates the endosomal pathway is important for the understanding of the pathophysiology of brain diseases, since the alteration or mutations in the expression of genes that allow endocytic transport are related to neurodegenerative diseases, such as Huntington's disease (HD), which regulates the intracellular trafficking of BDNF vesicle, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD), where axonal transport defects are due to defects in the proper organization of microtubules or in the binding of motor proteins to cargoes (Liu et al, 2012; Wang et al, 2013; Millecamps and Julien, 2013).

In this thesis, we were interested in studying two related processes, the first was to understand the role of BDNF/TrkB signaling on the dynamics of early endosomes by Rab5. The second

process was the role of long-distance axonal signaling of BDNF in cortical neurons evaluating key aspects within the post-endocytic trafficking of the TrkB in axons as well as cell bodies. The endocytic trafficking of membrane receptors is regulated by Rab5 that regulates the fusion of early endosomes, the anchoring of early endosomes to different molecular motors and the recruitment of adaptors for proper sorting of receptors along the endocytic pathway (Stenmark, 2009). It has been described that Rab5 is important for the maintenance of dendritic arborization in Drosophila and the regulation of neurotrophic signaling in mammals (Satoh et al, 2008; Liu et al, 2007). On the other hand, in the CNS neurons BDNF increases Rab11 activity in dendrites, increasing local recycling of TrkB and thus increasing BDNF signaling (Figure 10A chapter I) (Huang et al., 2013; Lazo et al., 2013). Here, we found that BDNF increases the dynamic and activity of Rab5 in dendrites and cell body of hippocampal neurons. Additionally, BDNF increases both Rab11 and Rab5 protein levels in an mTOR dependent manner. These results are particularly interesting, since mTOR regulates local protein synthesis (Schratt, et al, 2004), suggesting that BDNF can increase the availability of GTPases locally in dendrites and thus increasing their activity generating a positive loop for TrkB signaling (Figura 10B Chapter II). However, we still require more research to understand the mechanism by which BDNF regulates the activity and availability of Rab5 and Rab11 in dendrites and whether motor complex such as Hook1/dynein complex (Olenick et al, 2018) or myosin Vb (Lazo et al, 2013) plays a role for local and long-distance signaling.

Given the importance of BDNF signaling in the regulation of neuronal morphology and synaptic plasticity, it is important to understand the role of long-distance BDNF signaling, since it may have implication for wiring circuits in the CNS. Initial studies suggested a role of BDNF retrograde signaling, for example, the application of BDNF to axons of retinal ganglion cells in

Xenopus laevis studies (Du and Poo, 2004; Lom et al, 2002). In addition, several groups have shown that BDNF and TrkB is retrogradely transported in the axons of CNS neurons (Sobreviela et al, 1996; Zhou et al., 2012; Ramser, et al 2013; Zhao et al, 2014; Olenick et al., 2019). However, there were no definite evidences for neither the role of signaling endosomes in BDNF plastic changes in CNS neurons nor the role of TrkB signaling pathways in this process.

In the second part of this thesis, we contributed to this unsolved issue by studying the role of BDNF/TrkB signaling on dendritic growth using cortical neurons cultures in microfluidic chambers. We found that axonal stimulation with BDNF promotes an increase in dendritic arborization of rat and mouse neurons requiring TrkB-signaling but not p75 (Figure 1).

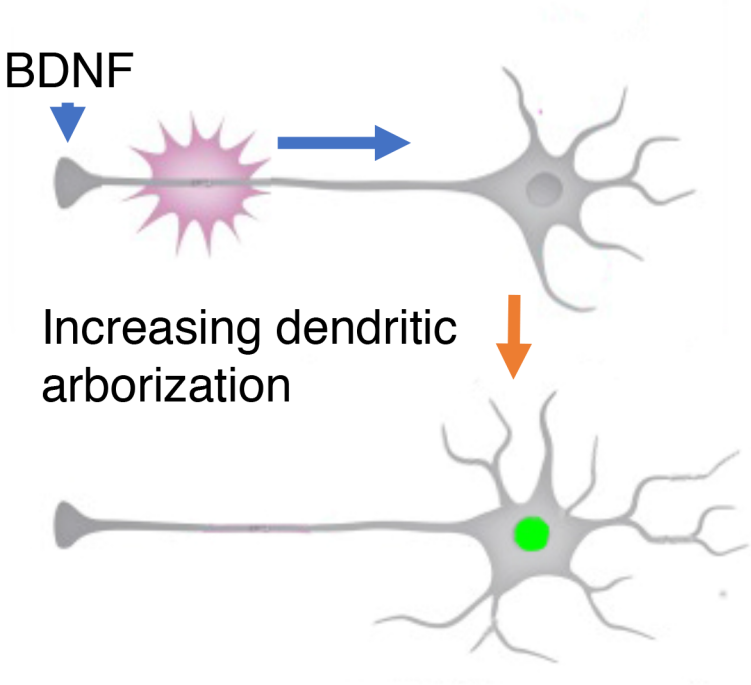

Figure 1. Axonal long-distance signaling of BDNF increases dendritic branching in cortical neurons. When BDNF is added to the axonal compartment activates TrkB. Then, TrkB is retrogradely transported and promotes an increase in dendritic arborization.

Then, we evaluate the role of BDNF signaling on endosomal activity and observed that BDNF increases Rab5 activity in axons of cortical neurons and that both dynein and Rab5 activity were required for long-distance BDNF/TrkB signaling (Figure 2A). These results confirm that the signal generated from axonal stimulation with BDNF has an endosomal component, which has a fundamental role in the regulation of long-distance dendritic arborization in neurons of the central nervous system. Interestingly, we observe that axonal stimulation produces an increase in the generation of intracellular calcium ion waves in axons in a PLC-dependent manner. Although, this finding in mammals has not been previously described, experiments performed in *Xenopus* suggested that a calcium wave that is TRPC and IP3R dependent can be transported retrogradely to mediate synaptic plasticity induced by BDNF (Du and Poo, 2004; Li et al 2005).

Together these results show that neurotrophic signaling has different types of pathways, which are activated together and converge in similar regulatory processes. For future experiments would be important to differentiate whether both signaling pathways, endosomal and calcium, act together or separately regulating axonal responses to neurotrophic factors.

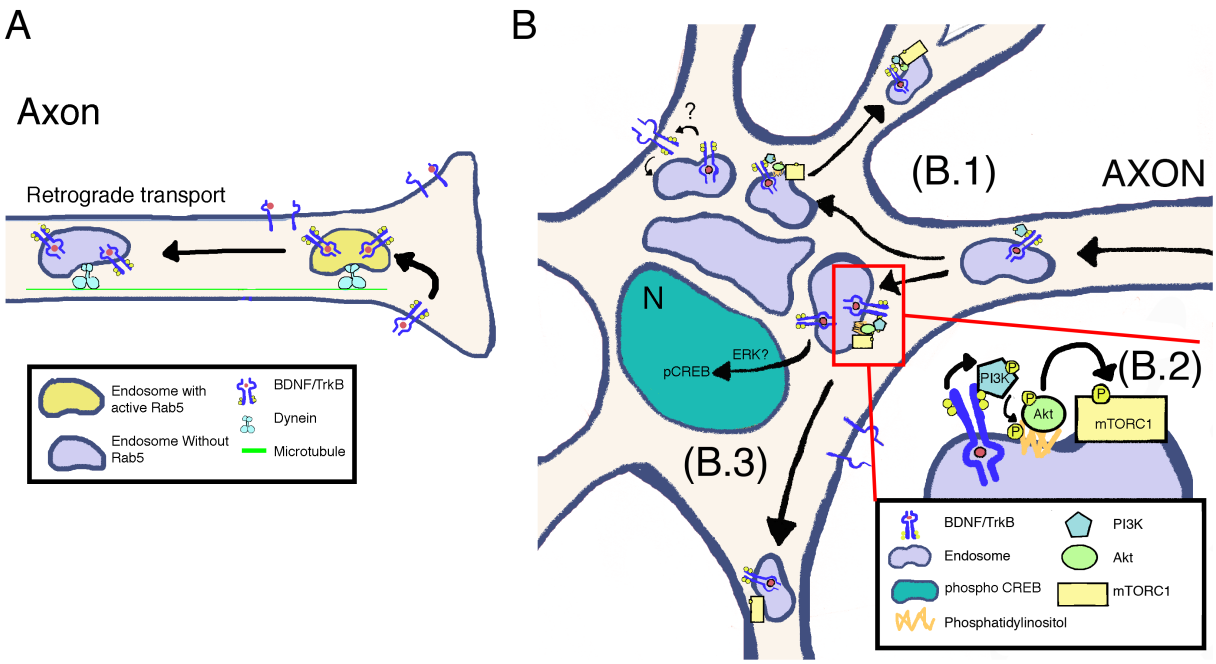

Figure 2. Scheme of BDNF local signaling in axon and cell bodies after axonal stimulation with BDNF. (A) When BDNF binds to TrkB in axons it is endocytosed and sorted to Rab5 positive endosome. Then, Rab5 is activated and the signaling endosome is retrogradely transported by dynein. (B) When the signaling endosome arrives to the cell body, (B.1) phosphorylated TrkB is found in the cell body and dendrites, (B.2) the kinase activity of PI3K is required for the activation of the mTOR (B.3) and CREB phosphorylation in the nucleus.

We observe that axonal stimulation with BDNF favors the arrival of the phosphorylated TrkB from the axon to the cell body and dendrites (Figure 2.B.1). Additionally, we observe that axonal BDNF promotes the phosphorylation of proteins associated with the mTOR signaling cascade in the cell body, in a manner that dependent on dynein and PI3K, suggesting that endosomal signaling promotes the activation of the mTOR kinase in the cell body (Figure 2.B.2). This result opens the doors to proteomic studies associated with BDNF activation of mTOR both at the level of the cell body and of the axons, since all the proteomic studies previously carried out have been performed in BDNF baths that completely cover the cell (Schratt, et al, 2004; Dijkhuizen and Ghosh, 2005; Kumar et al., 2005).

We also note that the cell body activity of TrkB receptors activated in axons by BDNF was required for CREB phosphorylation in the nucleus (Figure 2.B.3). The most studied role of CREB in long-distance signaling is the regulation of genes associated with neuronal survival in the peripheral nervous system. (Harrington and Ginty, 2013; Ye et al., 2003; Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018). In this work, we have shown that CREB activity is required for BDNF-induced protein expression, including Arc, and dendritic arborization induced by long-distance BDNF signaling, suggesting that the gene regulation exerted by CREB will depend on the cellular context to which it is found.

In conclusion, these results suggest that endosomal long-distance axonal signaling of BDNF favors the arrival to the body of active transduction pathways that increase the expression and synthesis of proteins associated with neuronal growth, so it would be interesting in the future to characterize the transcriptomic and proteomic associated with local signaling in axons and long distance in the cell body of neurons, as it could give clues to how neurotrophic signaling regulates each neuronal compartment.

REFERENCES

Akum, B.F., Chen, M., Gunderson, S.I., Riefler, G.M., Scerri-Hansen, M.M., and Firestein, B.L. (2004). Cypin regulates dendrite patterning in hippocampal neurons by promoting microtubule assembly. Nat Neurosci *7*, 145-152.

Alonso, M., Vianna, M.R., Depino, A.M., Mello e Souza, T., Pereira, P., Szapiro, G., Viola, H., Pitossi, F., Izquierdo, I., and Medina, J.H. (2002). BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. Hippocampus *12*, 551-560.

BasuRay, S., Mukherjee, S., Romero, E., Wilson, M.C., and Wandinger-Ness, A. (2010). Rab7 Mutants Associated with Charcot-Marie-Tooth Disease Exhibit Enhanced NGF-Stimulated Signaling. Plos One *5*.

Benraiss, A., Chmielnicki, E., Lerner, K., Roh, D., and Goldman, S.A. (2001). Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J Neurosci *21*, 6718-6731.

Bodmer, D., Ascano, M., and Kuruvilla, R. (2011). Isoform-Specific Dephosphorylation of Dynamin1 by Calcineurin Couples Neurotrophin Receptor Endocytosis to Axonal Growth. Neuron *70*, 1085-1099.

Bronfman, F.C., Escudero, C.A., Weis, J., and Kruttgen, A. (2007). Endosomal transport of neurotrophins: roles in signaling and neurodegenerative diseases. Dev Neurobiol *67*, 1183-1203.

Bronfman, F.C., Lazo, O.M., Flores, C., and Escudero, C.A. (2014). Spatiotemporal intracellular dynamics of neurotrophin and its receptors. Implications for neurotrophin signaling and neuronal function. Handb Exp Pharmacol *220*, 33-65.

Bucci, C., Alifano, P., and Cogli, L. (2014). The role of rab proteins in neuronal cells and in the trafficking of neurotrophin receptors. Membranes (Basel) *4*, 642-677.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci *4*, 299-309.

Chen, Y., Fu, W.Y., Ip, J.P., Ye, T., Fu, A.K., Chao, M.V., and Ip, N.Y. (2012). Ankyrin repeat-rich membrane spanning protein (kidins220) is required for neurotrophin and ephrin receptor-dependent dendrite development. J Neurosci *32*, 8263-8269.

Cohen, M.S., Bas Orth, C., Kim, H.J., Jeon, N.L., and Jaffrey, S.R. (2011). Neurotrophin-mediated dendrite-to-nucleus signaling revealed by microfluidic compartmentalization of dendrites. Proc Natl Acad Sci U S A *108*, 11246-11251.

Cosker, K.E., Couchesne, S.L., Segal, R.A., (2008). Action in the axon: generation and transport of signaling endosomes. Curr Opin Neurobiol *18*, 270-275.

Cosker, K.E., and Segal, R.A. (2014). Neuronal signaling through endocytosis. Cold Spring Harb Perspect Biol *6*.

Deinhardt, K., Salinas, S., Verastegui, C., Watson, R., Worth, D., Hanrahan, S., Bucci, C., and Schiavo, G. (2006). Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron *52*, 293-305.

Deogracias, R., Espliguero, G., Iglesias, T., and Rodriguez-Pena, A. (2004). Expression of the neurotrophin receptor trkB is regulated by the cAMP/CREB pathway in neurons. Mol Cell Neurosci *26*, 470-480.

Deppmann, C.D., Mihalas, S., Sharma, N., Lonze, B.E., Nieber, E., and Ginty, D.D. (2008). A model for neuronal competition during development. Int J Dev Neurosci *26*, 835-836.

Dijkhuizen, P.A., and Ghosh, A. (2005). BDNF regulates primary dendrite formation in cortical neurons via the PI3-kinase and MAP kinase signaling pathways. J Neurobiol *62*, 278-288.

Doherty, G.J., and McMahon, H.T. (2009). Mechanisms of endocytosis. Annu Rev Biochem *78*, 857-902.

Du, J.L., and Poo, M.M. (2004). Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature *429*, 878-883.

Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M.*, et al.* (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell *112*, 257-269.

Escudero, C.A., Cabeza, C., Moya-Alvarado, G., Maloney, M.T., Flores, C.M., Wu, C., Court, F.A., Mobley, W.C., and Bronfman, F.C. (2019). c-Jun N-terminal kinase (JNK)-dependent internalization and Rab5-dependent endocytic sorting mediate long-distance retrograde neuronal death induced by axonal BDNF-p75 signaling. Sci Rep *9*, 6070.

Fainzilber, M., Budnik, V., Segal, R.A., Kreutz, M.R. (2011). From synapse to nucleus and back again-communication over distance within neurons. J Neurosci *31*, 16045-16048.

Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., and Greenberg, M.E. (1997). CREB: a major mediator of neuronal neurotrophin responses. Neuron *19*, 1031-1047.

Fu, X.P., Yang, Y.R., Xu, C.C., Niu, Y., Chen, T.L., Zhou, Q., and Liu, J.J. (2011). Retrolinkin cooperates with endophilin A1 to mediate BDNF-TrkB early endocytic trafficking and signaling from early endosomes. Molecular Biology of the Cell *22*, 3684-3698.

Garza-Lombo, C., and Gonsebatt, M.E. (2016). Mammalian Target of Rapamycin: Its Role in Early Neural Development and in Adult and Aged Brain Function. Front Cell Neurosci *10*, 157.

Ginty, D.D., Segal, R.A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. Curr Opin Neurobiol *12*, 268-274.

Gonzalez, A., Lazo, O.M., and Bronfman, F.C. (2019). The Rab5-Rab11 endosomal pathway is required for BDNF-induced CREB transcriptional regulation in neurons. J Neurosci (Submitted).

Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billaut, C., and Bronfman, F.C. (2016). Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor. Cytoskeleton (Hoboken) *73*, 612-628.

Gorski, J.A., Zeiler, S.R., Tamowski, S., and Jones, K.R. (2003). Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. J Neurosci *23*, 6856-6865.

Harrington, A.W., and Ginty, D.D. (2013). Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci *14*, 177-187.

Hashimoto, R., Takei, N., Shimazu, K., Christ, L., Lu, B., and Chuang, D.M. (2002). Lithium induces brain-derived neurotrophic factor and activates TrkB in rodent cortical neurons: an essential step for neuroprotection against glutamate excitotoxicity. Neuropharmacology *43*, 1173-1179.

Heerssen, H.M., Pazyra, M.F., and Segal, R.A. (2004). Dynein motors transport activated Trks to promote survival of target-dependent neurons. Nature Neuroscience *7*, 596-604.

Hirokawa, N., Niwa, S., and Tanaka, Y. (2010). Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Neuron *68*, 610-638.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem *72*, 609-642.

Huang, S.H., Wang, J., Sui, W.H., Chen, B., Zhang, X.Y., Yan, J., Geng, Z., and Chen, Z.Y. (2013). BDNF-Dependent Recycling Facilitates TrkB Translocation to Postsynaptic Density during LTP via a Rab11-Dependent Pathway. Journal of Neuroscience *33*, 9214-9230.

Huotari, J., and Helenius, A. (2011). Endosome maturation. Embo J *30*, 3481-3500.

Ibanez, C.F., and Simi, A. (2012). p75 neurotrophin receptor signaling in nervous system injury and degeneration: paradox and opportunity. Trends Neurosci *35*, 431-440.

Jan, Y.N., and Jan, L.Y. (2010). Branching out: mechanisms of dendritic arborization. Nat Rev Neurosci *11*, 316-328.

Johansson, M., Rocha, N., Zwart, W., Jordens, I., Janssen, L., Kuijl, C., Olkkonen, V.M., and Neefjes, J. (2007). Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150(Glued), ORP1L, and the receptor beta III spectrin. Journal of Cell Biology *176*, 459-471.

Kalaidzidis, I., Miaczynska, M., Brewinska-Olchowik, M., Hupalowska, A., Ferguson, C., Parton, R.G., Kalaidzidis, Y., and Zerial, M. (2015). APPL endosomes are not obligatory endocytic intermediates but act as stable cargo-sorting compartments. J Cell Biol *211*, 123-144.

Kalil, K., and Dent, E.W. (2014). Branch management: mechanisms of axon branching in the developing vertebrate CNS. Nat Rev Neurosci *15*, 7-18.

Kang, H.J., and Schuman, E.M. (1995). Neurotrophin-induced modulation of synaptic transmission in the adult hippocampus. J Physiol Paris *89*, 11-22.

Kardon, J.R., and Vale, R.D. (2009). Regulators of the cytoplasmic dynein motor. Nat Rev Mol Cell Bio *10*, 854-865.

Kraemer, B.R., Yoon, S.O., and Carter, B.D. (2014). The biological functions and signaling mechanisms of the p75 neurotrophin receptor. Handb Exp Pharmacol *220*, 121-164.

Kumar, V., Zhang, M.X., Swank, M.W., Kunz, J., and Wu, G.Y. (2005). Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci *25*, 11288-11299.

Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H.H., and Ginty, D.D. (2004). A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell *118*, 243-255.

Kwon, M., Fernandez, J.R., Zegarek, G.F., Lo, S.B., and Firestein, B.L. (2011). BDNF-promoted increases in proximal dendrites occur via CREB-dependent transcriptional regulation of cypin. J Neurosci *31*, 9735-9745.

Lazo, O.M., Gonzalez, A., Ascano, M., Kuruvilla, R., Couve, A., and Bronfman, F.C. (2013). BDNF regulates Rab11-mediated recycling endosome dynamics to induce dendritic branching. J Neurosci *33*, 6112-6122.

Leal, G., Comprido, D., and Duarte, C.B. (2014). BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology *76 Pt C*, 639-656.

Lehigh, K.M., West, K.M., and Ginty, D.D. (2017). Retrogradely Transported TrkA Endosomes Signal Locally within Dendrites to Maintain Sympathetic Neuron Synapses. Cell Rep *19*, 86-100.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*, 1154-1162.
Lin, D.C., Quevedo, C., Brewer, N.E., Bell, A., Testa, J.R., Grimes, M.L., Miller, F.D., and Kaplan, D.R. (2006). APPL1 associates with TrkA and GIPC1 and is required for nerve growth factor-mediated signal transduction. Mol Cell Biol *26*, 8928-8941.

Li, Y., Jia, Y.C., Cui, K., Li, N., Zheng, Z.Y., Wang, Y.Z., and Yuan, X.B. (2005). Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature *434*, 894-898.

Lipton, J.O., and Sahin, M. (2014). The neurology of mTOR. Neuron *84*, 275-291.
Lom, B., Cogen, J., Sanchez, A.L., Vu, T., and Cohen-Cory, S. (2002). Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci *22*, 7639-7649.

Liu, J., Lamb, D., Chou, M.M., Liu, Y.J., and Li, G. (2007). Nerve growth factor-mediated neurite outgrowth via regulation of Rab5. Mol Biol Cell *18*, 1375-1384.

Liu, X. A., Rizzo, V. and Puthanveettil, S. V. (2012). Pathologies of axonal transport in neurodegenerative diseases. Transl Neurosci *3*, 355–372.

Lom, B., Cogen, J., Sanchez, A.L., Vu, T., and Cohen-Cory, S. (2002). Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci *22*, 7639-7649.

Matsuda, N., Lu, H., Fukata, Y., Noritake, J., Gao, H., Mukherjee, S., Nemoto, T., Fukata, M., and Poo, M.M. (2009). Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J Neurosci *29*, 14185-14198.

Millecamps S., and Julien J.P. (2013). Axonal transport deficits and neurodegenerative disease. Nat Rev *14*, 161-176.

Minichiello, L., Calella, A.M., Medina, D.L., Bonhoeffer, T., Klein, R., and Korte, M. (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron *36*, 121-137.

Moya-Alvarado, G., Gonzalez, A., Stuardo, N., and Bronfman, F.C. (2018). Brain-Derived Neurotrophic Factor (BDNF) Regulates Rab5-Positive Early Endosomes in Hippocampal Neurons to Induce Dendritic Branching. Front Cell Neurosci *12*, 493.

Olenick, M.A., Dominguez, R., and Holzbaur, E.L.F. (2019). Dynein activator Hook1 is required for trafficking of BDNF-signaling endosomes in neurons. J Cell Biol *218*, 220-233.

Panja, D., Dagyte, G., Bidinosti, M., Wibrand, K., Kristiansen, A.M., Sonenberg, N., and Bramham, C.R. (2009). Novel translational control in Arc-dependent long term potentiation consolidation in vivo. J Biol Chem *284*, 31498-31511.

Park, H., and Poo, M.M. (2013). Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci *14*, 7-23.

Park, M., Penick, E.C., Edwards, J.G., Kauer, J.A., and Ehlers, M.D. (2004). Recycling endosomes supply AMPA receptors for LTP. Science *305*, 1972-1975.

Philippidou, P., Valdez, G., Akmentin, W., Bowers, W.J., Federoff, H.J., and Halegoua, S. (2011). Trk retrograde signaling requires persistent, Pincher-directed endosomes. Proc Natl Acad Sci U S A *108*, 852-857.

Ramser, E.M., Gan, K.J., Decker, H., Fan, E.Y., Suzuki, M.M., Ferreira, S.T., and Silverman M.A. (2013). Amyloid-b oligomers induce tau-independent disruption of BDNF axonal transport via calcineurin activation in cultured hippocampal neurons. Mol Biol Cell *24*, 2494-2505.

Rao, Y., Ruckert, C., Saenger, W., and Haucke, V. (2012). The early steps of endocytosis: from cargo selection to membrane deformation. Eur J Cell Biol *91*, 226-233.

Ravindran, S., Nalavadi, V.C., and Muddashetty, R.S. (2019). BDNF Induced Translation of Limk1 in Developing Neurons Regulates Dendrite Growth by Fine-Tuning Cofilin1 Activity. Front Mol Neurosci *12*, 64.

Reichardt, L.F. (2006). Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci *361*, 1545-1564.

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A., and Ginty, D.D. (1999). Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science *286*, 2358-2361.

Riccio, A., Pierchala, B.A., Ciarallo, C.L., and Ginty, D.D. (1997). An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science *277*, 1097-1100.
Roux, P.P., and Barker, P.A. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol *67*, 203-233.

Ruit, K.G., and Snider, W.D. (1991). Administration or Deprivation of Nerve Growth-Factor during Development Permanently Alters Neuronal Geometry. J Comp Neurol *314*, 106-113.

Satoh, D., Sato, D., Tsuyama, T., Saito, M., Ohkura, H., Rolls, M.M., Ishikawa, F., and Uemura, T. (2008). Spatial control of branching within dendritic arbors by dynein-dependent transport of Rab5-endosomes. Nat Cell Biol *10*, 1164-1171.

Saxena, S., Bucci, C., Weis, J., and Kruttgen, A. (2005). The small GTPase Rab7 controls the endosomal trafficking and neuritogenic signaling of the nerve growth factor receptor TrkA. Journal of Neuroscience *25*, 10930-10940.

Schratt, G.M., Nigh, E.A., Chen, W.G., Hu, L., and Greenberg, M.E. (2004). BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. J Neurosci *24*, 7366-7377.

Scott-Solomon, E., and Kuruvilla, R. (2018). Mechanisms of neurotrophin trafficking via Trk receptors. Mol Cell Neurosci *91*, 25-33.

Sharma, N., Deppmann, C.D., Harrington, A.W., Hillaire, C.S., Chen, Z.Y., Lee, F.S., and Ginty, D.D. (2010). Long-Distance Control of Synapse Assembly by Target-Derived NGF. Neuron *67*, 422-434.

Sobreviela, T., Pagcatipunan, M., Kroin, J.S., and Mufson, E.J. (1996). Retrograde transport of brain-derived neurotrophic factor (BDNF) following infusion in Neo-and Limbic cortex in Rat: relationship to BDNF mRNA expressing neurons. J Comp Neurol *375*, 417-444.

Song, M., Giza, J., Proenca, C.C., Jing, D.Q., Elliott, M., Dincheva, I., Shmelkov, S.V., Kim, J., Schreiner, R., Huang, S.H.*, et al.* (2015). Slitrk5 Mediates BDNF-Dependent TrkB Receptor Trafficking and Signaling. Developmental Cell *33*, 690-702.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol *10*, 513-525.

Takei, N., Inamura, N., Kawamura, M., Namba, H., Hara, K., Yonezawa, K., and Nawa, H. (2004). Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci *24*, 9760-9769.

Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A.J., and Greenberg, M.E. (1998). Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron *20*, 709-726.

Valdez, G., Philippidou, P., Rosenbaum, J., Akmentin, W., Shao, Y., and Halegoua, S. (2007). Trk-signaling endosomes are generated by Rac-dependent macroendocytosis. Proc Natl Acad Sci U S A *104*, 12270-12275.

Van Aelst, L., and Cline, H.T. (2004). Rho GTPases and activity-dependent dendrite development. Curr Opin Neurobiol *14*, 297-304.

Villarroel-Campos, D., Schiavo, G., and Lazo, O.M. (2018). The many disguises of the signalling endosome. Febs Lett *592*, 3615-3632.

Voyvodic, J.T. (1989). Peripheral Target Regulation of Dendritic Geometry in the Rat Superior Cervical-Ganglion. Journal of Neuroscience *9*, 1997-2010.

Wandinger-Ness, A., and Zerial, M. (2014). Rab proteins and the compartmentalization of the endosomal system. Cold Spring Harb Perspect Biol *6*, a022616.

Wang, D., Chan, C. C., Cherry, S. and Hiesinger, P. R. (2013). Membrane trafficking in neuronal maintenance and degeneration. Cell Mol Life Sci *70*, 2919–2934.

Wang, X., and Proud, C.G. (2006). The mTOR pathway in the control of protein synthesis. Physiology (Bethesda) *21*, 362-369.

Wu, S.H., Arevalo, J.C., Sarti, F., Tessarollo, L., Gan, W.B., and Chao, M.V. (2009). Ankyrin Repeat-rich Membrane Spanning/Kidins220 protein regulates dendritic branching and spine stability in vivo. Dev Neurobiol *69*, 547-557.

Xing, J., Kornhauser, J.M., Xia, Z., Thiele, E.A., and Greenberg, M.E. (1998). Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol *18*, 1946-1955.

Xu, B., Zang, K., Ruff, N.L., Zhang, Y.A., McConnell, S.K., Stryker, M.P., and Reichardt, L.F. (2000). Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron *26*, 233-245.

Ye, H., Kuruvilla, R., Zweifel, L.S., and Ginty, D.D. (2003). Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron *39*, 57-68.

Ye, M.C., Lehigh, K.M., and Ginty, D.D. (2018). Multivesicular bodies mediate long-range retrograde NGF-TrkA signaling. Elife *7*.

Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V., and Bramham, C.R. (2002). Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. J Neurosci *22*, 1532-1540.

Zhao, X., Zhou, Y., Weissmiller, A.M., Pearn, M.L. Mobley, W.C., and Wu, C., (2014). Real-time imaging of axonal transport of quantum dot-labeled BDNF in primary neurons. J Vis Exp e51899.

Zheng, J., Shen, W.H., Lu, T.J., Zhou, Y., Chen, Q., Wang, Z., Xiang, T., Zhu, Y.C., Zhang, C., Duan, S.*, et al.* (2008). Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth. J Biol Chem *283*, 13280-13288.

Zhou, B., Cai, Q., Xie, Y., and Sheng, Z.H. (2012). Snapin recruits dynein to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Rep *2*, 42-51.

Zhou, P., Porcionatto, M., Pilapil, M., Chen, Y., Choi, Y., Tolias, K.F., Bikoff, J.B., Hong, E.J., Greenberg, M.E., and Segal, R.A. (2007). Polarized signaling endosomes coordinate BDNF-induced chemotaxis of cerebellar precursors. Neuron *55*, 53-68.

Zuccato, C., and Cattaneo, E. (2009). Brain-derived neurotrophic factor in neurodegenerative diseases. Nat Rev Neurol *5*, 311-322.