Myosin Va mediates BDNF-induced postendocytic recycling of full-length TrkB and its translocation into dendritic spines

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Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal survival, neurite outgrowth and synaptic plasticity by activating the receptor tropomyosin receptor kinase B (TrkB, also known as NTRK2). TrkB has been shown to undergo recycling after BDNF stimulation. We have previously reported that full-length TrkB (TrkB-FL) are recycled through a Rab11-dependent pathway upon BDNF stimuli, which is important for the translocation of TrkB-FL into dendritic spines and for the maintenance of prolonged BDNF downstream signaling during long-term potentiation (LTP). However, the identity of the motor protein that mediates the local transfer of recycled TrkB-FL back to the plasma membrane remains unclear.

Here, we report that the F-actin-based motor protein myosin Va (Myo5a) mediates the postendocytic recycling of TrkB-FL. Blocking the interaction between Rab11 and Myo5a by use of a TAT-tagged peptide consisting of amino acids 55–66 of the Myo5a ExonE domain weakened the association between TrkB-FL and Myo5a and thus impaired TrkB-FL recycling and BDNF-induced TrkB-FL translocation into dendritic spines. Finally, inhibiting Myo5a-mediated TrkB-FL recycling led to a significant reduction in prolonged BDNF downstream signaling. Taken together, these results show that Myo5a mediates BDNF-dependent TrkB-FL recycling and contributes to BDNF-induced TrkB spine translocation and prolonged downstream signaling.

KEY WORDS: BDNF, TrkB, NTRK2, Myosin Va, Myo5a, Recycling, Rab11

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) plays important roles in neuronal development and survival of the central nervous system (CNS). BDNF regulates neuronal survival, neurogenesis, neurite outgrowth and synaptic plasticity (Tessarollo, 1998; Huang and Reichardt, 2001; Chao, 2003). The BDNF Val66Met polymorphism, which leads to a decrease in activity-dependent BDNF secretion (Chen et al., 2004), has been reported to be associated with neuropsychiatric disorders, including depression and anxiety-related disorders (Shimizu et al., 2004; Chen et al., 2006; Yu et al., 2009; Verhagen et al., 2010; Yu et al., 2012). Tropomyosin receptor kinase B (TrkB, also known as NTRK2), as the high-affinity receptor for BDNF, are abundant in the nervous system and are crucial for synaptic plasticity in the CNS (Bramham and Messaoudi, 2005; Nagappan and Lu, 2005; Cohen and Greenberg, 2008). When activated by BDNF, TrkB receptors initiate three major signaling cascades, including those mediated by phosphoinositide 3-kinase (PI3K), phospholipase Cγ (PLCγ) and extracellular signal-regulated kinase 1 and 2 (ERK1/2; also known as MAPK3 and MAPK1, respectively) (Huang and Reichardt, 2003; Segal, 2003), thereby regulating synaptic transmission and membrane excitability in neurons (Poo, 2001; Rose et al., 2004).

As a transmembrane receptor, TrkB is transported through vesicles by motor proteins along microtubules or filamentous actin (F-actin). Several motor proteins have been reported to participate in the trafficking of TrkB receptors. Dynein and kinesin, respectively, mediate the retrograde and anterograde trafficking of TrkB-containing vesicles along microtubules (Yano et al., 2001; Arimura et al., 2009; Huang et al., 2011). It has been established that the TrkB receptors can be sorted to the recycling pathway after BDNF-triggered endocytosis (Grimes et al., 1997; Chen et al., 2005). Our previous work has shown that full-length TrkB (TrkB-FL) and the T1 isoform (TrkB.T1) are sorted into different recycling pathways after endocytosis: TrkB.T1 can rapidly recycle back to the neuronal surface through a Rab4-mediated ‘fast recycling’ pathway, whereas TrkB-FL goes into a Rab11-mediated ‘slow recycling’ pathway (Huang et al., 2009; Huang et al., 2013). Myosin VI has been found to mediate the internalization of TrkB receptors (Yano et al., 2006), but the motor proteins that mediate the postendocytic recycling of TrkB receptors have not yet been identified.

In our previous work, we performed a mass spectrometry assay to screen candidate proteins that immunoprecipitated in complex with TrkB-FL in lysate from cultured neurons (data not published), and myosin Va (Myo5a) was detected after BDNF stimulation (see Materials and Methods for details). Myo5a is known to be an F-actin-based motor, and it is highly expressed in the nervous system and contributes to the trafficking of many proteins or vesicles to the plasma membrane, including AMPA receptors, mRNA–protein complex and secretory granules (Naisbitt et al., 2000; Walikonis et al., 2000; Yoshimura et al., 2006; Correia et al., 2008; Hirokawa et al., 2010). In this study, we investigated the role of Myo5a in the intracellular trafficking of TrkB receptors.

RESULTS

Myo5a associates with TrkB-FL in a BDNF-dependent manner

We first investigated whether Myo5a could associate with TrkB in an exogenous co-immunoprecipitation assay. Because the cargo-binding domain of Myo5a is responsible for its association with cargos, we co-transfected HEK293 cells with a HA-tagged...
Myo5a cargo-binding domain (Myo5aCBD) and Flag-tagged TrkB-FL or TrkB.T1. Interestingly, Myo5aCBD could specifically associate with TrkB-FL but not TrkB.T1 (Fig. 1A). To exclude any artifacts caused by overexpression, we performed endogenous co-immunoprecipitation experiments in cultured hippocampal neurons and found an association between endogenous Myo5a and TrkB upon BDNF treatment (50 ng/ml) for 30 min (Fig. 1B), which suggests that this association is dependent on BDNF.

Next, we performed an immunocytochemistry assay to assess the subcellular colocalization of endogenous TrkB receptors with Myo5a in cultured hippocampal neurons after BDNF treatment (Fig. 1C). We found that more TrkB-FL colocalized with Myo5a after BDNF stimulation than in the no-BDNF group (Fig. 1D). This result confirmed that BDNF enhances the association between TrkB-FL with Myo5a in cultured hippocampus neurons.

The TrkB-FL kinase domain is necessary and sufficient for the interaction with Myo5a

Our results have shown that Myo5a could associate with TrkB-FL but not TrkB.T1. Next, we mapped the domains underlying the interaction between Myo5aCBD and TrkB-FL. TrkB.T1 has the same extracellular and transmembrane domains as TrkB-FL, but it contains a short isoform-specific cytoplasmic domain (Fig. 2A). We made various TrkB truncated constructs: TrkB-ACT (C-terminal deleted), TrkB-ΔTK (C-terminal and kinase domain both deleted), TrkB-ΔJM (C-terminal, kinase and juxtamembrane domain all deleted) (Fig. 2A). Both the co-immunoprecipitation assay and the GST pulldown assay showed that the loss of the kinase domain (TrkB-ΔTK) abolished the association between TrkB-FL and Myo5a, suggesting that the kinase domain in TrkB-FL was essential for its association with Myo5aCBD (Fig. 2B,C). Next, we used the TrkB kinase dead mutant (TrkB-KD) in co-immunoprecipitation assays with Myo5aCBD and found that TrkB-KD could not associate with Myo5aCBD (Fig. 2D). This result was consistent with our above endogenous co-immunoprecipitation experiment and suggested that kinase activity was necessary for the interaction between TrkB-FL and Myo5aCBD. In addition, we found that the overexpression of TrkB-FL in HEK293 cells led to TrkB self-transphosphorylation and significantly activated the ERK and PI3K signaling pathway (supplementary material Fig. S1), which explains why we observed an association between Myo5a and TrkB-FL in the co-immunoprecipitation assay in HEK293 cells without BDNF treatment. Then, we investigated whether the downstream

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Fig. 1. Myo5a associates with TrkB-FL in a BDNF-dependent manner. (A) Co-immunoprecipitation was performed in HEK293 cells expressing Flag-tagged TrkB-FL (FL) or TrkB.T1 (T1) and HA-tagged Myo5aCBD. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody and analyzed by immunoblotting (IB). (B) Endogenous association of Myo5a and TrkB receptors was detected using co-immunoprecipitation in cultured hippocampal neurons (10–14 DIV) under the indicated conditions. Neurons were serum starved overnight and BDNF (50 ng/ml) was added for 0 min or 30 min. Cell lysates were immunoprecipitated with anti-TrkB antibody. A pre-immunised IgG (rabbit) was used as a negative control. Each co-immunoprecipitation experiment (A,B) was repeated three times and representative data are shown. (C) Colocalization of endogenous TrkB-FL receptors (red) with Myo5a (green) in cultured hippocampal neurons (10–14 DIV). Higher magnification images of dendritic branches are shown in the lower panels. Arrows indicate colocalization. Arrowheads indicate TrkB-FL puncta. Scale bar: 20 μm. (D) Quantification of colocalization between TrkB-FL and Myo5a. n>15 dendrites from 5–6 cells for each condition per independent experiment. Data are shown as the mean±s.e.m. from three independent experiments. *P<0.05, Student’s t-test.
partners of TrkB were also necessary for the association. TrkB-ΔCT does not lose the C-terminal domain that activates the PLCγ pathway but we found it could still associate with Myo5aCBD (Fig. 2B). This result suggests that the PLCγ pathway is unnecessary for the association. Therefore, we used U0126 (a MEK1/2 inhibitor that inhibits upstream components of the ERK1/2...
pathway) or LY294002 (a PI3K inhibitor) to block the ERK1/2 and Akt signaling pathways, respectively, and found that neither the ERK1/2 nor the Akt pathway was necessary for the interaction between TrkB-FL and Myo5aCBD (Fig. 2E), which suggests that the TrkB kinase domain but not the downstream partners are necessary for the association between TrkB-FL and Myo5a. To address whether the kinase domain was sufficient for the interaction, we generated chimaeras that transplanted the kinase domain to TrkB.T1 (TrkB.T1TK, Fig. 2A). According to the co-immunoprecipitation assay, TrkB.T1TK associated with Myo5aCBD (Fig. 2F), demonstrating that the kinase domain is sufficient for the interaction. In conclusion, the TrkB receptor kinase domain is necessary and sufficient for the interaction between TrkB-FL and Myo5a.

Myo5a is not involved in BDNF-dependent TrkB-FL internalization

Because Myo5a associated with TrkB-FL in a BDNF-dependent manner, we next investigated whether Myo5a was involved in the endocytosis of TrkB-FL. We constructed a small interfering RNA (siRNA) targeting Myo5a (siMyo5a, Fig. 3A) to knockdown endogenous Myo5a. To control for off-target effects, we also expressed Myo5aCBD, which acts as a dominant-negative form because it associates with TrkB-FL but lacks the motor domain (Brown et al., 2001). First, we used a live-cell ratiometric fluorescence-based endocytosis assay (Kotowski et al., 2011) to analyze TrkB-FL internalization in cultured hippocampal neurons (Fig. 3B, see the Materials and Methods for details). Quantification of the fluorescence by ratiometric analysis
showed that neither siMyo5a nor Myo5aCBD influenced the levels of TrkB-FL internalization (Fig. 3C), indicating that Myo5a does not participate in TrkB-FL internalization.

Next, we performed a cleavable biotinylation assay to detect the internalization levels of endogenous TrkB-FL (Fig. 3D,E, see the Materials and Methods for details). Quantification of the results revealed that there was no significant change in TrkB-FL internalization levels when expressing siMyo5a or Myo5aCBD (Fig. 3F), which further confirmed that Myo5a did not participate in the BDNF-dependent internalization of endogenous TrkB-FL.

**Myo5a mediates postendocytic recycling of TrkB-FL but not TrkB.T1**

Our above experiments suggested that Myo5a is not involved in the internalization of TrkB-FL. Next, we investigated whether Myo5a participated in the postendocytic recycling of TrkB-FL. We used a live-cell ratiometric fluorescence-based recycling assay, which has been widely used in our previous studies (Chen et al., 2005; Huang et al., 2009; Huang et al., 2013), to measure the recycling levels of TrkB-FL (Fig. 4A). Quantification of these results by ratiometric analyzes revealed that when blocking endogenous Myo5a function by siMyo5a or Myo5aCBD, the levels of TrkB-FL recycling were significantly reduced (from ~38% to ~17%, Fig. 4B), indicating that Myo5a participates in postendocytic recycling of TrkB-FL. In contrast, we found that Myo5a was not involved in the recycling of TrkB.T1 (Fig. 4A,B), suggesting that Myo5a specifically mediated the postendocytic recycling of TrkB-FL but not TrkB.T1.

To exclude any artifacts caused by overexpression, we performed a cleavable biotinylation assay to detect the levels of postendocytic recycling of endogenous TrkB receptors (Fig. 4C). Quantification of these results revealed that when expressing siMyo5a or Myo5aCBD, the remaining un-recycled TrkB-FL levels increased significantly compared to the control group (CON, from ~57% to ~87%, Fig. 4E), indicating that the levels of TrkB-FL recycling decreased, whereas TrkB.T1 recycling levels were not changed. All of these results demonstrate that Myo5a mediates the postendocytic recycling of TrkB-FL but not TrkB.T1. It has been previously reported that TrkB-FL is degraded or recycled after endocytosis (Huang et al., 2009). Here, we performed surface biotin assays to study the levels of TrkB-FL degradation under control or siMyo5a conditions. Surface proteins were labeled with biotin, and the cells were then subjected to BDNF stimuli for 60 min. Biotin-labeled proteins were then pulled down with avidin beads and the levels of TrkB-FL were assessed (Fig. 4F). Quantification revealed that more TrkB-FL was degraded in the siMyo5a condition than in the control group, which suggests that more TrkB-FL is degraded once the recycling pathway is blocked (Fig. 4G).

**Rab11 regulates the interaction between Myo5a and TrkB-FL**

In our previous studies, we found that Rab11 associates with TrkB-FL and mediates its postendocytic recycling (Huang et al., 2013). It has also been reported that Rab11 associates with Myo5a and mediates the trafficking cargos such as AMPA receptors (Correia et al., 2008; Roland et al., 2009; Lindsay et al., 2013). Therefore, we investigated whether Rab11 was involved in the interaction between Myo5a and TrkB-FL. We first examined the interaction of endogenous Myo5a and TrkB under conditions where Rab11 was knocked down by siRNA (siRab11, Fig. 5A). As shown by the quantitation of the co-immunoprecipitation result in Fig. 5B, knock-down of Rab11 by siRNA significantly inhibited the association between endogenous TrkB receptors and Myo5a (to ~40% of control), suggesting that Rab11 was involved in the association between TrkB and Myo5a.

Although numerous studies have reported that Myo5a could associate with Rab11 (Correia et al., 2008; Roland et al., 2009; Lindsay et al., 2013), the specific motif in Myo5a responsible for its interaction with Rab11 has remained unclear. Previous reports have indicated that the splice variant of Myo5a present in the brain is the form that contains exons A, B, C, E and G (hereafter denoted 5aExonABCEG) (Fig. 5C), which is important for the recognition of specific cargo and the association with Rab proteins (Seperack et al., 1995; Huang et al., 1998; Roland et al., 2009). Therefore, we constructed a plasmid containing both 5aExonABCEG and the cargo-binding domain, which we named Myo5aECBD (Fig. 5C). Then, we performed co-immunoprecipitation assays, which showed that Myo5aECBD but not Myo5aCBD associated with Rab11 (Fig. 5D), suggesting that ExonABCEG in Myo5a is essential for the interaction between Rab11 and Myo5a. Both GTP- and GDP-bound forms of Rab11 exist in vivo. To investigate whether the GTP- or GDP-bound form of Rab11 showed different binding affinity with Myo5a, we performed co-immunoprecipitation assay of the wild-type (WT), the constitutively active GTP-bound form (Q70L) and the constitutively inactive GDP-bound form (S25N) of Rab11 with Myo5a. We found that the GTP-bound form of Rab11 had increased binding affinity with Myo5a whereas the GDP-bound Rab11 had reduced the binding affinity (Fig. 5E). To further confirm the role of Rab11 in the Myo5a-mediated recycling of TrkB-FL, we performed co-immunoprecipitation assays using exogenous TrkB-FL receptors and Myo5aECBD under Rab11 knockdown or overexpression conditions, and we found that the overexpression of Rab11 WT or the Q70L mutant enhanced the binding between Rab11 and Myo5a whereas Rab11 knockdown reduced their interaction (Fig. 5F), indicating that GTP-bound form of Rab11 enhances the interaction between TrkB-FL and Myo5a. As it has been reported that BDNF can significantly increase the levels of GTP-bound form of Rab11 (Lazo et al., 2013), we next investigated whether BDNF treatment could influence the endogenous interaction between Rab11 and Myo5a in neurons by performing a co-immunoprecipitation assay. The result revealed that BDNF enhanced the association between Rab11 and Myo5a (Fig. 5G,H).

**Identification of the key motif in Myo5a that mediates its association with Rab11**

We found that 5aExonABCEG was necessary for the Rab11-regulated association between TrkB-FL and Myo5aECBD, but the precise Rab11-interacting region in Myo5a remained unclear. To address this question, we first used a GST pulldown assay to examine whether the 5aExonABCEG region was also sufficient for the interaction between Rab11 and Myo5a. We observed that GST–5aExonABCEG could associate with Myc–Rab11 (Fig. 6A), which suggests that 5aExonABCEG is sufficient for the interaction.

We next tried to find the minimal region in Myo5a ExonABCEG that was responsible for the interaction with Rab11. First, we constructed mutants in which the Myo5a exon A, B, C, E or G regions were, respectively, deleted (5aExonΔA, ΔB, ΔC, ΔE and ΔG) and performed GST pulldown assays as above. We found that deleting Myo5a exon E (GST–5aExonΔE) led to the abolishment of the interaction with Rab11 (Fig. 6B), suggesting that Myo5a exon E is responsible for the interaction...
Fig. 4. See next page for legend.
Myo5a mediates the postendocytic recycling of TrkB-FL but not TrkB-T1. (A) Cultured hippocampal neurons were transfected with Flag–TrkB-FL or Flag–TrkB-T1 with siMyo5a or Myo5aSCBD as indicated. 594-M1 was used to label surface TrkB receptors. BDNF was added for 15 min followed by an EDTA wash to remove the surface-bound antibodies (Internalized, green). Neurons were then incubated with Cy5-conjugated secondary antibody for 45 min to detect the recycled TrkB (Recycled, red).

Representative, epifluorescence images of neurons with recycling are shown (see the Materials and Methods for details). Scale bar: 20 μm. (B) Quantitative analysis of recycled TrkB receptors as described in A. Graphs represent mean ± s.e.m. determined from results of more than three independent experiments (n = 25 cells for each condition per independent experiment). *P < 0.05 (one-way ANOVA followed by post hoc tests). (C) Schematic of the experiment to measure the levels of TrkB recycling levels using the cleavable surface biotinylation assay (see the Materials and Methods for details). (D) Lane 1 shows the 50% input of total biotinylated TrkB receptors at neuronal surface; lane 2 shows the efficiency of the stripping procedure; lane 3 shows the internalized biotinylated TrkB receptors; lane 4 shows the un-recycled TrkB receptors during the rewarming period. The efficiency of siMyo5a and the overexpression of Myo5aSCBD were detected by immunoblotting (IB) with anti-Myo5a antibody. Representative data from one lane (total group) are shown in the lower panel. (E) Densitometric quantitation of recycled TrkB receptors levels in D. Bar graphs represent mean ± s.e.m. (n = 4). *P < 0.05, one-way ANOVA followed by post hoc tests. (F) Cultured hippocampal neurons infected with control or siMyo5a lentivirus were serum starved overnight and labeled with biotin. After BDNF stimuli for 15 min, neurons were changed into fresh medium at 37˚C for 45 min. Then, remained surface TrkB-FL were detected by immunoblotting. Representative data are shown. (G) Densitometric quantitation of remained biotinylated TrkB-FL levels in F. Bar graphs represent mean ± s.e.m. (n = 3). **P < 0.05 (one-way ANOVA followed by post hoc tests).

Inhibiting the association between Myo5a and TrkB by use of TAT–5aExonE55–66 reduced the recycling of TrkB-FL

Because TAT–5aExonE55–66 inhibited the interaction between Myo5a and TrkB-FL, we first performed the live-cell ratiometric fluorescence-based recycling assay as described above to determine whether TAT–5aExonE55–66 influenced the postendocytic recycling of TrkB-FL (Fig. 7A). In our previous studies, we observed that postendocytic recycling contributed to the translocation of TrkB-FL receptors to the synapse, thereby contributing to the BDNF-induced synaptic plasticity (Huang et al., 2013). It has been reported that synaptic TrkB plays an important role in synaptic transmission and LTP (Poo, 2001), suggesting that the distribution of TrkB-FL in dendritic spine would be particularly important. Therefore, we measured the recycling levels of TrkB-FL in the total cell, soma and dendrites separately. We used MAP2 staining to show the dendrites. Quantification of this assay revealed that TrkB recycling in neurons occurred mostly in dendrites (Fig. 7B, TAT–scramble, dendrites compared to soma). TAT–5aExonE55–66 significantly reduced the levels of TrkB-FL recycling compared to the TAT–scramble group, especially in the dendrite group (Fig. 7B). Next, we performed the surface biotinylation assay to analyze the surface levels of TrkB-FL after BDNF stimulation upon pretreatment with TAT–scramble or TAT–5aExonE55–66 (Fig. 7C). Quantification of this experiment revealed that TAT–5aExonE55–66 significantly reduced the surface levels of TrkB-FL after BDNF stimulation compared to the scramble group (Fig. 7D, from ~0.46 to ~0.28). In combination with the results above, we concluded that TAT–5aExonE55–66 inhibits the Myo5a-mediated postendocytic recycling of TrkB-FL by reducing the association between TrkB-FL and Myo5a, therefore decreasing the surface levels of TrkB-FL after BDNF stimulation.

Myo5a-mediated postendocytic recycling of TrkB-FL contributes to the BDNF-triggered translocation of TrkB-FL into dendritic spines and sustained downstream signaling

Our previous studies have shown that BDNF-dependent recycling facilitates TrkB-FL translocation into dendritic spines (Huang et al., 2013). To determine whether Myo5a-mediated postendocytic recycling of TrkB-FL was involved in this process, cultured hippocampal neurons were transfected with GFP to show the spine shape, and endogenous TrkB-FL was visualized by immunocytochemistry after stimulation with BDNF for 60 min (Fig. 8A). Monensin (10 μM) was used as an inhibitor of endocytic recycling. Quantification of TrkB-FL localization in spines indicated that BDNF facilitated TrkB-FL uptake into spines, whereas monensin could significantly block this effect (Fig. 8B). Moreover, TAT–5aExonE55–66 could also block this process compared to the TAT–scramble condition (Fig. 8B). These results revealed that the Myo5a-mediated postendocytic recycling of TrkB-FL contributes to the translocation of the recycled TrkB-FL to dendritic spines.

ERK1/2 and PI3K–Akt are well-established downstream targets of TrkB-FL activation. We have previously shown that the Rab11-dependent recycling of TrkB-FL affects the sustained activation of TrkB-FL but does not affect transient TrkB-FL activation or the downstream ERK1/2 and Akt signaling (Huang et al., 2013). Given that 5aExonE55–66 could associate with Rab11 and Myo5a (Fig. 6D), demonstrating that this region is sufficient for the interaction between Rab11 and Myo5a. Next we assessed whether 5aExonE55–66 was also sufficient for this interaction. GST–5aExonE55–66 could pull down Rab11 but not Rab3a (Fig. 6D), demonstrating that this region is sufficient for the interaction between Rab11 and Myo5a.

Given that 5aExonE55–66 could associate with Rab11 in the GST pulldown assay, we next investigated whether 5aExonE55–66 could block the interaction between Rab11 and Myo5a in a competitive manner. We therefore fused the 5aExonE55–66 peptide with biotin and the membrane permeability domain (TAT, GRTRRRRRRRR) at the N-terminus (TAT–5aExonE55–66), using a TAT–scramble peptide as a control. We prestreated cultured hippocampal neurons with TAT–scramble or TAT–5aExonE55–66 (1 μM) for 30 min and performed co-immunoprecipitation assay for endogenous Rab11 and Myo5a. The association between endogenous Rab11 and Myo5a was clearly blocked by TAT–5aExonE55–66 compared with the scrambled peptide (Fig. 6E), demonstrating that TAT–5aExonE55–66 is sufficient to block the association between Myo5a and Rab11. Next, we examined whether this inhibition affected the interaction between TrkB-FL and Myo5a. By comparing the amount of Myo5a immunoprecipitated with TrkB from cultured hippocampal neurons in the presence of TAT–scramble or TAT–5aExonE55–66 (Fig. 6F), we found that TAT–5aExonE55–66 could significantly weaken the interaction between Myo5a and TrkB receptors (Fig. 6G). All of these results indicate that 5aExonE55–66 can inhibit the interaction between TrkB-FL and Myo5a. Next, we constructed several deletion constructs within 5aExonE to identify the amino acid residues that were crucial for this association (Fig. 6C left, 5aExonEΔ1–Δ5). When the last 12 amino acid residues in 5aExonE were deleted, the association between 5aExonE and Rab11 disappeared (GST–5aExonEΔ5 in Fig. 6C right), suggesting that these 12 amino acids (we named this region 5aExonE55–66) were necessary for the interaction between Rab11 and Myo5a. Next we assessed whether 5aExonE55–66 was also sufficient for this interaction. GST–5aExonE55–66 could pull down Rab11 but not Rab3a (Fig. 6D), demonstrating that this region is sufficient for the interaction between Rab11 and Myo5a.

These results revealed that the Myo5a-mediated postendocytic recycling of TrkB-FL contributes to the BDNF-triggered translocation of TrkB-FL into dendritic spines and sustained downstream signaling.
et al., 2013). Therefore, we investigated whether the Myo5a-mediated postendocytic recycling of TrkB-FL could also affect BDNF–TrkB signaling. We chose two time points (5 min and 60 min) to distinguish between transient and sustained signaling.

BDNF-triggered TrkB-FL phosphorylation and its downstream signaling events (phosphorylation of Akt1 or ERK1/2) under different conditions in cultured hippocampal neurons were detected by immunoblotting (Fig. 8C,E). Quantification of these
results revealed that the sustained but not the transient TrkB-FL activation and downstream ERK and Akt signaling were reduced by TAT–5aExonE 55–66 (Fig. 8D,F), suggesting that the Myo5a-mediated postendocytic recycling of TrkB-FL contributes to the maintenance of prolonged BDNF downstream signaling. All of these results confirm that the Myo5a-mediated postendocytic recycling of TrkB-FL is important for the BDNF-triggered translocation of TrkB-FL to dendritic spines and for sustained signaling downstream of BDNF.

DISCUSSION

The recycling of TrkB-FL is closely regulated and can be reprogrammed under different physiological conditions; these processes can regulate the synaptic translocation of TrkB and signaling downstream of BDNF (Huang et al., 2013). For instance, more TrkB-FL is recycled back to the plasma membrane under LTP conditions. However, the motor protein mediating the postendocytic recycling of TrkB receptors remained unclear. In this study, we demonstrate that Myo5a mediates the postendocytic recycling of TrkB-FL but not TrkB.T1. Moreover, we found that Rab11 regulates the association between TrkB-FL and Myo5a. Finally, we found that the Myo5a-mediated postendocytic recycling of TrkB-FL contributes to the translocation of TrkB-FL into dendritic spines and to the sustained downstream signaling after BDNF stimulation, suggesting that the Myo5a-mediated postendocytic recycling of TrkB-FL plays important roles in BDNF function.

Our results provide several new insights into the mechanism and significance of postendocytic recycling of TrkB-FL. First, we report that the motor protein Myo5a mediates the intracellular trafficking of TrkB-FL in postendocytic recycling but does not mediate its internalization. It has been reported that kinesin-1 and dynein are involved in TrkB anterograde and retrograde transport, respectively (Yano et al., 2001; Arimura et al., 2009; Huang et al., 2011). The internalization of TrkB has been reported to be mediated by myosin VI (Yano et al., 2006). However, the motor proteins mediating the postendocytic recycling of TrkB remained unknown. We found that Myo5a could interact with TrkB-FL but not TrkB.T1 in a BDNF-dependent manner. Myo5a loss-of-function studies using siRNA or a dominant-negative construct revealed that Myo5a serves as the motor protein for TrkB postendocytic recycling. Because Myo5a did not associate with TrkB-FL without kinase activity, we speculated that Myo5a does not mediate the biosynthesis and initial membrane insertion of TrkB. Our results, together with those from previous reports, indicate that the trafficking of TrkB in different subcellular compartments is differentially regulated by different motor proteins. This is conducive to the precise regulation of the intracellular distribution of TrkB receptors. Moreover, our previous work has shown that TrkB-FL is sorted to a ‘slow recycling pathway’, whereas TrkB.T1 is sorted to a ‘fast recycling pathway’ (Huang et al., 2013). Here, we found that Myo5a associated with TrkB-FL but not with TrkB.T1, suggesting that motor proteins also play crucial roles in the selection among different recycling pathways for TrkB receptors. Here, we proved that Myo5a mediates the postendocytic recycling of TrkB-FL, but the motor protein that mediates the recycling of TrkB.T1 requires further investigation. These results indicate that different isoforms of TrkB receptors, which are recycled through different pathways, could be transported by different motors. Myosin Vb (Myo5b), another isoform of myosin V, has been reported to participate in BDNF-induced dendriticbranching (Lazo et al., 2013), suggesting that Myo5b might also regulate TrkB intracellular trafficking. However, the detailed role of Myo5b in mediating TrkB intracellular trafficking, perhaps through the biosynthetic or postendocytic trafficking pathway, requires further investigation. Here, our results show evidence of Myo5a specifically mediating the postendocytic recycling of TrkB-FL, further elucidating the mechanism of TrkB recycling in neurons.

Furthermore, we found that Rab11 could interact with Myo5a through the 5aExonE 55–66 region and that Rab11 regulated the association between Myo5a and TrkB-FL. Rab11 has been reported to associate with Myo5a. We present the first evidence of a specific region, 5aExonE 55–66, in Myo5a as necessary and sufficient for the interaction between Myo5a and Rab11. We also demonstrate that TAT–5aExonE 55–66 can block the association between Rab11 and Myo5a. In our previous work, we observed that Rab11 mediated the recycling of TrkB-FL (Huang et al., 2013); however, the specific function of Rab11 in TrkB-FL recycling remains unclear. Here, we found that Rab11 could modulate the interaction between TrkB-FL and Myo5a. Blocking the interaction between Rab11 and Myo5a using TAT–5aExonE 55–66 weakened the association between TrkB-FL and Myo5a and therefore decreased the level of TrkB-FL recycling. All of these results revealed that the association between Myo5a and TrkB-FL is essential for TrkB-FL recycling, and they further suggest that Myo5a mediates the postendocytic recycling of TrkB-FL.

In our previous studies, we found that LTP facilitated the recycling of TrkB-FL (Huang et al., 2013). It has been reported that the activity of Myo5a is increased under LTP conditions, primarily owing to the increased Ca 2+ concentration. Increased intracellular Ca 2+ can directly bind to and activate Myo5a. Signaling downstream of Ca 2+, including that mediated by calmodulin-dependent kinase II (CaMKII), can also regulate the activity of Myo5a (Nascimento et al., 1996; Li et al., 2004; Wang et al., 2004; Lu et al., 2006). Moreover, more Rab11 is activated...
Fig. 6. Identification of the key motif in Myo5a that mediates the association with Rab11. (A) Lysates from HEK293 cells overexpressing Myc–Rab11 were incubated with GST or GST–ABCEG beads overnight. The beads were then washed, and bound proteins were detected by immunoblotting (IB) using anti-Myc antibody. GST-fusion beads were detected by Coomassie blue staining. (B) Lysates from HEK293 cells overexpressing Myc–Rab11 were incubated with GST–5aExonABCEG mutant (5aExonΔA–5aExonΔG)-fused beads as indicated overnight. The GST pulldown assay was performed as described above. (C) Left, schematic of 5aExonE deletion mutants (5aExonEΔ1–5aExonEΔ5). EFL, full-length 5aExonE, which contains 66 amino acids. Right, the GST pulldown assay, performed as described above. (D) Lysates from HEK293 cells overexpressing Myc–Rab11 or HA–Rab3a were incubated overnight with GST, GST–5aExonE or GST–5aExonE55–66 beads, as indicated. The GST pulldown assay was performed as described above. (E) Cultured hippocampal neurons were serum starved overnight and pretreated with TAT–scramble (1 μM) or TAT–5aExonE 55–66 (1 μM) for 30 min. Then, BDNF was added for 60 min before cells were lysed. Cell lysates were immunoprecipitated with anti-Rab11 antibody. A preimmune IgG (mouse) was used as a negative control. (F) Cultured hippocampal neurons were serum starved overnight and pretreated with TAT–scramble (1 μM) or TAT–5aExonE 55–66 (1 μM) for 30 min. Then, BDNF was added for 60 min before cells were lysed. Cell lysates were immunoprecipitated with anti-TrkB antibody. A preimmune IgG (Rabbit) was used as a negative control. (G) Densitometric quantitation of Myo5a co-immunoprecipitation with TrkB in F, normalized to scramble. Bar graphs represent mean ± s.e.m. (n=3). *P<0.05 (Student’s t-test). All of these experiments (A–G) were repeated at least three times independently and representative data are shown.
Fig. 7. Inhibiting the association between Myo5a and TrkB by TAT-5aExonE55–66 reduces the recycling of TrkB-FL. (A) Cultured hippocampal neurons were transfected with Flag–TrkB-FL 48 h before the experiment. Alexa-Fluor-488-conjugated mouse anti-Flag M1 (488-M1) antibody was used to label surface Flag–TrkB-FL receptors. TAT–scramble (1 μM) or TAT–5aExonE55–66 (1 μM) was added for 30 min. Then, the postendocytic recycling assay was performed as described in Fig. 4A. MAP2 was used to label the dendrites after the recycling assay was performed. Representative, epifluorescence images of neurons in the recycling groups are shown. Scale bar: 20 μm. (B) Quantitative analysis of TrkB-FL recycling levels in soma and dendrites in A. Graphs show mean ± s.e.m. determined from the results of more than three independent experiments (n>25 cells for each condition per independent experiment). * P<0.05 (one-way ANOVA followed by post hoc tests). (C) Cultured hippocampal neurons were serum starved overnight and pretreated with TAT–scramble (1 μM) or TAT–5aExonE55–66 (1 μM) for 30 min. Then, neurons were stimulated with BDNF (50 ng/ml) for 60 min followed by labeling with biotin. Next, neurons were lysed, and the lysates were pulled down with anti-α-tubulin antibody, and pulled down proteins were detected by anti-TrkB immunoblotting (IB). (D) Normalized densitometric quantification of the results in C. Bar graphs represent mean ± s.e.m. (n=3). * P<0.05 versus TAT-scramble No BDNF group; ** P<0.01 versus TAT–5aExonE55–66 no BDNF group; # P<0.05 (one-way ANOVA followed by post hoc tests).
neuronal development and synaptic plasticity, including in spine formation and LTP (Korte et al., 1995; Patterson et al., 1996; Minichiello et al., 2002). Here, we used TAT–5aExonE\textsuperscript{55–66} to inhibit the postendocytic recycling of TrkB-FL and found impairments in TrkB-FL translocation to spines and the maintenance of BDNF downstream signaling, which revealed that the Myo5a-mediated recycling of TrkB-FL was crucial for BDNF–TrkB signaling and further suggested that, to some extent, the impaired synaptic plasticity in Myo5a-defective neurons might be due to the deficiency in TrkB recycling. This provides another insight into the importance of Myo5a in the nervous system and indicates that Myo5a-mediated recycling of TrkB-FL is important for functional synapses in the nervous system.

Fig. 8. See next page for legend.
Plasmid constructs and siRNA
Plasmid constructs of rat TrkB-FL, TrkB.T1, TrkB mutant constructs, Myc–Rab11, HA–Rab3a were kept in our laboratory (Zhao et al., 2009; Huang et al., 2013). Constructs of the brain-spliced isoform of Myo5a (NCBI, XM_006510832) mutants described below were subcloned from cDNA from mouse brain. Myo5aCBD (amino acids 1420–1828) was a gift from José A Esteban (Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas/Universidad Autónoma de Madrid, Spain); Myo5a ExxonABCEG (amino acids 1208–1419) was as described previously (Seperack et al., 1995). All GST fusion plasmids were subcloned into the pGEX4T-1 expression vector with a GST tag at the N-terminus. Myo5aCBD and siMyo5a were separately subcloned into the pUltra vector (Addgene) to generate lentiviruses. All of the constructs made by PCR were confirmed by DNA sequencing (Genewiz, Beijing, China) to exclude potential PCR-introduced mutations. Target sequences for siRab11 using specific siRNA oligonucleotides are as follows: rat Rab11a, 5′-GGGAGACGUCCUAGAUGG-3′; rat Rab11b, 5′-GAAACGAGUCCUACCCAGA-3′. Target sequences for siMyo5a are as follows: rat Myo5a, 5′-CGCUACAGAGCUCUCAUA-3′.

Hippocampal neuron transfection
Neurons were transfected using Lipofectamine RNAiMAX reagent for RNA oligonucleotides or Lipofectamine 2000 transfection reagent for plasmids following the manufacturer’s instructions (Invitrogen). The hippocampal neurons were cultured for 10–14 days in vitro (DIV) or 18–21 DIV for the experiments as indicated below in neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, and 100 units/ml penicillin-streptomycin. The experiments were performed at 48 or 72 h after transfection as indicated.

Lentivirus infection, TAT–scramble and TAT–5aExonE55–66 pretreatment was performed by infecting neurons with the indicated lentivirus for 6 h, after which the medium was replaced with fresh medium. All experiments were performed 5 days after infection. TAT–scramble and TAT–5aExonE55–66 were applied for 30 min before the experiments were performed.

Biotinylation assay
For assessing the internalization of TrkB receptors, cultured hippocampal neurons (10–14 DIV) were infected with lentivirus and incubated for 5 days. Lentivirus expressing GFP was used as a control. After serum starvation overnight, neurons were labeled with sulfo-NHS-SS-biotin and then treated with vehicle (Fig. 3E, lane 1 and 2) or BDNF (50 ng/ml, Fig. 3E, lane 3) for 15 min. Afterward, the remaining surface biotin was removed using stripping solution (0.01 g/ml BSA, 50 mM glutathione, 100 units/ml penicillin-streptomycin). The experiments were performed at 48 or 72 h after transfection as indicated.

Lentivirus infection, TAT–scramble and TAT–5aExonE55–66 pretreatment was performed by infecting neurons with the indicated lentivirus for 6 h, after which the medium was replaced with fresh medium. All experiments were performed 5 days after infection. TAT–scramble and TAT–5aExonE55–66 were applied for 30 min before the experiments were performed.

Materials and methods
Label-free quantitative mass spectrometry
Cultured hippocampal neurons were labeled with TrkB antibodies after being serum starved overnight. Then neurons were treated with BDNF (50 ng/μl) for 5 or 60 min. Neurons were lysed for a Protein A pulldown assay. The immunoprecipitates were sent for label-free quantitative mass spectrometry. Some of the unpublished mass spectrometry data will be prepared for publication elsewhere. We confirm that there is no overlap of the mass spectrometry data used here and the data to be submitted elsewhere.

Reagents and antibodies
Goat anti-TrkB antibody was purchased from R&D (Minneapolis, MN). Rabbit anti-TrkB (C-14) and mouse anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-TrkB antibody was obtained from Millipore (San Jose, CA). Rabbit anti-Rab11 antibody was purchased from BD Biosciences Pharringen (San Jose, CA). Rabbit anti-Myo5a, rabbit anti-ERK1/2, and mouse anti-ERK1/2 and rabbit anti-phospho-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). TAT–scramble and TAT–5aExonE55–66 peptides were synthesized by GL Biochem Ltd (Shanghai, China). The scramble (QHETRTNLTE) and 5aExonE55–66 (QHETRLTENL) peptides were fused to cell-permeable TAT (GRTRRRRRRRRRR). Biotin and avidin beads were obtained from Thermo (Rockford, IL).

In summary, our studies provide evidence that Myo5a mediates the postendocytic recycling of TrkB-FL. The region 5aExonE55–66 in Myo5a is responsible for the interaction between Rab11 and Myo5a. The Myo5a-mediated recycling of TrkB-FL is important for the translocation of TrkB-FL into dendritic spines and for the maintenance of BDNF downstream signaling (supplementary material Fig. S2). Not only do these results provide new insights into the mechanism of TrkB trafficking, they also suggest us a mechanism by which BDNF–TrkB and Myo5a affect neuronal synaptic plasticity through recycling and thus contribute to higher-order behaviors.


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were lysed with TNE as described above. TrkB-FL pulled down by avidin beads was detected by SDS-PAGE and immunoblotting.

For assessing the surface levels of TrkB receptors after BDNF stimulation (Fig. 7C), cultured hippocampal neurons (10–14 DIV) were infected with lentivirus and incubated for 5 days. After serum starvation overnight, BDNF (50 ng/ml) was added for 60 min. Subsequently, surface proteins were labeled with biotin at 4˚C, and neurons were then lysed with TNE as described above. TrkB-FL pulled down by avidin beads was detected by SDS-PAGE and immunoblotting.

Fluorescence ratio microscopy analysis of the levels of TrkB receptor internalization and postendocytic recycling

The fluorescence images described below were acquired with an Eclipse TE2000-U (Nikon) using an HQ2 cooled CCD camera and a 60x objective lens. The plasmids for both siMyo5a and Myo5aCBD were GFP tagged, and we only captured images of cultured hippocampal neurons positive for GFP fluorescence; GFP images are not shown. Lentivirus expressing GFP was used as a control.

The internalization of TrkB-FL was assessed as described previously (Kotowski et al., 2011). Cultured hippocampal neurons (10–14 DIV) were transfected with the indicated constructs (Flag−TrkB-FL construct, Flag epitope fused to the N-terminal, extracellular) 48 h before. Neurons were serum starved overnight. Then, the total pool of TrkB-FL initially present at the cell surface was labeled with anti-Flag antibody (M2) conjugated to Alexa Fluor 594 (594-M2) at 4˚C for 15 min. Afterwards, neurons were stimulated with BDNF (50 ng/ml) at 37˚C for 15 min. Then, neurons were fixed with 4% PFA and stained under permeabilizing conditions. The ratio of Cy5 to Alexa Fluor 594 fluorescence was calculated. The mean ratio of the internalized group (I) represents the un-international TrkB-FL. For each experiment, there was a parallel control group (data not shown) in which neurons were fixed immediately after the 15 min incubation with 594-M2 and labeled with Cy5-conjugated anti-mouse IgG antibody, and the mean Cy5 to Alexa Fluor 594 ratio of this group (C) represents the 100% surface TrkB-FL. Thus, the percentage of internalized TrkB receptors was calculated using the following formula: (1−I/C)×100.

The level of TrkB recycling was assessed as described previously (Huang et al., 2009). Cultured hippocampal neurons (10–14 DIV) were transfected with the indicated constructs using Lipofectamine 2000 at 48 h before the recycling experiment. Neurons were serum starved overnight and then incubated with anti-Flag antibody (M1, calcium sensitive) conjugated to Alexa Fluor 594 (594-M1) at 4˚C for 15 min. Then, neurons were stimulated with BDNF (50 ng/ml) at 37˚C for 15 min followed by three quick washes with cold PBS containing EDTA (1 mM) to dissociate the 594-M1 bound to un-internalized TrkB receptors. Next, neurons were incubated in fresh medium containing Cy5-conjugated anti-mouse-IgG secondary antibody at 37˚C for 45 min to label recycled receptors with 594-M1. Then, neurons were immediately fixed. Images were acquired using epifluorescence microscopy. The Cy5 to Alexa Fluor 594 fluorescence ratio was calculated using MetaMorph software. The mean Cy5 to Alexa Fluor 594 ratio of the recycling group described above is defined as E. For each experiment, two parallel control groups (data not shown) were assessed: in the first, neurons were fixed immediately after the 15 min incubation with 594-M1 and subsequently labeled with Cy5-conjugated anti-mouse-IgG antibody, the 100% surface control, with the mean ratio of this group designated as C; in the second, neurons were fixed immediately after the EDTA-stripping step and then labeled with Cy5-conjugated anti-mouse-IgG antibody, as the 0% recycled control, and the mean ratio of this group is designated as Z. Thus, the percentage of recycled TrkB receptors was calculated using the following formula: (E−Z)/(C−Z)×100.

GST pulldown assay

All of the GST fusion proteins were expressed using pGEX4T-1 in the BL21 Escherichia coli (DE3 strain; Novagen). Then, BL21 cells were lysed and immobilized on glutathione-Sepharose-4B beads (GE Healthcare). The purified GST fusion proteins were incubated with lysates from HEK293 cells expressing the indicated plasmid constructs (electroporated) at 4˚C overnight. After three or four washes with TNE buffer, the bound proteins were detected using SDS-PAGE and immunoblotting with the indicated antibodies.

Localization analysis of internalized TrkB receptors in spines using fluorescence microscopy

This assay was performed as described previously (Huang et al., 2013). Cultured hippocampal neurons (10 DIV) were transfected with constructs expressing GFP. Neurons were then raised to 18–21 DIV and serum starved overnight, followed by pretreatment with monensin (10 μM), TAT−scramble or TAT−5aExon55−66 for 30 min. BDNF was added for 60 min. Images were captured using a Zeiss LSM780 confocal microscope with a 63x objective lens (Microstructural Platform of Shandong University).

Quantification of colocalization between TrkB-FL and Myo5a

Cultured hippocampal neurons (10–14 DIV) were serum starved overnight and stimulated with BDNF for 0 or 60 min. Then neurons were fixed immediately after the EDTA-stripping step and then incubated with Alexa Fluor 405, TrkB-FL was labeled with Alexa Fluor 594 and Myo5a was labeled with Alexa Fluor 488. The same threshold was set to optimize the representation of puncta in the 488 and 594 nm channels. Random dendrites with a length of ~20 μm and with a distance of more than 20 μm away from the cell body were chosen for quantification. Dendrites were circled as the region of interest (ROI). The puncta in the 594 channel (total TrkB-FL) were labeled with ‘1’, and then the images were combined with the 488 channel (total Myo5a) to determine overlaps. The overlaps were labeled with ‘2’ to show the colocalization of TrkB with Myo5a. The percentage of TrkB puncta colocalized with Myo5a puncta relative to total number of TrkB puncta in the same image was calculated as the number labeled 1 divided by the number labeled 2. Three independent experiments were performed; n>15 dendrites from 5–6 cells for each condition were used per independent experiment.

Statistical analysis

All statistical analyses were calculated using SPSS software. Comparisons of multiple groups were performed using one-way ANOVA followed by post hoc tests. Comparisons of two groups were performed with the Student’s t-test. The percentage of TrkB-FL translocation to spines was assessed by using a χ² analysis. Data are presented as the mean±s.e.m., and the significance was set at P<0.05.

Competing interests

The authors declare no competing or financial interests.

Author contributions

W.H.S., S.H.H. and Z.Y.C. designed the study. W.H.S., S.H.H. and Z.Y.C. wrote the manuscript. J.W. carried out the experiment of recycling of TrkB in Fig. 4A. Q.C. and T.L. carried out the GST-pulldown assay. W.H.S. performed the rest experiments and data analysis. All authors approved the final manuscript.

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Figure S1. HEK293 expressing TrkB-FL, T1 or T1TK were serum starved overnight and cell lysates were immunoprecipitated by anti-Flag antibody and detected by pY99 (anti-Tyr antibody). The downstream ERK and Akt signaling pathways were also detected by their respective antibodies as indicated. Overexpressing TrkB-FL or T1TK in HEK293 led to transphosphorylation of themselves and significantly activated ERK and PI3K signaling pathway. This explained why there was no BDNF in HEK293 medium but there was association between overexpressed TrkB-FL and Myo5aCBD.
Figure S2. (A) Schematic diagram of Myo5a mediating postendocytic recycling of TrkB-FL in dendritic spines. Internalized TrkB-FL receptors are transported to sorting endosomes. Some of the internalized TrkB-FL receptors are sorted to degradation and retrograde transport pathways while the rest are sorted to the recycling pathway in which the postendocytic recycling of TrkB-FL is mediated by Myo5a. (B) Schematic diagram of Myo5a-Rab11-TrkB-FL complex. TrkB-FL associated with cargo binding domain of Myo5a under BDNF stimulation. Rab11 associated with ExonE\textsuperscript{55-66} motif in Myo5a. The association of Rab11 with Myo5a facilitated the binding between TrkB-FL and Myo5a.