Dynein activator Hook1 is required for trafficking of BDNF-signaling endosomes in neurons

Mara A. Olenick1,2,3,4, Roberto Dominguez1,2,3, and Erika L.F. Holzbaur1,2,3

Axonal transport is required for neuronal development and survival. Transport from the axon to the soma is driven by the molecular motor cytoplasmic dynein, yet it remains unclear how dynein is spatially and temporally regulated. We find that the dynein effector Hook1 mediates transport of TrkB–BDNF-signaling endosomes in primary hippocampal neurons. Hook1 comigrates with a subpopulation of Rab5 endosomes positive for TrkB and BDNF, which exhibit processive retrograde motility with faster velocities than the overall Rab5 population. Knockdown of Hook1 significantly reduced the motility of BDNF-signaling endosomes without affecting the motility of other organelles. In microfluidic chambers, Hook1 depletion resulted in a significant decrease in the flux and processivity of BDNF-Qdots along the mid-axon, an effect specific for Hook1 but not Hook3. Hook1 depletion inhibited BDNF trafficking to the soma and blocked downstream BDNF- and TrkB-dependent signaling to the nucleus. Together, these studies support a model in which differential association with cargo-specific effectors efficiently regulates dynein in neurons.

Introduction
Axonal transport is vital for the maintenance and survival of neurons. Axons have a uniformly polarized microtubule array in which the faster-growing plus ends of microtubules are oriented toward the distal terminal. These microtubules serve as a highway for fast organelle trafficking mediated by molecular motors. While multiple plus end–directed kinesins are responsible for delivery of cargo to the distal end of the axon, the minus end–directed motor cytoplasmic dynein is solely responsible for trafficking a wide variety of cargo back to the soma including autophagosomes, endosomes, and mitochondria (Maday et al., 2014). These organelles not only differ in their lipid and protein compositions, but they also display distinct motility properties. It remains unclear how cytoplasmic dynein attaches to each of its cargos and how the motor is regulated to facilitate the precise trafficking of organelles to the soma.

Cytoplasmic dynein 1 (referred to in this study as dynein) is a 1.4-MD AAA+ motor complex that drives the majority of minus end–directed motility in the cell. Alone, dynein is a flexible dimer with low processivity, taking many sideways or backward steps along the microtubule lattice (Reck-Peterson et al., 2006; Ross et al., 2006). Processive motility is enhanced when dynein binds to dynactin, a 1-MD multisubunit complex that recruits two dynein dimers to a single dynactin, which further stabilizes the dynein–dynactin-effector complex. Some dynein effectors can enhance the force and velocity of the motor complex (Grotjahn et al., 2015) and the dynein light intermediate chain 1 (LIC1; Schroeder and Vale, 2016; Redwine et al., 2017). BICD2, the best characterized of these dynein effectors, has been shown to increase the affinity of dynein–dynactin interaction and induce superprocessive motility (McKenney et al., 2014; Schlager et al., 2014; Olenick et al., 2016; Schroeder and Vale, 2016; Redwine et al., 2017). BICD2 also interacts with the N-terminal tail of the dynein heavy chain (Chowdhury et al., 2015; Urnavicius et al., 2015) and the dynein light intermediate chain 1 (LIC1; Schroeder et al., 2014; Lee et al., 2018), leading to a stabilization of the dynein–dynactin-effector complex. Some dynein effectors can recruit two dynein dimers to a single dynactin, which further enhances the force and velocity of the motor complex (Grotjahn et al., 2018; Urnavicius et al., 2018).

Hook proteins (HookA or Hok1) are dynein effectors first characterized in filamentous fungi and shown to link dynein to early endosomes (Bielska et al., 2014; Zhang et al., 2014). In

1Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; 2The Pennsylvania Muscle Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; 3Biochemistry and Molecular Biophysics Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA; 4Center for Engineering Mechanobiology, University of Pennsylvania, Philadelphia, PA.

Correspondence to Erika L.F. Holzbaur: holzbaur@pennmedicine.upenn.edu.

© 2018 Olenick et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms/). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 International license, as described at https://creativecommons.org/licenses/by-nc-sa/4.0/).
mammalian cells, three highly conserved Hook proteins are expressed: Hook1, Hook2, and Hook3. These proteins are characterized by an N-terminal Hook domain, which binds LIC1 of dynein (Schroeder and Vale, 2016; Lee et al., 2018). The Hook domain is followed by a central coiled-coil region and a less well-conserved C-terminal cargo-binding domain (Bielska et al., 2014; Zhang et al., 2014). In vitro studies show that the binding of either Hook1 or Hook3 enhances the dynein–dynactin interaction, leading to significant increases in velocity and run lengths (McKenney et al., 2014; Olenick et al., 2016; Schroeder and Vale, 2016).

While Hook1 and Hook3 have been identified as dynein activators in vitro, the role of these proteins in dynein-mediated cargo transport in mammalian cells is less clear. Hook2 has been linked to centrosomal function and homeostasis (Szebenyi et al., 2007; Guthrie et al., 2009; Moynihan et al., 2009), while Hook1 and Hook3 have been implicated in a variety of endosomal trafficking pathways, although there is still no clear consensus on the specific roles of each isoform (Luiro et al., 2004; Xu et al., 2008; Maldonado-Báez et al., 2013). The highly polarized nature and spatial compartmentalization of neurons provide an excellent system to study the role of Hook proteins in endosomal transport. Initial work from Guo et al. (2016) suggested that Hook1 and Hook3 colocalize with retrograde Rab5a vesicles in hippocampal neurons and that knockdown (KD) of Hook1 and Hook3 reduced the retrieval of transferrin receptor from the axon (Guo et al., 2016). These data support a potential role for Hook proteins in dynein-mediated trafficking in axons, prompting us to investigate this question in more detail.

In this study, we investigated the role of Hook1 in the dynein-driven transport of endosomes along the axons of hippocampal neurons. We found that Hook1 comigrates with subpopulations of Rab5- and Rab7-positive endosomes. While loss of Hook1 did not significantly change the overall motility of Rab5- or Rab7-positive endosomes, Hook1 siRNA depletion significantly reduced the motility of a specific endosomal compartment that we identified as TrkB–brain-derived neurotrophic factor (BDNF)-signaling endosomes. The motility of TrkB–BDNF-signaling endosomes is also lost if the interaction of Hook1 with dynein is disrupted by targeted mutations at the Hook1-LIC1 interface. In addition, Hook1 is enriched in the distal axon, distinct from the cellular distribution of other dynein effectors like Rab-interacting lysosomal protein (RILP) or BICD2, suggesting a specific function in trafficking from the distal axon. Using microfluidic chambers to model the distal axonal transport of BDNF-signaling endosomes, we found that KD of Hook1 significantly reduced the flux and the processivity of BDNF transport from the distal axon to the soma. In contrast, KD of Hook3 did not affect BDNF uptake or transport. Loss of Hook1 also produced a functional block in downstream BDNF-dependent signaling to the nucleus, which is vital for neuronal survival and maintenance. Overall, this work supports a model in which Hook1 acts as a specific dynein effector for BDNF-signaling endosomes trafficking from the distal axon to induce downstream signaling in the soma of primary hippocampal neurons.

Results

Hook1 comigrates with the endosomal markers Rab5 and Rab7

To investigate the role of Hook1 in endosomal trafficking, we first expressed the early endosome marker Rab5-GFP or the late endosome marker Rab7-GFP in primary rat hippocampal neurons to observe endosomal motility. Neurons were imaged 7–8 d in vitro (DIV) at 48 h after transfection using live-cell confocal microscopy. Focusing on the axon, Rab5-GFP endosomes were found to be enriched in the distal axon, while Rab7-GFP endosomes were found throughout the distal and mid-axon. In the axon, Rab5-GFP endosomes were mainly stationary or bidirectional, with ∼80% moving <10 μm in any net direction (Fig. S1 B). In contrast, 50% of Rab7-GFP endosomes displayed net retrograde motility (Fig. S1 E).

Next, Hook1-Halo was coexpressed with Rab5-GFP or Rab7-GFP to assess whether Hook1 comigrates with a specific population of endosomes since previous studies had found Hook proteins on Rab5-positive endosomes (Bielska et al., 2014; Zhang et al., 2014; Guo et al., 2016). Surprisingly, we observed comigration of Hook1 with both a subpopulation of Rab5-endosomes and a subpopulation of Rab7 endosomes (Fig. 1, A and B). Previous in vitro research indicates that Hook1 enhances the processivity of dynein (Olenick et al., 2016), so we asked whether Hook1-positive endosomes displayed a distinctive retrograde bias in their motility or were significantly more processive than the overall population of axonal endosomes. We found that Hook1-positive Rab5 endosomes had an increased retrograde bias as seen by an increased retrograde:anterograde ratio as compared with neurons expressing Rab5-GFP only (Fig. 1, C and D). Hook1-positive Rab5 endosomes also showed an increase in retrograde-directed instantaneous velocity as compared with the overall population of Rab5-GFP-positive endosomes (Fig. 1 E). In comparison, Hook1-positive Rab7-endosomes had the same retrograde bias and retrograde-directed instantaneous velocities seen for the overall population of Rab7-GFP endosomes, where ∼50% of organelles displayed fast retrograde motility (Fig. 1, F–H). Overall, this analysis indicates that motile endosomes positive for Hook1 were primarily fast, retrogradely moving organelles, indicative of processive dynein-mediated motility.

Hook1 KD reduces the motility of BDNF-positive signaling endosomes

As Hook1 comigrates with a subpopulation of Rab5- and Rab7-positive endosomes, we next asked whether Hook1 depletion would alter the motility of these endosomes. We used a rat siRNA pool to KD Hook1 in cultured neurons; 60% depletion of Hook1 was observed in PC12 cells using this approach (Fig. S1, G and H). Rab5-GFP or Rab7-GFP was transfected along with Hook1 siRNA and imaged 48 h after transfection at DIV 7–8 along the mid- or distal regions of the axon. Hook1 KD did not induce significant differences in the motile fraction or the directionality of either Rab5- or Rab7-positive endosomes at a population level (Fig. S1, A–F). Hook1 KD did induce a trend toward decreased flux of Rab5 endosomes, but the effect was not significantly different than control (Fig. S1 C).
We hypothesized that Hook1 might play a more specific role in the transport of a subpopulation of neuronal endosomes, so we focused on signaling endosomes, which initiate distally and mature through the Rab5 and Rab7 endosomal pathway (Deinhardt et al., 2006; Ye et al., 2018). In neurons, neurotrophic factors such as BDNF bind to their transmembrane kinase receptors and are endocytosed to form signaling endosomes that undergo retrograde transport toward the nucleus, leading to changes in gene expression (Cosker and Segal, 2014; Scott-Solomon and Kuruvilla, 2018). To focus on a signaling endosomal population in neurons, we expressed the neurotrophic receptor TrkB-RFP and GFP-Hook1 and found that the labeled proteins comigrated in the axons of hippocampal neurons (Fig. 2A). Consistent with this observation, immunoisolation of TrkB vesicles from mouse brain lysates demonstrated the coprecipitation of Hook1 along with subunits of both dynein and dynactin (Fig. S2, B and C). In live neurons, we also found that GFP-Hook1 colocalized with BDNF-conjugated quantum dots (BDNF-Qdots; Fig. 2, C and D). Next, we investigated the motility of BDNF-Qdots in control or Hook1 KD neurons. Neurons with Hook1 KD displayed significantly reduced retrograde BDNF-Qdot motility compared with control neurons (Fig. 2, E and F). This motility defect could be rescued by expression of siRNA-resistant human Hook1-Halo (Fig. 2, E and F).

To assess whether the observed effects of Hook1 depletion were specific for signaling endosomes or instead represented a more generalized inhibition of organelle motility, we imaged mitochondrial motility as well as another highly processive retrograde cargo, LC3B-positive autophagosomes. Comparisons of the motility of mitochondria and autophagosomes in Hook1 KD and control neurons demonstrate that no differences were induced by Hook1 depletion (Fig. S1, G–L). Together with the observations that Hook1 depletion does not induce changes in the motility of the overall population of either Rab5-positive or Rab7-positive endosomes, these results indicate that Hook1 plays an essential and specific role in TrkB–BDNF–signaling endosome motility in axons.

A direct interaction of Hook1 with dynein is important for signaling endosome motility

Dynein subunit LIC1 interacts with several dynein effectors including BICD2, RILP, and FIP3 (Schroeder et al., 2014). Recent
studies have found that the Hook domain conserved in both Hook1 and Hook3 also mediates a direct interaction with LIC1 and is important for Hook-mediated dynein processivity (Schroeder and Vale, 2016; Lee et al., 2018). To determine whether the Hook1–LIC1 interaction is important for signaling endosome motility in neurons, we analyzed two constructs: Hook1(Q149A, I156A), based on previous mutations in Hook3 shown to diminish the interaction with LIC1 (Schroeder and Vale, 2016), and Hook1(M146D, I156D), based on our recent structure of a Hook3–LIC1 complex (Fig. 3, A and B; Lee et al., 2018). We used total internal reflection fluorescence (TIRF) microscopy to perform motility assays with single-molecule resolution to test these mutant constructs and found that both Hook1(Q149A, I156A) and Hook1(M146D, I156D) significantly inhibited dynein-driven motility along microtubules (Fig. 3, C and D).

Next, we tested whether mutating the binding interface of Hook1 and LIC1 would disrupt signaling endosome motility in hippocampal neurons. Similar to our results with Hook1 siRNA KD, BDNF-Qdot motility was significantly reduced in neurons expressing GFP-Hook1(Q149A, I156A) (Fig. 3, E and F). These results indicate that the interaction of Hook1 with LIC1 is important not only for in vitro motility but also during cargo transport of BDNF-signaling endosomes in neurons.

The C-terminal domain of the Hook proteins is thought to specify cargo binding. Comparisons of C-terminal Hook1 sequences from several species identify a high degree of sequence conservation (Fig. S3). There is more limited sequence conservation between Hook1 and Hook3, but we noted that immediately following a conserved sequence there was a pair of charged/polar residues in Hook1 not found within Hook3.
We engineered a double mutation to generate Halo-tagged Hook1(K672A,S673A) and asked whether this construct could rescue BDNF-Qdot motility in Hook1-depleted neurons. As shown in Fig. 3 G, this construct could not rescue signaling endosome motility. Next, we focused on a 10-aa residue insertion in the C-terminal domain of Hook1 relative to Hook3 and again mutated a pair of polar/charged residues to generate Hook1(S693A,K694A)-Halo. We tested this construct in KD-rescue experiments assaying for BDNF-Qdot motility and found no rescue (Fig. 3 H), further supporting the importance of the C-terminal domain of Hook1 in mediating organelle-specific dynein-driven motility.

Hook1 is enriched in the distal axon and is distinctly localized from other dynein effectors

There are now several dynein effectors implicated in the regulation of dynein-driven motility in cells (Kardon and Vale, 2009; Fu and Holzbaur, 2014; Reck-Peterson et al., 2018). We hypothesized that dynein effectors might display differential localizations in neurons, reflecting distinct roles in intracellular transport. To address this question, we individually expressed Hook1-Halo, RILP-GFP, and BICD2-GFP in hippocampal neurons for 48 h and then fixed the cells on DIV 7–8. Confocal z stacks were captured of the distal axon, mid-axon, and soma of neurons. Images were deconvolved, and the somal localization was scored as either punctate or cytoplasmic.
Over 80% of BICD2- and RILP-expressing neurons displayed an accumulation of these effectors in the soma, associated with either large puncta or clusters, while the distribution of Hook1 was predominantly cytoplasmic within the soma (Fig. 4, A and B). In axons, dynein effectors were imaged either in the mid- or distal axon and puncta quantified per unit length, comparing levels along the same axon to assess relative enrichment of individual effectors. BICD2 had a generally sparse distribution in axons compared with RILP and Hook1. In contrast, RILP was enriched in the mid-axon, and Hook1 was enriched in the distal axon (Fig. 4, A and C). We also quantified the density of puncta for each dynein effector within the growth cone and found there was significantly more Hook1 puncta in the growth cone as compared with BICD2 and RILP (Fig. 4 D). The differential localization of these effectors is consistent with their proposed roles in organelle transport. BICD2 is linked to Rab6 vesicles (Matanis et al., 2002; Matsuto et al., 2015), which are mainly localized to the soma. RILP is a Rab7 adaptor (Cantalupo et al., 2001; Wu et al., 2005; Johansson et al., 2007; Rocha et al., 2009), and Rab7 vesicles are enriched in the mid-axon and soma. In contrast, the enrichment of Hook1 in the distal axon suggests that this effector is involved early in the pathway of neurotrophic factor uptake and trafficking via signaling endosomes.

To further visualize the Hook1–dynein interaction, we used a proximity ligation assay to image dynein–Hook1 colocalization. Neurons expressing Hook1-Halo were stained with antibodies to the Halo-tag and to endogenous dynein (anti–dynein intermediate chain [DIC]) and visualized using secondary antibodies conjugated to complementary oligonucleotides to visualize complex formation. Using this assay, Hook1–dynein complexes were localized to the distal axon, a distribution not observed in control reactions.
lacking anti-DIC or anti-Halo antibody (Fig. 4, E and F). In contrast, we did not see the same distal axon signal using the proximity ligation assay to visualize dynein complexed with either RILP or BICD2. These results show that Hook1 is enriched in the distal axon and primed for motility as it is in a complex with dynein.

Hook1 depletion decreases the flux and processivity of BDNF-signaling endosomes from the distal axon

To better model the uptake and transport of BDNF from the distal axon, we used microfluidic devices in which axons grow through microchambers to reach the fluidly isolated axonal chamber, permitting BDNF application only to the distal axons (Fig. 5 A). Hippocampal neurons were electroporated with GFP fill and Hook1 siRNA before plating in the microfluidic devices. Neurons were cultured for 7–8 d, allowing the axons to extend to the distal chamber. Prior to imaging, BDNF-Qdots were added to the axonal chamber of the device. We compared the motility properties of BDNF-Qdots in Hook1 KD and control neurons (see Videos 1 and 2). While BDNF-Qdots still exhibited a retrograde bias in motility upon Hook1 KD, the flux was greatly reduced compared with control conditions (Fig. 5, B–D). In contrast, neurons transfected with Hook3 siRNA did not show this decrease in BDNF-Qdot flux (Fig. 5 D). In addition to decreased flux, we also noticed a difference in the size of BDNF-Qdot vesicles. Previous research has shown that multiple neurotrophic factor–bound Qdots may be internalized into a single signaling endosome (Cui et al., 2007). Thus, we measured the apparent size of BDNF-Qdot organelles as a measure of the number of internalized BDNF-Qdots. We found a significant reduction in organelle area in Hook1 KD neurons (0.39 µm², with 0.30–0.48 95% confidence interval [CI]) compared with control neurons (0.62 µm², with 0.51–0.74 95% CI), suggesting less BDNF-Qdots are being endocytosed per vesicle. Overall, this work suggests that Hook1 depletion reduces not only the number of BDNF organelles trafficked down the axon but also the load of individual organelles.

While significantly fewer BDNF-Qdots were observed to traffic along the axon in Hook1-depleted neurons, we analyzed the motility properties of these organelles to see whether loss of the dynein activator would reduce the processivity of signaling endosomes from the distal axon. In Hook1 KD cells, BDNF-Qdots showed less directed motility along the axon, with significantly more directional switching within individual runs (Fig. 5 E). In addition, retrograde events also displayed increased pause duration and reduced net velocity (Fig. 5, F and G). These results suggest that Hook1 does act as a dynein activator to increase the processivity of retrograde BDNF-signaling endosomes once they have been endocytosed.

Since we observed reduced flux and impaired motility of signaling endosomes in Hook1 KD neurons, we hypothesized there...
might be a resulting accumulation of BDNF-Qdots at the distal ends of axons due to loss of dynein transport (Fig. 6 A). We imaged the distal regions of axons after several washes to remove excess, surface-associated BDNF-Qdots. Quantification of BDNF-Qdot signal per unit area of axon showed no difference between Hook1 KD and control neurons (Fig. 6, B and C). Similar results were seen when dynein motility was blocked with the dynein inhibitor Cilobrevin D (Fig. 6, B and C), suggesting there is down-regulation of TrkB endocytosis when motility is impaired, preventing distal accumulation. One way to reduce endocytosis of BDNF is to reduce the amount of its receptor TrkB at the plasma membrane. We measured the amount of plasma membrane–associated TrkB on the surface of Hook1 KD or control axons using an antibody to the TrkB extracellular domain, which is not conserved in other Trk proteins. Neurons were fixed and stained with anti-TrkB (aa 54–67) without permeabilization. There was no significant change in surface TrkB levels at the axon tips with Hook1 depletion, suggesting that a reduction in TrkB levels is not contributing to the reduced flux we observed with Hook1 KD (Fig. 6, D and E). Together, these results suggest that endocytosis of BDNF is down-regulated when dynein motility is impaired, which may constitute a potential mechanism to reduce the distal accumulation of cargos in axons.

**Figure 6.** Impaired signaling endosome motility reduces BDNF endocytosis. (A) Schematic model of two possible effects of Hook1 KD on distal axons. (B) Representative images of distal tips of axons with BDNF-Qdots. Arrows indicate BDNF-Qdots, and the dashed line is the cell outline. (C) Area of Qdots in the distal axons normalized by area of axon quantified. Scatter plot shows mean ± SEM; Kruskal–Wallis one-way ANOVA (ns, P > 0.082). Mock: n = 58 neurons; Hk1 KD: n = 46 neurons; Ciliobrevin D (CilioD): n = 3–6 neurons. (D) Surface TrkB staining with anti-TrkB in distal axon of fixed neurons. Bottom panel (anti-Rabbit594) shows the secondary antibody only (as a control). (B and D) Bars, 5 µm. (E) Area of surface TrkB per micrometer axonal length. The anti-Rabbit 594 condition is a control for the secondary antibody. Scatter plot shows mean ± SEM; one-way ANOVA (***, P = 0.0004; **, P = 0.0055; ns, P = 0.9741). Mock: n = 40 neurons; Hk1 KD: n = 32 neurons; anti-Rabbit594: n = 10 neurons.

**Discussion**

In this study, we found that Hook1 comigrates with a subpopulation of Rab5- and Rab7-positive endosomes. Previous studies in fungi have implicated Hook proteins in early endosomal transport marked by Rab5, but these systems only express one Hook protein.
isoform (Bielska et al., 2014; Zhang et al., 2014). In mammalian systems, Hook1 has been linked to different aspects of the endosomal pathway. In HeLa cells, Hook proteins were found to interact with members of the HOPS complex and to be important for timely trafficking of EGF through endosomal compartments marked by EEA1, CD63, and LAMP1, but this study simultaneously knocked down all three Hook isoforms, making it difficult to determine their individual roles (Xu et al., 2008). Another study using HeLa cells suggested that Hook1 interacts with CD147 to facilitate sorting into Rab22-positive recycling tubules (Maldonado-Báez et al., 2013). In COS-1 cells, Hook1 was found to interact with Rab7, Rab9, and Rab11 using immunoprecipitation (Luiro et al., 2004). The variety of results seen in these studies is likely due to the fluid nature of endosomal pathways and differential cellular demands on these pathways. In our work, Hook1 comigrates primarily with fast, retrograde Rab5-positive vesicles in primary neurons, which suggests a role for Hook1 in activating the motility of these vesicles. Yet Hook1 depletion produced only subtle effects on the dynamics of the total Rab5-positive endosomal population in axons, significantly affecting only signaling endosome motility, suggesting a higher level of specificity than previously observed for dynein effectors that interact directly with Rab proteins such as the interaction of BICD proteins with Rab6 (Schlager et al., 2010; Matsuto et al., 2015; Terawaki et al., 2015; Huynh and Vale, 2017) or RILP with Rab7 (Cantalupo et al., 2001; Wu et al., 2005; Johansson et al., 2007). Instead of cargo attachment through a Rab protein, Hook1 has been suggested to attach to cargo through C-terminal interactions with Fused Toes (FTS) and FTS-Hook interacting (FHIP) proteins (Xu et al., 2008; Yao et al., 2014; Guo et al., 2016). It remains to be determined whether Hook1 is linked to signaling endosomes by FTS-FHIP or another protein complex in neuronal systems. Of note, mutating pairs of residues conserved within the C-terminal domain of Hook1, but not found in Hook3, was sufficient to disrupt the ability of Hook1 to activate the transport of signaling endosomes (Fig. 3, G and H), supporting a key role for the C-terminal of Hook1 in mediating organelle-specific dynein-driven motility.

In this study, we found that Hook1 plays a role in signaling endosome processivity. Our previous work has shown that Hook1 increases dynein-dynactin processivity in vitro, with Hook1-bound dynein displaying higher velocities and longer run lengths than BICD2-associated motors (Olenick et al., 2016). Recent cryo-EM structures have shown Hook3 can recruit two dynein dimers per dynactin complex (Urnavicius et al., 2018). Due to the high sequence similarity in the Hook domain and coiled-coil regions, it is likely that Hook1 functions in a similar manner. It is possible that the relatively high velocities observed for signaling endosome transport (averaging 1.4 µm/s) are due to the incorporation of two dynein dimers into the dynein-dynactin-Hook1 complex. We also found that the interaction of Hook1 with dynein subunit LIC1 is essential for signaling endosome motility, a mechanism...
Olenick et al.

Hook1 activates BDNF-endosome motility

that is likely conserved in other dynein effectors regulating cargo transport in the cell. Recent structural work showed that a helix (aa 433–458) within the otherwise unstructured C-terminal region of LIC1 is a conserved interface for the binding of dynein effectors including BICD2, Spindly, and Hook proteins (Lee et al., 2018). Since the LIC1 interaction region is conserved among dynein effectors, it is likely that competition for this binding site plays a role in regulating cargo transport. During our investigation of Hook1, we observed differential localization of the dynein effectors Hook1, BICD2, and RILP in hippocampal neurons. It is possible that the compartmentalization of neurons and the differential localization of dynein effectors locally regulates the competition of dynein effectors for dynein–dynactin binding sites.

In this study, we found that Hook1 plays a key role early in the transport of TrkB–BDNF endosomes as reflected by Hook1 enrichment in the distal axon. This enrichment is not seen for RILP or BICD2. In contrast, RILP was enriched in the mid-axon and soma, consistent with its role in mediating Rab7-endosome motility (Cantalupo et al., 2001; Johansson et al., 2007). It remains to be investigated whether there is a transition or handoff of dynein effectors during endosomal maturation and conversion from Rab5- to Rab7-positive organelles (Fig. 8) in line with the key role proposed for Rab7-positive multivesicular bodies in mediating the long-distance trafficking of TrkA in sympathetic neurons (Ye et al., 2018). It is also possible that a given cargo could have a mixture of dynein effectors. While Hook and BICD proteins are dynein effectors that modulate dynein processivity, there is as yet no direct evidence of RILP acting as a dynein activator, but other studies have shown that it acts as an adaptor to recruit dynein to Rab7 cargo (Johansson et al., 2007; Rocha et al., 2009). Similarly, Snapin has been reported to recruit dynein to BDNF–TrkB-positive signaling endosomes (Zhou et al., 2012), although this protein lacks the secondary structure expected for a dynein activator (Reck-Peterson et al., 2018). It is possible that a complement of dynein effectors and activators may be required to effectively recruit motors and activate transport; it remains to be seen whether there is coordination between dynein activators and adaptors to help maintain a constant processive dynein pool on a given organelle.

Using microfluidic devices, we showed that loss of Hook1 reduces the flux and processivity of BDNF-signaling endosomes. However, this reduced flux to the cell body did not result in an accumulation of BDNF-Qdots in the distal axon, suggesting that endocytosis is down-regulated when endosomal motility is inhibited. BDNF has been reported to be a self-amplifying autocrine factor, which can signal to promote BDNF expression and increase TrkB membrane levels (Cheng et al., 2011). Therefore, loss of BDNF signaling could reduce TrkB surface levels and regulate endocytosis to prevent a buildup of BDNF endosomes at the distal tip. However, we detected no significant change in plasma membrane–associated TrkB levels at the axon tip upon Hook1 depletion, suggesting that another mechanism might be at work. An alternative possibility is that the internalization of the TrkB–BDNF complex via endocytosis is tightly linked to the formation of a high-speed, highly processive Hook1-dependent transport compartment. Thus, if transport is blocked either by Hook1 depletion or dynein inhibition, internalization may also be down-regulated, preventing the distal accumulation of stalled signaling endosomes. Hook1 binding partners such as FTS and FHIP proteins (Xu et al., 2008; Yao et al., 2014; Guo et al., 2016) may mediate this coordination of uptake and transport, an interesting question for future studies.

TrkB–BDNF signaling is important for neuronal survival, and disruption of signaling endosome trafficking has been found in models of neurodegenerative diseases including Huntington’s or Parkinson’s disease (Millecamps and Julien, 2013). In Huntington’s disease, the polyQ-expanded huntingtin protein has been shown to impair BDNF retrograde trafficking, leading to reduced neuronal survival (Gauthier et al., 2004). α-Synuclein has also been shown to impair BDNF transport in a mouse model of Parkinson’s disease (Fang et al., 2017). Currently, it is unclear whether Hook proteins play a role in these neurodegenerative diseases, but Hook1 and Hook3 have been localized to tau aggregates, a pathological hallmark of Alzheimer’s disease, frontotemporal dementia, and other tauopathies (Herrmann et al., 2015). With our new understanding of Hook1 as a dynein effector for BDNF transport in nonpathological states, the role of Hook1 in disease states can now be investigated in more detail in future studies.

Materials and methods

Plasmids and reagents

Halo-tagged Hook1 constructs were generated from the human Hook1 sequence (UniProt code Q9UJC3) and using the HaloTag from the pHTN or pHTC Halo tag CMV-neo vector (Promega).
GFP-Hook1 constructs were generated in the pEGFP vector. Rab5-GFP was provided by M. Zerial (Max Planck Institute, Dresden, Germany). Rab7-GFP was purchased from Addgene. TrkB-mRFP was provided by M. Chao (New York University, New York, NY). RILP-GFP was provided by J. Neefjes (Leiden University Medical Center, Leiden, Netherlands). BICD2-GFP was provided by A. Akhmanova (Utrecht University, Utrecht, Netherlands). GFP-Hook1 or Hook3 was purchased from GE Healthcare. Olenick et al.

Antibodies used for biochemistry and Western blotting included anti-Hook1 (rabbit; 1:250; ab104514; for detection of rat protein; Abcam), anti-Hook1 (rabbit; 1:500; ab150397; for detection of mouse protein; Abcam), anti-Hook3 (rabbit; 1:1,000; ProteinTech), anti-actin (mouse; 1:1,000; EMD Millipore), anti-DIC (mouse; 1:1,000; EMD Millipore), anti-TrkB (rabbit; 1:4,000; ab187041; Abcam), and anti-Halo (rabbit; Promega). For immunofluorescence experiments, anti-pCREB(Ser133) (rabbit; 1:1,000; EMD Millipore), anti-TrkB (rabbit; 1:4,000; ab187041; Abcam), and anti-Halo (rabbit; Promega). For immuno-precipitation experiments, anti-β-tubulin (1:40; mouse; T5201; Sigma-Aldrich). An ON-TARGETplus siRNA SMA RTpool of four siRNAs was used. For immunofluorescence experiments, anti-pCREB(Ser133) (rabbit; 1:1,000; Cell Signaling) and anti-TrkB (aa 55–67; rabbit; 1:1,000; ProteinTech), anti-actin (mouse; 1:1,000; EMD Millipore, anti-TRK-B (isoform B; rabbit; 1:1,000; Cell Signaling) and anti-TRK-B (isoform A; 1:4,000; ab187041; Abcam), and anti-Halo (rabbit; Promega). For immunofluorescence experiments, anti-pCREB(Ser133) (rabbit; 1:1,000; Cell Signaling) and anti-TRK-B (isoform B; rabbit; 1:1,000; Cell Signaling) and anti-TRK-B (isoform A; 1:4,000; ab187041; Abcam), and anti-Halo (rabbit; Promega). For immunoprecipitation experiments, anti-β-tubulin (1:40; mouse; T5201; Sigma-Aldrich). An ON-TARGETplus siRNA SMARTpool of four siRNAs for rat Hook1 or Hook3 was purchased from GE Healthcare. Neuronal culture

E18 Sprague–Dawley rat hippocampal neurons were obtained in suspension from the Neuron Culture Service Center at the University of Pennsylvania and plated on 35-mm glass-bottom dishes (MatTek) or 25-mm coverslips (World Precision). Dishes or coverslips were precoated with 0.5 mg/ml poly-L-lysine (Sigma-Aldrich). An ON-TARGETplus siRNA SMARTpool of four siRNAs for rat Hook1 or Hook3 was purchased from GE Healthcare.

Neuronal imaging

Imaging was done at DIV 7–8 with neurons transfected 24–48 h before imaging. Neurons were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, with 0.3–1 μg of each DNA plasmid and, for siRNA conditions, 45 pmol siRNA. Control siRNA labeled with Cy5 was used to confirm transfection of siRNA with Lipofectamine in cultured neurons. Neurons were imaged in low-fluorescence media (HibertaneE; Brain Bits) supplemented with 2% B27 and 1% GlutaMAX. For experiments with Halo-Tag constructs, neurons were labeled with tetramethyl rhodamine (TMR) Halo-Tag ligands according to the manufacturer’s instructions (Promega). For mitochondrial imaging, TMR ethyl ester (TMR dared) was added according to the manufacturer’s protocol. Neurons were imaged in an environmental chamber at 37°C on a spinning-disk confocal UltraView VOX (PerkinElmer) on an inverted Nikon Eclipse Ti microscope with the Prefocus Focus system using Apochromat 100× 1.49 NA oil-immersion objective and a C9100-50 electron-multiplying charge-coupled device camera (Hamamatsu Photonics) controlled by Velocity software (PerkinElmer). Axons and dendrites were identified based on morphological criteria as outlined elsewhere.

where (Kaeche and Banker, 2006). The distal axon is defined as the area <100 μm from the axon terminal, while the mid-axon is defined as the area >100 μm from the axon terminal and >100 μm from the soma.

Microfluidic experiments

Round microfluidic devices of 450 μm (Xona microfluidics) were used for axon isolation experiments. Devices were UV sterilized and attached to poly-L-lysine-coated imaging dishes (Fluoro-Dish; World Precision Instruments) before plating. On the day of plating, neurons were nucleofected with an Amaza Nucleofector machine (Lonza) with DNA/siRNA in similar quantities as described above. Cells were plated to one side of microfluidic device at 4 × 10⁶ cells per dish. Fresh maintenance media was added every 2 d (~30%).

BDNF-Qdot experiments

Neurons were serum starved in unsupplemented Neurobasal (Gibco) for 2–4 h before BDNF-Qdot addition. 50 nM hBDNF-biotin (Alomone Labs) was combined with 50 nM Quantum Dot ITK 655 Streptavidin conjugate (Invitrogen) for 1 h on ice to generate BDNF-Qdots. After conjugation, BDNF-Qdots were added to neurons in unsupplemented Neurobasal to a final concentration of 0.25 nM for 1–2 h. In microfluidic experiments, BDNF-Qdots were only added to the axon side. For pCREB experiments, 1 nM unconjugated BDNF-biotin was added for 1 h before fixing. For Ciliobrevin D (EMD Millipore) conditions, 20 μM Ciliobrevin D was added 10 min before BDNF-Qdot addition and was present throughout BDNF-Qdot treatment and imaging, with the same timescale as control conditions.

Immunofluorescence

Neurons cultured on 25-mm glass coverslips were fixed at DIV 7–8 in PBS containing 4% paraformaldehyde and 4% sucrose for 8 min. Coverslips were washed three times in PBS and blocked with cell block (PBS with 5% normal goat serum and 1% BSA). Primary antibodies were incubated for 2 h at RT in cell block. After removing the primary antibodies, the coverslips were washed with PBS and incubated for 1 h at RT with fluorophore-conjugated secondary antibodies diluted in cell block. Following washes with PBS, the coverslips were mounted in ProLong Gold Antifade Mountant (Thermo Fisher Scientific) on glass slides. For proximity ligation assays, Duolink PLA kit with red detection reagents was used according to manufacturer’s protocol (Sigma-Aldrich). TIRF motility assay

Single-molecule TIRF motility assays were performed as previously described in detail (Olenick et al., 2016). In brief, HeLa cells 18–20 h after transfection of Halo-Hook1 constructs were labeled with the Halo ligand TMR (Promega) and lysed in buffer containing 40 mM Hepes, 1 mM EDTA, 120 mM NaCl, 0.1% Triton X-100, and 1 mM magnesium ATP, pH 7.4, supplemented with protease inhibitors. Cell lysates were diluted in assay buffer containing 10 mM magnesium ATP, 0.3 mg/ml BSA, 0.3 mg/ml casein, 10 mM DTT, and an oxygen-scavenging system. Diluted cell lysates were then flowed into imaging chambers with Taxol-stabilized microtubules immobilized to the coverslip with a tu-
bulin antibody. TIRF videos were acquired at RT at four frames per second using the Nikon TIRF system (Perkin Elmer) on an inverted Ti microscope with a 100× objective and an ImageEM C9100–13 camera (Hamamatsu Photonics) controlled by Velocity software.

**Immunoisolation of TrkB vesicles**

Using three mouse brains per immunoprecipitation experiment, lysates were homogenated in Hepes buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, and protease inhibitors) and then centrifuged at 800×g for 10 min and 3,000×g for 10 min. The supernatant was then added to Dynabeads protein G (Thermo Fisher Scientific) with anti-TrkB (Abcam) or anti-Halo (Promega) as a rabbit IgG control. Lysates were incubated for 15 min at RT and then washed three times with Hepes buffer. Proteins were then eluted in denaturing buffer and boiled 5 min before running on a SDS-PAGE gel.

**Western blotting**

To test siRNA efficiency, PC12 cells were transfected at 70–80% confluency with 45 pmol of a pool of Hk1 or Hook3 siRNAs using Lipofectamine RNAiMAX (Invitrogen) and lysed 48 h later. PC12 cells were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, and protease inhibitors) and centrifuged at 13,200 rpm for 10 min at 4°C. For all Western blot experiments, samples were boiled in denaturing buffer for 5 min and run on a SDS-PAGE gel to separate proteins.

**Imaging analysis**

For motility analysis, kymographs were generated using the MultipleKymograph plugin for Fiji (ImageJ; National Institutes of Health) and analyzed using custom MATLAB software (Math-Works) or by measurement tools in Fiji. For effector localization, images were deconvolved using Huygens Professional software. Effector puncta were then counted by hand, and axon length was measured in Fiji. Area of BDNF-Qdot was measured using measurement and analysis functions in Volocity. The pCREB signal was measured by outlining the nucleus with a Hoechst measurement and analysis functions in Volocity. The pCREB signal was measured by outlining the nucleus with a Hoechst signal was measured by outlining the nucleus with a Hoechst

**Statistical methods**

Statistics were performed in GraphPad Prism. A Student’s t test or Mann–Whitney test was used when comparing two datasets as indicated, while a ANOVA was used with multiple datasets. For all experiments, data were analyzed from at least three independent replicates. Statistical significance is noted as follows: NS, P > 0.05; *, P ≤ 0.05; ***, P ≤ 0.01; and ***, P ≤ 0.001. Figure legends contain specific P value for that figure.

**References**


Olenick et al. Hook1 activates BDNF-endosome motility


