## Stable Kinesin and Dynein Assemblies Drive the Axonal Transport of Mammalian Prion Protein Vesicles

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## SUMMARY

Kinesin and dynein are opposite-polarity microtubule motors that drive the tightly regulated transport of a variety of cargoes. Both motors can bind to cargo, but their overall composition on axonal vesicles and whether this composition directly modulates transport activity are unknown. Here we characterize the intracellular transport and steady-state motor subunit composition of mammalian prion protein (PrP<sup>C</sup>) vesicles. We identify Kinesin-1 and cytoplasmic dynein as major PrP<sup>C</sup> vesicle motor complexes and show that their activities are tightly coupled. Regulation of normal retrograde transport by Kinesin-1 is independent of dynein-vesicle attachment and requires the vesicle association of a complete Kinesin-1 heavy and light chain holoenzyme. Furthermore, motor subunits remain stably associated with stationary as well as with moving vesicles. Our data suggest a coordination model wherein PrP<sup>C</sup> vesicles maintain a stable population of associated motors whose activity is modulated by regulatory factors instead of by structural changes to motor-cargo associations.

## INTRODUCTION

The viability and proper function of neurons depend on the active axonal transport of diverse cargoes (Goldstein et al., 2008; Hirokawa and Takemura, 2005; Verhey and Hammond, 2009). The microtubule (MT)–based motors driving these movements are kinesin and cytoplasmic dynein, which use the energy of ATP hydrolysis to translocate along MT tracks in plus-end (anterograde) and minus-end (retrograde) directions. Cytoplasmic dynein consists of a core processive dynein heavy chain (DHC) motor that interacts with a large assembly of accessory subunits and with dynactin, to drive most retrograde transport (Kardon and Vale, 2009; Karki and Holzbaur, 1999). Kinesin-1 is a heterotetramer consisting of a homodimer of one of three kinesin heavy chains (KHC; Kinesin-1A, -1B, and -1C, formerly KIF5A, -B, and -C; Xia et al., 1998) that can interact in vitro with a homodimer of either of two accessory kinesin light chains (KLC1 and KLC2; Rahman et al., 1998). It is unknown what complexes of heavy and light chains form in vivo to drive the movement of any vesicular cargo studied to date (DeBoer et al., 2008; Rahman et al., 1998).

Intracellular transport is often bidirectional because cargoes regularly reverse course en route to their final destinations. These dynamics have been observed for mitochondria, peroxisomes, melanosomes, endosomes, lipid droplets, synaptic vesicle precursors, and viral particles, where transport of opposite polarity motors is often coordinated (Gross et al., 2002; Kural et al., 2005; Lyman and Enquist, 2009; Plitz and Pfeffer, 2001; Sato-Yoshitake et al., 1992; Shubeita et al., 2008; Soppina et al., 2009; Welte, 2004). An important question in transport regulation is how motor activity is controlled in cells to achieve bidirectionality. Because Kinesin-1 and dynein are unidirectional motors, coordination could occur either by the alternating association/dissociation of motors of either polarity to/from cargo, which generates motor activation by cargo binding, by the modulation of activity of both types of motors that simultaneously bind to cargo, or by generation of opposing forces of simultaneously cargo-bound motors in a tug of war (TOW) (Gross, 2004; Welte, 2004). It has been proposed that motor regulation by association/dissociation might be a generalized mechanism of transport regulation because motors can exist in inactive, unbound forms, and autoinhibition can be released by binding to cargo (Akhmanova and Hammer, 2010; Verhey and Hammond, 2009). Alternatively, there is evidence that certain neuronal cargoes in vitro or nonneuronal cargoes in vivo experience opposing TOW forces such that the total number of motors associated with cargo determines activity (Hendricks et al., 2010; Soppina et al., 2009). However, in coordination models of axonal transport, the extent of plus- and minus-end motor association with cargo and whether cargo association relates to changes in motor activity remain unclear. To test whether motor-cargo association modulates motor activity in axons and to build an in vivo model of bidirectional transport, it is imperative to characterize the steady-state composition of total motor assemblies on a single type of vesicular cargo and to relate this analysis to live movement data for the same cargo.

Analyzing motor composition of cargo in vivo has been experimentally challenging because of the difficulty in isolating populations of a single type of cargo and the absence of quantitative methods to characterize motor composition on them. Biochemical purifications of heterogeneous membrane populations or of melanosomes have yielded estimates of cofractionating plusand minus-end motors (Gross et al., 2002; Hendricks et al., 2010). However, these represent indirect estimates of average levels of bound motors, because motor-cargo associations could vary from cargo to cargo and over time. Likewise, stallforce measurements have provided estimates of numbers of active motors (Kural et al., 2005; Shubeita et al., 2008; Soppina et al., 2009), but it is unclear whether bidirectionality is dictated by the rapid association/dissociation of these active motors or whether these engaged motors represent a subgroup of a regulated but stable assembly of cargo-bound motors. Thus, the steady-state composition of motor assemblies on any single type of cargo remains undefined.

To distinguish between regulatory versus association/dissociation models of bidirectional transport, we characterized the mechanism of axonal transport of vesicles containing the cellular mammalian prion protein (PrP<sup>C</sup>). PrP<sup>C</sup> is a neuronally enriched glycosyl-phosphatidylinositol (GPI)-anchored protein that follows the secretory pathway inside the lumen of vesicles toward the cell surface (Caughey et al., 2009; Harris, 2003). PrP<sup>C</sup> can convert to a pathogenic form called PrP-scrapie (PrP<sup>Sc</sup>), which has been implicated in neurological disorders, including Creutzfeldt-Jakob disease in humans (Caughey et al., 2009). The function of PrP<sup>C</sup> is unclear, but evidence suggests that while it is at the cell surface it can interact with proteins involved in cell adhesion and signaling (Malaga-Trillo et al., 2009; Mouillet-Richard et al., 2000), as well as with PrPSc (Caughey et al., 2009). Thus, trafficking of PrP<sup>C</sup> to the plasma membrane via an intact transport system might be relevant to PrP<sup>C</sup> function and for the initiation of neurodegenerative pathologic abnormalities. Although PrP<sup>C</sup> is transported in nerves (Butowt et al., 2006; Moya et al., 2004; Rodolfo et al., 1999), the mechanism of intracellular PrP<sup>C</sup> vesicular transport is unknown. Here, we developed assays to characterize relative motor subunit composition on individual PrP<sup>C</sup> vesicles and used live imaging to identify Kinesin-1C/KLC1 and DHC1 as the major axonal motor complexes driving PrP<sup>C</sup> vesicle transport. Live tracking and motor composition analyses demonstrate that opposing motors positively coordinate each other's activities independently of cargo-association mechanisms. This coordination mechanism requires the formation and vesicle association of an intact Kinesin-1 complex composed of heavy and light chains.

## RESULTS

## Mammalian PrP<sup>C</sup> Vesicles Move Bidirectionally in Hippocampal Axons

Previous studies showed that mammalian and avian PrP<sup>C</sup> are transported along peripheral and central nervous system nerves in anterograde and retrograde directions (Borchelt et al., 1994;

Butowt et al., 2007; Moya et al., 2004; Rodolfo et al., 1999). We confirmed these observations using a protein accumulation paradigm in mouse sciatic nerves (see Figures S1A and S1B available online).

To characterize the intracellular transport of  $PrP^{C}$  vesicles in live neurons, we tracked individual moving vesicles in 10-dayold cultured mouse hippocampal axons from neurons transfected with a YFP-PrP<sup>C</sup> fusion construct (Borchelt et al., 1996) (Figures S1C–S1E). We restricted analyses to axons at day 10 after plating, which have a largely uniform microtubule polarity with plus ends directed toward axonal termini and minus ends toward cell bodies (Baas et al., 1988). To quantify YFP-PrP<sup>C</sup> vesicular movement, we used a MATLAB-based custom particle tracking software (LAPTrack; G.F. Reis, G. Yang, L.S., S.B. Shah, J.T. Robinson, T.S. Hays, G. Danuser. L.S.B.G., unpublished data), to generate a comprehensive dataset of trajectories at a spatial and temporal resolution of 0.126 µm, and 10Hz, respectively.

In wild-type neurons, YFP-PrP<sup>C</sup> vesicles moved in anterograde and retrograde directions, and a large percentage were stationary (Figures 1A and 1B; Movie S1). The remaining vesicles reversed directions at a mean (± standard error of the mean [SEM]) frequency of 0.027 ± 0.004 switches/s. Vesicle trajectories were broken into segments, defined as uninterrupted periods of movement framed by pauses (Extended Experimental Procedures). Mean (± SEM) anterograde and retrograde segmental velocities were 0.85  $\pm$  0.036  $\mu\text{m/s}$  and 0.86  $\pm$ 0.06 µm/s (Figure 1C), respectively, similar to those reported for Kinesin-1 and cytoplasmic dynein in vitro (Howard, 2001; Mazumdar et al., 1996). Analysis of segmental velocity distributions showed a wide range of anterograde and retrograde velocities that included maximal velocities of 2.8 and 2.6 µm/s, respectively (Figure 1D). Retrograde particles had shorter mean ( $\pm$  SEM) run lengths (4.8  $\pm$  0.4  $\mu$ m) than did anterograde ones (6.2  $\pm$  0.5  $\mu$ m), but paused as long and as frequently (Figures 1E–1G). These run lengths were longer than the 1–2 µm reported for Kinesin-1 and dynein in vitro (King et al., 2003; Thorn et al., 2000). Thus, vesicles containing YFP-PrP<sup>C</sup> move bidirectionally en route to the synapse in primary hippocampal neurons with dynamics that are consistent with MT-dependent fast axonal transport mediated by kinesin and dynein motor proteins.

## Kinesin-1 and Cytoplasmic Dynein Associate with PrP<sup>C</sup> Vesicles In Vivo

To understand the mechanism of axonal transport of  $PrP^{C}$  vesicles, we sought to identify the motor proteins moving these vesicles in axons. Because  $PrP^{C}$  and Kinesin-1 are predominantly expressed in brain, we tested the hypothesis that Kinesin-1 is a  $PrP^{C}$  vesicle motor protein. We first tested whether the KLC1 cargo-binding subunit associated biochemically with  $PrP^{C}$  vesicles in floated membrane fractions (Figure 2A). Using an antibody against KLC1 to pull down associated membrane components, we found that  $PrP^{C}$  and KHC (as detected by an antibody that recognizes primarily Kinesin-1C) immunoprecipitated with KLC1, as did the amyloid precursor protein (APP), which was previously identified in a complex with KLC1 (Kamal et al., 2000) (Figure 2B). Because  $PrP^{C}$  is in the vesicular lumen, the reverse immunoprecipitation experiment using  $PrP^{C}$ 



#### Figure 1. PrP<sup>C</sup> Vesicles Are Transported Bidirectionally in Wild-Type Hippocampal Axons

(A) Top panels: sequential images of YFP-PrP<sup>C</sup> vesicle movement in a hippocampal axon. Vesicles moving bidirectionally (\*), in a retrograde direction (•), and a stationary one ( $\diamond$ ) are followed for a period of 14 s. Middle panel: kymograph generated from movie in (A). Bottom panel: same kymograph depicting individual particle traces generated by particle tracking software.

(B) Population breakdown of YFP-PrP<sup>C</sup> vesicles.

(C) Mean segmental velocity.

(D) Segmental velocity histograms. Red lines show mean.

(E–G) Panels show run length (E), pause duration (F), and pause frequency (G) of YFP-PrP<sup>C</sup> vesicles.

Nv = # vesicles; Np = # pauses; Nt = # tracks; Ns = # segments. All values are shown as mean ± SEM. See also Figure S1 and Movie S1.

antibodies to pull down KLC1 was not possible without breaking vesicular membranes.

To further test whether Kinesin-1 subunits interact with PrP<sup>C</sup> vesicles, we imaged fixed hippocampal cell axons stained with antibodies against PrP<sup>C</sup> and KLC1, Kinesin-1C or Kinesin-1A. The fluorescent signal observed was punctate, suggesting that these proteins were localized to vesicular structures (Figure 2C). We observed significant colocalization between PrP<sup>C</sup> and KLC1 (58%  $\pm$  1.5% PrP<sup>C</sup> vesicles colocalized with KLC1; N<sub>vesicles</sub> = 510), and PrP<sup>C</sup> and Kinesin-1C (35%  $\pm$  1.5% PrP<sup>C</sup> vesicles colocalization was not expected as Kinesin-1 also mediates transport of other cargoes, and because other kinesin motors might also transport PrP<sup>C</sup> vesicles in addition to Kinesin-1.

To test whether cytoplasmic dynein transports PrP<sup>C</sup> vesicles, we quantified YFP-PrP<sup>C</sup> vesicle movement in hippocampal cells cotransfected with YFP-PrP<sup>C</sup> and with a short hairpin RNA (shRNA)-mCherry construct targeted to reduce the amount of dynein heavy chain 1 (DHC1; referred to as DHC1 shRNA). Imaging was restricted to axons coexpressing YFP and mCherry markers. Using the live imaging assay, we found that 2 days after cotransfection, reduction of DHC1 (mRNA reduced by 80%–90%, protein reduced by 66%) (Figures S2A and S2B and Figures S5D and S5E), disrupted bidirectional YFP-PrP<sup>C</sup> vesicle transport, decreasing run lengths and increasing the frequency of pauses (Figures 2D and 2E). Mean segmental velocities remained unchanged (Figure S2C). To confirm DHC1 association with PrP<sup>C</sup> vesicles, we stained hippocampal axons and found that DHC1 partially colocalized with PrP<sup>C</sup> vesicles







#### Figure 2. PrP<sup>C</sup> Vesicles Associate with Kinesin-1 and Dynein

(A) Schematic diagram of a membrane flotation experiment showing the 8/35 fraction used as starting material for the vesicle immunoisolation in (B). Wild-type post-nuclear supernatant (PNS) obtained from wild-type mouse brain homogenate was bottom loaded. Buffers used did not contain detergent to prevent breaking of membranes.

(B) An antibody against KLC1 was used to pull down associated membrane components from 8/35 fractions, including PrP<sup>C</sup>-containing vesicles. KHC antibody recognizes mostly Kinesin-1C. UNB = unbound fraction; imm = immunoisolation. Anti-GFP was used as a control.

(C) Deconvolved images of vesicles stained with antibodies against PrP<sup>C</sup> and KLC1, Kinesin-1C, Kinesin-1A, or DHC1. Arrows point to some colocalization events.

(D and E) Panels show run length (D) and pause frequency (E) in DHC1 shRNA axons. All values are shown as mean  $\pm$  SEM. \*\*p < 0.01, \*p < 0.05, permutation t test. (F) Segmental velocity histograms (shown as percent of segments) of YFP-PrP<sup>C</sup> transport in wild-type and DHC1 shRNA axons. Red and light blue curves represent the overall and predicted Gaussian modes, respectively.

Ns = # segments; Nt = # tracks. See also Figure S2 and Table S1.

(43%  $\pm$  2.9% PrP<sup>C</sup> vesicles colocalized with DHC1; N<sub>vesicles</sub> = 388) (Figure 2C). Thus, KLC1, Kinesin-1C, and DHC1, but not Kinesin-1A, associate with PrP<sup>C</sup> vesicles in vivo. The interaction

between Kinesin-1 and PrP<sup>C</sup> is not a direct one because immunoisolations from vesicular fractions using a KLC1 antibody in the presence of detergent did not pull down PrP<sup>C</sup> (data not shown). Furthermore, disruptions of dynein inhibited bidirectional transport, indicating that dynein is required for normal retrograde movement and is involved in the activation of the plus-end anterograde transport of these vesicles.

## Kinesin-1 Light Chains Mediate Anterograde Transport and Activate Retrograde Movement of PrP<sup>C</sup> Vesicles

Previous work showed that either of two KLCs can form complexes with any of the three KHCs (Rahman et al., 1998). However, it is unknown what combinations of KLC and KHC subunits interact in vivo to transport any cargo. Having shown that KLC1 and Kinesin-1C interact with PrP<sup>C</sup> vesicles, we next tested whether these physical interactions translated into functional transport requirements. Thus, we systematically reduced the function of each Kinesin-1 subunit and assayed for defects in PrP<sup>C</sup> vesicle transport.

We tested KLC1 by analyzing YFP-PrP<sup>C</sup> vesicle transport in hippocampal cells from mice homozygous for a gene-targeted KLC1 deletion (referred to as  $KLC1^{-/-}$  neurons) (Rahman et al., 1999). We tested KLC2 in wild-type hippocampal neurons cotransfected with YFP-PrP<sup>C</sup> and a KLC2 shRNA-mCherry construct, which reduced KLC2 by  $\sim$ 83% (referred to as KLC2 shRNA neurons) (Extended Experimental Procedures). Reducing the function of each KLC subunit caused a significantly decreased percentage of anterograde-moving vesicles and a higher frequency of stalled particles (Figures 3A and 3B). Noticeably, the percentage of retrograde moving PrP<sup>C</sup> particles was also reduced in the absence of KLC1, suggesting that this subunit might be involved in promoting dynein-based movement. Observed and estimated run lengths were reduced in KLC1<sup>-/-</sup> and shRNA KLC2 axons, respectively (Figures 3C and 3D; Extended Experimental Procedures), and vesicles paused more often (Figures 3E). Given the pronounced reductions of bidirectional movement, mean segmental velocities were surprisingly largely unaffected in KLC mutants, with the exception of slight increases in KLC1<sup>-/-</sup> neurons contributed solely by the small number of reversing vesicles (Figures S3A-S3C). Thus, KLC1 and KLC2 mediate anterograde transport of YFP-PrP<sup>C</sup> vesicles and also activate retrograde motility.

## Activation of Bidirectional Transport by a Neuronal Kinesin-1 Heavy Chain

To test whether KHC is required for the transport of PrP<sup>C</sup> vesicles, we analyzed YFP-PrP<sup>C</sup> vesicle movement in hippocampal neurons from Kinesin-1A<sup>-/-</sup> (Xia et al., 2003) and Kinesin-1C<sup>-/-</sup> mice (Figures S4A and S4B) and from conditional Kinesin-1B mice (Cui et al., 2010). Conditional Kinesin-1B neurons were treated after plating with a cre-recombinase adenovirus at a multiplicity of infection (MOI) of 100 or 400, to remove Kinesin-1B genomic DNA flanked by two loxP sites and to create functional null cells (Figures S4C and S4D and Extended Experimental Procedures).

In Kinesin- $1C^{-/-}$  axons, the proportion of anterograde-moving  $PrP^{C}$  vesicles declined, run lengths in both directions were significantly decreased, and these vesicles paused more frequently when moving in both directions (Figures 4A–4C). As was the case for DHC1 and KLC reduction, mean segmental velocities were unchanged (Figure 4D). In Kinesin-1B-cre axons, increasing adenoviral-cre MOI resulted in an increase in

stationary vesicles (Figure 4E). However, anterograde movement was either unchanged (pause frequencies; Figure 4G), or activated as demonstrated by longer runs and strikingly faster mean anterograde segmental velocities (Figures 4F and 4H). Although the basis for the enhanced mean velocities is uncertain, perhaps a faster Kinesin-1C motor, which we showed above is required for normal YFP-PrP<sup>C</sup> anterograde motion, could be responsible for these increases. We did not observe major changes in YFP-PrP<sup>C</sup> transport in Kinesin-1A<sup>-/-</sup> axons because only a minor decrease in retrograde run lengths and slight changes in segmental velocity distributions were observed (Figures 4A-4D and 4I; see next section). These results suggest that Kinesin-1A and -1B are not major components of the PrP<sup>C</sup> vesicular transport machinery, consistent with our immunofluorescence data showing no significant colocalization between PrP<sup>C</sup> and Kinesin-1A. We conclude that Kinesin-1C is required for normal anterograde YFP-PrP<sup>C</sup> transport and can act as an activator of retrograde movement. The requirements of both the neuronal-specific Kinesin-1C and of DHC1 to activate each other's transport suggest that the activities of these motors are tightly coupled.

## Reduction of Kinesin-1 Does Not Affect Global Transport in Axons

To test whether transport defects caused by reducing Kinesin-1 were specific to YFP-PrP<sup>C</sup> vesicles and not due to global disruptions of axonal transport, we characterized the movement of synaptophysin, a synaptic vesicle protein previously identified as a Kinesin-3 cargo (Okada et al., 1995). We tracked the movement of synaptophysin-mCherry vesicles in hippocampal cultured cells following identical conditions as described above for YFF-PrP<sup>C</sup> vesicles. Reducing the function of any of the Kinesin-1 subunits either did not change or modestly stimulated bidirectional synaptophysin-mCherry transport, as observed by increased mean segmental velocities and reduced percentage of stationary vesicles (Figures S3D–S3I). Thus, although Kinesin-1 reduction alters transport dynamics of synaptophysin-mCherry transport.

## Disrupting Kinesin-1 or Dynein Decreases Velocity Distributions Consistent with Downregulation of Motor Activity

Reducing Kinesin-1 or DHC1 results in bidirectional decreases in run lengths, and increases in pause frequencies, consistent with downregulation of opposing motor activity (Figure 3 and Figure 4). Surprisingly however, mean segmental velocity, a parameter also influenced by motor activity, was largely unaffected (Figure 4D, Figure S2C, and Figure S3A). Because possible redundancy among Kinesin-1 subunits or incomplete removal of DHC1 might mask differences in average velocities, we analyzed segmental velocity distributions to test whether these reflected reductions in opposing motor activity.

Wild-type anterograde and retrograde segmental velocity distributions were nonnormal and showed a right-skewed bias (Figure 1D). To analyze these distributions further, we performed cluster mode analysis by fitting nonnormal distributions observed in wild-type and in Kinesin-1 and DHC1 mutant axons,



#### Figure 3. PrP<sup>C</sup> Vesicular Transport Is Inhibited in Kinesin Light Chain Mutant Axons

(A) Representative kymographs of YFP-PrP<sup>C</sup> vesicle movement in wild-type (top panel), KLC1<sup>-/-</sup> (middle panel), and KLC2 shRNA (bottom panel) hippocampal axons.

(B–E) Transport parameters in KLC1<sup>-/-</sup> and KLC2 shRNA axons. Shown are population breakdown of YFP-PrP<sup>C</sup> vesicles (B) (Nv = # vesicles), run length (C), estimated run length (D), and pause frequency (E). Numbers inside bars are segments (run length in C) and tracks (pause frequency in E). All values are shown as mean  $\pm$  SEM. \*\*\*p < 0.001, \*\*p < 0.05, permutation t test.

(F) Segmental velocity histograms (shown in percentage of segments) in wild-type, KLC1<sup>-/-</sup>, and KLC2 shRNA axons. Red and light blue curves represent the overall and predicted Gaussian modes, respectively.

(G) Anterograde and retrograde wild-type segmental velocity histograms (shown as percentage of segments) were reconstituted from adding together histograms of KLC1<sup>-/-</sup> and KLC2 shRNA axons (in F).

See also Figure S3 and Table S1.

with predicted Gaussian modes using the MCLUST package in the R statistical computing environment (Fraley, 1999). Optimal mode fits were generated by the Bayesian Information Criterion (BIC) (Extended Experimental Procedures). In wild-type, three and two modes best fit the anterograde and retrograde distributions, respectively (Figure 2F, Figure 3F, and Figure 4I; Table S1). Strikingly, reduction of KLC1 or Kinesin-1C reduced velocity distributions in both directions, suggesting that these two subunits pair to form the main holoenzyme that normally drives anterograde and activates retrograde PrP<sup>C</sup> vesicle movement.



#### Figure 4. PrP<sup>C</sup> Vesicular Transport Is Inhibited in Kinesin-1C Mutant Axons

(A–H) Transport parameters in Kinesin-1A<sup>-/-</sup>, Kinesin-1C<sup>-/-</sup>, and Kinesin-1B-cre axons. Shown are population breakdown of YFP-PrP<sup>C</sup> vesicles (A and E) (Nv = # vesicles) run length (B and F), pause frequency (C and G), and segmental velocity (D and H). \*\*\*p < 0.001, \*p < 0.05, permutation t test (black asterisks), Wilcoxon-Mann-Whitney test (red asterisks). Numbers inside bars are segments (run length in B and F, segmental velocity in D and H), and tracks (pause frequency in C and G). All values are shown as mean ± SEM.

(I) Segmental velocity histograms (shown as percentage of segments) in wild-type, Kinesin-1A<sup>-/-</sup>, and Kinesin-1C<sup>-/-</sup> axons. Red and light blue curves represent the overall and predicted Gaussian modes, respectively. See also Figure S4 and Table S1.

Decreasing DHC1 also resulted in bidirectional shifts from higher to lower velocity modes and in a decreased number of modes (Figure 2F; Table S1). Notably, reducing Kinesin-1A and -1B also slightly reduced a mode or the proportion of vesicles within higher anterograde modes (Figure 4I; Table S1), suggesting that these motors might also play a role in the anterograde transport of PrP<sup>C</sup> vesicles, albeit a minor one because we did not observe major defects in other movement parameters. We conclude that segmental velocities represent a measure of the activity of motors and that velocity distributions are reduced as a result of removal or decreases of opposite polarity motors, suggesting that regulation of motors can contribute to these activity changes.

## Kinesin-1C Is Not Required for the Vesicle Association of DHC1 and KLC1

Our tracking data suggest that Kinesin-1C and DHC1 activities are tightly coupled because disruption of either inhibited opposite-polarity PrP<sup>C</sup> vesicle transport. Moreover, both KLC1 and Kinesin-1C are required for normal retrograde motion. To investigate the role of Kinesin-1 in bidirectional motion, we tested whether reduction of retrograde activity following removal of Kinesin-1C was the result of the dissociation of the primary retrograde motor DHC1, from PrP<sup>C</sup> vesicles. We also tested whether KLC1 association with vesicles is needed for normal retrograde motion. We developed a robust imaging method to quantify association of motor subunits on individual endogenous PrP<sup>C</sup> vesicles of hippocampal axons in the presence or absence of Kinesin-1C (Extended Experimental Procedures). Wild-type and Kinesin-1C<sup>-/-</sup> neurons were fixed and stained with antibodies against PrP<sup>C</sup>, DHC1, and KLC1 (Figure 5A), and immunofluorescence images of diffraction-limited PrP<sup>C</sup>, DHC1, and KLC1 vesicle point sources were fitted with 2D Gaussians to estimate their point spread function and to precisely map their coordinates and intensity amplitudes (Jaqaman et al., 2008). A custom-built "motor colocalization" algorithm guantified presence or absence and intensity amplitudes of each detected DHC1 and/or KLC1 puncta within 300 nm of each PrP<sup>C</sup> vesicle (L.S. and S.E.E., unpublished data). DHC1 and KLC1 antibody specificities were evaluated as described in Figure S2B and Figures S5B–S5E and Extended Experimental Procedures.

We designated four PrP<sup>C</sup> vesicle categories, those that had only DHC1, only KLC1, both motor subunits, or no motor subunits associated with them (Figure 5B). We found that in wild-type axons, 43%  $\pm$  2.9% of  $\text{Pr}\text{P}^{\text{C}}$  vesicles colocalized with DHC1, 57%  $\pm$  1.5% of PrP<sup>C</sup> vesicles associated with KLC1, and 25% ± 1.5% of PrP<sup>C</sup> vesicles had both motor subunits. We did not detect motor subunits on 23%  $\pm$  1.8% of PrP<sup>C</sup> vesicles. Removing Kinesin-1C resulted in almost identical DHC1and KLC1-associated PrP<sup>C</sup> vesicle pools (Figure 5B). In addition, relative amounts of PrP<sup>C</sup> vesicle-associated DHC1 and KLC1, as measured by intensity distributions, were very similar between wild-type and Kinesin-1C<sup>-/-</sup> axons (permutation t test, p =0.04 and p = 0.0681 for DHC1 and KLC1 comparisons, respectively) (Figures 5C and 5D). Thus, although the DHC1 intensity distribution was borderline significantly different in Kinesin-1C<sup>-/-</sup> axons as compared to wild-type, DHC1 certainly did not appear to dissociate from PrP<sup>C</sup> vesicles. We conclude that Kinesin-1C is not required for DHC1 or KLC1 association with PrP<sup>C</sup> vesicles. Thus, impairment of retrograde movement observed after removing Kinesin-1C is likely a result of coordination between Kinesin-1C and DHC1 activities, rather than by altering DHC1-vesicle associations. Furthermore, because our tracking data show that retrograde movement is reduced after removal

of KLC1 or Kinesin-1C, but removal of Kinesin-1C did not change the association of KLC1 with vesicles, we conclude that KLC1 is necessary but not sufficient to activate normal retrograde transport of PrP<sup>C</sup> vesicles. Thus, in the absence of KLC1 or Kinesin-1C, retrograde movement is impaired, suggesting that maximal activation of retrograde motion requires the formation and association of a complete KLC1-Kinesin-1C holoenzyme with PrP<sup>C</sup> vesicles. It is possible that redundancy with KLC2 and Kinesin-1A or Kinesin-1B might stimulate residual retrograde motion still observed in KLC1 and Kinesin-1C mutant axons.

## PrP<sup>C</sup> Vesicles Associate with Heterogeneous but Stable Motor Subunit Assemblies

To further confirm the presence of stable motor assemblies on PrP<sup>C</sup> vesicles and to characterize the nature of this motor composition, we asked whether motor subunits of both polarities were distributed evenly and stably on these vesicles. The nonnormal distributions of wild-type KLC1 and DHC1 intensity amplitudes detected on PrP<sup>C</sup> vesicles were mode-fitted and selected using MCLUST and BIC (Figures 5E and 5F). The predicted modes on each KLC1 and DHC1 distributions showed three peaks corresponding to 1×, 2×, and 3× increments of intensities. Because the fluorescent intensity distribution of single molecules has a single Gaussian peak (Sugiyama et al., 2005) and the intensity of fluorescently labeled proteins has been shown to increase with increasing molecule concentration (Dixit et al., 2008), the multiple predicted modes in our data suggest the presence of a heterogeneous population of PrP<sup>C</sup> vesicles associated with 1×, 2×, and 3× multiples of KLC1 or DHC1 motor subunits. We also tested that the KLC1 and DHC1 antibody signals scaled linearly with copy number and were in linear range (Figures S5A–S5E and Extended Experimental Procedures). The KLC1 and DHC1 quantal intensity modes did not change after removal of Kinesin-1C (Figures 5E and 5F), indicating that a stable motor subunit population on vesicles is not affected by the presence or absence of other motors.

To further assess the distribution of motor subunits on vesicles. we divided PrP<sup>C</sup> vesicles into those associated with a single motor subunit (either KLC1 or DHC1), or with both, and quantified their intensity distributions and the percentage of vesicles within each predicted mode. When PrP<sup>C</sup> vesicles were associated only with KLC1, vesicles were distributed evenly in each of the  $1 \times 2 \times$ , and 3× motor subunit number modes (Figure 5G), whereas the majority of PrP<sup>C</sup> vesicles associated only with 1× DHC1. No changes to these distributions were observed when both KLC1 and DHC1 were associated simultaneously with vesicles or when Kinesin-1C was removed, suggesting that presence of KLC1 on the vesicle did not influence the association of DHC1 and vice versa (Figure 5H; Figures S5F-S5H). We conclude that the population of endogenous PrP<sup>C</sup> vesicles in axons is heterogeneous, having vesicles with overall associated motor subunit amounts in guantal multiples of 1x, 2x, and 3x. However, this composition remained stable, and the presence of motor subunits on vesicles did not affect the binding of other subunits.

## Motor Subunits Are Associated with Stationary and Moving PrP<sup>C</sup> Vesicles

Many cargo-bound proteins and organelles stop and function at specified axonal microdomains. Thus, mitochondria are largely



#### Figure 5. Composition of Motor Subunits on PrP<sup>C</sup> Vesicles

(A) Representative immunofluorescence image of a hippocampal axon stained with antibodies against PrP<sup>C</sup>, KLC1, and DHC1. Insets show enlargement with arrows pointing to three and two point sources in KLC1 and DHC1 channels, respectively, that associate with PrP<sup>C</sup> vesicles. Dots represent the location of fitted Gaussian functions.

(B) Percentage of PrP<sup>C</sup> vesicles that have only KLC1, only DHC1, both, or no motor subunits associated with them. Inside bars are the numbers of vesicles for each category. All values are shown as mean ± SEM.

(C and D) Gaussian intensity amplitude distributions comparing the frequency of PrP<sup>C</sup> vesicles associated with DHC1 (C) and KLC1 (D) intensities, in wild-type and Kinesin-1C<sup>-/-</sup> axons.

(E and F) Histograms of the same Gaussian intensity amplitude distributions shown in (C and D), depicting percentage of  $PrP^{C}$  vesicles associated with DHC1 (E) and KLC1 (F). Red and light blue curves represent the overall and predicted Gaussian modes, respectively. Red open circles point to intersections between modes. (G and H) Distribution of  $PrP^{C}$  vesicles with one (G) or both (H) associated motor subunits. Numbers in boxes are percentages of  $PrP^{C}$  vesicles in each category. Color gradient represents higher to lower percentage of  $PrP^{C}$  vesicles in each category.

Nv = # vesicles. See also Figure S5.



Figure 6. Association of Motor Subunits with Stationary and Moving YFP-PrP<sup>C</sup> Vesicles

(A) Left panel: schematic diagram of a microfluidic chamber device. Transfected neurons are shown in green. Right panel: inset of two axons transfected with YFP-PrP<sup>C</sup> growing through a single microchannel (outlined with dotted lines).

(B) Kymograph of YFP-PrP<sup>C</sup> movement in the wild-type hippocampal axon shown in (A). Time of paraformaldehyde application is indicated by green dotted line. The panel below the kymograph is of the deconvolved image of the same fixed axon showing the YFP-PrP<sup>C</sup> channel. Red squares correspond to the same anterograde-moving vesicles in the kymograph that have been mapped to those in the fixed deconvolved image. Images (inset) were taken of all three fixed/ stained channels. Point sources were fitted with Gaussian functions (colored dots).

(C and D) Scatter plots of KLC1 versus DHC1 Gaussian intensity amplitudes of all moving and stationary mapped vesicles from n = 7 axons (C) (with and without associated motor subunits), and of stationary vesicles with detected motor subunits (D).

See also Movie S2.

stationary in axons at sites where there is a high demand for ATP (Kang et al., 2008). Although stoppage can be achieved via changes in external factors such as  $Ca^{2+}$  levels or via interaction with docking adaptors (Kang et al., 2008), it is unknown whether a motionless state is achieved via dissociation of motors from cargo. Our system afforded us the opportunity to test this hypothesis because although PrP<sup>C</sup> vesicles move robustly in both directions, the majority are stationary (~70%) (Figure 1B).

To ask whether motor subunits are differently associated with *individual* stationary versus moving vesicles in vivo, we developed a "vesicle mapping" technique to characterize relative amounts of KLC1 and DHC1 on YFP-PrP<sup>C</sup> vesicles following recording of their individual live motion (Extended Experimental Procedures). Hippocampal cells were plated in microfluidic chambers to promote the growth of straight axons and were transfected with YFP-PrP<sup>C</sup> (Taylor et al., 2005) (Figure 6A).

Movement of vesicles was imaged, and cells were fixed with paraformaldehyde for subsequent staining with KLC1 and DHC1 antibodies, but the trajectories of vesicles before and after fixation were recorded (Figure 6B). Fixed images were superimposed to live movement kymographs to map (colocalize) fixed vesicles to their trajectories, after obtaining their precise Gaussian position coordinates and intensity amplitudes (Figure 6B). The advantage of this method is that we are able to assess motor subunit composition on vesicles for which we have recorded individual vesicular trajectories (anterograde, retrograde, or stationary).

Our data revealed that both KLC1 and DHC1 associated with stationary as well as moving vesicles (Figure 6C). Interestingly, we observed a correlation between KLC1 and DHC1 motor association on stationary vesicles, suggesting simultaneous increasing association of KLC1 and DHC1 (Figure 6D). Our data show that motor subunits associate to vesicles regardless of whether they were moving, indicating that motor subunit association is necessary but not sufficient for active translocation along microtubules.

#### DISCUSSION

We identified Kinesin-1C and DHC1 as major anterograde and retrograde motors required to transport PrP<sup>C</sup> vesicles in mammalian axons and showed that they reciprocally promote their activity independently of motor-association mechanisms. We developed an assay to robustly assess the relative amounts of motors on vesicular cargo and, thus, provided the characterization of motor composition in vivo on a single type of vesicle. We report that PrP<sup>C</sup> vesicles have a stable complement of motor subunits regardless of changes in motor activity, suggesting that regulation of activity, and not motor-vesicle attachment, determines directionality.

## Differential Requirements of Kinesin-1 Subunits in PrP<sup>C</sup> Vesicle Transport

Previous studies showed that PrP<sup>C</sup> moved in anterograde and retrograde directions in nerves, but the mechanism of intracellular movement was unknown (Borchelt et al., 1994; Butowt et al., 2006; Moya et al., 2004; Rodolfo et al., 1999). Using a combination of genetic, live imaging, biochemical, and immunofluorescence approaches, we dissected the requirements of each Kinesin-1 subunit and of dynein, and identified Kinesin-1C and DHC1 as the main plus- and minus-end motors, respectively, with a minor role attributed to Kinesin-1A and Kinesin-1B. Reduction of any one KHC did not result in complete disruption of anterograde transport, suggesting either some redundancy, or the requirement of another as yet unidentified motor. Interestingly, Kinesin-1C mRNA expression was upregulated in Kinesin-1B null extraembryonic membranes, pointing to a possible redundant function between these KHCs (Tanaka et al., 1998). Whether higher transcript levels result in increased vesicle association of Kinesin-1C in Kinesin-1B $^{-/-}$  cells is unknown.

Our data also provide evidence for a role of KLC1 and KLC2 in PrP<sup>C</sup> vesicle transport. KLCs have been implicated in cargo binding and transport regulation through their binding to KHCs but are not always required. Mitochondria, for example, are

transported by KHC and a complex formed by Milton and Miro but lack KLC association (Glater et al., 2006). For PrP<sup>C</sup> vesicles, redundancy between KLC1 and KLC2 is likely because disruption of either KLC did not result in complete blockage of transport. We tested this idea by combining velocity distributions after reducing each KLC and observing the partial reconstitution of the wild-type anterograde distribution but not the retrograde one (Figure 3G; Table S1). Our data further point toward the pairing of neuronally enriched KLC1 and Kinesin-1C as a primary PrP<sup>C</sup> vesicle transport complex: vesicle immunoisolations with KLC1 brought down Kinesin-1C, and disrupting either of these motor subunits resulted in almost identical bidirectional phenotypes, more severe than seen with other subunits, including the decrease in bidirectional velocity distributions (Table S1). Thus, our work establishes KLC1, KLC2, Kinesin-1C, and DHC1 as main motor subunits for PrP<sup>C</sup> vesicle transport. These requirements appear specific, as movement of synaptophysin vesicles is not inhibited following their disruption. Differential use of Kinesin-1 components might be important for the selective targeting of different types of PrP<sup>C</sup> vesicles to distinct axonal domains. Whether the subunits are used in different cellular contexts is unknown, and functional experiments to address this issue for PrP<sup>C</sup> vesicles still need to be performed.

## Uncoupling Motor-Association from Motor-Activation Mechanisms to Drive Bidirectional Transport

To characterize transport mechanisms, we assessed  $\mathsf{Pr}\mathsf{P}^\mathsf{C}$ vesicle movement by live imaging, as well as motor-vesicle associations using immunofluorescence assays. Our data support the hypothesis that the total composition of motor subunits on PrP<sup>C</sup> vesicles is constant even when pronounced changes in motor activity are detected. Activity changes were revealed after impairing Kinesin-1 and DHC1, which resulted in PrP<sup>C</sup> vesicles that traveled shorter distances, paused more frequently, and had altered velocity distributions (Figure 3 and Figure 4). The lower distribution of velocities observed here are in contrast to at least one in vivo study on Drosophila embryo lipid droplets that showed that average velocities were slightly higher following reductions in anterograde motor copy number (Shubeita et al., 2008). Although inherent differences between Drosophila lipid droplets versus axonal transport systems might explain the contrasting observations, it is also possible that mean velocities might not reveal differences that can be detected only in analyses of velocity distributions. Indeed, mean velocities were generally unaffected after reduction of motors in our system (Figure 4D; Figure S3A).

Are the changes in observed motor activity directly correlated to the amount and composition of motors associated to those vesicles? Previous in vitro studies estimated the number of cargo-bound motors and suggested that these directly correlate to level of movement activity during TOW (Hendricks et al., 2010; Shubeita et al., 2008; Soppina et al., 2009). However, during in vivo axonal transport of cargoes undergoing regulatory coordination, although cargoes can be moved by multiple motors, it is unclear whether all motors associated with cargo are active. Our data show that in axons in vivo, removing or reducing a motor reduces parameters of opposite-polarity transport, thus providing support for regulatory motor coordination and against





## Figure 7. Stable Motor Association and Coordination Model of PrP<sup>C</sup> Vesicle Transport

(A) A stable motor subunit composition on anterograde, retrograde, or stationary vesicles is depicted, but only a subset of those are active to drive transport in either direction. Our data suggest a coordination model whereby Kinesin-1 and dynein act as alternating activators of opposite polarity transport. Number of motors depicted is arbitrary. See Discussion for details.

(B) Activation of retrograde motility requires the vesicle association of a complete Kinesin-1 holoenzyme comprised of both KLC1 and Kinesin-1C. Removal of either subunit downregulates activation of DHC1, but does not dissociate DHC1 from PrP<sup>C</sup> vesicles. Thus, Kinesin-1C can activate DHC1 via interaction with KLC1.

simple TOW scenarios. In this regulatory coordination setting, we show that a stable motor subunit complement associates with PrP<sup>C</sup> vesicles regardless of their activity level or directionality. A combination of motor-interactions and regulatory coordination could be at play in vivo, as motors of opposite-polarities can mechanically interact to influence their motility (Ally et al., 2009). Thus, the mechanism of motor activity regulation in axons in vivo appears to be uncoupled from the one that regulates motor-vesicle associations. Consistent with this stable motor association model, previous work showed that Kinesin-2 and dynein levels from purified melanosomes did not change during directionality switches, although these were bulk estimates and therefore it was unclear whether motor levels were unchanged on a per cargo basis (Gross et al., 2002).

We thus propose a coordination model in which a stable population of Kinesin-1 subunits and DHC1 associate with PrP<sup>C</sup> vesicles and a subset of these activate bidirectional movement, while the rest remain vesicle bound but inactive (Figure 7A). Stationary states are likely achieved by regulatory inhibition of bound motors. Thus, this model does not support cargo binding as the sole mechanism of motor activation (Akhmanova and Hammer, 2010). Indeed, in vitro work has shown that inactive motors can bind cargo and diffuse along the MT lattice (Lu et al., 2009). The ability of inactive motors to remain vesicle bound could allow them to be activated "on the spot" according to cues specific to the cargo being transported.

#### **Coordination of Bidirectional Transport in Axons**

Our data show that coordination of retrograde activity by Kinesin-1C is independent of DHC1-vesicle association but involves the simultaneous attachment of both types of motors to vesicles (Figure 5B). Kinesin-1C thus appears to perform a dual function as a mediator of anterograde movement and as an activator of retrograde transport, but is not required for cargo binding by dynein. How might this retrograde activation occur? Our data suggest that it does so by the formation and vesicle association of an intact Kinesin-1C/KLC1 complex, which is required for proper retrograde activity. The absence of either Kinesin-1C or KLC1 precludes normal retrograde activation (Figure 7B). However, KLC1 remains vesicle associated in Kinesin-1C mutants, so its presence alone is not sufficient to activate normal retrograde motion. Likewise, Kinesin-1C is decreased but still present in KLC1<sup>-/-</sup> cells (Rhiannon Killian, personal communication), suggesting that this KHC subunit alone cannot induce normal retrograde activity. It is possible that Kinesin-1C can bind to KLC2 in KLC1-/- axons because our data show that KLC2 is also required for normal levels of bidirectional movement and is likely responsible for the residual retrograde motion observed. However, such a putative interaction is clearly not sufficient to rescue normal retrograde transport. A possible outcome of requiring a complete vesicle-bound Kinesin-1 complex is that it could facilitate rapid activation and/or autoinhibition, which has been shown to occur via KLC with KHC interactions (Cai et al., 2007; Verhey et al., 1998). Changes in Kinesin-1 autoregulation could translate to changes in dynein activity. Thus, our data are consistent with a model for bidirectional coordination in which activities of Kinesin-1 and dynein might be linked via physical contacts (Martin et al., 1999) and points to a role of KLCs in this linkage. Whether contact occurs via KLC interactions with dynein accessory subunits, dynactin, and/or other unidentified components has been suggested but is unclear (Ligon et al., 2004; Martin et al., 1999). Alternatively, direct motor-motor interactions could coordinate movement because mechanical pulling of plus- and minus-end motors against each other has been suggested to be necessary and sufficient to activate opposing motor activity (Ally et al., 2009). In the case of PrP<sup>C</sup> vesicles, coordination of a stable population of simultaneously bound motors is likely to be important for their efficient transport and delivery to various axonal regions or to the cell surface, where PrP<sup>C</sup> has been implicated in signal transduction and cell adhesion (Malaga-Trillo et al., 2009; Mouillet-Richard et al., 2000). This mechanism might also be a strategy for efficient distribution of many other cargoes along axons, with stable complexes of motors loading onto vesicles presumably at or near the cell body, where motors are produced. Stable associations would allow differential regulation and coordination of subsets of motors, either by themselves or by factors specific to the vesicular cargo.

#### **EXPERIMENTAL PROCEDURES**

#### Mice and Cell Culture

Mice used throughout this study were in the C57/Bl6 background. KLC1, Kinesin-1A<sup>-/-</sup>, and conditional Kinesin-1B mice were described previously (Cui et al., 2010; Rahman et al., 1999; Xia et al., 2003). Generation of Kinesin-1C<sup>-/-</sup> mice is detailed in Extended Experimental Procedures. Hippocampal cultures were plated from either embryonic day (E)15–E18 or 1-day-old pups (Falzone et al., 2009).

#### **Transfection and Adenovirus Cre-Recombinase Transduction**

Transfections of hippocampal neurons were done 10 days after plating following a standard Lipofectamine 2000 protocol (Invitrogen). Cells were imaged or fixed 18–24 hr later. Plated hippocampal cells from conditional homozygous Kinesin-1B II/II mice were treated 10 days after plating with 0, 100, or 400 MOI adenovirus cre-recombinase (Ad5CMVCre from the University of Iowa, Gene Transfer Vector Core), corresponding to 0,  $1.1 \times 10^7$ , and  $4.4 \times 10^7$  plaque-forming units (PFU), respectively.

#### **Vesicle Immunoisolation**

Vesicle immunoisolations were performed with antibodies against KLC1 or GFP to pull down vesicular membrane components obtained from floated membrane fractions (Extended Experimental Procedures).

#### Immunofluorescence and Microscopy

Hippocampal neurons and N2a cells were fixed with 4% paraformaldehyde, permeabilized, and stained with antibodies against KLC1 and DHC1. Fixed immunofluorescence images were taken on a Deltavision RT deconvolution system, and live images were taken with a Nikon Eclipse TE2000-U inverted microscope (Extended Experimental Procedures).

#### **Data and Statistical Analysis**

Trajectories of individual YFP-PrP<sup>C</sup> or synaptophysin-mCherry vesicles (i.e., tracks) were detected using a custom-made semiautomated particle tracking software written in MATLAB (Mathworks) and C++ (G.F. Reis, G. Yang, L.S., S.B. Shah, J.T. Robinson, T.S. Hays, G. Danuser, L.S.B.G., unpublished data). Definitions and calculations for each parameter are detailed in Extended Experimental Procedures.

"Motor colocalization" and "vesicle mapping" analyses are detailed in Extended Experimental Procedures. All tracking parameters reported here were first tested for normality using the Lilliefors test implemented in the *nortest* package of R. Most parameters were not normally distributed so a nonparametric permutation t test was used for comparison between genotypes (Moore and McCabe, 2005). Differences in medians were also compared between genotypes for all parameters using the Wilcoxon-Mann-Whitney rank-sum test.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, one table, and two movies and can be found with this article online at doi:10.1016/j.cell.2011.01.021.

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# **Supplemental Information**

## **EXTENDED EXPERIMENTAL PROCEDURES**

#### **Sciatic Nerve Ligations**

We confirmed earlier observations that the mammalian prion protein is transported in the sciatic nerve by using a protein accumulation paradigm in which nerves were ligated at the midsection, and proximal and distal sections were dissected and analyzed for  $PrP^{C}$  immunofluorescence 6 hr post-ligation (Figure S1A). In this assay, proteins that travel in anterograde and retrograde directions accumulate at the proximal and distal side of the ligations, respectively. For each wild-type mouse, one sciatic nerve was ligated approximately at the midsection while the other was left un-ligated. Six hours post-ligation, mice were sacrificed and either 3 mm of nerve at either side of the ligation, or 6 mm of unligated nerve were removed. Nerves were embedded with Histo Prep (Fisher) and immersed immediately in methanol/dry ice and maintained at  $-20^{\circ}C$ . Nerves were cut longitudinally in a cryostat at  $-20^{\circ}C$ , fixed with 4% paraformaldehyde and stained for antibodies against  $PrP^{C}$  and APP. We found that  $PrP^{C}$  accumulated primarily in the proximal side of nerves suggesting that net movement in the nerve is toward the terminal (Figure S1B).

#### **Molecular Cloning**

As the signal peptide (SP) and GPI anchor sequences are cleaved off from the 5' and 3' ends of PrP<sup>C</sup> during normal processing, YFP and linker sequences were inserted between the signal peptide and the rest of the mouse PrP<sup>C</sup> sequence to avoid cleavage of YFP (Figure S1C). YFP was annealed by PCR amplification to the 5' end of amino acid 23 of the PrP<sup>C</sup> sequence with a Xhol site at the 3' end (YFP-PrP<sup>C</sup>-Xhol). The signal peptide (amino acids 1–22, nucleotides 1–66) was amplified by PCR together with the adjacent 10 nucleotides 5' of the coding region (ATCAGTCATC), and with the addition of Xhol sites 5' of the amplicon (Xhol-SP). These two PCR fragments (YFP-PrP<sup>C</sup>-Xhol and Xhol-SP) were annealed together by overlapping PCR amplification which resulted in the addition of a linker between the two amplicons. The resulting sequence (Xhol-SP-linker-YFP-PrP-Xhol) was cloned into the Xhol site of vector MoPrP.Xho which contains the promoter, 5' intronic, and 3' untranslated sequences of the murine prion protein gene (Borchelt et al., 1996).

To determine whether our construct behaved similarly to endogenous  $PrP^{C}$ , we transfected differentiated mouse neuroblastoma (N2a) cells with YFP-PrP<sup>C</sup>, and fixed and stained them with antibody Hum-D13 against  $PrP^{C}$ . We found that ~90% of endogenous  $PrP^{C}$  vesicles colocalized with YFP-PrP<sup>C</sup> (Figure S1D). We further tested YFP-PrP<sup>C</sup> for the common posttranslational addition of glycosylation groups that endogenous  $PrP^{C}$  undergoes in the Golgi. We found that treatment of N2a cells with the peptide N-glycosidase PNGase F removed the glycosylated isoforms both in endogenous (nontransfected) and N2a cells transfected with YFP-PrP<sup>C</sup>, showing that YFP-PrP<sup>C</sup> is posttranslationally glycosylated (Figure S1E). Finally, the levels of YFP-PrP<sup>C</sup> in transiently transfected N2a cells was ~88% that of endogenous  $PrP^{C}$ , thus indicating that transient transfection does not result in highly overexpressed levels of this transgene (Figure S1E). The high degree of colocalization, deglycosylation profiles, and overall expression levels indicate that YFP-PrP<sup>C</sup> behaves and is being processed similarly to the endogenous protein.

## Mice

#### **Generation of Kinesin-1C Null Mice**

Homozygous Kinesin-1C null mice were generated in our lab by replacement targeting. Adult Kinesin- $1C^{-/-}$  mice do not exhibit any gross morphological abnormalities and live through adulthood as homozygous null mutants.

Constructing the Kinesin-1C Targeting Vector. A Kinesin-1C genomic clone (KIF5g-G) isolated from a mouse 129SVJ/Lambda FIX II genomic library (Stratagene) was used to construct a simple replacement targeting vector. The targeting vector was made as follows: the right arm was a 3.6 kb Smal-Scal-Scal fragment. This fragment was made by subcloning Smal-Scal and Scal-Scal fragments into pBluescript SK vector, then being ligated together, the right arm was subcloned into the BamHI site of pPNT vector by blunt ligation. A 4.5 kb Smal-Smal fragment was blunt ligated into Xhol site of right-arm/pPNT, and this fragment containing two exons (encoding amino acid #132 to the first two nucleotides of aa #196) with an ~2 kb pGK-neo cassette, and was named as KIF5C-KO/pPNT.

Generating Chimeric Mice. The Kinesin-1C targeted ES cells were generated as described by Klein et al. (Klein et al., 1993). The KIF5C-KO/pPNT plasmid DNA was linearized with Notl, and transfected into RI ES cells by electroporation, 200 ~g/ml G418 was added ~36 hr after transfection, and 2 uM gancyclovir was added 1 day later for the negative selection. Surviving clones were screened by Southern blot analysis of HindIII-digested ES DNAs with a 3'-external probe. Two positive clones were isolated by two independent transfections: K128 (one out of 240 clones screened), and #157 (one out of 180 clones). Clone K128 was injected into C57BL/6J blastocysts to produce chimeras, and germline transmitted heterozygous Kinesin-1C mice were generated by crossing male chimeras to C57Bl/6J mice.

#### Kinesin-1B Mice

Kinesin-1B null/Pflox mice (type I deletion; hereafter referred as Kinesin-1B I/+), and conditional homozygous Kinesin-1B pflox mice (type II deletion; hereafter referred as Kinesin-1B II/II), were generated by Nancy Jenkings and JianDong Huang (Cui et al., 2010). These mice were maintained as heterozygous (Kinesin-1B I/+), or as conditional homozygotes (Kinesin-1B II/II). Kinesin-1B I/+ intercrosses produced no Kinesin-1B I/I mice (n = 87; Nancy Jenkins, unpublished data), likely due to early embryonic lethality, which is in agreement with earlier reports of Kinesin-1B homozygote lethality at E9.5 (Tanaka et al., 1998). Genotyping of cre-treated cells showed a shift from the II/II to the I/I null mutant genotype 3 days after adenovirus treatment (Figure S4C). This shift was confirmed by qPCR, which showed 80% and 88% reduction of II/II genotype after treatment with 100 and 400 MOI, respectively. Immunobloting of treated cell lysates also showed reduction in protein levels by  $\sim$ 50% (Figure S4D).

#### **Cell Culture**

Hippocampal cells were dissected from 1-day-old neonates for all mice except from  $KLC1^{-/-}$  and Kinesin- $1A^{-/-}$  mice. Because most  $KLC1^{-/-}$  and all Kinesin- $1A^{-/-}$  neonate pups die immediately upon birth, hippocampal neurons were dissected from E18-E21 embryos (Rahman et al., 1999; Xia et al., 1998). Hippocampi were dissected, treated with Papain (Worthington), dissociated by trituration, and plated on poly-L-lysine treated glass coverslips, as described previously (Falzone et al., 2009). Cells were plated on DMEM medium containing 10% fetal bovine serum (FBS) for 1–3 hr, and switched to Neurobasal-A media containing B27 complement and Glutamax (Invitrogen). Neurons were cultured for 10 days at  $37^{\circ}C$  and in a 5.5% CO<sub>2</sub> atmosphere prior to transfection and imaging. For live tracking imaging studies, cells were plated in 24-well glass coverslips at a density of ~125,000 cells per well.

## Antibodies

The antibody against PrP<sup>C</sup> (Hum-D13) was obtained from InPro and also as a gift from A. Williamson and Laura Solforosi (The Scripps Research Institute; (Peretz et al., 1997). Antibodies against KLC1 (V-17) and DHC1 (sc-9115) were from Santa Cruz Biotechnology. Anti-Kinesin-1A, and anti-Kinesin-1C antibodies were described previously (Rahman et al., 1998; Xia et al., 2003). Kinesin-1B antibody is a rabbit polyclonal raised against amino acids 499–783.

Primary antibodies used for motor colocalization immunofluorescence experiments PrP<sup>C</sup> (Hum-D13), KLC1 (V-17), DHC1 (sc-9115), were used at a 1:100 dilution. The corresponding secondary chicken anti-human (FITC), donkey anti-goat (Alexa 647), and donkey anti-rabbit (Alexa 568) antibodies were used at a dilution of 1:200, respectively. These antibody dilutions were chosen after obtaining antibody saturation curves using different primary and secondary antibodies dilutions to determine the optimal combinations. Primary and secondary antibody-only controls were done for all possible antibody combinations to test for antibody crossreactivity. We observed only background levels in axons with primary and secondary antibody-only staining.

For the motor colocalization studies, to assess KLC1 antibody specificity and whether there was a linear correlation between KLC1 intensities and KLC1 copy number by immunofluorescence, KLC1<sup>+/+</sup>, KLC1<sup>+/-</sup>, and KLC1<sup>-/-</sup> hippocampal cells were stained with antibody KLC1 (V-17). KLC1 levels were quantified on all KLC1-associated vesicles after estimation of Gaussian curves for each point source. Mean KLC1 intensity in KLC1<sup>-/-</sup> vesicles was reduced by >60% as compared to KLC1<sup>+/+</sup> vesicles, after background subtraction (93.82 arbitrary units [au] in KLC1<sup>+/+</sup>; 65.42 au in KLC1<sup>+/-</sup>; and 36.94 au in KLC1<sup>-/-</sup> axons). Furthermore, the KLC1 intensity signal increased with increasing KLC1 copy number, and this relationship was linear with a high regression coefficient (R<sup>2</sup> = 0.99), suggesting that relative quantitation of KLC1 levels is possible to assess with this antibody (Figure S5A). Furthermore, although it is possible that the residual KLC1 signal observed in KLC1<sup>-/-</sup> axons might be attributed to unspecific binding of the antibody, western blot assays from KLC1<sup>-/-</sup> mouse brain homogenates show high antibody specificity (Figure S5B), suggesting that the remaining signal corresponds to background fluorescent levels. We also observed a linear relationship between amounts of KLC1 detected with the same KLC1 antibody (V-17) and KLC1 copy number in western blots of mouse brain homogenates (Figure S5C). In addition, we costained hippocampal neurons with antibodies against KLC1 and mitochondrial Cox1, and found no significant colocalization between the two markers, in agreement with reports that mitochondrial transport is independent of KLC1 function (Glater et al., 2006), and further suggesting that the KLC1 antibody was specific.

For motor colocalization studies, to assess DHC1 (sc-9115) specificity, we stained hippocampal cells transfected with a scrambled control or a DHC1 shRNA-mCherry construct to reduce the function of DHC1 (see shRNA section below). Quantitation of immuno-fluorescent staining of DHC1 levels of transfected versus nontransfected cell bodies 2 days post-transfection indicated that DHC1 signal was reduced from 50%–80%, for an average of 66% (n = 13; Figure S2B). Furthermore, DHC1 mRNA levels were reduced by >80% from N2a cells transfected with the same DHC1 shRNA-mCherry construct (Figure S2A, see shRNA section below). To test that our DHC1 antibody was in a linear range, increasing amounts of DHC1 shRNA-treated and scrambled-treated N2a cell homogenates were loaded and analyzed by western blot using a DHC1 antibody (Figure S5D). Quantitation of DHC1 levels normalized to alphatubulin (DM1 $\alpha$ ) showed a linear range and that relative quantitation of DHC1 levels is possible to assess with this antibody (Figure S5E).

#### **Vesicle Immunoisolations**

Wild-type mouse brains were homogenized in a detergent-free buffer (8% sucrose and 3mM imidazole, pH 7.4), and post-nuclear supernatant (PNS) was bottom-loaded on a sucrose step gradient consisting of 35%, and 8% sucrose. After centrifugation at 200,000 g for 2 hr at 4°C, the 8/35 interphase was harvested and incubated overnight with antibodies against KLC1 (V-17, Santa Cruz Biotechnology), or GFP (A6455, Molecular Probes). Protein A or G agarose beads (Roche) were added and fractions were incubated for 2 hr at 4°C. Washed pellets were eluted with beta-mercaptoethanol (BME) and boiled, and eluted samples were analyzed by SDS-PAGE and western blot.

#### shRNA

Three shRNA constructs for each KLC2, DHC1, and scrambled D11 targets were built as part of a kinesin and dynein lentiviral minilibrary, in a pLL3.7 GW lentiviral vector with gateway entry modifications and a mCherry marker (S.E.E., unpublished data). Target sequences were designed by Tony Orth at the Genomics Novartis Foundation using proprietary software that identified and selected sequences after comparison of each to the entire mouse genome to ascertain a high degree of specificity (or no-specificity in the case of the scrambled sequence). Validation of shRNA constructs for reduction of KLC2, and DHC1 function was done by transfecting N2a cells separately with the 3 sequences of each target using Lipofectamine 2000 (Invitrogen). Transfection rates varied from 50%–70%, and were ascertained by counts of mCherry fluorescence on cell bodies. Cells were harvested 2-4 days post-transfection and lysates were immunoblotted with antibodies against KLC2 (63–90), and DHC1 (sc-9115, Santa Cruz Biotechnology). N2a cells were also harvested at the same time points and RNA made by reverse-transcriptase (RT) PCR using a SuperScript First Strand kit (Invitrogen). Quantitative PCR was performed to test for reduced mRNA of KLC2 and DHC1 using 3 sets of primers designed for each. One sequence was selected for each target and cotransfected with either YFP-PrP<sup>C</sup> or synaptophysin-YFP using Lipofectamine 2000, in 10-day-old hippocampal neurons 18–24 hr prior to imaging. Only cells with mCherry fluorescence were imaged for YFP-PrP<sup>C</sup> or synaptophysin-YFP vesicle transport. To further validate decreased levels of DHC1 protein in hippocampal cells transfected with the chosen DHC1 shRNA-mCherry construct, we fixed and stained transfected cells with an antibody against DHC1. Quantitation of immunofluorescent staining of DHC1 levels of transfected versus non-transfected cells was done as detailed above.

The sequences used in shRNA constructs were the following: KLC2 (forward 5'-GTGGAATACTACTACCGGA-3'; reverse 5'-AGGTGGAATACTACTACCGGAGA-3'); DHC1 (forward 5'- GTGATGCCATACGAGAGAA-3'; reverse 5'- CAGTGATGCCATACGA GAGAAGA-3'); scrambled D11 (forward 5'-GCACACGTATCGACGTATC-3'; reverse 5'-CGCACACGTATCGACGTAT-3'). We observed nearly ~95% cotransfection (n = 25 cells) between YFP-PrP<sup>C</sup> and KLC2 or DHC1 shRNA-mCherry, and 50%–70% transfection efficiency in N2a cells, which produced an estimated ~83% and ~89% reduction of KLC2 and DHC1 mRNA expression, respectively, 2 days after transfection, as confirmed by RT-QPCR (Figure S2A and data not shown). Protein levels were reduced by >90% and >66% for KLC2 and DHC1, respectively (Figure S5D, and data not shown).

Mouse N2a cells used for validation of KLC2, and DHC1 shRNAs were cultured in DMEM containing 10% FBS and 5% penicillinstreptomycin at 37°C in 5% CO<sub>2</sub>. Cells were plated at 40%–50% confluency and transfected with shRNA target constructs 18–24 hr later with Lipofectamine 2000.

#### Live-Imaging Microscopy, Motor Colocalization, and Vesicle Mapping

Live imaging of YFP-PrP<sup>C</sup>, synaptophysin-mCherry, or synaptophysin-YFP was done on 10- or 11-day-old hippocampal neuron axons, within 24 hr after transfection. Transfection rates were  $\sim$ 3%, which facilitated imaging of single axons. Plates were maintained at 37°C and in a 5.5% CO<sub>2</sub> environment throughout the imaging period, for a maximum of 40 min. Images were taken exclusively from axons and we distinguished axons from dendrites by morphology. Axons are thinner and uniform in diameter, are longer, have fewer branches, and exhibit prominent growth cones (Baas et al., 1989; Baas et al., 1988). Axons were traced from termini to cell body and imaged within a region > 180 um from either end. Live images were taken with a Nikon Eclipse TE2000-U inverted microscope equipped with a Coolsnap HQ camera (Roper Scientific) and a 100X/1.4 NA oil objective. Movies were 15 s long and collected at 10 frames per second at 100 ms exposure (10 Hz), at a resolution of 0.126um using Metamorph stream acquisition software (MDS Analytical Technologies).

For motor colocalization studies, cells were fixed with 4% paraformaldehyde plus 4% glucose, for 30 min at 37°C and in a 5.5%  $CO_2$ . Cells were incubated for 5 min at room temperature with 0.1% Triton X-100 (TX-100) for permeabilization, and followed by a 45 min incubation in block consisting of 10% donkey serum, 3% BSA, 0.1% TX-100 in PBS. Images of fixed wild-type and Kinesin-1C<sup>-/-</sup> hippocampal cells stained with antibodies against PrP<sup>C</sup>, KLC1 and DHC1 were taken on the same day and under the exact exposure conditions for each experimental condition. Fixed immunofluorescence images were taken on a Deltavision RT deconvolution system mounted on an Olympus IX70 inverted microscope equipped with a mercury lamp, with bandpass excitation and emission filters for FITC, Rhodamine and Cy-5 and a 100X objective/1.4 NA PlanApo. Images of 0.5 mm TetraSpeck fluorescent microspheres (Invitrogen), which were used to calibrate the X-Y-Z alignment of the microscope to account for spherical and chromatic aberration of the fluorescent channels, were also collected on the same day. For some colocalization analyses, fluorescent microspheres from an InSpeck Microscope Image Intensity Calibration Kit (Invitrogen).

For vesicle mapping analyses, hippocampal neurons (~275,000) were plated in microfluidic chambers as described previously (Taylor et al., 2005). Movies of intracellular YFP-PrP<sup>C</sup> movement from transfected cells were taken as described above, from axonal regions inside microchannels. While live imaging, culture media was removed from chambers and replaced with 4% paraformalde-hyde to fix neurons and stain with KLC1 and DHC1 antibodies. Images of YFP-PrP<sup>C</sup>, KLC1 and DHC1 channels from fixed axons were taken from the exact location where live movies were acquired, and these images were subsequently mapped back onto kymographs of the live YFP-PrP<sup>C</sup> movement using Adobe Photoshop. Individually mapped YFP-PrP<sup>C</sup> vesicles were classified according to transport class (anterograde, retrograde or stationary), and were fitted with Gaussians. The X-Y coordinates of each mapped PrP<sup>C</sup> vesicle were used to determine co-localization with KLC1 and/or DHC1 Gaussian-fitted point sources. The combination of live and fixed analyses was used because simultaneous live visualization of motor and vesicle movement via coexpression of fluorescent tags

attached to motors is challenging due to the overwhelming signal of presumably inactive soluble motors that results in a non-localized cytoplasmic fluorescence.

#### Data Analysis

## **Particle Tracking Definitions**

For each genotype, trajectories were classified as anterograde, retrograde, stationary or reversing, and five parameters describing the dynamics of transport were calculated: percent cargo population, segmental velocity, run length, pause frequency, and pause duration. Switch frequency and estimated run length were also calculated for some genotypes. We used a custom MATLAB based particle tracking software (LAPTrack) to track the trajectories of vesicles (Reis et al., unpublished data). The following definitions were used for the calculation of transport parameters:

*Track*. A cargo trajectory represented by its X and Y coordinate series, that lasts through all frames of the imaging period (15 s, 150 frames).

*Track Segment*. An uninterrupted period of anterograde or retrograde movement with a speed of >0.095 μm/s framed by pauses and/or the beginning of an imaging period.

Pause. A cargo undergoes a pause when, at a particular position, has an instantaneous velocity of <0.095  $\mu$ m/s, within a sliding window width of 7 frames.

Stationary Vesicle. A particle that is paused for the entire duration of the imaging period (15 s).

*Reversal (Switch) Vesicle.* We define a cargo as undergoing a reversal at coordinates X, Y, if the two vectors that meet at that location (a) point in opposite directions, and (b) are located a minimum of 550 nm away from X, Y. This minimum threshold distance allows us to avoid false positives in reversal detection caused by small local random movement.

Segmental Velocity (in  $\mu$ m/s). The total distance of a track segment divided by the segment duration. Anterograde or retrograde segmental velocities include those from segments moving uniquely in either direction, as well as those segments from the anterograde or retrograde direction of reversal tracks.

Run Length (in  $\mu$ m). Is the distance moved by a particle in a defined segment. Run length is calculated for individual segments and then pooled together for each genotype. Standard error is calculated separately for anterograde and retrograde directions pooling data from all tracks.

Duration-Weighted Segmental Velocity (in  $\mu$ m/s). Is a weighted average of segmental velocities by the duration of movement. Thus, they are calculated as the sum of the segmental velocities per track, multiplied by their durations, and divided by the sum of the durations for that particular track. This parameter is calculated for anterograde and retrograde segments (including reversals), and mean and standard error are computed pooling all tracks per genotype. This parameter was used to calculate estimated run length.

Estimated Run Length (in µm). Is the average duration-weighted segmental velocity divided by the corrected pause frequency. Since observed run lengths were in many instances invariably truncated due to the limits of a fixed microscope field-of-view, and due to the finite time of image acquisition, to test whether KLC2 shRNA neurons also had decreased run lengths, we calculated estimated run lengths as the average duration-weighted segmental velocity divided by the average corrected pause frequency. These values are more representative of the true run lengths that particles would travel if we were to image them without sampling constraints. This parameter is a bulk value, so no standard errors can be calculated.

Corrected Pause Frequency (in times/s). Pause frequency is calculated for individual segments as one divided by the moving time of the segment immediately preceding that pause. For each genotype, pause frequency is calculated for each anterograde and retrograde segment pause (including in reversals), and averaged. Standard error is calculated for all segmental pause frequencies per genotype.

Switch Frequency (times/s). The number of reversals a particle undergoes per second. Switch frequency is calculated for each track and averaged per genotype. Standard error is calculated per track.

#### Motor Colocalization and Vesicle Mapping Data Analyses

Hippocampal cells stained with antibodies against PrP<sup>C</sup>, KLC1, and DHC1 were assessed for colocalization. Image preprocessing for "motor colocalization" and "vesicle mapping" analyses of stained vesicles was done in ImageJ (NIH). We then fit 2D Gaussian distributions (Thomann et al., 2002) to all point sources from each channel using a custom algorithm (Jaqaman et al., 2008). This resulted in precise estimates of point source positions in each channel. We then designed a method to assess colocalization between PrP<sup>C</sup> and KLC1 and/or DHC1 by first taking the Gaussian-fitted coordinates in the PrP<sup>C</sup> channel, and determining colocalization with the other channels within a 300 nm radius of each Gaussian-fitted PrP<sup>C</sup> puncta (L.S. and S.E.E., unpublished data). We defined colocalization within a 300 nm cut-off based on the diffraction-limits of the microscope, and assuming that the size of PrP<sup>C</sup> vesicles is below this limit. Cluster mode-fitting of non-normal Gaussian intensity amplitudes was done in MATLAB.

#### Gaussian Fitting and Cluster Analysis of Segmental Velocity Distributions and Intensity Amplitudes

Intensity amplitudes for all PrP<sup>C</sup> vesicles associated with KLC1 and/or DHC1, and segmental velocity distributions of YFP-PrP<sup>C</sup> vesicles were non-normal and were fitted with two or three Gaussian modes using the MCLUST package in the R statistical computing environment. Optimal fits were selected using the Bayesian Information Criterion (BIC) analysis, a robust statistic based on model-based clustering, that allows comparison of models with differing clusters (Fraley, 1999). To test the internal consistency of the BIC method, we bootstrapped the original intensity and velocity distributions to create 40 resampled distributions. We obtained the same

number of predicted cluster modes for 68%–80% of the distributions. Generating a higher number of bootstrapped distributions will likely increase the percent of distributions with the original mode designations.

#### **Gaussian Intensity Amplitude Mode Designation**

Intensity amplitudes for all PrP<sup>C</sup> vesicles associated with KLC1 and/or DHC1, and segmental velocity distributions were non-normal and were fitted with three Gaussian modes. The assignment of vesicles to each mode was done after calculating threshold values for the intersection between two Gaussian curves (red open circles in Figures 5E and 5F), using published threshold equations (Moore and McCabe, 2005).

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## Figure S1. PrP<sup>C</sup> Is Transported along Axons, Related to Figure 1

(A) Schematic diagram of a sciatic nerve ligation experiment. After ligation, proteins moving in an anterograde direction accumulate in the proximal side of the ligature, while those traveling in the retrograde direction accumulate in the distal side.

(B) Confocal images of longitudinally sectioned ligated and unligated mouse sciatic nerves fixed and stained with antibodies against PrP<sup>C</sup> and APP. PrP<sup>C</sup> and APP accumulated primarily at the proximal end of the ligature. Arrows point to ligation site.

(C) Schematic diagram of the MoPrP.Xho::YFP-PrP (referred to as YFP-PrP<sup>C</sup>) construct made to image YFP-PrP<sup>C</sup> vesicle transport in hippocampal mouse cells. Top panel shows a diagram of PrP<sup>C</sup> protein structure indicating the signal peptide (SP) sequence, coding sequence (PrP<sup>C</sup>), and GPI anchor sequence. Both SP and GPI sequences are cleaved post-translationally. The letters between the SP and YFP are linker amino acids. The numbers below indicate position of amino acid residues (2-227 for YFP; 23-231 for PrP<sup>C</sup> coding sequence).

(D) Confocal image of a mouse N2a cell transfected with YFP-PrP<sup>C</sup> and fixed and stained with an antibody against PrP<sup>C</sup> and an Alexa-568 secondary antibody. Cell was permeabilized prior to staining. Arrowheads indicate vesicles in both channels that colocalize. Some vesicles reside in a different optical slice in either one or the other channel.

(E) Extracts of N2a cells transfected and non-transfected with the YFP-PrP<sup>C</sup> construct. For each condition, cells were either treated or not with PNGase F to deglycosylate PrP<sup>C</sup> and YFP-PrP<sup>C</sup>.

20 u

hRNA DHC1 + DHC1 ar



#### Figure S2. Reduction of DHC1 by shRNA Does Not Change Segmental Velocities, Related to Figure 2

DHC1 s

(A) N2a cells were transfected with a lentiviral construct containing a shRNA against DHC1 or a scrambled control. Cells were harvested 2 days post-transfection and RNA was extracted to perform qRT-PCR. Percentage of DHC1 mRNA for both conditions using two different primer sets is shown.

(B) Deconvolved images of hippocampal cells transfected with a DHC1 shRNA-mCherry construct to reduce DHC1 levels and stained with antibodies against DHC1 and PrP<sup>C</sup>. (a) Red arrow points to mCherry signal in transfected cell; (b) Green arrow points to untransfected cell stained with an antibody against DHC1 showing higher levels than the shRNA DHC1-mCherry transfected cell in (a); (c) Same cells stained with an antibody against PrP<sup>C</sup> to show cell shape; (d) Merge of mCherry and DHC1 antibody signals from (a) and (b). In cells expressing DHC1 shRNA-mCherry, DHC1 protein levels were reduced by 50%–80%, for an average of 66%, as compared to non-mCherry cells.

(C) Anterograde and retrograde mean segmental velocities of PrP<sup>C</sup> vesicles moving in axons are not affected after treatment with the DHC1 shRNA-mCherry construct validated in (A). All values are shown as mean ± SEM.

YFP-PrP<sup>C</sup>:







F

segmental velocity (µm/sec)

I

average % population

0.9

0.8

0.7

0.6

0.5

0.4

0.3

0.2

0.1





G

70

60

50 20

10

0

anterograde

werage % population











Figure S3. Differential Requirements of Kinesin-1 Subunits in the Intracellular Transport of YFP-PrP<sup>C</sup> and Synaptophysin-mCherry or Synaptophysin-YFP Vesicles, Related to Figure 3

total Nv WT = 293

total Nv Kinesin-1A -/- = 894

total Nv Kinesin-1C -/- = 383

PrP<sup>C</sup> in WT

PrP<sup>c</sup> in Kinesin-1A -/-

PrP<sup>c</sup> in Kinesin-1C -/-

(A–C) Anterograde and retrograde mean segmental velocities for (A) all moving YFP-PrP<sup>C</sup> vesicles (bi- and uni-directional), (B) YFP-PrP<sup>C</sup> vesicles moving unidirectionally (anterograde or retrograde), and (C) only YFP-PrP<sup>C</sup> vesicles reversing at least once during the duration of our recording.

(D–F) Anterograde and retrograde mean segmental velocities of synaptophysin<sup>a</sup> vesicles in (D) KLC mutants, (E) Kinesin-1A and Kinesin-1C mutants, and (F) Kinesin-1B mutants. All moving (unidirectional and reversals) vesicles are included.

(G-I) Average percent synaptophysin<sup>a</sup> cargo population for anterograde, retrograde, reversing and stationary cargo.

Nv = # vesicles; Numbers of segments are indicated inside bars.

reversing

total Ny WT = 293

PrP<sup>c</sup>in WT

PrP<sup>c</sup>in KLC1 -/-

PrP<sup>C</sup>in KLC2 shRNA

total Nv KLC1 -/- = 428

total Nv KLC2 shRNA = 211

retrograde

stationary

All values are shown as mean ± SEM. \*\*\*p < 0.001, \*\*p < 0.01, \*p < 0.05, permutation t test (black asterisks), Wilcoxon-Mann-Whitney test (red asterisks). <sup>a</sup>Data correspond to the movement dynamics of synaptophysin-mCherry for all conditions except for KLC2 shRNA-mCherry, in which case the behavior of synaptophysin-YFP was recorded.



#### Figure S4. Generation of a Kinesin-1C Deletion Null Mutant Mouse, and Kinesin-1B Mutant Cells, Related to Figure 4

(A) Schematic diagram of Kinesin-1C gene targeting vector, which was constructed as a deletion mutant. In the targeted allele, two Kinesin-1C exons encoding amino acid 132 to the first two nucleotides of amino acid 196 were replaced with a pGK-neo cassette. PCR genotyping primers are indicated by the red arrows.
(B) Kinesin-1C mRNA (Northern blot, left panel), and protein (western blot, right panel) were absent from homozygous mutant Kinesin-1C mice brain lysates. Kinesin-1A and 1B protein levels were unchanged in the Kinesin-1C null mutant.

(C) DNA gel showing genotyping bands for Kinesin-1B hippocampal cell extracts treated with 0, 100, or 400 multiplicity of infection (MOI) units of cre-recombinase adenovirus. The Kinesin-1B II/II product is excised upon cre-treatment to convert to a Kinesin-1B I/I product. The larger PCR product of the Kinesin-1B I/I band is detected because of the position of the primers. Bottom arrow shows wild-type Kinesin-1B.

(D) Western blot showing reduction in the expression of Kinesin-1B protein in 100, and 400 MOI cre-treated hippocampal cells.



Figure S5. Intensity Distributions of Motor Subunits on PrP<sup>C</sup> Vesicles Reveal a Heterogeneous Composition, Related to Figure 5

(A) Scatter plot showing a linear relationship between normalized KLC1 Gaussian intensity amplitudes and KLC1 copy number. Gaussian amplitudes were obtained from the 'motor colocalization' program following KLC1 immunofluorescent staining of individual vesicles in hippocampal axons.  $KLC1^{-/-}$  axons have 0 KLC1 copy number ( $N_{axons} = 10$ ; Nvesicles = 237),  $KLC1^{+/-}$  axons have 1 KLC1 copy number ( $N_{axons} = 10$ ; Nvesicles = 326), and  $KLC1^{+/+}$  axons have 2 KLC1 copy numbers ( $N_{axons} = 10$ ; Nvesicles = 391).

(B) Western blot showing specificity of KLC1 antibody for increasing loaded amounts of KLC1 protein from mouse brain homogenates. KLC1 antibody recognizes a single band in wild-type (KLC1<sup>+/+</sup>), but no band is recognized in KLC1<sup>-/-</sup> homogenates.

(C) Scatter plot showing a linear relationship between KLC1 intensities and increasing loaded amounts of KLC1<sup>+/+</sup> mouse brain homogenate. Intensities were measured from the western blot in (B).

(D) Western blot showing DHC1 and tubulin (control) staining of N2a cell homogenates from cells transfected with DHC1 shRNA-mCherry or scrambled control constructs.

(E) Scatter plot showing linear relationships between DHC1 intensities (divided by tubulin intensities for the same loadings), and increasing loaded amounts of N2a cell homogenate from cells transfected with scrambled or DHC1 shRNA-mCherry constructs (to reduce DHC1 levels). Intensities were measured from the western blot in (D).

(F and G) Intensity distributions of the percentage of PrP<sup>C</sup> vesicles with (A) KLC1, when only KLC1 or both KLC1 and DHC1 are present; and with (B) DHC1, when only DHC1 or both motor KLC1 and DHC1 are present. Nv = # vesicles.

(H) Distribution of  $PrP^{C}$  vesicles in Kinesin- $1C^{-/-}$  cells with one or both KLC1 and DHC1. Numbers in boxes are percentages of  $PrP^{C}$  vesicles that fall within each category. Color gradient represents higher to lower percentage of  $PrP^{C}$  vesicles in each category.