THE EMBO JOURNAL

Manuscript EMBO-2009-72422

Kinesin-1/Hsc70 dependent mechanism of slow axonal transport and its relation to fast axonal transport

Sumio Terada, Masataka Kinjo, Makoto Aihara, Yosuke Takei, Nobutaka Hirokawa

Corresponding author: Nobutaka Hirokawa, Univ Tokyo Grad Sch Med

Review timeline:

Submission date:	01 April 2009
Editorial Decision:	11 May 2009
Rebuttal:	20 May 2009
Invitation to resubmit:	25 May 2009
New submission received:	02 September 2009
Editorial Decision:	05 October 2009
Revision received:	08 November 2009
Editorial Decision:	26 November 2009
Accepted:	26 November 2009

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 May 2009

Thank you for submitting your manuscript to the EMBO journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see from these comments, while referee #3 is more supportive of the analysis, referee #1 and 2 also raise significant concerns with different aspects of the analysis. In particular, referee #1 and 2 find that there is not sufficient direct evidence in support of the proposed model that DnaJ domain plays an active role in switching between SAT and FAT. This opinion is also expressed in the comments to the editor. Overall, while I recognize that there is a potential interest in the study, there are also many concerns raised with the present analysis and it is unclear if these issues can be fully resolved. With such significant reservation, I can therefore not offer to commit to a revised manuscript and I see no other choice but to reject the manuscript at this stage.

I thank you in any case for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript by Terada et al examines slow axonal transport by the microtubule-based motor Kinesin-1. Previous work by the authors has been instructive in deciphering the mechanisms of slow axonal transport, particularly for tubulin molecules. In this work, the authors examine the hypothesis that the KLC subunit of Kinesin-1 acts via an interaction with Hsc70 to switch Kinesin-1 transport between fast transport of vesicles and slow transport of cytoplasmic proteins. This is a very interesting hypothesis but unfortunately, the experiments in this manuscript do not directly test this hypothesis and are lacking in depth.

Previous work by the authors utilized fluorescently-marked creatine kinase as a marker for slow axonal transport (Terada Cell 2000) but did not show that Kinesin-1 was the motor responsible for this transport. Thus, the authors should confirm that Kinesin-1 is required for creatine kinase transport before continuing with studies on the DnaJ-like domain of KLC.

In the first section of the results, the authors state "to examine the relevance of the DnaJ-like domain of KLC as a scaffold for cytoplasmic transport through Hsc70 binding..." yet none of the experiments directly address whether the DnaJ-like domain is scaffolding an interaction of KLC with Hsc70 or creatine kinase. The authors should provide biochemical evidence to support their claim of a KLC/Hsc70/creatine kinase complex. They should show that the DnaJ peptide disrupts the KLC/Hsc70 complex. Finally, they should also provide evidence for a role for Hsc70 and its ATP/ADP cycle in slow axonal transport.

The studies in extruded squid axoplasm are also lacking in depth. I don't see any evidence that "lots of vesicular organelles were moving both anterogradely and retrogradely". In addition, "Fast organelle movement" cannot be quantified by the "number and longer axial length of the organelles" but only by actual measurements of movement. And what is the evidence that "movement" or "length" of the longer organelles is by Kinesin-1 and thus relevant to these studies?

The author's conclusions would be greatly strengthened by showing that the DnaJ peptide and/or Hsc70 have an effect on slow axonal transport of tubulin.

I don't find the data in Figure 4 very convincing. In most cases, the darkest bands are found in the darkest lanes, suggesting that maybe more protein overall was loaded in those lanes. And it would be helpful if the authors looked at creatine kinase in this assay to tie this figure together with the previous work in the manuscript.

The authors want to conclude from these studies that the DnaJ-like domain of KLC switches Kinesin-1 between fast and slow axonal transport. There is no evidence that the DnaJ domain plays an active role in switching. A more plausible model is that the DnaJ-like region serves merely to anchor KLC to Hsc70 for slow transport and that it is Hsc70 that switches Kinesin-1 between fast and slow axonal transport.

Referee #2 (Remarks to the Author):

This study proposes to find a "change-over system" by which kinesin-1 is switched between its proposed roles in fast and slow axonal transport. It is not completely clear why such a mechanism is being proposed, or what unexplained aspect of axonal transport it would clarify. Even if kinesin-1 is responsible for force generation for both FAT and SAT, it is expressed at very high levels in neurons and should be adequate to accomplish both simultaneously. Some of the experiments should be clarified, and the text should be revised to avoid starting with conclusions and then proving them. Specific issues follow:

(1) "Change-over regulation." It is not clear what motivates this line of inquiry, particularly why one would seek a "change-over" mechanism for motor protein deployment between slow and fast axonal transport cargoes. Both SAT and FAT operate essentially continuously throughout the life of the neuron, and it is easy to envision motor proteins, even those of the same type, being distributed between the two transport systems.

(2) Figure 1. Please list the exact peptide sequences used here: the DNAJ-like peptide and its reverse sequence, and the two c-terminal Hsc70 reverse sequences. In 1D, where are the data for CK alone? How do late phase velocities compare? "After time for the peptide to diffuse away" means how much time? Is the result consistent with a reasonable estimate of the diffusion time for a peptide of this size in the axoplasmic volume ?

(3) Figure 2. The conclusion in the last sentence of p 3 in the text is premature. Are the autocorrelation values significantly different? If the autocorrrelation curves for CK are best fit by a "one-component model of small/readily movable molecules" are you really measuring a SAT component? Using a one-component model, how can you explain a fraction of the CK being "complexed" with Hsc70? How likely is the assumption that if the CK and Hsc70 correlate, they are actually complexed? For readers unfamiliar with these methods, please explain how to interpret the data on DnaJ-injected samples showing that "6 samples out of 84 were fitted by a one-component model." On p.6, 2nd para, the issue of "change-over" of motor usage is again raised and does not make sense. See point 1, above.

(4) Figure 3. With a number of well-established methods available for quantifying organelle transport, why have the authors used the axial length of the organelles? I assume that this rather odd measure assumes that distortion of shape will reveal the velocity of transport? This is, to say the least, an indirect measure of movement. Please use organelle velocity or % of time spent moving. Can the authors estimate how the concentrations of DnaJ-like domain peptide and Hsc70 introduced here compare to exogenous levels?

(5) Figure 4. The introduction to the transgenic mouse experiments states that the thy1.2-driven DnaJ-like domain was an unsuccessful approach and "deduces" that the "construct showed too much toxicity by blocking the slow axonal transport system." This begs the entire question being approached in this study! Please be specific about the evidence for this or drop this "deduction." Likewise for the statement that the experiments were to "check the dominant-negative effect of KLC that lacked the DnaJ-like domain...on slow axonal transport..." This again introduces an experiment with a conclusion. The concept of a "dominant negative" construct assumes that we understand the mechanism by which this domain binds and affects its partners, which we expressly do not.
Why was KIF5A used as a FAT marker rather than a non-motor cargo? How can the proximal:distal ratio at a ligation be used to determine accurately the rates or flux of FAT? Even if we accept these methods of measurement, both the SAT and FAT measurements show rather modest differences between control and K-D nerves.

(6) Figure 5. The legend of this figure begins with "Impaired slow axonal transport induces..." which is only one possible conclusion. The deterioration could also result from impaired SAT, or from the apparent accelerated FAT, or from another effect of the transgene. In 5A, can the morphologic differences be quantified? What % of the axons were "slightly dilated"? What is the "position of gravity centre" here? How were axons classified as having a biased vs normal MT distribution? How frequent were the large swollen fibres?

(7) Discussion. The first sentence is not really a fair description of our current knowledge of the mechanism of SAT movement. Work from several laboratories has established that SAT and FAT involve similar force generation mechanisms with a large difference in duty cycle. This critical finding has significant explanatory power! Is there any evidence from any published studies that motors must be shifted between these two cargoes?
What does the last sentence of the first para of the discussion mean?
"Molecular change-over" - see above. Exactly what property of SAT or FAT does a postulated KLC "switch" explain?
The connection between the FAT/SAT effects and axonal degeneration remains indirect. Other, unexamined properties of the transgene could also be involved in the long-term survival of the neurons.

Referee #3 (Remarks to the Author):

This paper from Hirokawa's group reports an interesting study on a novel mechanism of kinesin-based regulation of slow and fast anterograde transport. This molecular switch operates via a

specific interaction between the DNA-J like domain of KLC and the chaperone Hsp70. This interaction prevents the binding of kinesin 1 with axonal organelles and their fast transport towards the neuron periphery. Importantly, Hsp70 links conventional kinesin with soluble cargoes that are known to be transported by a slow kinetic. Both pathways are essential for neuronal homeostasis, and therefore this work is both important and of general interest. However, this manuscript requires some amendments prior to publication. In particular:

1. Although this is not unheard of for squid axoplasm-based studies, the concentration of J-domain peptide used in this assay is very high. In addition to the control peptide, it would be reassuring to know that this effect is dose-dependent by a note either in the main text or as data not shown/supplemental material.
2. The profile movements shown in Figure 1B and 1C are difficult to associate with their correspondent time of analysis shown by different colours. To improve clarity, best fitting curves should be omitted or alternatively dashed lines should be used.
3. To improve clarity, average diffusion time estimates may be reported in an inset (i.e. of Figure 2B).
4. The results obtained using video-enhanced contrast/differential interference diffusion and related videos are difficult to rationalise. Why should the attachment of an active molecular motor cause such a deformation in this pool of organelles? Have the authors controlled for unspecific peptide-induced fusion in this preparation? Furthermore, the quality of the video is rather low and borderline for publication. The authors should consider alternative strategies to strengthen this area of the manuscript.
5. In the experiments shown in Figure 4D, the Hsp70 signal has been used as internal control to correct for sample volume variations. However, Hsp70 binds to KIF5A and may undergo itself to localised regulation. Therefore, an alternative independent marker better representing axoplasm or plasma membrane volume should be used.
6. In Figure 5, the wild-type optic nerve seems to have patches deprived of axons as well. Quantification of this pathological landmark should be provided together with a statistical analysis of the results.
7. The text is populated with large sections in parenthesis (e.g. page 10 and 14), which decreases the overall readability. These parentheses should be removed.

Rebuttal 20 May 2009

The authors appealed the original editorial decision, and after communication an agreement was reached that a revised manuscript would be considered if they were to provide additional independent data that could address all the major concerns raised by the referees.

Invitation to resubmit 25 May 2009

Thank you for your letter asking me to reconsider the decision taken on the manuscript. I am sorry for the slight delay in getting back to you, but things have been very busy here. I have now had a chance to take a look at your manuscript, the comments of the referees and your rebuttal. Both referees #1 and 2 find the analysis potentially interesting, but they also raised significant concern with the analysis and find that the manuscript does not provide sufficient direct support for an active role of Dna-J like domain of KLC in switching. Having taken a careful look at your rebuttal, it is difficult to fully anticipate if your response will address their raised concerns. Given the potential interest in the topic, I am willing to consider a resubmission and to run a revised version by referees again. Should you choose to submit a resubmission please address their raised concerns as complete as possible. I will try to involve the same set of referees, but I might also bring in new referee(s) if needed or involve an additional editorial advisor. I should point out that for resubmissions that we consider the novelty of the data at the time of resubmission. I should also add that the referees would have to come back with clear support for that the study should be published in the EMBO journal in order for us to consider the manuscript for publication here. It is this aspect that is at present rather difficult to anticipate, but as said above I am willing to proceed with such an approach and will take

a careful and fair decision based upon all the available input.

If you send us a resubmission, please indicate in the cover letter that the resubmission is linked to MS 70996 and that I handled the previous submission. Please also include a detailed point-by-point response.

New submission received 02 September 2009

Responses to Referee #1

We thank the Referee for both admitting the significance of our working hypothesis and his/her constructive comments. According to the suggestions, we added more direct experimental supports and revised the manuscript. We hope we could address all of the Referee's concerns.

Comment 1

Previous work by the authors utilized fluorescently-marked creatine kinase as a marker for slow axonal transport (Terada Cell 2000) but did not show that Kinesin-1 was the motor responsible for this transport. Thus, the authors should confirm that Kinesin-1 is required for creatine kinase transport before continuing with studies on the DnaJ-like domain of KLC.

We added more direct data by functional blocking antibody experiment, showing Kinsein-1 supports the transport of fluorescently-marked creatine kinase. (From page 5, l. 21 to page 6, l. 11.) We had unpublished data that various cytoplasmic proteins are transported in squid giant axons in the same way as creatine kinase or tubulin. As far as we are concerned, functional blocking antibody against Kinesin-1 blocked every transport, including creatine kinase.

Comment 2

In the first section of the results, the authors state "to examine the relevance of the DnaJ-like domain of KLC as a scaffold for cytoplasmic transport through Hsc70 binding..." yet none of the experiments directly address whether the DnaJ-like domain is scaffolding an interaction of KLC with Hsc70 or creatine kinase. The authors should provide biochemical evidence to support their claim of a KLC/Hsc70/creatine kinase complex. They should show that the DnaJ peptide disrupts the KLC/Hsc70 complex. Finally, they should also provide evidence for a role for Hsc70 and its ATP/ADP cycle in slow axonal transport.

To show the interaction among the possible complex of KLC/Hsc70/creatine kinase, we performed immunoprecipitation experiment (page 5, ll. 2-13 and new Figure 1). Both anti-KLC antibody and anti-Hsc70 antibody co-immunoprecipitated with the other two of the complex and this interaction is reduced when the DnaJ-like domain peptide of KLC was added in the solution in the presence of ATP. When ATP is depleted by apyrase treatment, the DnaJ-like domain peptide effect of creatine kinase dissociation from the complex was reduced, suggesting the ADP form of Hsc70, i.e., DnaJ domain protein binding form, carry the slow axonal transport cargoes more effectively. We also revised the discussion briefly (page 19, ll. 5-10).

Comment 3

The studies in extruded squid axoplasm are also lacking in depth. I don't see any evidence that "lots of vesicular organelles were moving both anterogradely and retrogradely". In addition, "Fast organelle movement" cannot be quantified by the "number and longer axial length of the organelles" but only by actual measurements of movement. And what is the evidence that "movement" or "length" of the longer organelles is by Kinesin-1 and thus relevant to these studies?

We added the quantitative data by counting the number of vesicles passing across the fixed line with a unit length, both antero- and retrogradely (from page 10, l. 11 to page 11, l. 4, and new Figure 4A), and more video data in the supplementary information. When we added DnaJ-like domain peptide, we observed enhanced vesicular movements, especially in anterograde direction, compared

with those of the cases with control peptide or untreated axoplasm. On the other hand, when we added Hsc70, the vesicular movements were blocked, especially in anterograde direction, compared with those of the cases with control peptide or untreated axoplasm. Both DnaJ-like domain peptide and Hsc70 did not affect the vesicular transport of retrograde direction. We deleted the previous counting data, according to the Refereeís suggestion.

Comment 4

The author's conclusions would be greatly strengthened by showing that the DnaJ peptide and/or Hsc70 have an effect on slow axonal transport of tubulin.

Tubulin (or other cytoskeletal proteins such as actin) does not bind to Hsc70. Our preliminary results indicate that DnaJ-like domain peptide has less effect on tubulin transport and we consider that the molecular mechanism of axonal transport of general cytoplasmic proteins is different from that of cytoskeletal proteins. We added the comments in the Discussion section on this point (Page 19, ll. 11-15).

Comment 5

I don't find the data in Figure 4 very convincing. In most cases, the darkest bands are found in the darkest lanes, suggesting that maybe more protein overall was loaded in those lanes. And it would be helpful if the authors looked at creatine kinase in this assay to tie this figure together with the previous work in the manuscript.

In metabolic labelling studies, the background density of lanes does not reflect the protein quantity loaded in the lanes. In general, the background signal around heavily labelled bands is dark, and this might reflect very small amount of labelled proteins that are not properly separated by electrophoresis. We attached some of the raw data for the Referee's inspection. As proteins are transported down the axon, the background density of lanes gradually transport to the same direction. As one can see in these data, we strictly arranged the samples to be in the same loading condition as much as possible. To avoid possible sample variation, we normalized the radioactivity of each segment by dividing with the total radioactivity value of 5 segments. We have repeated the experiments, and differences are statistically significant. We could not use creatine kinase here because there are several other proteins of similar molecular weight values such as aldolase or actin that are metabolically labelled. As we wrote in the manuscript, Hsc71, GAPDH, NFL and tubulin are some of the few examples that have radioactive spots of unique molecular weight values on 1D electrophoresis gels. These proteins do not have other metabolically labelled proteins of similar molecular size, thus can be identified as single protein origin. Creatine kinase cannot be treated as such.

Comment 6

The authors want to conclude from these studies that the DnaJ-like domain of KLC switches Kinesin-1 between fast and slow axonal transport. There is no evidence that the DnaJ domain plays an active role in switching. A more plausible model is that the DnaJ-like region serves merely to anchor KLC to Hsc70 for slow transport and that it is Hsc70 that switches Kinesin-1 between fast and slow axonal transport.

Following the suggestion by the Referee, we rewrote the manuscript (page 4, ll. 9-10; page 11, ll. 17-20; from page 19, l. 16 to page 20, l. 6), including the (running) title and abstract (page2, l. 13).

Responses to Referee #2

We thank the Referee for his/her constructive comments. We tried to address the Referee's concerns as much as possible, and believe that this time the referee would admit the significance of our paper.

Comment 1 and 7

This study proposes to find a "change-over system" by which kinesin-1 is switched between its proposed roles in fast and slow axonal transport. It is not completely clear why such a mechanism is

being proposed, or what unexplained aspect of axonal transport it would clarify. Even if kinesin-1 is responsible for force generation for both FAT and SAT, it is expressed at very high levels in neurons and should be adequate to accomplish both simultaneously. Some of the experiments should be clarified, and the text should be revised to avoid starting with conclusions and then proving them...

(1) "Change-over regulation." It is not clear what motivates this line of inquiry, particularly why one would seek a "change-over" mechanism for motor protein deployment between slow and fast axonal transport cargoes. Both SAT and FAT operate essentially continuously throughout the life of the neuron, and it is easy to envision motor proteins, even those of the same type, being distributed between the two transport systems.

(7) Discussion. The first sentence is not really a fair description of our current knowledge of the mechanism of SAT movement. Work from several laboratories has established that SAT and FAT involve similar force generation mechanisms with a large difference in duty cycle. This critical finding has significant explanatory power! Is there any evidence from any published studies that motors must be shifted between these two cargoes?

What does the last sentence of the first para of the discussion mean?
"Molecular change-over" - see above. Exactly what property of SAT or FAT does a postulated KLC "switch" explain?

We revised all of the manuscript (page 4, ll. 9-10; page 10, ll. 11-20; page 11, ll. 17-20; from page 19, l. 16 to page 20, l. 6), and the title to clear up the Referee's concerns. (The last sentence of the first paragraph of the previous Discussion meant that by utilizing the non-specific biding and chaperone activity of chaperone molecules, SAT could convey various cargos safely by the same motor, Kinesin-1.) We are afraid that our previous manuscript might be misleading, especially on the function of Hsc70 regarding FAT/SAT switching. First, we found that SAT of general cytoplasmic proteins are dependent on Hsc70. Second, we unexpectedly found that Hsc70 switches Kinesin-1 between FAT/SAT in a DnaJ-like domain dependent manner.
Kinsin-1 is considered to play the roles both in FAT/SAT, but how the same motor plays the different roles has been unknown. A preceding report from Dr. Brady's lab demonstrated that Hsc70 inhibits FAT, and our experimental result suggests that Hsc70 support SAT. Surprisingly, the treatment for inhibiting the interaction between Hsc70 and Kinesin-1 motor complex, induced both deteriorated SAT and promoted FAT. Our finding on this exclusive deployment of Kinesin-1 between FAT/SAT was an unexpected experimental outcome, and had not been anticipated. It is not clear why such mechanism exists or what property of each transport is regulated in this fashion.
Regarding the SAT mechanism, this is the first report pointing out its Hsc70 dependency. The molecular mechanism linking Kinesin-1 motor and its cytoplasmic cargos has been totally enigmatic. We also believe that the preceding finding of Kinesin-1 as a common motor for both FAT/SAT does not explain the whole figure without accumulating the knowledge of the regulatory mechanism linking the motor complex with different cargos, cytoplasmic proteins or membranous organelles.
We would like to ask the Referee to admit the significance of our study on the molecular mechanism of SAT and FAT/SAT regulation.

Comment 2

(2) Figure 1. Please list the exact peptide sequences used here: the DNAJ-like peptide and its reverse sequence, and the two c-terminal Hsc70 reverse sequences. In 1D, where are the data for CK alone? How do late phase velocities compare? "After time for the peptide to diffuse away" means how much time? Is the result consistent with a reasonable estimate of the diffusion time for a peptide of this size in the axoplasmic volume?

We added the peptide sequences in the figure (new Figure 2). We also added the data for CK alone in the new Figure 2D. We showed the late phase data to demonstrate that transport inhibition by the peptide was reversible, and to deny the possibility of non-specific toxic effect of the peptide. From our data, the transport regains after 30 minutes from injection, thus we consider that the local concentration of the peptide seems to decrease under the effective concentration in half an hour. We could consistently observe the transport recovery during 30 to 60 minutes after injection, but the

late phase velocities during this period varies considerably due to the variance of giant axon size and injection volume. Previous electrophysiological experiments using squid neurons indicate that microinjected functional peptides (0.1-1 pl of 2-20 mM solution) of the similar size show effects immediately after injection for about 30 minutes. (See J Neurosci 25; 2658-69, 2005, PNAS 106; 5901-6, 2009, for example.) Regarding the time-course of peptide effect due to diffusion in the squid cytoplasm, our experimental results are well comparable with these findings. We added and revised the manuscript (page 6, l. 21- page 7, l. 7).

Comment 3

(3) Figure 2. The conclusion in the last sentence of p 3 in the text is premature. Are the autocorrelation values significantly different? If the autocorrelation curves for CK are best fit by a "one-component model of small/readily movable molecules" are you really measuring a SAT component? Using a one-component model, how can you explain a fraction of the CK being "complexed" with Hsc70? How likely is the assumption that if the CK and Hsc70 correlate, they are actually complexed? For readers unfamiliar with these methods, please explain how to interpret the data on DnaJ-injected samples showing that "6 samples out of 84 were fitted by a one-component model." On p.6, 2nd para, the issue of "change-over" of motor usage is again raised and does not make sense. See point 1, above.

Fluorescence correlation spectroscopy (FCS) measures diffusion behavior of biomolecules collectively. The model fitting estimates rough grouping of molecular behavior, thus very small amount of specific molecular populations might be overlooked. As we indicated in the manuscript, the autocorrelation curves for CK are both best fit by one-component model, and their estimated diffusion time was not different. These results indicate that single FCS cannot detect the small population on SAT of CK, if any. Both the SAT component and CK-Hsc70 complex was successfully and definitely measured by FCCS. Theoretically and thoroughly, cross-correlation signal indicates the existence of biomolecular interaction, between CK and Hsc70, in our case. Together with the single FCS results, we can deduce that CK-Hsc70 complex does exist, but its quantity is small (page 9, ll. 7-14).
The fact that 6 samples out of 84 in the presence of DnaJ-like domain peptide were fitted by a one-component model means that in majority of the samples, FCCS signal reflecting CK-Hsc70 complex disappeared. On the other hand, 47 samples out of 56 in the presence of control peptide were fitted by a one-component model means that in majority of the samples, FCCS signal reflecting CK-Hsc70 complex does exist (from page 9, l. 21 to page 10, l. 3). As this reviewer pointed out, the description on page 6, 2nd paragraph in the previous version is not appropriate. We have revised to make our point clear. (Please see response to Comment 1 and 7.)

Comment 4

(4) Figure 3. With a number of well-established methods available for quantifying organelle transport, why have the authors used the axial length of the organelles? I assume that this rather odd measure assumes that distortion of shape will reveal the velocity of transport? This is, to say the least, an indirect measure of movement. Please use organelle velocity or % of time spent moving. Can the authors estimate how the concentrations of DnaJ-like domain peptide and Hsc70 introduced here compare to exogenous levels?

Following the previous publication, we added the quantitative data by counting the number of vesicles passing across the fixed line with a unit length, both antero- and retrogradely (from page 10, l. 11 to page 11, l. 4, and new Figure 4A). When we added DnaJ-like domain peptide, we observed enhanced vesicular movements, especially in anterograde direction, compared with those of the cases with control peptide or untreated axoplasm. On the other hand, when we added Hsc70, the vesicular movements were blocked, especially in anterograde direction, compared with those of the cases with control peptide or untreated axoplasm. Both DnaJ-like domain peptide and Hsc70 did not affect the vesicular transport of retrograde direction. We deleted the previous counting data, according to the Refereeís suggestion. We consider the concentrations of DnaJ-like domain peptide and Hsc70 introduced here is well controlled within the effective dose range. To monitor the diffusion of Hsc70 into the axoplasm, Cy5-labelled Hsc70 was applied to the axoplasm to inhibit organelle movement. Fluorescent Hsc70 was found to penetrate the axoplasm with a time course similar to that found for the appearance of the inhibitory effect. The effect by Hsc70 was first

detected within 5 minutes after protein addition and became apparent throughout the axoplasm by 30 minutes. The effects by peptide addition were apparent within a minute after peptide addition, and detected throughout the axoplasm by 5 minutes, reflecting the faster penetration of peptide than Hsc70. Though the exact estimation of local concentrations of Hsc70 or peptide in axoplasm is difficult, we consider that they are similar to, or a little bit less than exogenous levels because the image by VEC-DIC microscopy was collected from the axoplasm near the external surface (i.e., less than about 50 m from the surface). We have revised the manuscript and added the explanation (page 11, ll. 4-15).

Comment 5

(5) Figure 4. The introduction to the transgenic mouse experiments states that the thy1.2-driven DnaJ-like domain was an unsuccessful approach and "deduces" that the "construct showed too much toxicity by blocking the slow axonal transport system." This begs the entire question being approached in this study! Please be specific about the evidence for this or drop this "deduction." Likewise for the statement that the experiments were to "check the dominant-negative effect of KLC that lacked the DnaJ-like domain...on slow axonal transport..." This again introduces an experiment with a conclusion. The concept of a "dominant negative" construct assumes that we understand the mechanism by which this domain binds and affects its partners, which we expressly do not.
Why was KIF5A used as a FAT marker rather than a non-motor cargo? How can the proximal:distal ratio at a ligation be used to determine accurately the rates or flux of FAT? Even if we accept these methods of measurement, both the SAT and FAT measurements show rather modest differences between control and K-D nerves.

To show the mechanism by which DnaJ domain of KLC binds and affects its partners, Hsc70 and creatine kinase in our case, we performed immunoprecipitation experiment (page 5, ll. 2-13 and new Figure 1). Both anti-KLC antibody and anti-Hsc70 antibody co-immunoprecipitated with the other two of the complex and this interaction is reduced when the DnaJ-like domain peptide of KLC was added in the solution in the presence of ATP. When ATP is depleted by apyrase treatment, the DnaJ-like domain peptide effect of creatine kinase dissociation from the complex was reduced, suggesting the ADP form of Hsc70, i.e., DnaJ domain protein binding form, carry the slow axonal transport cargoes more effectively. Together with the results of experiments using squid giant axons, we believe our model has gained more experimental basis to justify the transgenic mice construct for dominant-negative experiments. According to the suggestion, we dropped the part of transgenic mice expressing the thy1.2-driven DnaJ-like domain to avoid possible confusion (from page 11, l. 21 to page 12, l. 5). (We explained why we had to use dominant-negative construct instead of DnaJ-like domain itself in supplementary section. Both of the constructs are intended to suppress slow axonal transport, although the extent of suppression was supposed to be different. The phenotype of DnaJ-like domain construct was lethal, and that is why we had changed our strategy to dominant-negative construct.)
We used KIF5A as a FAT marker because previous studies (Zhao et al., 2001) demonstrated that it is a very good marker for FAT. The contrast of the band on Western blotting is more than the other FAT markers (for example, APP and KIF1A), and we can measure the density of the stained band more exactly than others (page 13, ll. 14-18). Because the ligation time is sufficiently short for SAT, but long for FAT, we consider the band density of Hsc70 could serve as a good internal control for assessing FAT to normalize the quantity of the samples loaded. As the referee pointed out, we admit the difference between wild-type and transgenic mice is moderate. We revised and explained above situation in the manuscript (from page 13, l. 18 to page 14, l. 2).

Comment 6 and 7

(6) Figure 5. The legend of this figure begins with "Impaired slow axonal transport induces..." which is only one possible conclusion. The deterioration could also result from impaired SAT, or from the apparent accelerated FAT, or from another effect of the transgene. In 5A, can the morphologic differences be quantified? What % of the axons were "slightly dilated"? What is the "position of gravity centre" here? How were axons classified as having a biased vs normal MT distribution? How frequent were the large swollen fibres?
(7) ... The connection between the FAT/SAT effects and axonal degeneration remains indirect. Other, unexamined properties of the transgene could also be involved in the long-term survival of the neurons.

According to the reviewer's suggestion, we revised the manuscript and figure legend to describe and interpret the experimental results more accurately. Strictly speaking, as our experimental result indicates that both slow and fast axonal transport systems are inter-dependent, analyzing the two separately is impossible. But the late-onset time course of the pathological change indicates that it is more plausible to interpret the findings based on slow axonal transport mechanism (page 20, ll. 12-19). We had to admit the fact that every genetic experiment cannot escape from the necessities of interpretation in the context of mutual dependence of biomolecular interactions.
Regarding Figure 5A, we counted darkly stained structures and patches of empty space without axons in peripheral areas and added the quantitative data (page 14, ll. 11-17). The quantification data on the axon-diameter difference between transgenic and wild-type mice are demonstrated most accurately in the diameter distribution curves shown in the supplementary figure S1. Some of the axons, especially those with 0.6-0.9 μm in diameter, seemed to be reduced, while those with 0.9-2 μm in diameter increased, compared with those of wild-type mice, suggesting the slight dilation in diameter. We added quantitative data on these distribution curves and revised the expression (from page 14, l. 24 to page 15, l. 10). Regarding the position of gravity centre, we defined the 'gravity centre' more accurately, and described the way to determine the position in more detail in the supplementary information ("The putative gravity centre of each component was determined as follows. First, the circumference of each component was fitted with a quadrant ellipse by least squares method and intercepts of both major and minor axes are determined. When the intercept values are expressed (a, 0) and (0, b) as coordinates relative to the centre of the axon, the coordinates of the gravity centre is defined as ($\sqrt{}4\pi a/4$, $\sqrt{}\pi b/4$)."). The standard for classifying biased or normal MT distribution is based on the existence of wide MT-free spaces (more than 0.5 μm diameter circle) in axoplasm. When MT distribution is heavily biased in large-diameter axons with more than 0.7 μm-minor diameters, we can find these wide MT-empty spaces, and judged the axons as "biased". We added the detailed explanation on these criteria more conspicuously in the supplementary information. ("The standard for classifying biased or normal microtubule distribution is based on the existence of wide microtubule-free spaces (more than 0.5 μm diameter circle) in axoplasm. When microtubule distribution is heavily biased in large-diameter axons with more than 0.7 μm-minor diameters, we can find these wide microtubule-empty spaces, and judged the axons as "biased." All of the inspected axons are less than 2 μm in major diameter."). We counted the frequency of large swollen axons with more than 10 μm-major diameter. Those axons are found only in transgenic mice. We have added the data and revised the manuscript to explain these matters (page 14, ll. 17-22).

Responses to Referee #3

First of all, we thank the Referee for highly evaluating the significance of our working hypothesis (This paper from Hirokawa's group reports an interesting study on a novel mechanism of kinesin-based regulation of slow and fast anterograde transport. This molecular switch operates via a specific interaction between the DNA-J like domain of KLC and the chaperone Hsp70. This interaction prevents the binding of kinesin 1 with axonal organelles and their fast transport towards the neuron periphery. Importantly, Hsp70 links conventional kinesin with soluble cargoes that are known to be transported by a slow kinetic. Both pathways are essential for neuronal homeostasis, and therefore this work is both important and of general interest.) and his/her constructive comments. Following the suggestion, we amended the manuscript.

Comment 1

1. Although this is not unheard of for squid axoplasm-based studies, the concentration of J-domain peptide used in this assay is very high. In addition to the control peptide, it would be reassuring to know that this effect is dose-dependent by a note either in the main text or as data not shown/supplemental material.

We consider that the concentration of the peptide used here is within the range of previous electrophysiological experiments using squid neurons. Microinjected functional peptides (0.1-1 pl of 2-20 mM solution) of the similar size show effects immediately after injection for about 30 minutes. (See J Neurosci 25; 2658-69, 2005, PNAS 106; 5901-6, 2009, for example.) We also experienced dose-dependency effect by the peptide, and added the note on these points in the manuscript (page 6,

l. 21- page 7, l. 7 and page 11, ll. 4-15).

Comment 2

2. The profile movements shown in Figure 1B and 1C are difficult to associate with their correspondent time of analysis shown by different colours. To improve clarity, best fitting curves should be omitted or alternatively dashed lines should be used.

We have corrected the figure, as indicated by the Referee.

Comment 3

3. To improve clarity, average diffusion time estimates may be reported in an inset (i.e. of Figure 2B).

We added the diffusion time estimate in the figure.

Comment 4

4. The results obtained using video-enhanced contrast/differential interference diffusion and related videos are difficult to rationalise. Why should the attachment of an active molecular motor cause such a deformation in this pool of organelles? Have the authors controlled for unspecific peptide-induced fusion in this preparation? Furthermore, the quality of the video is rather low and borderline for publication. The authors should consider alternative strategies to strengthen this area of the manuscript.

To dissolve the Reviewer's concern, we added the quantitative data by counting the number of vesicles passing across the fixed line with a unit length, both antero- and retrogradely (from page 10, l. 11 to page 11, l. 4, and new Figure 4A). When we added DnaJ-like domain peptide, we observed enhanced vesicular movements, especially in anterograde direction, compared with those of the cases with control peptide or untreated axoplasm. On the other hand, when we added Hsc70, the vesicular movements were blocked, especially in anterograde direction, compared with those of the cases with control peptide or untreated axoplasm. Both DnaJ-like domain peptide and Hsc70 did not affect the vesicular transport of retrograde direction. We did not observe any fusion of separate organelles to form an elongated vesicle in our preparation. We deleted the previous counting data, according to the Referee's suggestion, and added more video data in the supplementary information.

Comment 5

5. In the experiments shown in Figure 4D, the Hsp70 signal has been used as internal control to correct for sample volume variations. However, Hsp70 binds to KIF5A and may undergo itself to localised regulation. Therefore, an alternative independent marker better representing axoplasm or plasma membrane volume should be used.

Strictly speaking, there are slim pickings in marker molecule candidates for internal control that is independent from transport phenomena. Every cytosolic protein possibly binds to Hsp70. To the best of our knowledge, almost every protein is transported by FAT/SAT. Membrane lipids are not exception and cannot escape from transport phenomena. We tried the measurement of sample weight, but our effort was in vain, because data varied more widely. Because the ligation time is sufficiently short for SAT, but long for FAT, we consider that the band density of Hsc70 could serve practically as a good internal control for assessing FAT to normalize the quantity of the samples. Our preliminary experiment using other cytosolic protein such as creatine kinase indicated that the signal strength did not altered during 6-hour ligation in both transgenic and wild-type animals. We have added the explanation on these points (from page 13, l. 18 to page 14, l. 2).

Comment 6

6. In Figure 5, the wild-type optic nerve seems to have patches deprived of axons as well.

Quantification of this pathological landmark should be provided together with a statistical analysis of the results.

We added morphometric data, following the suggestion (page 14, ll. 11-17).

Comment 7

7. The text is populated with large sections in parenthesis (e.g. page 10 and 14), which decreases the overall readability. These parentheses should be removed.

Following the Refereeís suggestion, we have removed the parentheses.

2nd Editorial Decision 05 October 2009

Thank you for submitting your manuscript to the EMBO Journal. This submission is an invited resubmission of MS 70996 that was rejected after review earlier this year. I asked the original referees #1 and 2 to review the manuscript and while referee #1 was able to do so, referee #2 was not. I also involved a new referee (referee #2) and asked this referee to comment on the suitability of the manuscript for publication here. I have now heard back from both referees and I am pleased to say that both referees are supportive of publication here. Referee #1 has a few outstanding concerns that should not involve too much additional work to address. Both referees however also find that the present data does not provide strong enough support for a "switch over" mechanism. They both find that this issue can be resolved by a more careful and balanced discussion (plus change of title). Given the overall positive evaluation of your manuscript, I would like to ask you to address the remaining concerns in a final round of revision. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The revised manuscript by Terada et al provides several new pieces of data to support the role of kinesin-1 in slow axonal transport of Creatine Kinase. The authors have also changed the text to avoid over-interpretation of their data and a role for the DnaJ domain of kinesin-1 in a switch-over mechanism. Although improved, I still have several concerns about this manuscript.

The authors have added two pieces of data in response to my concerns (Comment 1) about kinesin-1 and its role in Creatine Kinase transport: coimmunoprecipitation of KLC, Hsc70 and Creatine Kinase (new Figure 1) and inhibition of Creatine Kinase transport upon injection of an anti-KHC antibody (data not shown). Although this is an improvement, the new Figure 1 lacks critical controls. Specifically, they need to show the amount of KLC in the anti-KLC precipitation, etc. That is, each panel should have three blots: the protein being precipitated and the two interacting proteins. As shown, it is not clear whether the differences in coimmunoprecipitation are due to differences in protein-protein interaction or differences in precipitation efficiency in the presence of the peptides and/or ATP/ADP. Also, a graphical representation of the effects of the anti-kinesin antibody would be helpful.

In my previous Comment 2, I asked the authors to "provide evidence for a role for Hsc70 and its ATP/ADP cycle in slow axonal transport." This concern does not appear to have been addressed. The evidence that Hsc70 plays a role in slow axonal transport of Creatine Kinase appears to be based on the fact that the DnaJ peptide disrupts the KLC - Hsc70 interaction and Creatine Kinase transport. But the data in the new Figure 1 show that addition of the DnaJ peptide in the presence of ATP decreases the interaction between Hsc70 and Creatine Kinase. In subsequent experiments where this peptide is used, the effects of the peptide on Creatine Kinase transport and Hsc70/Creatine Kinase cross-correlation can be explained by a dissociation of Hsc70 and Creatine Kinase. Unless I am missing something, there is no evidence that Hsc70 actually plays a role in slow axonal transport, an idea that authors put forth in the Introduction.

I appreciate the authors providing a CD of the videos of the squid axoplasm experiments. As someone who is not used to viewing this kind of data, I still find the videos very hard to evaluate. I still see only a few moving particles in each of the videos and can't tell if I just don't know what to look for or there is very little movement. To support their claim of "robust and durable vesicular movement", it would be helpful if the authors provided a supplementary figure that provides the final frame of each movie with the maximum-value projection or life history of each moving particle drawn in color on top of the image. Other notations on these images could be used to indicate other points of interest such as "increased number of organelles with longer axis."

In the previous submission, the authors over-interpreted their data in suggesting that the DnaJ domain of KLC plays a role in "switch over" between fast and slow axonal transport. The authors have improved the manuscript by removing their emphasis on this point. I suggested that a more plausible model may be that Hsc70 plays a role in "switch over" between fast and slow axonal transport. While I appreciate their acceptance of my suggestion, there not yet enough evidence to support this model. That is, the data in the paper do not directly support this model either. Addition of Hsc70 does increase the number of membranous vesicles undergoing transport (Figure 4A) but what effect does it have on slow axonal transport? Maybe Hsc70 is acting in a dominant-interfering and/or non-specific way to interfere with all transport. As a chaperone, Hsc70 is likely to have many effects.

Overall, although I think the idea is interesting, I still do not see any evidence of a "switch over" mechanism. The data in the manuscript only show that if you interfere with the slow axonal transport of Creatine Kinase, then you see an increase in the movement of membranous vesicles that may or may not be carried by kinesin-1. A switch over is not needed; it may be just that interfering with slow axonal transport frees more kinesin-1 motors to participate in fast axonal transport. What evidence is there that kinesin-1 is limiting and that there is competition for its usage in different transport events? Unless the authors can show that the amount of kinesin-1 is limiting for transport and that Hsc70 actually switches the motor between populations, I think the authors should limit their proposal about a switch over to a discussion of this as one possible mechanism in the Discussion and instead leave the focus of this manuscript on a role for kinesin-1 and Hsc70 in slow axonal transport.

Referee #2 (Remarks to the Author):

I only had the chance to read the corrected version of this manuscript (and answers to all reviewers queries). Overall I found the work quite good, certainly within the standards to be published at The EMBO Journal. The reasons are twofold: the concept of the possibility of switching between fast and slow axonal transport based on the activity of a chaperone/DnaJ-like domain interaction is conceptually novel and provocative and the experiments that lead to that notion are overall (but not always) clear-cut. I found quite convincing the biochemical and microscopy data in squid giant axons dissecting Hsc70/Kinesin-1/DnaJ-like domain-cargo selection. I also found convincing the DIC and fluorescence cross-correlation spectroscopy showing the effect of Hsc70 and DnaJ-like peptide injection on membrane transport. However, must say that the data presented to prove the switchover mechanism is not all that clear to me and it is possible that Hsc70 might affect fast transport indirectly (in which case increased slw would be by default), through a stress response, possibly via the CHIP pathway. The stress response may affect at first events with higher energy demand and so it would advisable that longer time points are considered in this type of analysis. Alternatively, they should discuss this possibility and consequently tone down their conclusions in this respect. The mice data are nice in regard to the consequences of affecting chaperone binding

yet, again, the possibility that the effects are the sum of consequences and that impaired transport might arise from a disregulation of pathways away from cargo/MT binding should be more properly discussed. Overall, I support acceptance of this work in The EMBO Journal.

3rd Revision - authors' response 08 November 2009

Responses to Referee #1

We thank the Referee for his/her constructive comments. According to the suggestions, we added some experimental data and revised the manuscript.

Comment 1
The authors have added two pieces of data in response to my concerns (Comment 1) about kinesin-1 and its role in Creatine Kinase transport: coimmunoprecipitation of KLC, Hsc70 and Creatine Kinase (new Figure 1) and inhibition of Creatine Kinase transport upon injection of an anti-KHC antibody (data not shown). Although this is an improvement, the new Figure 1 lacks critical controls. Specifically, they need to show the amount of KLC in the anti-KLC precipitation, etc. That is, each panel should have three blots: the protein being precipitated and the two interacting proteins. As shown, it is not clear whether the differences in coimmunoprecipitation are due to differences in protein-protein interaction or differences in precipitation efficiency in the presence of the peptides and/or ATP/ADP. Also, a graphical representation of the effects of the anti-kinesin antibody would be helpful.

We added the requested control data and refined the data presentation in the new Figure 1. We also supplemented the anti-kinesin antibody data in the new Figure 2A (ìIn the case for creatine kinase, functional blocking antibody (Fab fragment) against Kinesin-1 (H2, 0.4 mg/ml) suppressed the movement during the first 20 minutes from injection (P-value 0.00 < 0.05, Mann-Whitney test). Control normal mouse IgG Fab fragment did not block the transport and depletion of H2 Fab fragment by recombinant Kinesin-1 beads cancelled the inhibition by this antibody.î Page 6, ll. 1-5).

Comment 2
In my previous Comment 2, I asked the authors to "provide evidence for a role for Hsc70 and its ATP/ADP cycle in slow axonal transport." This concern does not appear to have been addressed. The evidence that Hsc70 plays a role in slow axonal transport of Creatine Kinase appears to be based on the fact that the DnaJ peptide disrupts the KLC - Hsc70 interaction and Creatine Kinase transport. But the data in the new Figure 1 show that addition of the DnaJ peptide in the presence of ATP decreases the interaction between Hsc70 and Creatine Kinase. In subsequent experiments where this peptide is used, the effects of the peptide on Creatine Kinase transport and Hsc70/Creatine Kinase cross-correlation can be explained by a dissociation of Hsc70 and Creatine Kinase. Unless I am missing something, there is no evidence that Hsc70 actually plays a role in slow axonal transport, an idea that authors put forth in the Introduction.

We intended to address the previous Comment 2 by immunoprecipitation experiment (Figure 1). As we explained in the manuscript (page 8, ll. 6-9, and page 18, l.19 to page 19, l. 1) and Figure 3C, the DnaJ-like domain peptide inhibited slow axonal transport by disrupting the transporting complex consisting of Kinesin-1, Hsc70 and creatine kinase, in this case. By associating with DnaJ-like domain of KLC, the ADP-form of Hsc70 binds to Creatine Kinase, and the injected DnaJ-like domain peptide competes this association. Since the peptide binding is weak and the axoplasm is ATP-rich, dissociated ADP-form Hsc70 is converted into the ATP-form, thus releasing Creatine Kinase. Both biochemical data shown in Figure 1 (addition of the DnaJ peptide in the presence of ATP decreases the interaction between Hsc70 and Creatine Kinase) and in vitro FCCS measurements using Hsc70 and Creatine Kinase supported this mechanism, since DnaJ-like domain peptide effect of Creatine Kinase dissociation was dependent on ATP, and the cross-correlation signal increased with ATP consumption over time. Please note that a dissociation of Hsc70 and Creatine Kinase is caused by the conversion of Hsc70, from ADP-form to ATP-form.

Comment 3

I appreciate the authors providing a CD of the videos of the squid axoplasm experiments. As someone who is not used to viewing this kind of data, I still find the videos very hard to evaluate. I still see only a few moving particles in each of the videos and can't tell if I just don't know what to look for or there is very little movement. To support their claim of "robust and durable vesicular movement", it would be helpful if the authors provided a supplementary figure that provides the final frame of each movie with the maximum-value projection or life history of each moving particle drawn in color on top of the image. Other notations on these images could be used to indicate other points of interest such as "increased number of organelles with longer axis."

Following the Referee's suggestion, we prepared a supplementary figure (New Supplementary Figure S1).

Comment 4
I suggested that a more plausible model may be that Hsc70 plays a role in "switch over" between fast and slow axonal transport. While I appreciate their acceptance of my suggestion, there not yet enough evidence to support this model. That is, the data in the paper do not directly support this model either. Addition of Hsc70 does increase the number of membranous vesicles undergoing transport (Figure 4A) but what effect does it have on slow axonal transport? Maybe Hsc70 is acting in a dominant-interfering and/or non-specific way to interfere with all transport. As a chaperone, Hsc70 is likely to have many effects.
Overall, although I think the idea is interesting, I still do not see any evidence of a "switch over" mechanism. The data in the manuscript only show that if you interfere with the slow axonal transport of Creatine Kinase, then you see an increase in the movement of membranous vesicles that may or may not be carried by kinesin-1. A switch over is not needed; it may be just that interfering with slow axonal transport frees more kinesin-1 motors to participate in fast axonal transport. What evidence is there that kinesin-1 is limiting and that there is competition for its usage in different transport events? Unless the authors can show that the amount of kinesin-1 is limiting for transport and that Hsc70 actually switches the motor between populations, I think the authors should limit their proposal about a switch over to a discussion of this as one possible mechanism in the Discussion and instead leave the focus of this manuscript on a role for kinesin-1 and Hsc70 in slow axonal transport.

When we blocked the interaction between Hsc70 and Kinesin-1 motor complex by KLC DnaJ like domain peptide (Hsc70 cannot bind Kinesin-1 motor complex in this condition.), Creatine Kinase transport was inhibited, while membranous organelles transport was promoted. When we applied Hsc70, membranous organelles transport was inhibited, and this observation confirmed the previous experimental results by Dr. Bradyís group. They say that the effected movements of membranous organelles are carried by Kinesin-1. (Please note that addition of Hsc70 does NOT increase the number of membranous vesicles undergoing transport. We are afraid that the Referee might misunderstand the situation.) Following the previous suggestion by this Referee, we interpreted and expressed these observations might be based on the Hsc70 ìswitch overî role between fast and slow axonal transport. As far as we have observed, Hsc70 has at least no interfering effect on Creatine Kinase transport. We co-injected Hsc70 and Creatine Kinase and measured the cross-correlation signal between the two many times, and in these conditions, transport profile movement of Creatine Kinase was not inhibited by the existence of Hsc70. From these consequences, we consider that the argument by the Referee (Maybe Hsc70 is acting in a dominant-interfering and/or non-specific way to interfere with all transport.) does not decline our hypothesis.
The reason why we used the expression ìswitch-overî is based on the fact that putative Hsc70 binding domain within KLC, i.e. DnaJ binding domain almost correspond to the TPR domain, which binds to membranous organelles. This suggests that Hsc70 and membranous organelles bindings to KLC are mutually exclusive, as previously described by Dr. Bradyís group. As Creatine Kinase transport is dependent on Hsc70 binding to KLC, Creatine Kinase and membranous organelles transport events are mutually exclusive. This concept has nothing to do with the notion that Kinesin-1 is limiting and that there is competition for its usage in different transport events.
We do not intend to stick to the word ìswitching-overî and we admit that our experimental results themselves allow other interpretations. As we know that majority of Kinesin-1 is within cytoplasmic pools, the working motor population along microtubule track is presumed to be small in quantity, but the exact quantity of Kinesin-1 on duty is obscure. From these consequences, we accepted the Reviewer's suggestion again and revised the discussion section (from page 19, l. 12 to page 20, l.

2), the title (ìKinesin-1/Hsc70 dependent mechanism of slow axonal transport and its relation to fast axonal transport,î page 1, ll. 1-2) and running title (ìSlow axonal transport by Hsc70,î page 1, l. 17) to focus more on the role of Kinesin-1 and Hsc70 in slow axonal transport. We also changed the expression in the Abstract ("this domain might play a role as a switchover system between slow and fast transport by Hsc70," page2, ll. 12-13).

Responses to Referee #2

First of all, we deeply thank the Referee for his/her clear support of our paperís significance and data quality (Overall I found the work quite good, certainly within the standards to be published at The EMBO Journal. The reasons are twofold: the concept of the possibility of switching between fast and slow axonal transport based on the activity of a chaperone/DnaJ-like domain interaction is conceptually novel and provocative and the experiments that lead to that notion are overall ... clear-cut. I found quite convincing the biochemical and microscopy data in squid giant axons dissecting Hsc70/Kinesin-1/DnaJ-like domain-cargo selection. I also found convincing the DIC and fluorescence cross-correlation spectroscopy showing the effect of Hsc70 and DnaJ-like peptide injection on membrane transport... Overall, I support acceptance of this work in The EMBO Journal.). Following the suggestion, we amended the manuscript.

Comment 1
...the experiments that lead to that notion are overall (but not always) clear-cut... must say that the data presented to prove the switchover mechanism is not all that clear to me and it is possible that Hsc70 might affect fast transport indirectly (in which case increased slw would be by default), through a stress response, possibly via the CHIP pathway. The stress response may affect at first events with higher energy demand and so it would advisable that longer time points are considered in this type of analysis. Alternatively, they should discuss this possibility and consequently tone down their conclusions in this respect. The mice data are nice in regard to the consequences of affecting chaperone binding yet, again, the possibility that the effects are the sum of consequences and that impaired transport might arise from a disregulation of pathways away from cargo/MT binding should be more properly discussed.

As the Referee pointed out, it still remains the possibility that Hsc70 might affect the transport indirectly through a stress response. The experimental system using acute preparation of squid giant axons cannot escape from this possible drawback because longer experiments more than two hours are challenges. Although the effects of Hsc70 on transport were indistinguishable within this experimental time-window, we revised the discussion ("The system using acute preparation of squid giant axons cannot escape from the stress response induced by the experimental intervention, because longer experiments more than two hours are challenges. Although the effects of Hsc70 on transport were indistinguishable within this experimental time-window, it is possible that Hsc70 might affect the transport indirectly through a stress response, possibly via the CHIP pathway. Accumulating the knowledge of the regulatory mechanism linking the motor complex with different cargos, cytoplasmic proteins or membranous organelles is indispensable to explain the whole figure of intracellular transport." Page 20, ll.3-10) and tone down our conclusion. We also amended our description on our mice data, along the lines suggested by the Referee ("Though the late-onset time course of the pathological change indicates that it might be more plausible to interpret the findings based on slow axonal transport mechanism, the possibility remains that the effects are the sum of consequences and that impaired transport might arise from a dysregulation of pathways away from the mechanism we have postulated." Page 20, ll.21-25).

Editorial Decision 26 November 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 to review the revised manuscript and I have now heard back from this referee. As you can see below, the referee appreciates the added changes and supports publication here. I am therefore very pleased to proceed with the acceptance of your paper for publication here. You will receive the formal acceptance letter shortly.

Editor
The EMBO Journal

REFEREE REPORT

Referee #1:

The authors have done a very nice job responding to the reviewer's concerns. I particularly like the notations on Supplementary figure 1 to indicate the moving particles. In my opinion the manuscript is now suitable for publication.

