THE EMBO JOURNAL

Mitochondrial entry into damaged axons is restricted by impaired disassembly of the axon initial segment

Sumiko Kiryu-Seo, Reika Matsushita, Yoshitaka Tashiro, Takeshi Yoshimura, Yohei Iguchi, Masahisa Katsuno, Ryosuke Takahashi, and Hiroshi Kiyama

DOI: 10.15252/embj.2021110486

Corresponding authors: Sumiko Kiryu-Seo (skiryu@med.nagoya-u.ac.jp) , Hiroshi Kiyama (kiyama@med.nagoya-u.ac.jp)

Review Timeline:

Submission Date:	5th Jan 22
Editorial Decision:	10th Mar 22
Revision Received:	3rd Jun 22
Editorial Decision:	7th Jul 22
Revision Received:	15th Jul 22
Accepted:	20th Jul 22

Editor: Kelly Anderson

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Kiryu-Seo,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

Given the referees' comments, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. It would be good to discuss your plan for addressing the reviewers concerns and I am available to do so either by zoom or email in the next few weeks.

I have attached a guide for revisions for your convenience.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (8th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

The manuscript by Kiryu-Seo et al. describes an elegant mouse model, which takes advantage of Atf3 as an injury-responsive factor to achieve GFP labelling of mitochondria and simultaneous ablation of an essential proteasome subunit specifically upon nerve injury. Capitalizing on this mouse model and using a combination of tissue clearing, histology, biochemistry and in vivo time-lapse imaging, the study uncovers an interesting new compensatory mechanism in nerve-injured motor neurons. The cells are shown to transiently disassemble their axon initial segment (AIS) to facilitate entry of mitochondria into the axon and thereby meet the increased energy demand for nerve regeneration. This AIS disassembly depends on the ubiquitin-proteasome system, which degrades the major AIS organizer Ankyrin G. Interestingly, ALS-affected motor neurons are also known to show proteasome deficiency. Similarly to proteasome-ablated cells, they fail to disassemble the AIS, despite activating the injury response as evidenced by Atf3 induction. The described mechanism is therefore proposed to be relevant for ALS.
The study is well-designed, the paper is clearly written and nicely illustrated.

Main comments:
1. Rpt3 gene targeting strategy is not described in the paper - please provide the information on how the Rpt3flox knock-in was generated in Fig. 1A and in the Materials and Methods.
2. Fig. 3D: it would be better to perform statistics for N=number of mice, not number of imaged areas, as areas in the same mouse are not independent observations (same applies to other figures, e.g. Fig. 5E,K, Fig. 7J, etc.). In addition, the number of

areas stated in the figure legend (10-15) does not correspond to the number of data points for some of the bars.
3. The observation that GFP is predominantly expressed in more vulnerable neurons in Aft3:BAC;SOD1G93A mice (Fig. EV7) is interesting, but requires further characterization. The authors should perform a quantification of MMP-9 and Osteopontin expression in GFP-positive and GFP-negative motor neurons. Please also provide references reporting MMP-9 and Osteopontin as vulnerability markers.
4. The data presented in the study suggests that decreased mitochondrial influx into the axon could play a role in ALS pathology, and hints at transient AIS disassembly as a potential therapy for ALS. This, however, is not supported by data. To test this hypothesis, it would be necessary to disassemble the AIS in SOD1 mice, e.g. through AnkG knock-down, and then ask whether this manipulation improves disease phenotypes such as motor neuron loss and muscle denervation.
5. Most of the experiments in the paper are based on the new Atf3:BAC2 transgenic line generated in this study. However, the SOD1 ALS mice are crossed to a different Atf3:BAC line that the group used previously in Kiryu-Seo et al., Sci Rep 2016. As the paper claims that the Atf3:BAC2 model is new, the authors should provide an explanation what is different between the two BAC lines, and why the experiments in ALS mice were done with a different line than the rest of the study.

Minor comments:
1. Title: Based on the presented data, I think it would be more precise to say that "impaired proteasome-dependent disassembly of the AIS restricts mitochondrial entry into degenerating motor axons", as the presence of mitochondria rather than energy supply per se was analyzed in this study.
2. Results, p. 6: the authors first describe GFP expression in injured hypoglossal motor neurons (end of first paragraph), then introduce the hypoglossal nerve injury paradigm and its advantages (second paragraph). I recommend describing the experimental paradigm before showing the results.
3. Fig. EV1C: the labeling of the y-axis should probably be "% of Atf3(+) MNs"?
4. P. 6: "Rpt3 CKO mice grew reached adulthood" - the word "grew" should probably be deleted?
5. Where appropriate, it would be helpful if the authors referred to individual panels of EV figures in the text (e.g. Fig. EV3A-B on top of p. 7, Fig. EV3C-D in the middle of p. 7, etc.)
6. Fig. 4C: While the downregulation of AnkG protein in the hypoglossal nucleus looks convincing, the situation in the hypoglossal nerve is not clear. In the text (p. 10, middle) it is stated: "...injured nerves exhibited no signals for 480-kDa and 270-kDa forms of AnkG...", but as presented on the blots in the panel 4C, the 480-kDa isoform is absent in both control and injured conditions, while the 270-kDa isoform is present in both, and looks unchanged. Could you please clarify/rephrase?
7. The schemes in Fig. 6G and Fig. 8 appear redundant. In my opinion, one scheme in the last figure would be sufficient.
8. There is a typo in Discussion, p. 16, middle: "Injured motor neurons likely use this mechanism to meat urgent energy demand... " - should be "to meet".
9. Materials and methods, image analysis (p. 24) - it would be helpful to include an explanation how the AIS was defined (e.g. for looking at mitochondria within it) in case of an absence of clear AnkG staining.

Referee #2:

If a lot of work has been done on the genetic regulation of pro-regenerative and survival pathways, major gaps exist in our understanding of the cell biology of these pathways in vivo. This study is looking to fill this gap by investigating how motor neurons (MN) counteract neurodegenerative stress by modulating the disassembly of the axon initial segment (AIS). Kiryu-Seo and colleagues propose an interesting mechanism by which following an acute axonal injury, MN of the hypoglossal nuclei dismantle their AIS to allow mitochondria to enter the injured axons to support survival and axonal regeneration. Of significance, this phenomenon was observed in another type of neurons and injury (spinal MN, sciatic nerve injury). The authors use an elegant mouse model that labels mitochondria in an injury dependent manner by taking advantage of the regulatory element of the stress-activated gene ATF3. Furthermore, they expend this strategy to concomitantly express the Cre recombinase in a Rpt3flox/flox mouse line in order to delete an essential subunit of the 26S proteasome (Rpt3) in MN upon injury. Results obtain with this injury-induced proteasome-deficient mouse model led the authors to conclude that the AIS disassembly was regulated by the proteasome. Overall, results presented here are robust, experiments are high-powered and the authors deciphered a very novel and previously undescribed mechanism. Some conclusions are overreaching (especially in the ALS study, see below) but they are in general supported by data.

Major:

1) The authors convincingly showed that the mitochondrial staining in the MN of the hypoglossal nuclei from the Atf3:BAC2 mouse fully matches cytochrome c staining after injury which support their observation of a decrease amount of mitochondria in injured MN axons deleted for Rpt3. However, they haven't addressed whether the deletion of Rpt3 affect mitochondrial density without injury. Indeed, it is possible that Rpt3 deletion has an effect on axonal mitochondria per se which would not be due to an injury-related stress response.

2) In the figure 7, the authors are attempting to test whether the AIS disassembly mechanism is also occurring in progressive chronic neurodegenerative disease. A very elegant model is used that labelled vulnerable (or stressed) ATF3 positive spinal

MN. However, I do have several concerns about this last study. First of all, there is a lack of characterization of the Atf3:BAC2;SOD1G93A . For example, P70 is used as a pre-symptomatic stage but simple pathological study (spinal MN count, axonal integrity) hasn't been done to show that indeed these MN are not already affected by the disease. Next, my understanding is that the whole idea of this experiment is to show that vulnerable spinal ALS mutant MN fail to disassemble their AIS which would in turn fail to protect them from degeneration. However, it is hard to imagine that in such chronic condition MN would keep an unassembled AIS for weeks/months to protect them since a lack of AIS would likely provoke some pathological events independent of those caused by SOD1 mutation. A key experiment to make this point would be to find non-vulnerable/resistant spinal MN in this model and demonstrate that their AIS is lacking. The authors present a proxy for this experiment in figure 7H using an injured condition which I assume is a sciatic nerve injury although it is hard to understand what this condition really is by reading both the text and the figure legend. In this condition, the AIS is indeed disassembled, but it is very difficult to convincingly use a response to an acute injury as a positive control for a progressive chronic condition. Overall, the claim; "Intriguingly, the failure of this proteasome-mediated mechanism in response to pathological damage seems to cause the axonal pathology of ALS motor neurons (Fig 8)." is overreaching and data don't support this unambiguously.

3) For legitime reasons, the authors focus on mitochondria and elegantly demonstrate that upon injury, their density in proximal and distal axons is reduced when AIS fails to be dissembled in Rpt3 deleted MN. However, a key question is to whether this mechanism is specific to mitochondria. Since the AIS has been shown to act as a filter to permit the entry of different cargos in the axons, it is possible the AIS persistence in Rpt3 deleted MN also affect the transport of other cargos that could be beneficial for neuron repair. This important piece of data is lacking in the present version of the manuscript.

4) Although challenging, it would be interesting to assess the mitochondrial density in the soma of the Rpt3 deleted MN after injury. Indeed, if it is clear that the mitochondrial density is affected in MN axons of Rpt3 mutant, the potential effect of the Rpt3 deletion on a non-axonal population of mitochondria remains to be demonstrated. This result would greatly strengthen the axonal specificity of the mechanism described.

Minor:

5) Fig.1F: "peripheral" is not well defined. I would be useful to add on the scheme of Fig. 1B where this peripheral region of the axon is approximately located.

6) Fig.3: It is unclear what represents each dot on the bar graphs of Fig.3D and E. Based on the figure legend, I assume there are areas. If this is the case, then a) the legend is incorrect since it says "n=10-15 areas "and the CKO control in D has less than 10 (6) and b) the picture of Atf3:Bac2 Injured MN 5D in Fig. 3C is not representative since based on Fig.3D none of the area show 0 AnkG labeled AIS at 5D.

7) In Fig. 6E, the axis legend says "Motility %" and in the figure legend it is described as "Motility of motile mitochondria". Both are very vague. The author should modify these legends to precisely designate what is shown on this graph. Is it the percentage of time each motile mitochondria spend in motion or the percentage of motile mitochondria?

8) The results presented in Fig. 4 where AnkG is degraded after injury in the AIS but not in the node of Ranvier's axons is both fascinating and difficult to interpret. This result is worth to be discussed at length in the discussion and adding some speculations that could explain this phenomena.

Referee #3:

This is an ambitious study that appears to have a number of aims, however the initial hypothesis is unclear. The title of the paper suggests a study into motor axon degeneration, as does the initial paragraph of the abstract, which further suggests a study into ALS disease pathology. The abstract then indicates an injury model to study ALS, whilst at the same time mentioning a transcriptional programme for regeneration. This is confusing because ALS is not an acute injury-related phenomenon.
The abstract starts by stating that "The proteasome is essential for the repair of damaged motor neurons." I am not aware of any literature that specifically supports a role for the proteasome in motor neuron repair. The proteasome has been studied in relation to axon regeneration as well as developmental axon growth, but its role is not clear. This introductory statement is misleading.

(For the authors, here are some references regarding the proteasome and regeneration.
Verma, P.; Chierzi, S.; Codd, A.M.; Campbell, D.S.; Meyer, R.L.; Holt, C.E.; Fawcett, J.W. Axonal Protein Synthesis and Degradation Are Necessary for Efficient Growth Cone Regeneration. J. Neurosci. 2005, 25, 331-342.
Hsu, M.-T.; Guo, C.-L.; Liou, A.Y.; Chang, T.-Y.; Ng, M.-C.; Florea, B.I.; Overkleeft, H.S.; Wu, Y.-L.; Liao, J.-C.; Cheng, P.-L. Stage-Dependent Axon Transport of Proteasomes Contributes to Axon Development. Dev. Cell 2015, 35, 418-431

Knöferle, J.; Ramljak, S.; Koch, J.C.; Tönges, L.; Asif, A.R.; Michel, U.; Wouters, F.S.; Heermann, S.; Krieglstein, K.; Zerr, I.; et al. TGF-β 1 enhances neurite outgrowth via regulation of proteasome function and EFABP. Neurobiol. Dis. 2010, 38, 395-404.
Park, J.Y.; Jang, S.Y.; Shin, Y.K.; Suh, D.J.; Park, H.T. Calcium-dependent proteasome activation is required for axonal neurofilament degradation. Neural Regen. Res. 2013, 8, 3401.
Staal, J.A.; Dickson, T.C.; Chung, R.S.; Vickers, J.C. Disruption of the Ubiquitin Proteasome System following Axonal Stretch Injury Accelerates Progression to Secondary Axotomy. . Neurotrauma 2009, 26, 781-788.)

The introduction is lengthy and introduces several concepts, some of which are also misleading. In bullet points, the introduction suggests that previous studies have shown:

• ATF3 is a key factor that is activated in response to injury or in ALS related motor neuron degeneration, and that is also associated with protein accumulation.
• Mitochondrial dynamics and quality are altered in ALS disease pathology.
• The axon initial segment (AIS) contributes to maintaining an appropriate number of axonal mitochondria under normal neuronal activity.
• Traumatic injury, optic nerve injury and excitotoxic insults dismantle the AIS.
• Calpain is responsible for the degradation of the AIS, however, the proteasome is an additional candidate.

These are partially disparate concepts, but the introduction suggests the hypothesis that axonal injury might lead to proteasomal degradation of the AIS, which might alter axonal mitochondrial dynamics, (to cope with injury-related / regenerative energy demands) and that this is related to activation of the stress-related transcription factor ATF3.
There is however no obvious hypothesis other than (from the introduction): "Despite the accumulated evidence, the physiological significance and exact mechanisms for the AIS morphological change during brain damage and disease have not been well addressed." Whilst in the abstract it states: "The proteasome is essential for the repair of damaged motor neurons. Dysfunction of the proteasome is thought to be implicated in motor neuron degeneration of amyotrophic lateral sclerosis (ALS). However, it remains unclear how proteasome function impacts the stress resilience of damaged motor neurons."

There is not therefore a clear objective or hypothesis for the study, and the reader is unsure whether the study is investigating ALS disease mechanisms or axon regeneration after injury (these are two very different phenomena), however the introduction ends with: "Here we demonstrate a new mechanism by which motor neurons disassemble the AIS in a proteasome-sensitive manner in response to damage, which facilitates mitochondrial entry into axons. This regulatory mechanism of mitochondrial logistic system would be critical for damaged motor neurons to compensate enough energy to boost their regeneration and survival responses."

There are several statements in the introduction which are central to the study, but which are not well supported by the literature. This is crucial, because the strength of the findings and conclusions of the authors relies on concepts that have not been previously established.

Regarding ATF3, the authors state that "Stress-responsive activating transcription factor 3 (ATF3) is robustly and specifically induced in injured neurons to elicit transcriptional reprogramming, which changes the protein composition of cells through protein degradation and synthesis (Chandran et al, 2016; Lu et al, 2020; Nakagomi et al, 2003; Palmisano et al, 2019; Renthal et al, 2020; Seijffers et al, 2006)."
This suggests to the reader that ATF3 "changes the protein composition of cells through protein degradation". ATF3 is not, to my knowledge, known to regulate degradation. And my searches for information relating to ATF3 and protein degradation only find papers related to degradation of ATF3 as a regulatory mechanism. If this is the case, then the studies presented here, in which the proteasome is "deleted" conditionally in motor neurons, would be affected by altered regulation of ATF3 itself, which would impact the findings.

Regarding the proteasome: "Impaired proteostasis, in particular proteasomal dysfunction, has been implicated in ALS pathology (Picher-Martel et al, 2019; Tashiro et al, 2012)." These references do not support that statement.

Regarding mitochondria "Mitochondrial motility in axons is progressively reduced during axonal maturation". This is correct, but is written to imply a reduction in axonal mitochondria in mature axons, which is not the case. It is only their motility that is reduced.
Also: "The AIS may also contribute to maintaining an appropriate numbers of axonal mitochondria under normal neuronal activity." There is no evidence in the literature to support the idea that the axon initial segment has any control over axonal mitochondrial transport. Many studies have addressed how the AIS might control axon transport, but these have not focused on mitochondria. (see The Axon Initial Segment: An Updated Viewpoint Leterrier 2018." Also, many studies have addressed mechanisms regulating mitochondrial axon transport (focusing on Trak, Milton, Miro, Syntaphilin, Armcx1 etc), and none of these indicate regulation at or by the axon initial segment.

Regarding injury induced degradation of the AIS: "The cysteine protease calpain is considered to be responsible for the degradation of the AIS, including AnkG and voltage-gated sodium channels (Schafer et al., 2009; Zhao et al, 2020). However, the proteasome is an additional candidate, given its preferential localization at the AIS along with developmental maturation (Hsu

et al, 2015; Lee et al, 2020)." These papers do not support this statement. Firstly, all the studies that have examined AIS disassembly have identified calpain as the central regulator. Secondly, the proteasome is not known to localise preferentially to the AIS. In fact, the paper by Hsu et al supports the idea that axon growth relies on a reduction of axonal proteasomes, and that growth promoting signals trigger retrograde removal of proteasomes from axons. The paper by Lee et al reports on proteasomes in the early part of the axon during early development. It does not demonstrate an enrichment of proteasomes in the AIS in adult animals. On the contrary, a recent study published an AIS proteome, with an extensive list of enriched (or not) molecules at the AIS. In the supplementary datasheet for this paper, a number of proteasome related molecules are identified, however none of them are specifically enriched in the AIS (https://www.nature.com/articles/s41467-019-13658-5 Mapping axon initial segment structure and function by multiplexed proximity biotinylation, Hamdan et al, Nature Communications, 2020)

There are also some significant mechanistic issues regarding the study itself.
A key issue is the design of the study, using complicated transgenic mice which express GFP-tagged mitochondria under the control of the ATF3 promoter. The rational is that these mitochondria are only present after an injury, which leads to ATF3 activation. In the same genetic cassette, there is also Cre recombinase downstream of an IRES site. The authors cross these with mice floxed for Rpt3. This means that in these mice, after an injury, GFP-mitochondria are present whilst the proteasome is depleted.
The experimental conditions are therefore:
1. Uninjured mice.
2. Injured mice with GFP-mitochondria in the injured axons.
3. Injured mice with GFP-mitochondria in the injured axons, but no Rpt3 (no proteasome).
The authors find that after an injury, axons attempt to regenerate, except for condition 3, where the axons degenerate, and do not regenerate.
A considerable issue is that the authors have previously published a paper which demonstrates that conditional depletion of RPT3 in motor neurons leads to axon degeneration (Tashiro et al 2012). This means that the degeneration in condition 3 occurs even in the absence of the injury.
The experiment essentially already has a condition 4: Uninjured mice with no RPT3. This is an important control, and essentially changes the question being asked, which is actually "can an injury signal downstream of ATF3 (or even other transcription factors) overcome axonal degeneration caused by depleted proteasomes? And the answer is no. Even after upregulation of ATF3, axons still degenerate without proteasomes.

This axon degeneration in itself could impact on not only mitochondrial but any axonal transport mechanisms, especially of the degeneration is related to (for example) microtubule degeneration, where mitochondrial transport would not be possible.

Another issue is the compensatory upregulation in autophagy that occurs in response to proteasome deletion, as was recently demonstrated (Inducible Rpt3, a Proteasome Component, Knockout in Adult Skeletal Muscle Results in Muscle Atrophy, Kitajima et al Front Cell Dev Biol. 2020;). This seems to be quite a comprehensive response, involving numerous autophagy-related molecules (eg LC3II, p62, Atg5, Atg7, and Beclin-1), which will undoubtedly complicate mechanistic characterisation.

Each of the figures therefore addresses potential axonal mechanisms that are perhaps alluded to in previous literature, but which in fact have not been well studied. I do not think there is sufficient data here to support the proposed novel mechanisms, but rather that each of the studied steps needs further investigation in order to convincingly demonstrate mechanism.

Detailed point-by-point response

Referee #1:

The manuscript by Kiryu-Seo et al. describes an elegant mouse model, which takes advantage of Atf3 as an injury-responsive factor to achieve GFP labelling of mitochondria and simultaneous ablation of an essential proteasome subunit specifically upon nerve injury. Capitalizing on this mouse model and using a combination of tissue clearing, histology, biochemistry and in vivo time-lapse imaging, the study uncovers an interesting new compensatory mechanism in nerve-injured motor neurons. The cells are shown to transiently disassemble their axon initial segment (AIS) to facilitate entry of mitochondria into the axon and thereby meet the increased energy demand for nerve regeneration. This AIS disassembly depends on the ubiquitin-proteasome system, which degrades the major AIS organizer Ankyrin G. Interestingly, ALS-affected motor neurons are also known to show proteasome deficiency. Similarly to proteasome-ablated cells, they fail to disassemble the AIS, despite activating the injury response as evidenced by Atf3 induction. The described mechanism is therefore proposed to be relevant for ALS.

The study is well-designed, the paper is clearly written and nicely illustrated.

Response: We appreciate Reviewer #1's summary of our findings and positive assessment.

Main comments:
1. Rpt3 gene targeting strategy is not described in the paper - please provide the information on how the Rpt3flox knock-in was generated in Fig. 1A and in the Materials and Methods.

Response: We added a description of the *Rpt3* gene-targeting strategy in the Materials and Methods section, as well as a brief scheme for breeding in Figure EV1D, of the revised manuscript.

2. Fig. 3D: it would be better to perform statistics for N=number of mice, not number of imaged areas, as areas in the same mouse are not independent observations (same applies to other figures, e.g. Fig. 5E,K, Fig. 7J, etc.). In addition, the number of areas stated in the figure legend (10-15) does not correspond to the number of data points for some of the bars.

Response: We thank the reviewer for this advice. According to this advice and the Journal's requirements, we performed statistics again wherever necessary and corrected N values to indicate numbers of mice in all figures. For Figure 3D, some data seemed to be accidentally missed when we prepared the figure. We apologize for this error in the original version of our manuscript and have corrected it in the revised manuscript.

3. The observation that GFP is predominantly expressed in more vulnerable neurons in Aft3:BAC;SOD1G93A mice (Fig. EV7) is interesting, but requires further characterization. The authors should perform a quantification of MMP-9 and Osteopontin expression in GFP-positive and GFP-negative motor neurons. Please also provide references reporting MMP-9 and Osteopontin as vulnerability markers.

Response: We appreciate the reviewer's interest in *Atf3*:SOD1 mice. As Reviewers #1 and #2 suggested, the characterization of *Atf3*:SOD1 mice was insufficient in the original version of our manuscript. We added data showing lifespan, motor neuron (MN) survival, and age-dependent expression of GFP in *Atf3*:SOD1 mice in Figure EV5 of the revised manuscript. In addition, we show the percentage of GFP-expressing MNs relative to all ChAT-positive MNs, and percentages of MMP-9- and osteopontin-positive MNs relative to all GFP-positive MNs in Fig. 7D and E of the revised manuscript. GFP-positive MNs represented a small portion of vulnerable MNs, probably indicating stress-responsive vulnerable MNs are selectively labeled in *Atf3*:SOD1 mice. Studies regarding MMP-9 and osteopontin (Kaplan et el., *Neuron*, 2016 and Morisaki et al., *Sci Rep*, 2016) are mentioned on page 12, line 3 from the bottom of the revised manuscript.

4. The data presented in the study suggests that decreased mitochondrial influx into the axon could play a role in ALS pathology, and hints at transient AIS disassembly as a potential therapy for ALS. This, however, is not supported by data. To test this hypothesis, it would be necessary to disassemble the AIS in SOD1 mice, e.g. through AnkG knock-down, and then ask whether this manipulation improves disease phenotypes such as motor neuron loss and muscle denervation.

Response: We thank the reviewer for raising this important point. We agree that our data imply the potential of transient AIS disassembly but do not definitively support the idea in an ALS model. Unfortunately, we have not succeeded in manipulating only a specific type of spinal MNs, such as GFP(ATF3)-positive MNs. In the revised manuscript, we carefully rephrased this part to not overinterpret the conclusion.

However, we think reviewer's point is very important. Therefore, we added data showing that AnkG knockdown delayed degeneration of injured MNs in *Rpt3* CKO mice (Fig. 5L and M of revised manuscript). The reason why the rescue effect was not perfect may be because there are numerous proteasomal targets in damaged MNs.

5. Most of the experiments in the paper are based on the new Atf3:BAC2 transgenic line generated in this study. However, the SOD1 ALS mice are crossed to a different Atf3:BAC line that the group used previously in Kiryu-Seo et al., Sci Rep 2016. As the paper claims that the Atf3:BAC2 model is new, the authors should provide an explanation what is different between the two BAC lines, and why the experiments in ALS mice were done with a different line than the rest of the study.

Response: Both *Atf3*:BAC and *Atf3*:BAC2 show injury responsiveness. However, the progeny of *Rpt3* CKO mice crossed with *Atf3*:BAC mice had a shorter lifespan. This is one

of the reasons why we generated an additional transgenic line. When we crossed *Atf3*:BAC with other floxed mice carrying fundamental genes whose conventional knockout are embryonic lethal, they grew into adulthood and exhibited no differences in lifespan compared with wild-type mice. In the *Rpt3* CKO cross with *Atf3*:BAC, an unexpected germline recombination may have occurred or locus of transgene insertion may have caused the unexpected phenotype leading to short lifespan. We added this information in the Results section of the revised manuscript (page 6, line 1).

The reason we crossed *Atf3*:BAC with ALS mice is that *Atf3*:BAC Tg mice more effectively mimic expression of endogenous ATF3 in our examinations. For example, more than 90% of damaged MNs in *Atf3*:BAC mice express GFP, while only 60%–70% of injured motor neurons in *Atf3*:BAC2 mice express GFP. We added this information to the Results section of the revised manuscript (page 12, line 11).

Minor comments:
1. Title: Based on the presented data, I think it would be more precise to say that "impaired proteasome-dependent disassembly of the AIS restricts mitochondrial entry into degenerating motor axons", as the presence of mitochondria rather than energy supply per se was analyzed in this study.

Response: We thank the reviewer for this suggestion. We revised the title to not exceed 100 characters.

2. Results, p. 6: the authors first describe GFP expression in injured hypoglossal motor neurons (end of first paragraph), then introduce the hypoglossal nerve injury paradigm and its advantages (second paragraph). I recommend describing the experimental paradigm before showing the results.

Response: According to the reviewer's advice, we first described our experimental paradigm and then moved onto an explanation of the mice. With this change, we exchanged the order of panels A and B in Figure 1 of the revised manuscript.

3. Fig. EV1C: the labeling of the y-axis should probably be "% of Atf3(+) MNs"?

Response: Yes. Thank you for pointing out this error. It has been corrected in the revised manuscript.

4. P. 6: "Rpt3 CKO mice grew reached adulthood" - the word "grew" should probably be deleted?

Response: We thank the reviewer for identifying this mistake. It has been corrected in the revised manuscript.

5. Where appropriate, it would be helpful if the authors referred to individual panels of EV figures in the text (e.g. Fig. EV3A-B on top of p. 7, Fig. EV3C-D in the middle of p. 7, etc.)

Response: We added references to individual panels of EV figures in the text of the revised manuscript.

6. Fig. 4C: While the downregulation of AnkG protein in the hypoglossal nucleus looks convincing, the situation in the hypoglossal nerve is not clear. In the text (p. 10, middle) it is stated: "...injured nerves exhibited no signals for 480-kDa and 270- kDa forms of AnkG...", but as presented on the blots in the panel 4C, the 480-kDa isoform is absent in both control and injured conditions, while the 270-kDa isoform is present in both, and looks unchanged. Could you please clarify/rephrase?

Response: We apologize for the insufficient explanation. We rephrased this part of the text (p 9, line 8 from the bottom) in the revised manuscript.

7. The schemes in Fig. 6G and Fig. 8 appear redundant. In my opinion, one scheme in the last figure would be sufficient.

Response: We agree with the reviewer's opinion. The scheme presented in Fig. 6G of the original manuscript has been removed from the revised manuscript.

8. There is a typo in Discussion, p. 16, middle: "Injured motor neurons likely use this mechanism to meat urgent energy demand... " - should be "to meet".

Response: Thank you for pointing out this typo. It has been corrected in the revised manuscript.

9. Materials and methods, image analysis (p. 24) - it would be helpful to include an explanation how the AIS was defined (e.g. for looking at mitochondria within it) in case of an absence of clear AnkG staining.

Response: We have two ways to identify the disappeared AIS region after injury. One is to find injured MNs retaining very weak and fragmented AnkG staining. The other is to confirm that the neurite of the injured motor neuron exited from the hypoglossal nucleus and formed the hypoglossal nerve bundle. We described them in the Materials and Methods section of the revised manuscript (page 24, line 10).

Referee #2:

If a lot of work has been done on the genetic regulation of pro-regenerative and survival pathways, major gaps exist in our understanding of the cell biology of these pathways in vivo. This study is looking to fill this gap by investigating how motor neurons (MN) counteract neurodegenerative stress by modulating the disassembly of the axon initial segment (AIS). Kiryu-Seo and colleagues propose an interesting mechanism by which following an acute axonal injury, MN of the hypoglossal nuclei dismantle their AIS to allow mitochondria to enter the injured axons to support survival and axonal regeneration. Of significance, this phenomenon was observed in another type of neurons and injury (spinal MN, sciatic nerve injury). The authors use an elegant mouse model that labels mitochondria in an injury dependent manner by taking advantage of the regulatory element of the stress-activated gene ATF3. Furthermore, they expend this strategy to concomitantly express the Cre recombinase in a Rpt3flox/flox mouse line in order to delete an essential subunit of the 26S proteasome (Rpt3) in MN upon injury. Results obtain with this injury-induced proteasome-deficient mouse model led the authors to conclude that the AIS disassembly was regulated by the proteasome. Overall, results presented here are robust, experiments are high-powered and the authors deciphered a very novel and previously undescribed mechanism. Some conclusions are overreaching (especially in the ALS study, see below) but they are in general supported by data.

Response: We thank the reviewer for this summary and for highlighting the significance of our study.

Major:

1) The authors convincingly showed that the mitochondrial staining in the MN of the hypoglossal nuclei from the Atf3:BAC2 mouse fully matches cytochrome c staining after injury which support their observation of a decrease amount of mitochondria in injured MN axons deleted for Rpt3. However, they haven't addressed whether the deletion of Rpt3 affect mitochondrial density without injury. Indeed, it is possible that Rpt3 deletion has an effect on axonal mitochondria per se which would not be due to an injury-related stress response.

Response: We agree with the reviewer that the *Rpt3* deletion would affect mitochondrial density without injury. Indeed, a previous study showed that deletion of *Rpt3* in uninjured motor neurons led to motor neuron death (Tashiro et al. JBC, 2012). The death is not due to the failure of the AIS disassembly, because proteasome-sensitive disassembly of the AIS is not necessary in uninjured motor neurons. There are expected to be numerous proteasomal targets apart from AnkG, including targets not related with injury. We discussed the point in the text of revised manuscript (p16, line 2 from the bottom).

2) In the figure 7, the authors are attempting to test whether the AIS disassembly mechanism is also occurring in progressive chronic neurodegenerative disease. A very elegant model is used that labelled vulnerable (or stressed) ATF3 positive spinal MN. However, I do have several concerns about this last study. First of all, there is a lack of

characterization of the Atf3:BAC2;SOD1G93A . For example, P70 is used as a pre-symptomatic stage but simple pathological study (spinal MN count, axonal integrity) hasn't been done to show that indeed these MN are not already affected by the disease.

Response: We thank the reviewer for these comments. As suggested, the characterization of *Atf3*:SOD1 was insufficient in the original version of our manuscript. We have added new data showing lifespan, MN survival, and expression of GFP in *Atf3*:SOD1 mice in Figure EV5 of the revised manuscript. Our results suggest that the *Atf3*:BAC transgene does not affect the disease phenotype of $SOD1^{G93A}$ mice.

Next, my understanding is that the whole idea of this experiment is to show that vulnerable spinal ALS mutant MN fail to disassemble their AIS which would in turn fail to protect them from degeneration. However, it is hard to imagine that in such chronic condition MN would keep an unassembled AIS for weeks/months to protect them since a lack of AIS would likely provoke some pathological events independent of those caused by SOD1 mutation. A key experiment to make this point would be to find non-vulnerable/resistant spinal MN in this model and demonstrate that their AIS is lacking.

Response: We agree with the reviewer that the lack of an AIS would likely provoke some pathological events if chronic condition MNs maintained an unassembled AIS for weeks/months. We do not think long-term disassembly of the AIS is beneficial. Recovery of the AIS at an appropriate time is also important, as shown in Figure 3C–E. What we would like to emphasize is that, in response to emergency, ATF3-inducing MNs can increase axonal mitochondria by temporarily dismantling the AIS. In addition, we present the possibility that ATF3-inducing ALS MNs would be partially protected if they can transiently dismantle the AIS. All ALS MNs, including non-vulnerable/resistant MNs, have the AIS (Fig. 7F and G of revised manuscript). We think that non-vulnerable/resistant MNs do not need to dismantle the AIS because they do not induce ATF3.

The authors present a proxy for this experiment in figure 7H using an injured condition which I assume is a sciatic nerve injury although it is hard to understand what this condition really is by reading both the text and the figure legend. In this condition, the AIS is indeed disassembled, but it is very difficult to convincingly use a response to an acute injury as a positive control for a progressive chronic condition. Overall, the claim; "Intriguingly, the failure of this proteasome-mediated mechanism in response to pathological damage seems to cause the axonal pathology of ALS motor neurons (Fig 8)." is overreaching and data don't support this unambiguously.

Response: We apologize that our ambiguous and insufficient description was confusing to the reviewer. To explain the point more clearly, we summarize MNs as follows:

MN	Uninjured MN		Injured MN (5d)		ALS MN (pre-symp)	
	ATF3(-)		ATF3(+)		ATF3(+)	ATF3(-)
RPT3	+	-	+	-	↓	↓
AIS	+	+ (*)	-	+	+	+
axonal mitochondria	+	± (*)	++	+	±	±
axon	normal	degeneration(*)	regeneration	degeneration	degeneration	degeneration
mouse	*Atf3*:BAC2 *Thy1*-Mito	*Rpt3* CKO (x*ChAT*-cre)	*Atf3*:BAC2	*Rpt3* CKO (x*Atf3*:BAC2)	*Atf3*:SOD1	SOD1

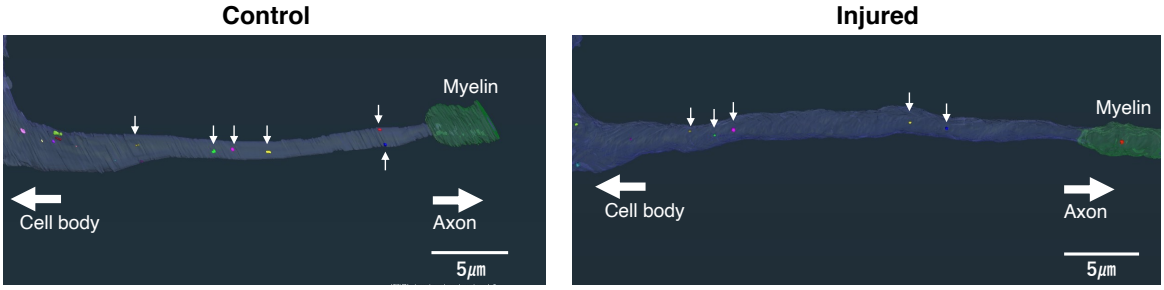
(*) expected

We carefully rewrote this part (page 12-13) in the revised manuscript. In Figure 7H, we mentioned the potential of ATF3-positive MNs by using injured MNs capable of regenerating after sciatic nerve injury. ALS MNs began to induce ATF3 around P60 or later. We added data showing time-dependent expression of GFP(ATF3) in *Atf3*:SOD1 MNs in Figure EV5E and F of the revised manuscript. Although ALS is a chronic disease, it would be reasonable to compare the AIS and mitochondria between GFP-positive injured MNs and GFP-positive ALS MNs as a stress-response after ATF3 induction. However, we agree with the reviewer's concern that the original statement is overreaching. Accordingly, we rephrased this statement (page 14, line 10) in the revised manuscript.

3) For legitime reasons, the authors focus on mitochondria and elegantly demonstrate that upon injury, their density in proximal and distal axons is reduced when AIS fails to be dissembled in Rpt3 deleted MN. However, a key question is to whether this mechanism is specific to mitochondria. Since the AIS has been shown to act as a filter to permit the entry of different cargos in the axons, it is possible the AIS persistence in Rpt3 deleted MN also affect the transport of other cargos that could be beneficial for neuron repair. This important piece of data is lacking in the present version of the manuscript.

Response: We show data indicating that numbers of Lamp1-positive lysosomes were similar between injured axons of *Atf3*:BAC2 and *Rpt3* CKO mice at 5 days after injury in Figure EV2B–D. Thereafter, lysosomes were increased in injured Rpt3-deficient axons at 10 days after injury, at which point Rpt3-deficient injured motor neurons are degenerating. Apart from this, we recently published an article describing the distribution of mitochondria in the AIS before and after injury using 3D electron microscopy (Focused Ion Beam Scanning Electron Microscopy) (Tamada et al., *J Comp Neurol*, 2021). In this study, we found that the distribution of lysosomes was not changed before and after injury, unlike that of mitochondria (see below, unpublished). As we have not examined all cargos, we cannot provide a complete answer to the reviewer's question. However, it is likely that

mitochondria and lysosomes move separately by different mechanisms in response to *Rpt3*-deletion or damage.



Arrows indicate lysosome

4) Although challenging, it would be interesting to assess the mitochondrial density in the soma of the Rpt3 deleted MN after injury. Indeed, if it is clear that the mitochondrial density is affected in MN axons of Rpt3 mutant, the potential effect of the Rpt3 deletion on a non-axonal population of mitochondria remains to be demonstrated. This result would greatly strengthen the axonal specificity of the mechanism described.

Response: It is indeed interesting. We attempted to obtain clear images of individual mitochondrion within injured motor neurons using super-resolution confocal microscopy. However, it was difficult to clearly observe all mitochondria in a whole cell body with higher resolution, owing to technical limitations. In revised the manuscript, we counted numbers of mitochondria residing on a line using the image with highest resolution (Fig. EV2E–H, Fig. 1J of revised manuscript). Although this method does not represent exact numbers of mitochondria in soma, it roughly indicates their density. We found that *Rpt3*-deficient injured motor neurons had higher numbers of mitochondria than injured motor neurons with *Rpt3.* These findings suggest that the limited entry of mitochondria into axons is not due to decreased numbers of mitochondria in soma of *Rpt3*-deficient injured motor neurons, although we cannot exclude the possibility the mitochondrial shape and GFP intensity affect the results.

Minor:

5) Fig.1F: "peripheral" is not well defined. I would be useful to add on the scheme of Fig. 1B where this peripheral region of the axon is approximately located.

Response: As the reviewer requested, we added the “peripheral” location in a red box on the schema in Fig. 1A of the revised manuscript.

6) Fig.3: It is unclear what represents each dot on the bar graphs of Fig.3D and E. Based on the figure legend, I assume there are areas. If this is the case, then a) the legend is incorrect since it says "n=10-15 areas "and the CKO control in D has less than 10 (6) and b) the picture of Atf3:Bac2 Injured MN 5D in Fig. 3C is not representative since based on Fig.3D none of the area show 0 AnkG labeled AIS at 5D.

Response: We thank the reviewer for this comment.

a) For Figure 3D, some data seemed to be accidentally missed when we prepared the figure. We apologize for this error in the original version of our manuscript. According to Reviewer #1 and the Journal's instructions, we performed statistics again wherever necessary and corrected N values to indicate numbers of mice in all figures.

b) We changed the image of the injured MN at 5 days after injury in Figure 3C of the revised manuscript.

7) In Fig. 6E, the axis legend says "Motility %" and in the figure legend it is described as "Motility of motile mitochondria". Both are very vague. The author should modify these legends to precisely designate what is shown on this graph. Is it the percentage of time each motile mitochondria spend in motion or the percentage of motile mitochondria?

Response: We apologize for the ambiguous description. It is the percentage of motile mitochondria. Accordingly, we changed the description in Figure 6E and accompanying figure legend in the revised manuscript.

8) The results presented in Fig. 4 where AnkG is degraded after injury in the AIS but not in the node of Ranvier's axons is both fascinating and difficult to interpret. This result is worth to be discussed at length in the discussion and adding some speculations that could explain this phenomena.

Response: We thank the reviewer for this comment. We added a brief discussion of this topic on page 17, line 15 of the revised manuscript. One possible explanation is that apparent demyelination does not occur in PNS injury, unlike CNS injury.

Referee #3:

This is an ambitious study that appears to have a number of aims, however the initial hypothesis is unclear. The title of the paper suggests a study into motor axon degeneration, as does the initial paragraph of the abstract, which further suggests a study into ALS disease pathology. The abstract then indicates an injury model to study ALS, whilst at the same time mentioning a transcriptional programme for regeneration. This is confusing because ALS is not an acute injury-related phenomenon.

Response: We thank Reviewer #3 for providing us the opportunity to reconsider our concept and underlying logic. We have improved the manuscript according to their helpful comments.

The abstract starts by stating that "The proteasome is essential for the repair of damaged motor neurons." I am not aware of any literature that specifically supports a role for the proteasome in motor neuron repair. The proteasome has been studied in relation to axon regeneration as well as developmental axon growth, but its role is not clear. This introductory statement is misleading.

(For the authors, here are some references regarding the proteasome and regeneration.
Verma, P.; Chierzi, S.; Codd, A.M.; Campbell, D.S.; Meyer, R.L.; Holt, C.E.; Fawcett, J.W. Axonal Protein Synthesis and Degradation Are Necessary for Efficient Growth Cone Regeneration. J. Neurosci. 2005, 25, 331-342.
Hsu, M.-T.; Guo, C.-L.; Liou, A.Y.; Chang, T.-Y.; Ng, M.-C.; Florea, B.I.; Overkleeft, H.S.; Wu, Y.-L.; Liao, J.-C.; Cheng, P.-L. Stage-Dependent Axon Transport of Proteasomes Contributes to Axon Development. Dev. Cell 2015, 35, 418-431
Knöferle, J.; Ramljak, S.; Koch, J.C.; Tönges, L.; Asif, A.R.; Michel, U.; Wouters, F.S.; Heermann, S.; Krieglstein, K.; Zerr, I.; et al. TGF-β 1 enhances neurite outgrowth via regulation of proteasome function and EFABP. Neurobiol. Dis. 2010, 38, 395-404.
Park, J.Y.; Jang, S.Y.; Shin, Y.K.; Suh, D.J.; Park, H.T. Calcium-dependent proteasome activation is required for axonal neurofilament degradation. Neural Regen. Res. 2013, 8, 3401.
Staal, J.A.; Dickson, T.C.; Chung, R.S.; Vickers, J.C. Disruption of the Ubiquitin Proteasome System following Axonal Stretch Injury Accelerates Progression to Secondary Axotomy. . Neurotrauma 2009, 26, 781-788.)

Response: We thank the reviewers for providing references. As the reviewers pointed out, *in vitro* studies show involvement of the proteasome in neurite extension as well as degeneration, but the role of the proteasome remains especially unclear *in vivo*. Accordingly, we changed the related statement in the abstract to not mislead readers by using inappropriate words.

The introduction is lengthy and introduces several concepts, some of which are also misleading. In bullet points, the introduction suggests that previous studies have shown:

• ATF3 is a key factor that is activated in response to injury or in ALS related motor neuron degeneration, and that is also associated with protein accumulation.
• Mitochondrial dynamics and quality are altered in ALS disease pathology.
• The axon initial segment (AIS) contributes to maintaining an appropriate number of axonal mitochondria under normal neuronal activity.

• Traumatic injury, optic nerve injury and excitotoxic insults dismantle the AIS.
• Calpain is responsible for the degradation of the AIS, however, the proteasome is an additional candidate.

Response: Thank you for summarizing these points. With the reviewer's comment, we realized that established concept, hypothesis, and results were presented in a confusing manner in the Introduction of the original manuscript. Accordingly, we rearranged the Introduction section and carefully rephrased statements in the revised manuscript.

These are partially disparate concepts, but the introduction suggests the hypothesis that axonal injury might lead to proteasomal degradation of the AIS, which might alter axonal mitochondrial dynamics, (to cope with injury-related / regenerative energy demands) and that this is related to activation of the stress-related transcription factor ATF3.

There is however no obvious hypothesis other than (from the introduction): "Despite the accumulated evidence, the physiological significance and exact mechanisms for the AIS morphological change during brain damage and disease have not been well addressed." Whilst in the abstract it states: "The proteasome is essential for the repair of damaged motor neurons. Dysfunction of the proteasome is thought to be implicated in motor neuron degeneration of amyotrophic lateral sclerosis (ALS). However, it remains unclear how proteasome function impacts the stress resilience of damaged motor neurons."

There is not therefore a clear objective or hypothesis for the study, and the reader is unsure whether the study is investigating ALS disease mechanisms or axon regeneration after injury (these are two very different phenomena), however the introduction ends with: "Here we demonstrate a new mechanism by which motor neurons disassemble the AIS in a proteasome-sensitive manner in response to damage, which facilitates mitochondrial entry into axons. This regulatory mechanism of mitochondrial logistic system would be critical for damaged motor neurons to compensate enough energy to boost their regeneration and survival responses."

Response: We agree with the reviewer's point. We carefully rewrote this part in the revised manuscript. Our central hypothesis is that an unknown proteasome-mediated stress-resilient mechanism exists in damaged motor neurons. We assume that the mechanism functions in regenerative damaged motor neurons but not degenerative damaged motor neurons.

As the reviewer mentioned, ALS disease mechanisms and axon regeneration after injury are different phenomena. However, both involve damaged motor neurons exhibiting numerous similar stress responses. ATF3 induction is one example of a common mechanism between ALS motor neurons and injured motor neurons. Using the advantage of our established mice, we found that proteasome-sensitive AIS disassembly functions in regenerative injured motor neurons but not damaged motor neurons, such as proteasome-deficient injured motor neurons and ALS motor neurons.

There are several statements in the introduction which are central to the study, but which are not well supported by the literature. This is crucial, because the strength of the findings and conclusions of the authors relies on concepts that have not been previously established.

Regarding ATF3, the authors state that "Stress-responsive activating transcription factor 3 (ATF3) is robustly and specifically induced in injured neurons to elicit transcriptional reprogramming, which changes the protein composition of cells through protein degradation and synthesis (Chandran et al, 2016; Lu et al, 2020; Nakagomi et al, 2003; Palmisano et al, 2019; Renthal et al, 2020; Seijffers et al, 2006)."
This suggests to the reader that ATF3 "changes the protein composition of cells through protein degradation". ATF3 is not, to my knowledge, known to regulate degradation. And my searches for information relating to ATF3 and protein degradation only find papers related to degradation of ATF3 as a regulatory mechanism. If this is the case, then the studies presented here, in which the proteasome is "deleted" conditionally in motor

neurons, would be affected by altered regulation of ATF3 itself, which would impact the findings.

Response: We apologize that our ambiguous and insufficient description was confusing to the reviewer and thank them for pointing out the grammatical error. It is out intent to introduce ATF3 as a highly stress-responsive transcription factor capable of initiating transcriptional reprograming as a hub transcription factor. We do not intend to prove an association between ATF3 and the proteasome. The explanation about ATF3 was not appropriately arranged in the introduction of the original manuscript. We rearranged the text and clarified the role of ATF3 (page 4, line 7) in the revised manuscript.

Regarding the proteasome: "Impaired proteostasis, in particular proteasomal dysfunction, has been implicated in ALS pathology (Picher-Martel et al, 2019; Tashiro et al, 2012)." These references do not support that statement.

Response: We thank the reviewer for pointing this out. We appropriately changed this reference in the revised manuscript.

Regarding mitochondria "Mitochondrial motility in axons is progressively reduced during axonal maturation". This is correct, but is written to imply a reduction in axonal mitochondria in mature axons, which is not the case. It is only their motility that is reduced. Also: "The AIS may also contribute to maintaining an appropriate numbers of axonal mitochondria under normal neuronal activity." There is no evidence in the literature to support the idea that the axon initial segment has any control over axonal mitochondrial transport. Many studies have addressed how the AIS might control axon transport, but these have not focused on mitochondria. (see The Axon Initial Segment: An Updated Viewpoint Leterrier 2018." Also, many studies have addressed mechanisms regulating mitochondrial axon transport (focusing on Trak, Milton, Miro, Syntaphilin, Armcx1 etc), and none of these indicate regulation at or by the axon initial segment.

Response: The reviewer’s comments are correct. The link between mitochondrial transport in the axon and AIS are not supported by the literature. To avoid misleading the readers, we removed this description in the revised manuscript. However, we maintained the reference in the Discussion section to explain about mitochondrial motility and axonal maturation (page 15, line 13) in the revised manuscript.

Regarding injury induced degradation of the AIS: "The cysteine protease calpain is considered to be responsible for the degradation of the AIS, including AnkG and voltage-gated sodium channels (Schafer et al., 2009; Zhao et al, 2020). However, the proteasome is an additional candidate, given its preferential localization at the AIS along with developmental maturation (Hsu et al, 2015; Lee et al, 2020)." These papers do not support this statement. Firstly, all the studies that have examined AIS disassembly have identified calpain as the central regulator. Secondly, the proteasome is not known to localise preferentially to the AIS. In fact, the paper by Hsu et al supports the idea that axon growth relies on a reduction of axonal proteasomes, and that growth promoting signals trigger retrograde removal of proteasomes from axons. The paper by Lee et al reports on

proteasomes in the early part of the axon during early development. It does not demonstrate an enrichment of proteasomes in the AIS in adult animals. On the contrary, a recent study published an AIS proteome, with an extensive list of enriched (or not) molecules at the AIS. In the supplementary datasheet for this paper, a number of proteasome related molecules are identified, however none of them are specifically enriched in the AIS (https://www.nature.com/articles/s41467-019-13658-5 Mapping axon initial segment structure and function by multiplexed proximity biotinylation, Hamdan et al, Nature Communications, 2020)

Response: We do not exclude the possibility that calpain is involved in AIS disassembly in damaged motor neurons, as discussed on page 16 (last line) of the revised manuscript. We would like to present the possibility that the proteasome can function in AIS in addition to calpain. As the reviewer pointed out, the original description was not accurate. According to the reviewer's instructions, we changed the statement to not mislead readers and cited the study by Dr. Rasband's group (page 4, line 9 from the bottom) in the revised manuscript.

There are also some significant mechanistic issues regarding the study itself.
A key issue is the design of the study, using complicated transgenic mice which express GFP-tagged mitochondria under the control of the ATF3 promoter. The rational is that these mitochondria are only present after an injury, which leads to ATF3 activation. In the same genetic cassette, there is also Cre recombinase downstream of an IRES site. The authors cross these with mice floxed for Rpt3. This means that in these mice, after an injury, GFP-mitochondria are present whilst the proteasome is depleted.
The experimental conditions are therefore:
1. Uninjured mice.
2. Injured mice with GFP-mitochondria in the injured axons.
3. Injured mice with GFP-mitochondria in the injured axons, but no Rpt3 (no proteasome).
The authors find that after an injury, axons attempt to regenerate, except for condition 3, where the axons degenerate, and do not regenerate.
A considerable issue is that the authors have previously published a paper which demonstrates that conditional depletion of RPT3 in motor neurons leads to axon degeneration (Tashiro et al 2012). This means that the degeneration in condition 3 occurs even in the absence of the injury.
The experiment essentially already has a condition 4: Uninjured mice with no RPT3. This is an important control, and essentially changes the question being asked, which is actually "can an injury signal downstream of ATF3 (or even other transcription factors) overcome axonal degeneration caused by depleted proteasomes? And the answer is no. Even after upregulation of ATF3, axons still degenerate without proteasomes.

Response: We apologize that our description misled the reviewer. To explain this point more clearly, we summarize MNs as follows;

MN	Uninjured MN		Injured MN (5d)		ALS MN (pre-symp)	
	ATF3(-)		ATF3(+)		ATF3(+)	ATF3(-)
RPT3	+	-	**+**	-	↓	↓
AIS	+	+ (*)	**-**	+	+	+
axonal mitochondria	+	± (*)	**++**	+	±	±
axon	normal	degeneration(*)	regeneration	degeneration	degeneration	degeneration
mouse	*Atf3*:BAC2 *Thy1*-Mito	*Rpt3* CKO (x*ChAT*-cre)	*Atf3*:BAC2	*Rpt3* CKO (x*Atf3*:BAC2)	*Atf3*:SOD1	SOD1

(*) expected

As the reviewer suggested, previous study showed that *Rpt3*-deficient uninjured (ATF3-) motor neurons died more rapidly than autophagy-deficient motor neurons (Tashiro et al. JBC, 2012). To our knowledge, they did not show any data about axon degeneration, mitochondrial dynamics, or AIS. It is expected that *Rpt3* deletion of uninjured motor neurons causes axon degeneration and alteration of mitochondrial dynamics. However, it is not due to the failure of AIS disassembly presented in our manuscript. Because uninjured (ATF3-) motor neurons keep the AIS in the presence of *Rpt3*, proteasome-sensitive AIS disassembly is not necessary in uninjured motor neurons. Only injured motor neurons (ATF3+) require transient disassembly of the AIS in the presence of *Rpt3* to increase axonal mitochondria. There are expected to be numerous proteasomal targets of in motor neurons apart from AnkG. Degeneration of uninjured motor neurons in the context of *Rpt3* deletion would depend on the lack of other proteasome-mediated mechanisms. It is indeed interesting to examine such mechanisms, but beyond the scope of our current work. To make this concept more easily understood by readers, we changed the illustration in Figure 8 and accompanying discussion (page 16, line 2 from the bottom) in the revised manuscript.

As the reviewer suggested, the effect of proteasome deletion is potent. However, we show data that AnkG knockdown increased axonal mitochondria and delayed degeneration of injured motor neurons in *Rpt3* CKO mice (Fig. 5I–M of revised manuscript). These results indicate that AIS disassembly in *Rpt3*-deficient injured motor neurons (ATF3+) partly contributes to their ability to overcome degeneration.

This axon degeneration in itself could impact on not only mitochondrial but any axonal transport mechanisms, especially of the degeneration is related to (for example) microtubule degeneration, where mitochondrial transport would not be possible.

Response: We cannot rule out this possibility. However, axon degeneration occurs at a later stage in our model. At the time point when injured motor neurons with *Rpt3* dismantle the AIS (5 days after injury), injured motor neurons without *Rpt3* have not yet degenerated and even show a regenerative response (Fig. 2A-D). As shown in Fig. EV2B-D of the

revised manuscript, the distribution of lysosomes was unaffected in *Rpt3*-deficient injured axons at 5 days after injury. In addition, mitochondrial density was increased in *Rpt3*-deficient injured axons after AnkG knockdown (Fig. 5I–K). These results suggest that axonal transport mechanisms are not severely affected at this time point after injury.

Another issue is the compensatory upregulation in autophagy that occurs in response to proteasome deletion, as was recently demonstrated (Inducible Rpt3, a Proteasome Component, Knockout in Adult Skeletal Muscle Results in Muscle Atrophy, Kitajima et al Front Cell Dev Biol. 2020;). This seems to be quite a comprehensive response, involving numerous autophagy-related molecules (eg LC3II, p62, Atg5, Atg7, and Beclin-1), which will undoubtedly complicate mechanistic characterisation.

Response: *Rpt3*-deficient injured motor neurons upregulate p62 as shown in Figure 2E, suggesting the presence of a compensatory autophagic mechanism. Regeneration-associated proteins, such as ChAT and NeuN, are successfully downregulated in the absence of a proteasome (Fig 2B). This is probably because of compensatory regulation of autophagy. Even though the autophagy system works in a compensatory way, AIS was not dismantled in proteasome-deficient injured motor neurons. This indicates that the proteasome is dominantly responsible for disassembly of the AIS in injured motor neurons.

Each of the figures therefore addresses potential axonal mechanisms that are perhaps alluded to in previous literature, but which in fact have not been well studied. I do not think there is sufficient data here to support the proposed novel mechanisms, but rather that each of the studied steps needs further investigation in order to convincingly demonstrate mechanism.

Response: We agree with the reviewer that further studies are necessary to elucidate the complete mechanism. But this study showed unexpected significance of proteasome-mediated AIS disassembly *in vivo*, which could not be achieved without our mouse system. We believe that our study opens a new direction for understanding stress-responsive mechanisms in traumatically and pathologically damaged motor neurons.

Dear Dr. Kiryu-Seo,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal. Thank you for your comprehensive response to the referees' concerns. You will notice that one referee has made two minor comments that should be addressed in a final version of the manuscript.

In addition, please update the following editorial issues in the final version:

- Please update the conflict of interest statement as "Disclosure Statement & Competing Interests" statement. Information about the updated competing interests statement is available in our author guidelines.
- Figure callouts should be in the order of the figures. Figure EV4A-E are called out in the text before Fig EV3E
- Appendix Figs S2 panel callouts are missing
- The movies should be ZIPed with their legends. The legends should be removed from the manuscript file.
- Please also check the .dox version of the "data edited ms file" and make any necessary changes described, for example there is a note on Figure 7.
- We encourage the publication of source data, particularly for electrophoretic gels and blots and graphs, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure or for graphs, an Excel spreadsheet with the original data used to generate the graphs. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight marker; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

Once you have submitted the updated manuscript, I will begin the final checks before submitting to the publisher. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire peer review process including referee concerns and your point-by-point response will be available to readers.

It has been a pleasure to work with you to get to the acceptance stage. I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

Link Not Available

Referee #1:

The revised manuscript by Kiryu-Seo et al. has, in my opinion, improved both in terms of the quality of the data as well as the clarity of writing. Most of my comments have been thoroughly addressed by the authors. I understand the difficulty of performing AnkG knockdown in ALS mice, and I appreciate the authors' effort to address this question by including new data on motor neuron survival in Rpt3 CKO mice upon AnkG knockdown (Fig. 5L-M). I also appreciate the characterization of Aft3:BAC x SOD1 mice added in Fig. EV5.
Taken together, the new data strengthen the paper, and I am supportive of publication, although I do have a couple of minor comments on the revised version of the manuscript:

1. Fig. EV2F: the labeling of the y-axis is not clear, I suppose it should be "GFP(+) MNs (% of ChAT(+) MNs)"
2. The new data on axonal lysosomes presented in Fig. EV2B, D reveal a striking increase in lysosomes in the Rpt3 CKO mice at 10 days after injury, which is not at all commented on in the text. As the effect is highly significant, the authors should include

some explanation about this new observation.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision **20th Jul 2022**

Dear Sumiko,

Thank you for addressing all of our editorial comments in your revised version. I will now begin the final checks on your manuscript before submitting to the publisher next week. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire review process including referee concerns and your point-by-point response will be available to readers. I notice you have figures in your response, please confirm that you are okay with these figures in the public version of your response to referee concerns.

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Yours sincerely,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

EMBO Press Author Checklist

Corresponding Author Name: Sumiko Kiryu-Seo	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: The EMBO Journal	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOJ-2021-110486	EMBO Reports - Author Guidelines
	Molecular Systems Biology - Author Guidelines
	EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Reagents and Tools Table and "Animals" in Materials and Methods section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools Table and "Immunohistochemistry" in Materials and Methods section

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S1 and Materials and Methods section

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Reagents and Tools Table
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Reagents and Tools Table and "Animals" in Materials and Methods section
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods: Housing and husbandary conditions included standard pellet food and water supply, 12-hour light-dark cycle at temperature

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Please see the statement in acknowledgement sction.

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Each figure legend
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods section
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Yes, all figures include a statemtn to the statistical tests. Statistical analysis were performed using Graphpad prism and each analysis provided us withan estimate of variation. Exact P-value and number of replication are provided. The 95% confidence intervals of all groups are shown in the graphs.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legend
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legend

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods: Animals
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	