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A CRMP4-Dependent Axon to Soma Retrograde Death Signal in ALS

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1st Editorial Decision 3rd Feb 2021

Dear Eran,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees appreciate the analysis. However, they also raise a number of important issues that need to be resolved. Should you be able to address the raised concerns then I would like to invite you to submit a suitably revised manuscript. I think it would be helpful to discuss the raised issues further and I am happy to do so via phone or video.

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

I thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 4th May 2021.

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Referee #1:

Impairments in axonal transport have been linked to many neurological conditions, including the severe and invariably fatal motor neuron disease, amyotrophic lateral sclerosis (ALS). Most reports suggest that reduced retrograde delivery of peripherally-sourced survival factors contribute to the demise of the ALS motor neurons (MNs). However, a few groups have reported that retrograde trafficking of negative signals may also be contributing to the disease. In the paper under review, Maimon et al. identify CRMP4 as a possible retrograde harbinger that contributes to motor neuron death in ALS. The authors highlight intriguing alterations in CRMP4 localisation in ALS model mice, where CRMP4 appears to go from being predominantly found at the NMJ in WT mice to being mostly in axons and at MN soma in mutant animals. This appears to be related to distal Sema3A signalling and require the interaction of CRMP4 with the motor protein cytoplasmic dynein. Reduced CRMP4 delivery to the soma looks like it preserves motor neuron health in vitro and possibly in vivo.

Overall, the manuscript provides a fairly convincing series of experiments to support a role for CRMP4 as a retrograde death signal that contributes to ALS motor neuron phenotypes. However, in several instances, I feel that provision of WT control data would strengthen the findings. Moreover, in its current state, the manuscript suffers considerably from pseudo-replication and the inclusion of several unsupported conclusions/assertions. I feel that several additional experiments are required for this manuscript to reach publication level. My specific comments are as follows:

Major
• I find the evident pseudo-replication present in several experiments to be a major weakness (e.g. Figures 1I, 2F-H, 4I, S4B), and worry that other experiments that don't show individual data points on graphs, may have been analysed in a similar manner. While the sample data are likely representative of the population, we should be building houses of brick rather than mansions of straw. I can understand why this is done with precious, limited samples from ALS patients, but

nearly all other experiments have sample sizes of three.
• The authors have reported the percentage of NMJs positive for CRMP4 (Fig. 1F-G) - did the CRMP4 negative synapses in ALS mice look like they were more denervated than CRMP4 positive NMJs? This analysis would lend weight to the theory that loss of CRMP4 at the NMJ is associated with subsequent denervation.
• Without the WT iPSC-MN control experiments to mirror those depicted in Figure 2, it is not currently certain (although probable) that transport of distal CRMP4 is the cause of the CRMP4 increases found in the soma and proximal axons (Fig. 2D-G) and the associated reduction in cell body area (Fig. 2H). The Ciliobrevin experiments indicate that the observations in ALS MNs are dynein-dependent, but alternative dynein-driven signals could be causing the phenotypes. Experiments in WT iPSC-MN, which showed no CRMP4 increase in distal axons, could alleviate these concerns. Furthermore, correlating somata areas with CRMP4 intensities in ALS somata would further clarify the relevance of CRMP4 levels to the area phenotype.
• For the sALS patient intra-muscular nerve analysis of CRMP4 levels (Figure 1H and I), the authors should provide confirmation that they were indeed looking at neuronal CRMP4, as opposed to Schwann cell expression. Alternatively, they should caveat that this expression may not be restricted to neurons. I mention this because of the very high CRMP4 expression in the myelin of mouse sciatic nerves (Fig. 1D) and the fact that the CRMP4 appears to localise outside of the NFH staining in Figure 1H.
• Assessment of CRMP4 levels/localisation in the AAV-treated mice (Fig. 6) is required, and should really be correlated with the Caspase 3 levels (per neuron) to confirm the importance of the CRMP4-dynein complex to MN health.

Minor
• There are non-motor neurons that are NeuN+ in the spinal cord ventral horn. The authors should be clearer on this point, because they refer to "control MNs" in relation to Figures 1B and C, which is inaccurate.
• The data in Supplementary Figure 4 are not comparable, i.e. WT iPSC-MNs without Sema3A are compared to C9 iPSC-MNs treated with Sema3A. The authors should perform the experiment correctly and provide appropriate comparisons.
• The left panels in Figure 4F are WT Sema3A-negative, whereas the right panels are SOD1-G93A Sema3A-positive: please compare like with like, as these figures do not provide a viable comparison.
• Please present the WT primary MN data to parallel the SOD1G93A data shown in Figure 5E. It is mentioned in the text, but not shown.
• The authors do a nice job to show that dynasore impairs internalisation of BDNF in Supplementary Figure 6; however, it would be more appropriate to confirm that dynasore restricts Sema3A internalisation in their model system. Alternatively, providing citations that support this idea will suffice.
• The I141V mutation in CRMP4 is stated to "enhance formation of the CRMP4-dynein complex" (lines 236-237), but upon inspection of the graph, the result is non-significant. Repetition of the experiment should be performed to confirm its relevance or the sentence should be caveated with this important detail. Moreover, dedicating an entire paragraph of the discussion to this non-significant finding is perhaps somewhat premature.
• Motor neuron counts post-treatment would provide the most convincing evidence for the importance of CRMP4-dynein interactions mediating motor neuron death.

Non-essential suggestions
• Details of the spinal cord level analysed for Figure 1, would be helpful in the main text.
• The authors should provide details of how many motor neurons, sciatic nerve axon fields, and NMJs were assessed per biological replicate in the CRMP4 expression analyses. Similarly, were

approximately equal numbers of intra-muscular MNs analysed in each of the n = 3 human samples? I ask the latter because the nerves (understandably) are used as the replicate rather than the human, and therefore oversampling from a single ALS patient could skew the data.
• Can the authors please speculate as to why the WT iPSC-MNs did not respond to Sema3A treatment in Figure 2B? Did the authors assess CRMP4 levels at the iPSC-MN growth cones (as a possible explanation)?
• The staining of CRMP4 in somas of C9 iPSC-MNs (Fig. 2D) appears nuclear, unlike the cytoplasmic staining observed in SC MNs from the mouse (Fig. 1B) - please can the authors confirm/discuss this finding?
• Given that WT iPSC-MNs were analysed, the statement that, "exposure to Sema3A led to CRMP4 elevation specifically in the ALS diseased MN soma..." (lines 161-162) is not true/assessable.
• Which marker was used to measure somata areas in Figure 2?
• The two purple colours of the CRMP4 peptides are hard to differentiate (Fig. 3A) - changing one to a different colour would help.
• Figure 4E-I: are these primary motor neurons? Please clarify in the text and figure legend.
• The legend of Figure 4, panel E requires a better explanation.
• The supplementary movies are not needed.
• The authors allude on several occasions to their data showing a role for CRMP4 in MN death/loss in vivo (Lines 41-42, 97-98, 307-308, 339-340); however, no in vivo MN counts are presented. Caspase 3 is used as a proxy in Figure 6; however, almost 100% of MNs are reported to be positive in SOD1G93A mice, and not all of these neurons will degenerate. Rewording is required.
• The title could be improved by changing the focus of the sentence.

Referee #2:

In this study, Maimon et al report on the role of CRMP4 as a degenerative retrograde signal in models of ALS. Their results indicate that CRMP4 expression is up regulated in motor neuron cell bodies and axons in SOD1 mutant mice and that there is a decrease in CRMP4 expression in the NMJ of these mice as well as in human ALS patient samples. Using both the SOD1 mutant mouse model and human iPSC-derived motor neurons from C9orf72 patients, they demonstrate that CRMP4 associates with the retrograde motor dynein and that this interaction is necessary for motor neuron degeneration. In addition, they find that Sema3a, which they previously reported is secreted from muscle of ALS models, increases the CRMP4-dynein interaction. Importantly, blocking the interaction with peptides that disrupt the binding of CRMP4 to dynein rescued motor neuron death induced by Sema3a in cultures from SOD1 mutant mice and iPSC-derived motor neurons from C9orf72 patients. In addition, expression of the disrupting peptide in motor neurons of SOD1 mutant mice in vivo provided significant protection from the loss of motor neurons. These findings are very exciting and provide novel insights into the pathology of ALS by identifying CRMP4 as a degenerative retrograde signal. Overall, the study is thorough and well controlled and there are just a few concerns that the authors should address.

In Fig. 1 H&I is there less CRMP4 staining because there are just fewer NMJs in these patients? A ChAT or BTX co-staining should be included.

Does CRMP4 mRNA increase in the motor neurons? The high expression in Schwann cells shown in Fig. 1D is striking. Given that Schwann cells communicate many factors to axons, including RNA (e.g. Lopez-Verilli and Court, 2012; Wei et al., 2019), is it possible that some or even most of the CRMP4 is coming from these glia? No experiments are needed here, but just something the

authors might consider.

In the results, lines 236-240, referring to Fig. 4D, and again in the Discussion, the authors state that the I141V mutation in CRMP4 increases interaction with dynein. However, based on the standard of $p<0.05$, there is no statistical difference between the complex formed by WT CRMP4 and the mutant. Therefore, these statements need to be changed.

In lines 169-171, the quantification of cell body data needs better description in the methods and results to help the reader with interpretation and implication of this data.

What do the white and red arrows indicate in Fig 5B?

The graph in Fig. 5G should be revised to match 5C-E. It is very confusing in its present form.

The orientation of the spinal cords in Fig. 6F should be indicated. Presumably, ventral is at the top, which is flipped from the usual convention of ventral down.

In supplementary Fig 2, the scalebar is missing.

In Fig. 6J, instead of an exclamation mark, a question mark seems more appropriate as the retrograde degenerative signal in motor neurons is yet to be identified.

The authors might want to include mention of DLK as possibly involved in ALS retrograde signaling in their discussion, given the extensive work of Lewcock and others in this area.

In the Discussion, lines 378-380, "p75NTR is retrogradely transported … a retrograde apoptotic signal that activates JNK (Kenchappa et al., 2010)". The Pathak et al reference should be moved to replace the final reference to Kenchappa et al.

Referee #3:

In this manuscript Maimon and collaborators build up on previous studies by their group and others on the possible involvement of CRMP4 in ALS related motor neuron death.

They first show that CRMP4 levels are altered in different ALS models. In G93A mice, they show an increase in MN cell bodies positive for CRMP4, associated with increased axonal content and decreased content at presynaptic sites. In ALS nerve biopsies, they observed decreased intraaxonal levels, and in C9ORF72 iPS derived motor neurons, they show increased axonal content upon Sema3A administration.

They further used cell biology techniques to demonstrate a direct interaction between CRMP4 and dynein, and identify interaction domains. They use this knowledge to set up two methods to interfere with CRMP4/Dynein interaction (peptides and GFP-50 aa, that encodes the dynein interaction domain). In cellular models, they show that ALS mutations increase CRMP4/dynein interactions, and provide evidence that disrupting this interaction might provide neuroprotection in ALS mice.

The study is generally interesting, and would deserve publication upon appropriate revision. In its current state, there are a number of controls that are crucially lacking, as well as discussion of other

relevant literature.

Major points:
1) Critical controls lacking. A number of experimental controls are lacking in this study which renders some of the key results not entirely interpretable.

a. In figures 2c-f and 4h-I, the experiments only include C9ORF72 patients cells, and not control patients. In figure 2c-h, the authors show that dynein inhibition decreases CRMP4 axonal levels, suggesting that the observed increased levels in these cells are due to dynein activity. However, it cannot be excluded that dynein inhibition generally decreases CRMP4 levels, independently of disease status. Similarly, in Figure 4, the authors show that administration of CBP peptides decrease co-localization of CRMP4 and dynein in C9ORF72 patients, but do not provide control experiments with healthy controls. Whether this is disease specific or not is important also in this case. Last, in Figure S4, they compare healthy controls with C9ORF72 cells treated with Sema 3. There lacks two control groups in this Figure S4 (controls treated and C9ORF72 untreated...). While it is possible that those controls were properly done, the presentation is currently confusing.

b. In Figure 4A, the authors show increased interaction between CRMP4 and DIC. However, they also previously show that total CRMP4 levels are increased in G93A nerves. How do they control for the increased CRMP4 amount? Is it the interaction that is increased or simply the total CRMP4 levels?

c. In figure 6, the authors seemingly performed their overexpression experiment of GFP and GFP50aa only in diseased mice, and not in wild type mice. Seemingly, Figure 6F shows results of uninjected mice. Their only control in Figure 6G appears thus G93A transduced with GFP, and they observe strong levels of caspase 3 positive motor neurons in this condition. It is possible that overexpression of GFP itself triggers toxicity to motor neurons, leading them to execute apoptosis. These experiments should be done in wild type mice in parallel to demonstrate that their expression levels of GFP is not toxic per se in motor neurons. Furthermore, it is not shown that GFP and GFP50aa overexpression levels are similar (especially because the two images provided show completely different saturation levels), nor do they provide results on CRMP4 accumulation in G93A motor neurons (which is expected to decrease upon intervention).

2) Specificity of the antibodies used: the authors observe a strong expression of CRMP4 in Schwann cells (Figure 1), yet this is at odds with publications of other groups ((eNeuro. 2020 Mar-Apr; 7(2): ENEURO.0479-19.2020.). Could the authors provide evidence for the specificity of their immunostaining for CRMP4?

3) Overinterpretation of conclusions in Figure 4D: the authors claim that the interaction between CRMP4 and DIC is increased by the mutant I141V, yet, the p-value obtained is of 0.0532. While this is a clear trend, the authors should repeat this experiment to demonstrate a truly significant effect.

4) Discussion of results:

a. Selectivity of the mechanism regarding ALS: it has been documented in a number of experimental models that CRMP4 is increased by neuronal injury (see eg Injury-induced CRMP4 expression in adult sensory neurons; a possible target gene for ciliary neurotrophic factor, Jang SY, Shin YK, Jung J, Lee SH, Seo SY, Suh DJ, Park HT; Neurosci Lett. 2010 Nov 12; 485(1):37-42.). It is thus entirely possible that the results of the authors are reflecting a response of moitor neurons to stress rather than an ALS-specific mechanism. While this does not necessarily decrease interest, this should be

clearly stated and discussed.

b. The authors also do not discuss the possibility that part of their results could be explained by proteolytic degradation of CRMP4 at the injured site of the neuron, as has been shown by others (eNeuro. 2020 Mar-Apr; 7(2): ENEURO.0479-19.2020.)

c. The authors should appropriately discuss the novelty of their findings as compared to the work of Duplan and collaborators, which previously showed the toxicity of CRMP4 to motor neurons. As such, the current discussion does not provide such critical discussion of novelty.

5) The western blot of Figure 4H is of poor quality, and the GFP-IgG well seems to show unspecific signal. Could another experiment be shown here? Also, all uncropped western blots should be provided in supplementary figures with molecular weight markers.

Response to Reviewers of Maimon et al; (EMBOJ-2020-107586)

General responses

We were pleased to see that each of the three reviewers found the work of interest and potentially appropriate for publication in *EMBO Journal.* We have now made major changes/additions which we believe successfully answer the concerns raised by the editor and each of the three reviewers (our detailed responses are appended below).

In the first submission:

- First, we identified spatial subcellular alterations of CRMP4 along the motor unit in MNs from sporadic ALS patients, familial C9orf72 human iPSC-derived MNs, and in the SOD1^{G93A} ALS mouse model.

- Second, we reveal that an increased interaction of CRMP4 with the retrograde motor protein dynein in ALS models triggers its transport from distal axons to the soma, and prompts MN loss.

- Third, we blocked the CRMP4-dynein interaction and thereby profoundly reduced MN loss both *in vitro* in C9orf72 mutant human iPSC-derived MNs, and *in vivo* in the SOD1^{G93A} ALS mouse model.

In the revised manuscript we have substantially expanded our evidence and:

- Extended the sporadic ALS human patient data, demonstrating consistent upregulation of CRMP4 in human spinal cord neurons, similar to what we observed in SOD1^{G93A} transgenic mice.

- Added additional controls for many experiments.

- Provided sample sizes and the number of biological replications for each experiment.

- Added new images and blots to improve clarity.

- Extended the discussion where requested by reviewers.

In so doing, we believe that we addressed each concern raised by the reviewers, and we are hoping that the reviewers and editor will find our rebuttal responsive. Our responses to the specific concerns raised by each reviewer are detailed below in a point-by-point manner (reviewers' comments are in blue, our responses in black).

Specific responses to Reviewer #1

Impairments in axonal transport have been linked to many neurological conditions, including the severe and invariably fatal motor neuron disease, amyotrophic lateral sclerosis (ALS). Most reports suggest that reduced retrograde delivery of peripherally-sourced survival factors contribute to the demise of the ALS motor neurons (MNs). However, a few groups have reported that retrograde trafficking of negative signals may also be contributing to the disease. In the paper under review, Maimon et al. identify CRMP4 as a possible retrograde harbinger that contributes to motor neuron death in ALS. The authors highlight intriguing alterations in CRMP4 localisation in ALS model mice, where CRMP4 appears to go from being predominantly found at the NMJ in WT mice to being mostly in axons and at MN soma in mutant animals. This appears to be related to distal Sema3A signalling and require the interaction of CRMP4 with the motor protein cytoplasmic dynein. Reduced CRMP4 delivery to the soma looks like it preserves motor neuron health in vitro and possibly in vivo. Overall, the manuscript provides a fairly convincing series of experiments to support a role for CRMP4 as a retrograde death signal that contributes to ALS motor neuron phenotypes.

We acknowledge reviewer #1 for her/his comprehensive response. We have now addressed all of the directions the reviewer has proposed. we sincerely thank the reviewer for her/his efforts in directing us to ways in which our effort could be significantly improved.

However, in several instances, I feel that provision of WT control data would strengthen the findings. Moreover, in its current state, the manuscript suffers considerably from pseudo-replication and the inclusion of several unsupported conclusions/assertions. I feel that several additional experiments are required for this manuscript to reach publication level. My specific comments are as follows:

We agree with reviewer #1 that provision of WT control data would strength the findings in this manuscript. In the revised manuscript we used healthy controls wherever possible (please refer to the point by point rebuttal below). We further agree that in the initial manuscript we were missing several key experiments. We now addressed all of reviewer #1's concerns and we hope that the reviewer will find our manuscript ready for publication in the EMBO Journal.

1. I find the evident pseudo-replication present in several experiments to be a major weakness (e.g. Figures 1I, 2F-H, 4I, S4B), and worry that other experiments that don't show individual data points on graphs, may have been analysed in a similar manner. While the sample data are likely representative of the population, we should be building houses of brick rather than mansions of straw. I can understand why this is done with precious, limited samples from ALS patients, but nearly all other experiments have sample sizes of three.

We agree with the reviewer that a more extensive and detailed report of the results were needed in the initial manuscript. We are sorry that we missed this important issue in the first submission and believe that in the current form of the manuscript we improved our consistency in reporting for each experiment. **We now added to the revised manuscript,**

for each experiment: 1) The number of biological repeats; 2) The number of the fields/cells that were analyzed in every condition of the experiment; and 3) The statistical analyses that were performed. This information can be found in every figure legend in the updated manuscript. Furthermore, we gathered this information into one document and attached it to the supplementary information. Lastly, we provided all the

raw data as supplementary material, including blots, images and quantifications. Here is an example from our supplementary file for the information in Figure 1 and Figure 2:

"Figure1-
A-B:

- *2 human controls and 3 ALS patients were used for the IHC assay. We analyzed 7 spinal cord sections of controls and 14 spinal cord sections of ALS patients.*
- *Scale bar: left images 20 µm, right insets 10 µm.*
- *P value- ***0.0003 ; Mann-Whitney test.*

C-D:

- *Spinal cords from 3 different mice in each condition were analyzed. We monitored CRMP4 expression in total of 108 cells in WT spinal cords and 123 cells in $SOD1^{G93A}$.*
- *Scale bar: 10µm.*
- *P value- * 0.0161 ; Student t.test*

Figure2-

A-B:

- *5 non-ALS controls and 4 sALS patients were used for monitoring CRMP4 in intramuscular nerves samples. We analyzed 40 terminal axons from the healthy samples (~8 axons per sample) and 36 terminal axons from ALS patients samples (~8 axons per sample).*
- *Scale bar: 20 µm.*
- *P value: * 0.0475 ; Student t.test*

C-D:

- *Gastrocnemius muscles tissues from 3 different mice in each condition (WT and $SOD1^{G93A}$) were analyzed. A number of 44 NMJ's in WT condition and 60 NMJ's in $SOD1^{G93A}$ condition monitored per each animal.*
- *Scale bar: 10µm.*
- *P value- * 0.0157 Student t.test*

E:

- *Gastrocnemius muscles tissues from 3 different mice ($SOD1^{G93A}$) were analyzed. We counted 24 NMJ's in $SOD1^{G93A}$ CRMP4 negatives and 67 NMJ's in $SOD1^{G93A}$ CRMP4 positives.*
- *P value- * 0.0352 Student t.test*

F-G:

- *Sciatic nerves from 3 different mice in each condition were analyzed. Total of 14 WT sections and 11 $SOD1^{G93A}$ sections were monitored.*
- *P value- **** p<0.0001 ; Student t.test*
- *Scale bar: 5µm."*

Similar summaries were compiled for all other figures (please refer to the supplementary material).

2. The authors have reported the percentage of NMJs positive for CRMP4 (Fig. 1F-G) - did the CRMP4 negative synapses in ALS mice look like they were more denervated than CRMP4 positive NMJs? This analysis would lend weight to the theory that loss of CRMP4 at the NMJ is associated with subsequent denervation.

We agree with reviewer #1 that understanding the relation between CRMP4 expression and the percent of NMJ denervation is highly relevant. Following this idea, we extended our initial experiment and counted the percentage of partially innervated NMJs in CRMP4 positive or negative NMJs. As one can observe in **Figure 1, Reviewer #1** in the WT condition almost all NMJs showed strong CRMP4 expression. However, in the P90 $SOD1^{G93A}$ mice we observed a ~25% decrease in the number of NMJs expressing

CRMP4. Strikingly, almost all of the CRMP4-negative/ChAT-positive NMJs were partially denervated. Thus, it seems that CRMP4 loss in the distal axons indicates early denervation in the SOD1^{G93A} mice. These data were added to the main manuscript and figure, and also discussed in the main text.

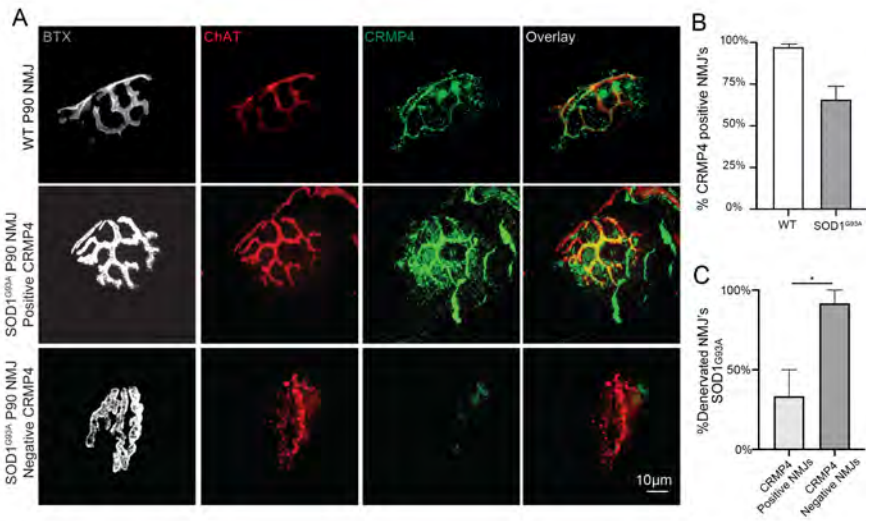

Figure 1, Reviewer #1 (Figure 2 C,D,E in the revised manuscript): (A) Representative images of SOD1$^{G93A/ChAT::tdTomato}$ or WT$^{ChAT::tdTomato}$ neuromuscular junctions at P90. White: denotes BTX, red denotes Chat, green denotes CRMP4, yellow denotes the co-localization area of CRMP4 and NFH. Scale bar: 10 μm. (B) Quantification of CRMP4 positive NMJ in WT or SOD1^{G93A} at P90. Student's t-test, n=3, *p = 0.016. (C) Quantification of the percent of partially denervated NMJ's in the presence or absence of immunostaining Student's t-test, n=3, *p = 0.016

3. Without the WT iPSC-MN control experiments to mirror those depicted in Figure 2, it is not currently certain (although probable) that transport of distal CRMP4 is the cause of the CRMP4 increases found in the soma and proximal axons (Fig. 2D-G) and the associated reduction in cell body area (Fig. 2H). The Ciliobrevin experiments indicate that the observations in ALS MNs are dynein-dependent, but alternative dynein-driven signals could be causing the phenotypes. Experiments in WT iPSC-MN, which showed no CRMP4 increase in distal axons, could alleviate these concerns.

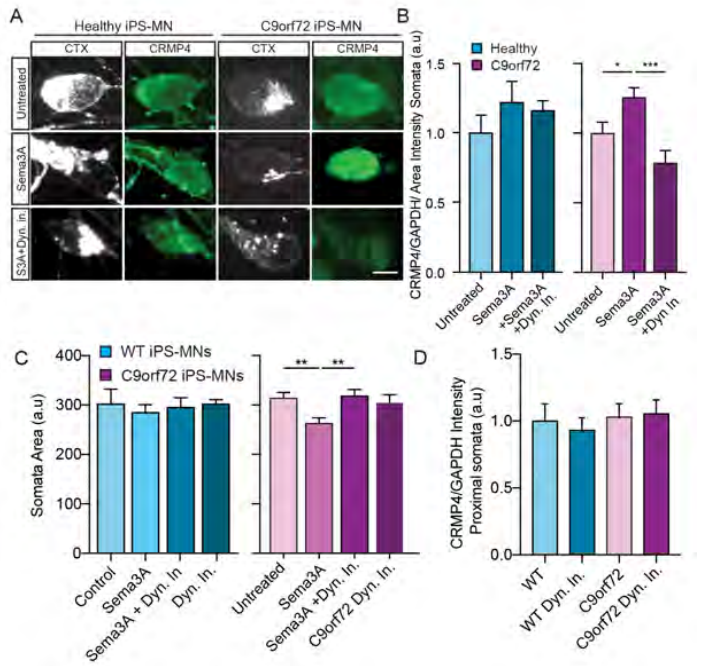

Figure 2, Reviewer #1 (Figure 3 D,E and Supplementary Figure 3 in the revised manuscript) (A) Representative images of healthy and C9orf72 human-derived MN cell somata after Sema3A, Sema3A + dynein inhibitor, or control treatment. Gray denotes CTX, green denotes CRMP4. Scale bar: 5um. (B-D) Quantification of CRMP4 intensity (B,D) levels and somata size (C) at the somata of healthy and C9orf72 human-derived MN after Sema3A treatment compared with untreated control or with dynein inhibitor applied prior to Sema3A treatment (n=3, One-way ANOVA, Tukey's multiple comparisons test; *p =0.013, *p=0.01).

Again, we highly agree with reviewer #1 and are thankful for the opportunity to address this concern. The revised manuscript includes new controls for many experiments. In order to verify that transport of distal CRMP4 is the cause of CRMP4 increase in the

cell body and proximal axons specifically in ALS, we used WT iPSC-derived MNs as a control as suggested by reviewer #1. We demonstrate in the revised manuscript that Dynein activity mediates CRMP4 upregulation in the cell somata and proximal axons of

C9orf72 iPSC-derived MNs, but not in WT iPSC-derived MNs (**Figure 2, Reviewer #1).** Moreover, we added another control and measured CRMP4 intensity in the somata and proximal axons of both WT and C9orf72 mutant iPSC-derived MNs in the presence of Dynein inhibitor alone. Our results (**Supplementary Figure 3 in the revised manuscript)** indicate that application of Dynein inhibitor by itself has no effect on CRMP4 without activation by Sema3A.

Furthermore, correlating somata areas with CRMP4 intensities in ALS somata would further clarify the relevance of CRMP4 levels to the area phenotype.

Indeed, we observed that elevated CRMP4 expression in cell somata corelated with smaller cell somata size. Here, too, the effect was specific to C9orf72 iPSC-derived MNs as we did not observe cell somata shrinkage in healthy iPSC-derived MNs post Sema3A treatment (**Supplementary Figure 3 in the revised manuscript)**.

4. For the sALS patient intra-muscular nerve analysis of CRMP4 levels (Figure 1H and I), the authors should provide confirmation that they were indeed looking at neuronal CRMP4, as opposed to Schwann cell expression. Alternatively, they should caveat that this expression may not be restricted to neurons. I mention this because of the very high CRMP4 expression in the myelin of mouse sciatic nerves (Fig. 1D) and the fact that the CRMP4 appears to localise outside of the NFH staining in Figure 1H.

Very interesting point. Following this concern, we first repeated our immunostaining of WT and SOD1^{G93A} sciatic nerves for CRMP4 with NFH (marking CRMP4 in neurons) but this time also added GFAP staining along with CRMP4 (for marking CRMP4 in spinal cord glial cells) and measured the colocalization of CRMP4 with both of these markers. Our results indicate that the elevations we observed in CRMP4 in SOD1^{G93A} sciatic nerves overlapped with both GFAP and NFH **(Figure 3, Reviewer #1).** Importantly, the colocalization of CRMP4 with NFH was 3 times higher in SOD1^{G93A} compared to WT nerves. In contrast, we found a modest ~70% increase in the colocalization of CRMP4 with GFAP **(Figure 3, Reviewer #1)**. Thus, it seems that the increase in CRMP4 in ALS is more pronounced in diseased neurons than glial cells, but

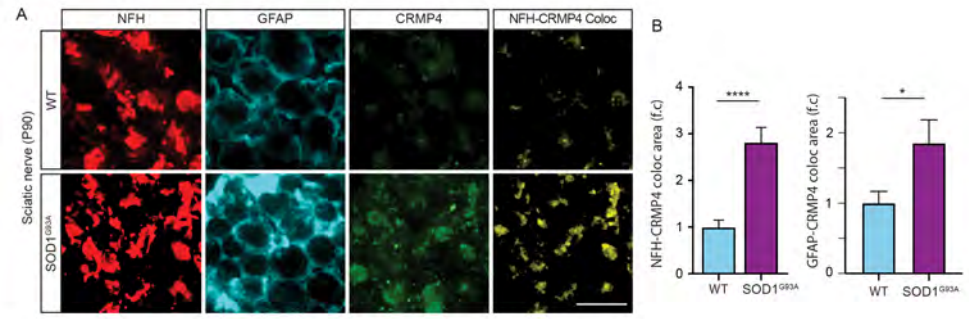

Figure 3, Reviewer #1 (Figure 2F,G and Supplementary figure 1 in the revised manuscript): (A) Representative images of P90 SOD1^{G93A} and WT sciatic nerves. Red: denotes NFH, Blue: denotates GFAP, Green: denotes CRMP4, Yellow is the co-localization channel of CRMP4 with NFH. scale bar: 10 µm. (B) Quantification of the co-localization area of CRMP4 with NFH (left) or GFAP (right) in the SOD1^{G93A} compared with the control. Student's t-test, n=3, *****p<0.000, *p<0.05

indeed as the reviewer noticed CRMP4 is upregulated both in neurons and glia in ALS spinal cords. Next, we further repeated our initial immunostaining of CRMP4 and NFH in human intramuscular nerves and this time also performed immunostaining of CRMP4 with MPB (marker for Schwann cells). In contrast to the significant decrease in CRMP4 in NFH positive terminal axons that we observed, our analysis revealed a non-significant trend

towards a reduction in colocalization of CRMP4 and MBP in intramuscular Schwann cells in ALS, compared to healthy controls **(Figure 4, Reviewer #1).**

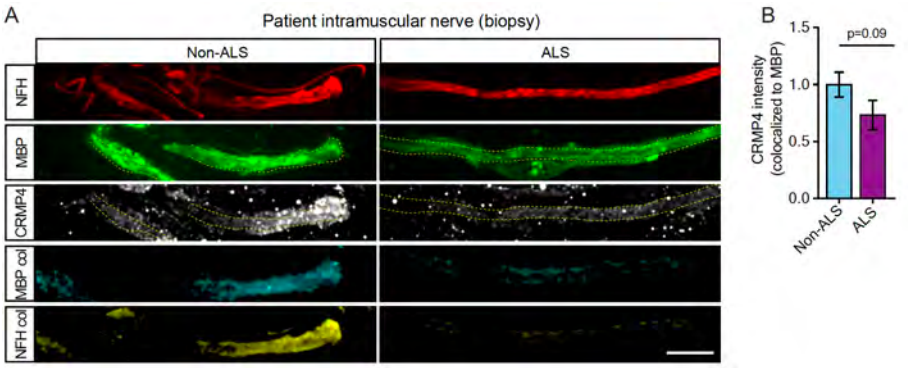

Figure 4, Reviewer #1 (Supplementary Figure 1 in the revised manuscript): (A) Representative images of ALS patient or non-ALS human control intra-muscular nerves. Red: denotes NFH, Green: denotes MBP, White: denotes CRMP4. Scale bar: 20 μm. (B) Quantification of CRMP4 intensity levels in 5 non-ALS controls and 4 sALS patients intra-muscular Schwann cells.

Lastly, in this version of the manuscript we extended our observations in the SOD1^{G93A} mouse model and now demonstrate that CRMP4 is upregulated specifically in the spinal cord of human sALS patients compared to human controls. Our immunohistochemistry for CRMP4 in sALS diseased and healthy controls revealed a significant 2.5 fold increase in CRMP4 protein levels specifically in spinal cord cell bodies **(Figure 5, Reviewer #1).**

Altogether, in the revised manuscript we 1) confirmed that the increase in CRMP4 we observed occurs in ALS diseased neurons; 2) show that CRMP4 upregulation also occurs in glia; 3) validated the decrease in CRMP4 we observed in sALS is indeed within terminal axons; and 4) extended our initial observation to demonstrate that CRMP4 is increased in sALS spinal cord cells. These data are now in the main manuscript and figures of the revised manuscript.

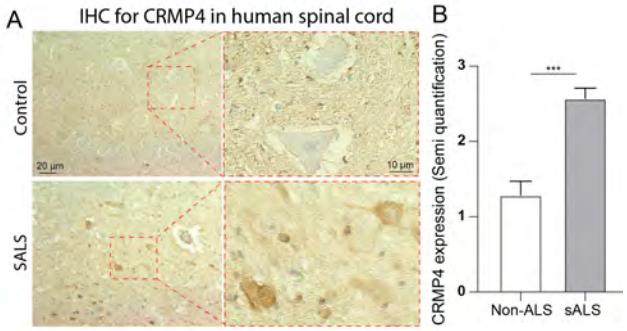

Figure 5, Reviewer #1 (Figure 1 in the revised manuscript): (A,B) Representative IHC images (A) and semi quantification (B) of CRMP4 protein in sALS patient or human control spinal cord cells. DAB label CRMP4. Scale bar: 10 μm.

5. Assessment of CRMP4 levels/localisation in the AAV-treated mice (Fig. 6) is required, and should really be correlated with the Caspase 3 levels (per neuron) to confirm the importance of the CRMP4-dynein complex to MN health.

Following reviewer #1's suggestion we assessed CRMP4 levels in the spinal cord of AAV-treated mice. We immunoassayed CRMP4 in GFP and GFP-50aa injected mice and measured CRMP4 intensity levels in spinal cord neurons one-month post injections. The result was complex **(Figure 6, Reviewer #1)**: While we observed that injection of GFP-50aa reduced CRMP4 levels in neurons expressing low amounts of CRMP4 (below 2000 a.u) we did not observe similar shift in neurons expressing high amounts of CRMP4 **(Figure 6, Reviewer #1)**. Surprisingly, we found an increase in the number of cells that express high levels of CRMP4 with GFP-50aa treatment, compared with GFP **(Figure 6, Reviewer #1)**. Thus, whereas the distribution of CRMP4 is normal in the GFP injected group, there are 2 different CRMP4 expressing cell populations in the GFP 50aa group. We assume that these unexpected results might be the result of: 1) Recognition of the 50aa by our antibody for CRMP4, even though the CRMP4 antibody was not raised against this epitope; 2) CRMP4 mislocalization is time-dependent. In this case, measuring CRMP4 expression at earlier time points post injection (i.e. 1 or 2 weeks) might result in higher shift; or 3) a CRMP4 homeostatic loop results in overproduction of CRMP4 in the cell body due to low levels of CRMP4 in this compartment. As discussed in the main text, since CRMP4 is not a transcription factor we assume that the CRMP4-Dynein complexes are enriched with other toxic transcription factors that induce cell death (DLK, c-Jun, etc.), thus CRMP4 elevation in the cell body will not result in cell death.

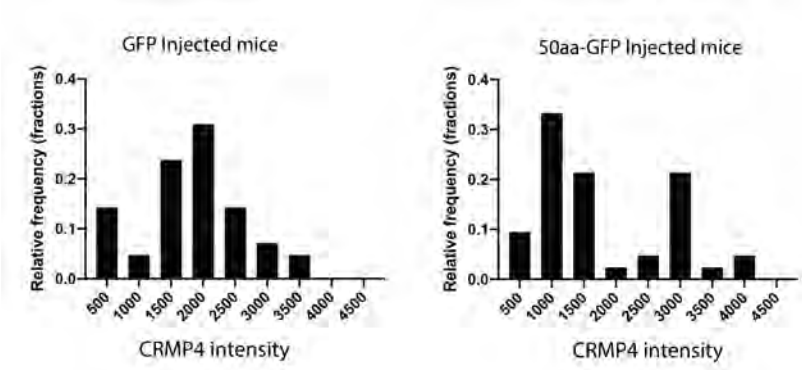

Figure 6, Reviewer #1: Distribution of CRMP4 intensity in spinal cord neurons of GFP injected mice (left) and 50aa-GFP injected mice (right)

We believe that the right measurement for the function of GFP-50aa is monitoring CRMP4-Dynein complexes post treatment rather than measurement of overall CRMP4 expression. Following this idea, we performed a proximity ligation assay for CRMP4 and Dynein *in vivo* (similar to what was done before *in vitro)* with the ultimate goal of measuring CRMP4-Dynein interaction post AAV injections. Unfortunately, the PLA experiments were technically not successful *in vivo.* However, our data demonstrated the ability of over expression GFP-50aa to reduce CRMP4 and Dynein complexes *in vitro*. Furthermore, we observed beneficial effect of overexpressing GFP-50aa on neuronal number and Caspase3 activation, compared with the GFP control, suggesting an active role for this construct.

6. There are non-motor neurons that are NeuN+ in the spinal cord ventral horn. The authors should be clearer on this point, because they refer to "control MNs" in relation to Figures 1B and C, which is inaccurate.

We agree with reviewer #1 and in the updated manuscript have considered this point. We narrowed our initial claim and changed the text both in the figure legends and main text to

"spinal cord neurons" rather than "motor neurons" when we used murine *in vivo* samples. Here is an example from the main text:

…"*The total number of neuronal cells expressing CRMP4 in the P90 SOD1^{G93A} spinal cord compared to their litter mate were also elevated (Figure 1C,D) (mean: WT 4.54% ± 4.54%; SOD1^{G93A} 26.43% ± 3.04%)".*

7. The data in Supplementary Figure 4 are not comparable, i.e. WT iPSC-MNs without Sema3A are compared to C9 iPSC-MNs treated with Sema3A. The authors should perform the experiment correctly and provide appropriate comparisons.

Again, we are very sorry for missing important controls in the first manuscript submission. The current version of the manuscript contains all necessary controls as reviewer #1 suggested. Please refer to **Figure 7, Reviewer #1** that specifically addresses this concern. Our new analysis of WT iPSC-derived MNs re-enforced our initial findings. We now

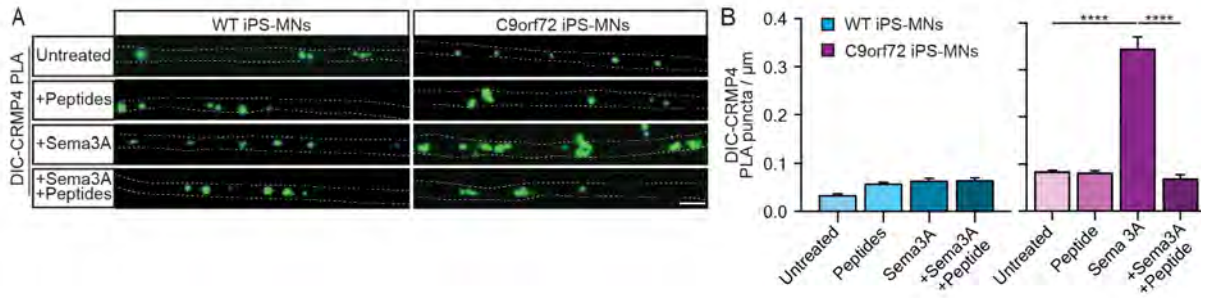

Figure 7, Reviewer #1 (Figure 5 H,I in the revised manuscript): (A) Representative images of the proximity ligation assay for CRMP4 and dynein in C9orf72 and healthy human-derived MN's in the presence or absence of Sema3A, and/or peptides application. (B) Quantification of the CRMP4-DIC puncta number per axon in each group (One way ANOVA, ****p=0.0001, n=3).

demonstrate that CRMP4-Dynein complexes are enhanced specifically in C9orf72 MNs but not in WT cells. The ability of the peptides to reduce this interaction is specific as well, since there was no change in WT MNs in which the CRMP4-Dynein interaction is minimal **(Figure 7, Reviewer #1)**. These data are now incorporated within main figure 5 of the revised manuscript.

8. The left panels in Figure 4F are WT Sema3A-negative, whereas the right panels are SOD1-G93A Sema3A-positive: please compare like with like, as these figures do not provide a viable comparison.

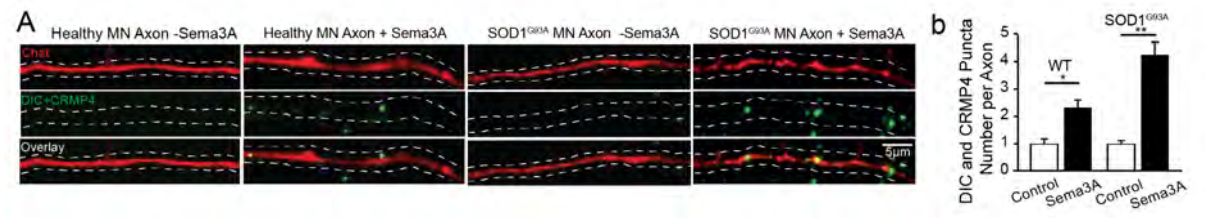

Figure 8, Reviewer #1 (Figure 5F in the revised manuscript): (A) Representative images from the proximity ligation assay for CRMP4 and dynein in SOD^{G93A} and control MNs axons that were exposed to either control or Sema3A 8h post treatment.
(B) Quantification of the CRMP4-DIC puncta number per axon in each group (One way Anova, n=3, **p=0.01 *p=0.04)

We now added the full matrix of representative images to Figure 5F **(Figure 8, Reviewer #1)** of the revised manuscript.

9. Please present the WT primary MN data to parallel the SOD1G93A data shown in Figure 5E. It is mentioned in the text, but not shown.

We now present in **Figure 9, Reviewer #1** (and supplementary figure 6 of the revised manuscript) representative images of the WT condition as well as all other related representative images which were used to quantify the graph in Figure 6 E.

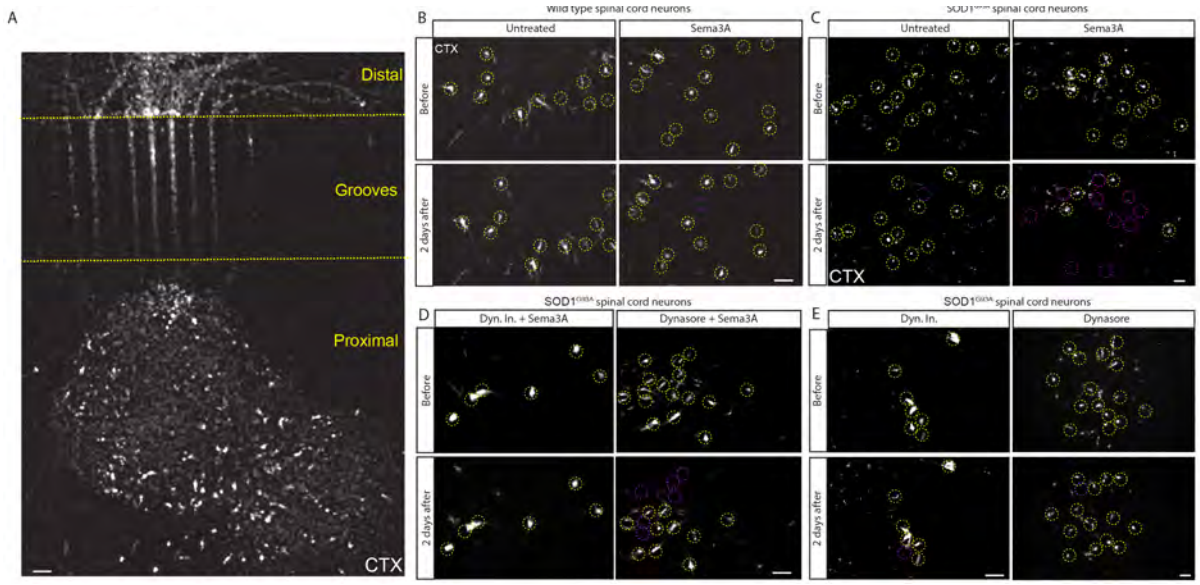

Figure 9, Reviewer #1 (Supplementary Figure 6 in the revised manuscript) (A) Representative image of CTX signal in WT spinal cord murine explant plated in microfluidic device. CTX signal detected in proximal compartment and labeled ultimately neurons that transverse their axons to the distal side. (B-E) Representative images of murine spinal cord primary neurons before and after Sema3A application. Gray denotes CTX-positive cells. Yellow circles label CTX positive cells. purple circles label CTX signal loss Scale bar: 50 µm for the left image and 30 µm for all the right images.

10. The authors do a nice job to show that dynasore impairs internalisation of BDNF in Supplementary Figure 6; however, it would be more appropriate to confirm that dynasore restricts Sema3A internalisation in their model system. Alternatively, providing citations that support this idea will suffice.

We thank reviewer #1 for this observation. Indeed, in the first submission we demonstrated that Dynasore treatment in distal axons impairs internalization of BDNF. These data were presented in supplementary movie 4 in the first manuscript. We also demonstrated in supplementary Figure 6 **(Figure 10, Reviewer #1)** that Plexin A1 (PLXA1 - the receptor for Sema3A that is internalized along with Sema3A)

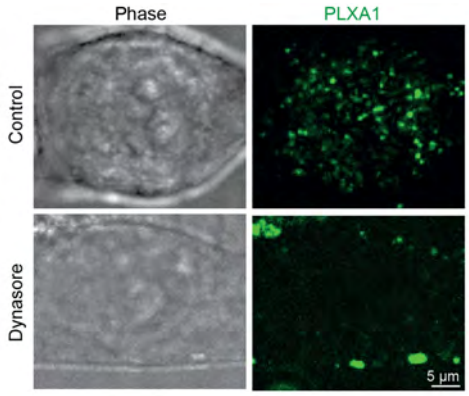

Figure 10, Reviewer #1 (Supplementary figure 7 in the main manuscript) Representative images of COS7 cells that were grown on glass dishes for 2DIV and then treated with FITC-PlexinA1 antibody with and without Dynasore application. Scale bar: 5 µm

internalization is similarly impaired after Dynasore treatment. We are sorry that this wasn't clear in the first submission and emphasized this in the main text of the revised manuscript. Furthermore, we added citations that support this idea in the main text:

…"To further determine whether endocytosis of Sema3A is important for death signaling, as was shown before in different neurons (Wehner et al., 2016), we applied Dynasore, a dynamin-dependent endocytosis inhibitor (Macia et al, 2006), to the distal axons prior to applying Sema3A - which was shown to internalize along with his receptor Plexin A1 (PLXNA1) via this pathway (Castellani et al, 2004; Fournier et al, 2000) (Supplementary Figure 7)".

11. The I141V mutation in CRMP4 is stated to "enhance formation of the CRMP4-dynein complex" (lines 236-237), but upon inspection of the graph, the result is non-significant. Repetition of the experiment should be performed to confirm its relevance or the sentence should be caveated with this important detail. Moreover, dedicating an entire paragraph of the discussion to this non-significant finding is perhaps somewhat premature.

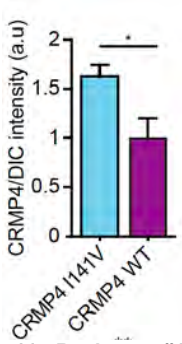

Figure 11, Reviewer #1 (Main Figure 5D in the main manuscript): Quantification of four repeated pull-down experiments with anti-DIC. The CRMP4 intensity band was normalized to the DIC intensity band for each repeat. (Ratio Paired t-test, n = 4, *p = 0.0393).

Following point number 11 of reviewer #1, we repeated the pull-down assay that was used to demonstrate an enhanced interaction of the ALS-associated CRMP4 mutant with Dynein. The fourth repeat of this assay indeed lead to a significant effect, confirming a strong interaction of the CRMP4 mutant with dynein in ALS. These data have been incorporated into the main text and presented in Figure 5D of the revised manuscript (**Figure 11, Reviewer #1**).

Note: We are extremely motivated by this result. We believe that the ALS-associated mutation in CRMP4 increases its interaction with Dynein, and that this effect may accentuate neuronal loss in ALS.

12. Motor neuron counts post-treatment would provide the most convincing evidence for the importance of CRMP4-dynein interactions mediating motor neuron death.

We agree that a count of motor neurons post treatment would provide convincing evidence for the importance of CRMP4-Dynein interactions mediating motor neuron death. Following this idea, we first immunostained for ChAT and NeuN in P90 spinal cords, 30 days post treatment. Despite the fact that the ChAT immunostaining was unsuccessful, our analysis showed a significant increase in NeuN positive cells in animals injected with GFP-50aa compared to GFP alone **(Figure 12, Reviewer#1).** Hence, in addition to a decrease in Caspase3 activation, we now demonstrate that overexpressing GFP-50aa in early symptomatic

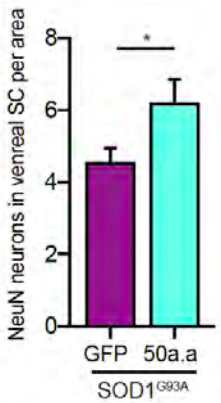

Figure 12, Reviewer #1 (Main Figure 7E in the main manuscript): Quantification of NeuN positive cells in the ventral horn of P90 SOD1^{G93A} injected with GFP or GFP-50 a.a (Student t.test *p=0.038)

stages of disease results in more surviving NeuN positive cells in the ventral spinal cord horn. These data have been incorporated into the revised manuscript as Fig. 7E.

13. Details of the spinal cord level analysed for Figure 1, would be helpful in the main text.

We added this information to the main text. Also as mentioned in point 1 above, we summarized all information for each figure within the supplementary material of the revised manuscript. The details reviewer #1 asked for in point 13 are below:

C-D:

- *Spinal cords from 3 different mice in each condition were analyzed. We monitored CRMP4 expression in total of 108 cells in WT spinal cords and 123 cells in $SOD1^{G93A}$.*
- *Scale bar: 10µm.*
- *P value- * 0.0161 ; Student t.test*

14. The authors should provide details of how many motor neurons, sciatic nerve axon fields, and NMJs were assessed per biological replicate in the CRMP4 expression analyses. Similarly, were approximately equal numbers of intra-muscular MNs analysed in each of the n = 3 human samples? I ask the latter because the nerves (understandably) are used as the replicate rather than the human, and therefore oversampling from a single ALS patient could skew the data.

As described in point 1 we added all of this information to figure legends and the supplementary material. Here is the specific information requested in point 14:

A-B:

- *5 non-ALS controls and 4 sALS patients were used for monitoring CRMP4 in intramuscular nerves samples. We analyzed 40 terminal axons from the healthy samples (~8 axons per sample) and 36 terminal axons from ALS patients samples (~8 axons per sample).*
- *Scale bar: 20 µm.*
- *P value: * 0.0475 ; Student t.test*

C-D:

- *Gastrocnemius muscles tissues from 3 different mice in each condition (WT and $SOD1^{G93A}$) were analyzed. A number of 44 NMJ's in WT condition and 60 NMJ's in $SOD1^{G93A}$ condition monitored per each animal.*
- *Scale bar: 10µm.*
- *P value- * 0.0157 Student t.test*

E:

- *Gastrocnemius muscles tissues from 3 different mice ($SOD1^{G93A}$) were analyzed. We counted 24 NMJ's in $SOD1^{G93A}$ CRMP4 negatives and 67 NMJ's in $SOD1^{G93A}$ CRMP4 positives.*
- *P value- * 0.0352 Student t.test*

F-G:

- *Sciatic nerves from 3 different mice in each condition were analysed. 14 WT sections and 11 $SOD1^{G93A}$ sections were monitored.*
- *P value- **** p<0.0001 ; Student t.test*
- *Scale bar: 5µm."*

15. Can the authors please speculate as to why the WT iPSC-MNs did not respond to Sema3A treatment in Figure 2B? Did the authors assess CRMP4 levels at the iPSC-MN growth cones (as a possible explanation)?

This is a very interesting point. Here, we demonstrated that WT iPSC-derived MNs did not respond to Sema3A treatment in the same way that C9orf72 MNs did **at this specific time point** (6-8 hrs post Sema3A treatment). Nonetheless, the data presented in the

revised manuscript shows minor CRMP4 increases for WT MNs at this time point (Figure 3D,E in the revised manuscript). We suspect that measuring activation of CRMP4 in both WT and C9orf72 MNs at earlier time points (5 min, 15 min, 2 hrs) will result in similar activation between condition. The difference between the conditions is probably due to distinct kinetics of CRMP4 translation and/or degradation. However, the mechanism responsible for the permanent elevation of CRMP4 specifically in C9orf72 MNs is unknown. miRNA-mediated downregulation and impaired local protein synthesis are common features in several ALS models (Haramati *et al*, 2010; Costa & Willis, 2018). Additionally, Sema3A was shown to induce axonal local synthesis in several neuronal systems (Manns *et al*, 2012; Wu *et al*, 2005; Campbell & Holt, 2001; Cagnetta *et al*, 2019, 2018). Thus, we hypothesize that the permanent elevation in CRMP4 that we observed in C9orf72 MN somata and proximal axons are possibly due to increased axonal protein synthesis. Another possibility for CRMP4 alterations in ALS is the proteolytic degradation of CRMP4 at the site of injury, as has been shown before (Jang *et al*, 2010). Further experiments are needed to test these ideas. This discussion is part of our revised manuscript. **Note**: We are working on a follow up manuscript pursuing this direction.

16. The staining of CRMP4 in somas of C9 iPSC-MNs (Fig. 2D) appears nuclear, unlike the cytoplasmic staining observed in SC MNs from the mouse (Fig. 1B) - please can the authors confirm/discuss this finding?

We agree with reviewer #1 that the representative images in Figure 2D of the original manuscript suggested nuclear CRMP4 localization. Following this concern, we first re-evaluated our raw data images and second, immunoassayed CRMP4 in WT and C9orf72 MNs and measured CRMP4 subcellular localization in each. Here, we monitored CRMP4 localization in every Z slice of the image stack rather than monitoring CRMP4 in the overall max projections. We observed that CRMP4 is not localized specifically in the nucleus of the cell in any condition or treatment, but rather it is localized diffusely within the cytoplasm **(Figure 13, reviewer #1)**. Our data further suggest that Sema3A facilitates cell shrinkage in C9orf72 human iPSC-derived MNs (Supplementary Figure 3 of the revised manuscript). Sem3A-treated cells that shrunk noticeably exhibited reduced cytoplasmic areas, resulting in an apparent nuclear localization of CRMP4 post Sema3A treatment. Although it is tempting to argue that CRMP4 enters the nucleus post Sema3A treatment (and perhaps triggers neuronal loss), this is not the case in our system. In the revised manuscript, we used more appropriate images in this panel as well as in every other new experiment that we performed, to eliminate any further confusions.

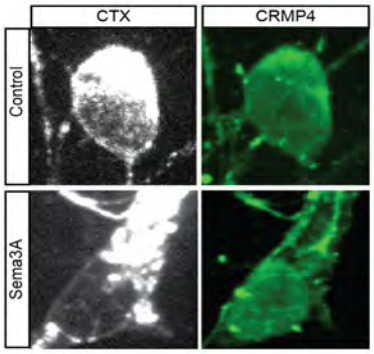

Figure 13, Reviewer #1 (Part of Figure 3 in the revised manuscript) Representative images of healthy human-derived MN cell somata in the presence or absence of after Sema3. Gray denotes CTX, green denotes CRMP4. Scale bar: 5um.

17. Given that WT iPSC-MNs were analysed, the statement that, "exposure to Sema3A led to CRMP4 elevation specifically in the ALS diseased MN soma..." (lines 161-162) is not true/assessable

We now completed the full matrix and analyzed both WT and C9orf72 MNs in the presence and absence of Sema3A. As described in point 3 and point 7 above we demonstrated

that indeed Sema3A exposure led to significant CRMP4 elevation only in C9orf72 neurons.

18. Which marker was used to measure somata areas in Figure 2?

We used CTX signal boundaries to measure somata area in Figure 2. We made this point clear in the text and figure legend of the revised manuscript.

19. The two purple colours of the CRMP4 peptides are hard to differentiate (Fig. 3A) - changing one to a different colour would help.

We agree with reviewer #1 that the two purple colors are too close and hard to differentiate. We made one of the peptides in the illustration green in order to help differ the motifs in CRMP4 protein.

20. Figure 4E-I: are these primary motor neurons? Please clarify in the text and figure legend.

We are sorry for the confusion. These are both murine (Figure 5 E,F) and human iPSC-derived motor neurons (Figure 5 G,H). We now made sure that this is clear in the main text, and marked each panel with the correct culture.

21. The legend of Figure 4, panel E requires a better explanation.

We now provide a better explanation of the technique used in Figure 4, panel E and refer the reader to the method section:

…"The proximity ligation assay (PLA) was used to visualize the co-localization of selected proteins; it was performed as previously described (Söderberg *et al*, 2008)*. Briefly, iPSc-derived MNs and murine-MN cultures were grown in the MFC on glass dishes for 18 and 5 DIV, respectively, and were then fixed in 4% PFA, at 4˚C for 20 minutes. Subsequently, the samples were blocked and permeabilized with 5% Donkey Serum, 1% BSA, and 0.1% Triton X-100 in PBS for 1h and incubated with anti-CRMP4 and anti-DIC antibodies overnight at 4˚C. Interactions (range ~40nm) were detected by the proximity ligation assay Duolink kit (Sigma: PLA probe anti-mouse minus DUO92004, anti-rabbit plus DUO92002, and the detection kit Far Red). PLA was performed according to the manufacturer's instructions. Coverslips were washed, mounted, and imaged by confocal microscopy. Half ligation samples were used as a negative control. The axonal PLA signal was quantified with ImageJ software using an axonal mask based on an endogenous m.Cherry/Rosa signal. The PLA puncta signal was quantified with the analyzed particle function of the software".*

22. The supplementary movies are not needed.

We removed the movies attached to this manuscript, as suggested by the reviewer.

23. The authors allude on several occasions to their data showing a role for CRMP4 in MN death/loss in vivo (Lines 41-42, 97-98, 307-308, 339-340); however, no in vivo MN counts are presented. Caspase 3 is used as a proxy in Figure 6; however, almost 100% of MNs are reported to be positive in SOD1G93A mice, and not all of these neurons will degenerate. Rewording is required.

As discussed before in points number 6 and 12 in this rebuttal, we agree that we cannot claim motor neuron loss in our experiments *in vivo.* We re-worded all mentions of motor neuron in the text, using 'spinal cord neurons' instead.

24. The title could be improved by changing the focus of the sentence.

We improved the title and changed the focus of the sentence. Now, the title of the article is: **CRMP4-Dynein Complex Formation Mediates Retrograde Death Signals in ALS.**

Specific responses to Reviewer #2

In this study, Maimon et al report on the role of CRMP4 as a degenerative retrograde signal in models of ALS. Their results indicate that CRMP4 expression is up regulated in motor neuron cell bodies and axons in SOD1 mutant mice and that there is a decrease in CRMP4 expression in the NMJ of these mice as well as in human ALS patient samples. Using both the SOD1 mutant mouse model and human iPSC-derived motor neurons from C9orf72 patients, they demonstrate that CRMP4 associates with the retrograde motor dynein and that this interaction is necessary for motor neuron degeneration. In addition, they find that Sema3a, which they previously reported is secreted from muscle of ALS models, increases the CRMP4-dynein interaction. Importantly, blocking the interaction with peptides that disrupt the binding of CRMP4 to dynein rescued motor neuron death induced by Sema3a in cultures from SOD1 mutant mice and iPSC-derived motor neurons from C9orf72 patients. In addition, expression of the disrupting peptide in motor neurons of SOD1 mutant mice in vivo provided significant protection from the loss of motor neurons.

These findings are very exciting and provide novel insights into the pathology of ALS by identifying CRMP4 as a degenerative retrograde signal. Overall, the study is thorough and well controlled and there are just a few concerns that the authors should address.

We want to thank reviewer for his/her interest in our work. We were highly encouraged by the reviewer inputs and positive attitude - **Thank you**. Please find our responses to your very important observations below.

1. In Fig. 1 H&I is there less CRMP4 staining because there are just fewer NMJs in these patients? A ChAT or BTX co-staining should be included.

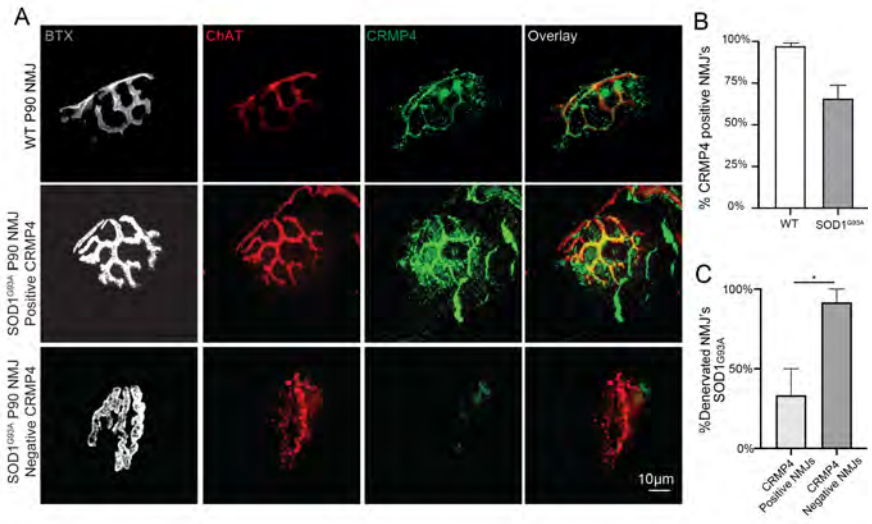

Figure 1, Reviewer #2 (Figure 2 C,D,E in the revised manuscript): (A) Representative images of SOD1$^{G93A/ChAT::tdTomato}$ or WT$^{ChAT::tdTomato}$ neuromuscular junctions at P90. White: denotes BTX, red denotes Chat, green denotes CRMP4, yellow denotes the co-localization area of CRMP4 and NFH. Scale bar: 10 μm. (B) Quantification of CRMP4 positive NMJ in WT or SOD1^{G93A} at P90. Student's t-test, n=3, *p = 0.016. (C) Quantification of the percent of partially denervated NMJ's in the presence or absence of immunostaining Student's t-test, n=3, *p = 0.016

Excellent point. Following this idea, we extended our initial experiment and counted the percent of partially denervated NMJs in CRMP4 positive or negative NMJs. As observed in **Figure 1, Reviewer #2,** in the WT condition almost all NMJs express CRMP4. However, in SOD1^{G93A} mice we observed a ~25% decrease in the number of NMJs expressing CRMP4 at P90. Strikingly, almost all of the CRMP4 negative NMJs were already partially denervated. Thus, it seems that CRMP4 loss can indicate early denervation in the SOD1^{G93A} mice.

In Figure 1 H, I of the initial manuscript we immunoassayed CRMP4 in the intramuscular nerves that reach the distal muscle. These nerves are not part of the end plate. To further validate this notion we stained the biopsies with BTX, as the reviewer suggested. We could not locate any end plates in this tissue. We further stained the biopsies with ChAT to specifically mark motor neuron axons. Unfortunately, ChAT staining is highly challenging and the signal we observed was not sufficient to differentiate motor neuron axons. However, NFH immunostaining marked neuronal axons and our analysis reveled CRMP4 reductions specifically within NFH positive axons. Interestingly, we repeated our initial immunostaining of CRMP4 and NFH in human intramuscular nerves and this time also performed immunostaining for CRMP4 and MPB (marker for Schwann cells). Our analysis

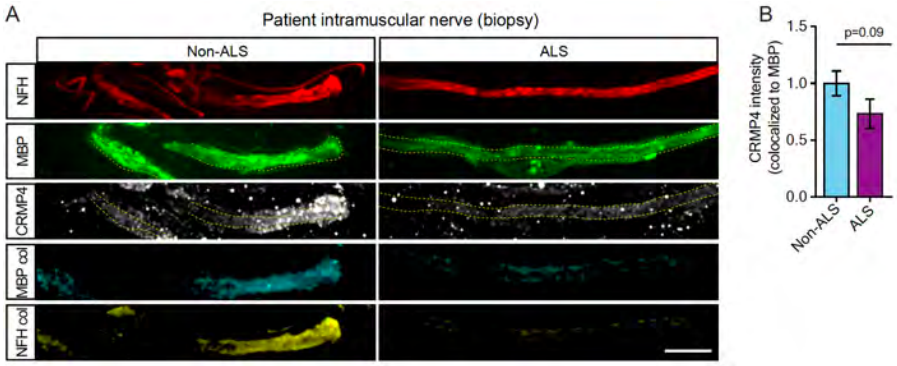

Figure 2, Reviewer #2 (Supplementary Figure 1 in the revised manuscript): (A) Representative images of ALS patient or non-ALS human control intra-muscular nerves. Red: denotes NFH, Green: denotes MBP, White: denotes CRMP4. Scale bar: 20 μm. (B) Quantification of CRMP4 intensity levels in 5 non-ALS controls and 4 sALS patients intra-muscular Schwann cells.

of CRMP4 intensity in MBP positive intramuscular Schwann cells revealed a non-

significant reduction in sALS samples compare with those from healthy controls **(Figure 2, Reviewer #2).** Altogether, it is unlikely that the reduction we observed in CRMP4 is due to fewer NMJs.

2. Does CRMP4 mRNA increase in the motor neurons? The high expression in Schwann cells shown in Fig. 1D is striking. Given that Schwann cells communicate many factors to axons, including RNA (e.g. Lopez-Verilli and Court, 2012; Wei et al., 2019), is it possible that some or even most of the CRMP4 is coming from these glia? No experiments are needed here, but just something the authors might consider.

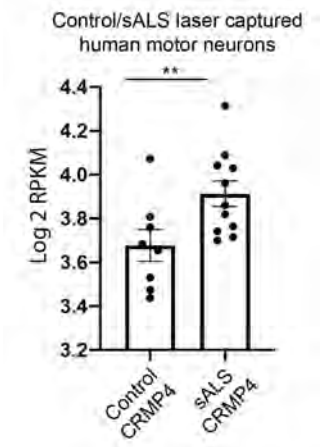

Figure 3, Reviewer #2: CRMP4 mRNA levels in sALS and control laser captured motor neurons. This data re-analysed from (Krach *et al*, 2018) mRNA data set.

Following reviewer #2's suggestion we monitored CRMP4 mRNA levels in both human and murine ALS diseased motor neurons. First, we re-analyzed the published mRNA sequencing data from our recent publication (Rotem *et al*, 2017) that aimed to compare compartment-specific mRNA levels in healthy and ALS diseased (TDP-43, SOD1^{G93A}) murine motor neurons. We found that CRMP4 is: 1) Found in motor neuron axons and somata of both WT and ALS diseased conditions; 2) increased in ALS motor neurons axons, compared with control axons; 3) ~3 times more abundant in the ALS diseased axons compare to their somata; 4) increased by 45% in ALS disease cell somata compared to controls. Next, we decided to analyze the mRNA expression levels of CRMP4 in a human context. For this, we re-analyzed published RNA-seq data from laser captured microdissected motor neurons (Krach *et al*, 2018) from sALS and control spinal cords. Similar to the murine cultures, our re-analysis demonstrates that CRMP4 mRNA is significantly higher in sALS spinal cord motor neurons compare to controls (**Figure 3, reviewer #2**). Altogether, it seems that CRMP4 mRNA is enriched in both motor neuron somata and axons in ALS. As reviewer #2 suggested, it is possible that some or all of the CRMP4 is coming from glia via cell-cell communication. However, the increase in CRMP4 mRNA can also arise from other processes in the motor neurons including: nuclear import-export dysfunction, a reduction in ribosome activity, and/or activation of a CRMP4 homeostatic loop. We are aiming to fill this gap in a follow up story.

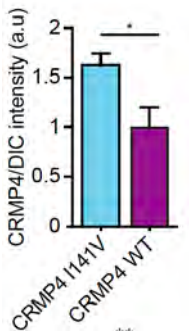

Figure 4, Reviewer #2 (Main Figure 5D in the main manuscript): Quantification of four repeated pull-down experiments with anti-DIC. The CRMP4 intensity band was normalized to the DIC intensity band for each repeat. (Ratio Paired t-test, n = 4, p = 0.0383).

3. In the results, lines 236-240, referring to Fig. 4D, and again in the Discussion, the authors state that the I141V mutation in CRMP4 increases interaction with dynein. However, based on the standard of $p<0.05$, there is no statistical difference between the complex formed by WT CRMP4 and the mutant. Therefore, these statements need to be changed.

We agree with the reviewer that in the first submitted manuscript we overstated the finding that the CRMP4 mutation increases the interaction of dynein with CRMP4. In order to validate this result, we repeated the pull-down assay that we originally used to demonstrate the enhanced interaction of mutant CRMP4 with Dynein. The fourth repeat of this assay also showed an effect (**Figure 4, Reviewer #2**), confirming the stronger interaction of mutant CRMP4 with Dynein in ALS. These data have been incorporated into the main text and presented in Figure 5D of the revised manuscript.

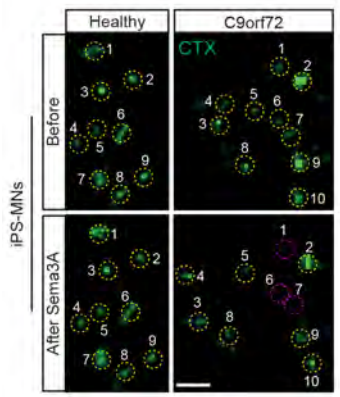

Figure 5, Reviewer #2 (Main Figure 6A in the main manuscript): Representative images of CTX signal in healthy and C9orf72 human IPSC-derived MNs before and after Sema3A application. Green denotes CTX-positive cells. Yellow circles are CTX positive cells. Purple circles are cells that are missing due to Sema3A treatment. Scale bar: 40 µm.

4. In lines 169-171, the quantification of cell body data needs better description in the methods and results to help the reader with interpretation and implication of this data.

We are sorry for the misunderstanding. In the revised manuscript we extended the methods and result sections and tried to report adequately every experiment that we performed. We hope that the additional information included within the supplementary material of the revised manuscript will help readers with the interpretation and implications of the data.

5. What do the white and red arrows indicate in Fig 5B?

Thanks to reviewer #2 for this comment—we now realize that the white and red arrows were confusing. In the current version of the manuscript we replaced the arrows with circles and numbers, marking the cultured neurons that sent their axons towards the distal side that have been tagged with CTX (**Figure 5, Reviewer #2)**. Yellow circles indicate CTX positive cells. Purple circles outline cells that are missing due to Sema3A treatment. Numbers indicate the neurons**.** This explanation was added to the revised manuscript in the figure legend.

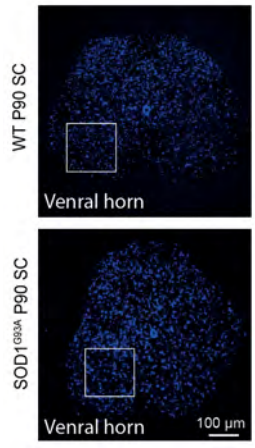

Figure 7, Reviewer #2 (Figure 7A in the main manuscript): Spinal cord sections marked with DAPI

6. The graph in Fig. 5G should be revised to match 5C-E. It is very confusing in its present form.

We revised Figure 5G to eliminate any further confusions **(Figure 6, Reviewer #2)**

7. The orientation of the spinal cords in Fig. 6F should be indicated. Presumably, ventral is at the top, which is flipped from the usual convention of ventral down.

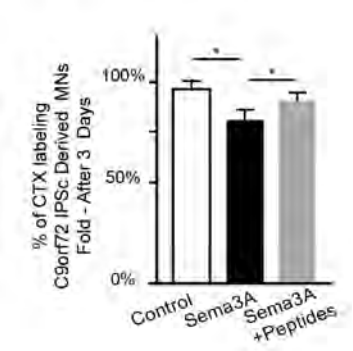

Figure 6, Reviewer #2 (Figure 6G in the revised manuscript): Quantification of C9orf72 iPSC-derived MNs in the proximal compartment of an MFC before and after Sema3A treatment with and without 10 ug CBP1-4.

Again, we are sorry for the confusion. We now flipped the images and indicated where the ventral horn is **(Figure 7, Review #2)**

8. In supplementary Fig 2, the scalebar is missing.

We added every scale bar that was missing. We thank the reviewer for helping us improve our manuscript.

9. In Fig. 6J, instead of an exclamation mark, a question mark seems more appropriate as the retrograde degenerative signal in motor neurons is yet to be identified.

We agree with the reviewer. Please refer to **Figure 8, Reviewer #2**

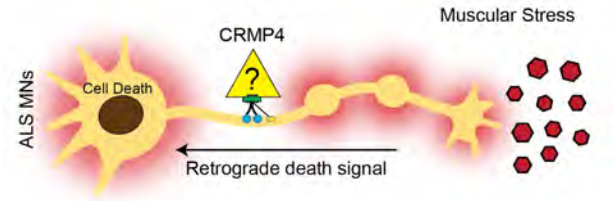

Figure 8, Reviewer #2 (Main Figure 7F in the main manuscript): our model

10. The authors might want to include mention of DLK as possibly involved in ALS retrograde signaling in their discussion, given the extensive work of Lewcock and others in this area.

We agree with the Reviewer. To address this, we added the following to the revised discussion: *…"For example, DLK regulation of JNK and c-Jun might also be part of this death signal mediated by Sema3A in ALS MNs, since it was previously shown that DLK and JNK signaling is elevated in ALS models and that it is a part of a retrograde death signal".*

11. In the Discussion, lines 378-380, "p75NTR is retrogradely transported ... a retrograde apoptotic signal that activates JNK (Kenchappa et al., 2010)". The Pathak et al reference should be moved to replace the final reference to Kenchappa et al.

Done. *…"and plays a role in generating a retrograde apoptotic signal that activates JNK (Kenchappa et al, 2010)".*

Specific responses to Reviewer #3

In this manuscript Maimon and collaborators build up on previous studies by their group and others on the possible involvement of CRMP4 in ALS related motor neuron death. They first show that CRMP4 levels are altered in different ALS models. In G93A mice, they show an increase in MN cell bodies positive for CRMP4, associated with increased axonal content and decreased content at presynaptic sites. In ALS nerve biopsies, they observed decreased intraaxonal levels, and in C9ORF72 iPS derived motor neurons, they show increased axonal content upon Sema3A administration.They further used cell biology techniques to demonstrate a direct interaction between CRMP4 and dynein, and identify interaction domains. They use this knowledge to set up two methods to interfere with CRMP4/Dynein interaction (peptides and GFP-50 aa, that encodes the dynein interaction domain). In cellular models, they show that ALS mutations increase CRMP4/dynein

interactions, and provide evidence that disrupting this interaction might provide neuroprotection in ALS mice.

The study is generally interesting, and would deserve publication upon appropriate revision. In its current state, there are a number of controls that are crucially lacking, as well as discussion of other relevant literature.

We want to thank reviewer #3 for his/her interest in our work and for the opportunity to revise our initial manuscript. We took very seriously each of the raised concerns. Please find our responses below.

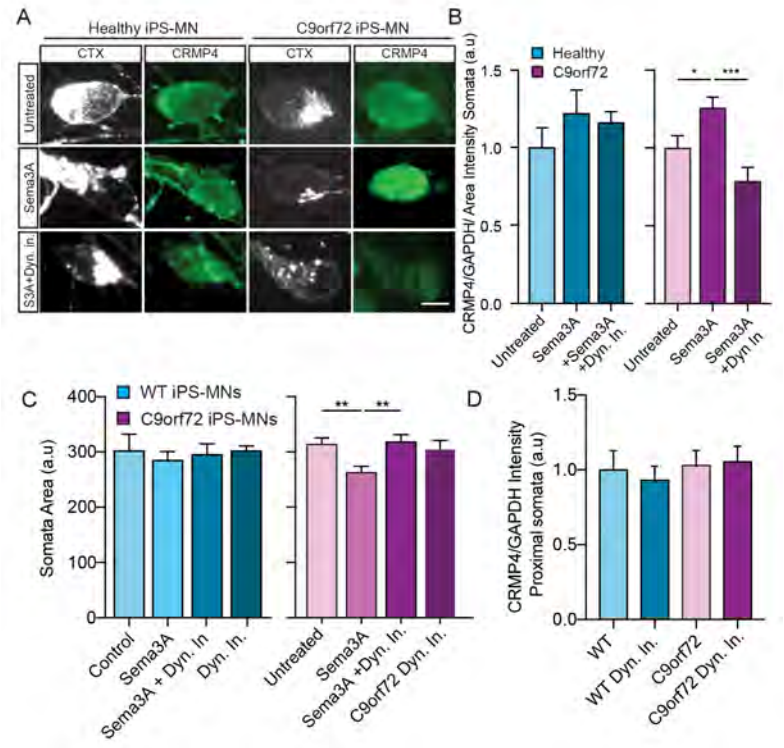

Figure 1, Reviewer #3 **(Figure 3 D,E and supplementary Figure 3 in the revised manuscript)** (A) Representative images of healthy and C9orf72 human-derived MN cell somata after Sema3A, Sema3A + dynein inhibitor, or control treatment. Gray denotes CTX, green denotes CRMP4. Scale bar: 5um. (B-D) Quantification of CRMP4 intensity (B,D) levels and somata size (C) at the somata of healthy and C9orf72 human-derived MN after Sema3A treatment compared with untreated control or with dynein inhibitor applied prior to Sema3A treatment (n=3, One-way ANOVA, Tukey's multiple comparisons test; *p =0.013, *p=0.01).

1. In figures 2c-f and 4h-l, the experiments only include C9ORF72 patients cells, and not control patients. In figure 2c-h, the authors show that dynein inhibition decreases CRMP4 axonal levels, suggesting that the observed increased levels in these cells are due to dynein activity. However, it cannot be excluded that dynein inhibition generally decreases CRMP4 levels, independently of disease status. Similarly, in Figure 4, the authors show that administration of CBP peptides decrease co-localization of CRMP4 and dynein in C9ORF72 patients, but do not provide control experiments with healthy controls. Whether this is disease specific or not is important also in this case. Last, in Figure S4, they compare healthy controls with C9ORF72 cells treated with Sema 3. There lacks two control groups in this Figure S4 (controls treated and C9ORF72 untreated...). While it is possible that those controls were properly done, the presentation is currently confusing.

We agree with reviewer #3 that we were missing important controls in the first submission and we are thankful for the opportunity to address these concerns. **We performed several new experiments and added these to the revised manuscript.** In order to verify that transport of distal CRMP4 is the cause of CRMP4 upregulation in the cell body and proximal axons we used WT iPSC-derived MNs as suggested by reviewer #3. We

demonstrate in the revised manuscript that whereases Dynein treatment upregulated CRMP4 in the cell somata and proximal axons of C9orf72 iPSC-derived MNs, this effect was not significant in WT iPSC-derived MNs (**Figure 1, Reviewer #3).** Moreover, we

added another control and measured CRMP4 intensity in the somata and proximal axons of both WT and C9orf72 iPSC-derived MNs in the presence of Dynein inhibitor alone. Our results (**Figure 1, Reviewer #3)** indicate that application of Dynein inhibitor alone have no effect on CRMP4 intensity in either condition.

Furthermore, our new analysis of WT iPSC-derived MNs re-enforced our initial findings. We now demonstrate that CRMP4-Dynein complexes are enhanced specifically in C9orf72 iPSC-derived MNs but not WT cells. The ability of the peptides to reduce this interaction is specific to C9orf72 MNs as well, as they have no effect in WT iPSC-derived MNs in which CRMP4-Dynein interactions are minimal **(Figure 2, Reviewer #3).** These data are now incorporated within figure 5 of the revised manuscript.

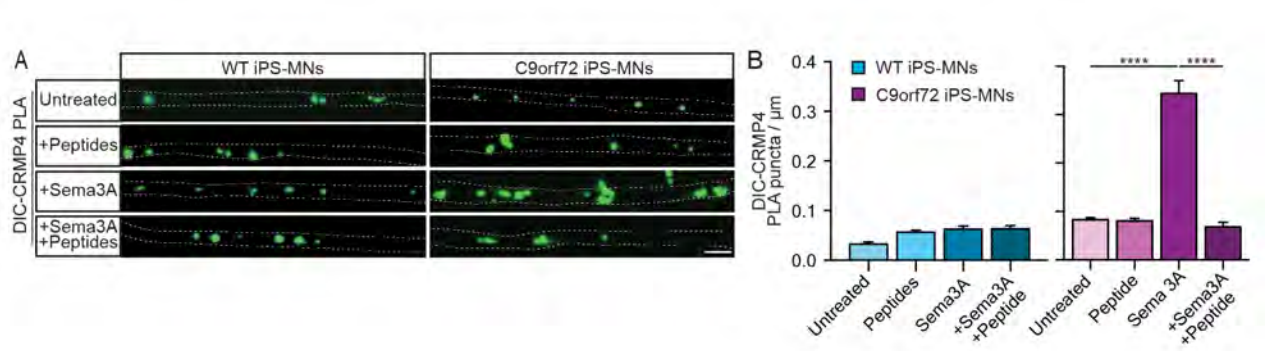

Figure 2, Reviewer #3 (Figure 5 H,I in the revised manuscript): (A) Representative images of the proximity ligation assay for CRMP4 and dynein in C9orf72 and healthy human-derived MN's in the presence or absence of Sema3A, and peptides application. (B) Quantification of the CRMP4-DIC puncta number per axon in each group (Student's t-test, p=0.0001, n=3).

2. In Figure 4A, the authors show increased interaction between CRMP4 and DIC. However, they also previously show that total CRMP4 levels are increased in G93A nerves. How do they control for the increased CRMP4 amount? Is it the interaction that is increased or simply the total CRMP4 levels?

In this manuscript we observed two phenomena related to CRMP4 in ALS. First, we observed elevation of CRMP4 in the cell somata and proximal axons, and decreased levels if CRMP4 in distal axons and NMJs in ALS. We found that these alterations in CRMP4 expression are mediated by Dynein. Second, we demonstrated that CRMP4 is upregulated by Sema3A. Since our recent publication suggested over secretion of Sema3A from ALS diseased muscles (Maimon *et al*, 2018) we hypothesize that the increase in CRMP4 that we observed in ALS is due to Sema3A.

Overall, we argue that both CRMP4 elevations in the distal axons as well as CRMP4-Dynein interactions are occurring in parallel in ALS disease.

3. In figure 6, the authors seemingly performed their overexpression experiment of GFP and GFP50aa only in diseased mice, and not in wild type mice. Seemingly, Figure 6F shows results of uninjected mice. Their only control in Figure 6G appears thus G93A

transduced with GFP, and they observe strong levels of caspase 3 positive motor neurons in this condition. It is possible that overexpression of GFP itself triggers toxicity to motor neurons, leading them to execute apoptosis. These experiments should be done in wild type mice in parallel to demonstrate that their expression levels of GFP is not toxic per se in motor neurons.

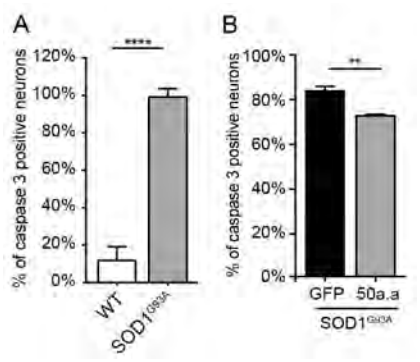

Figure 3, Reviewer #3 (Figure 7 in the revised manuscript) (A) Quantification of active caspase 3 positive cells in P90 WT and $SOD1^{G93A}$ SC (Student's t-test, n=3, ***p<0.0001) (B) Quantification of caspase 3 positive cells in P90 $SOD1^{G93A}$ mice SC cross sections that were injected with either AAV9-GFP or AAV9-50aa-GFP (Student's t-test, n=3, **p=0.0015).

To address this issue, we first compared the Caspase 3 positive cells between all 4 groups: WT, un-injected $SOD1^{G93A}$, $SOD1^{G93A}$ + GFP, $SOD1^{G93A}$ + GFP-50aa Our analysis revealed similar Caspase 3 activation in the un-injected $SOD1^{G93A}$ mice compared with the $SOD1^{G93A}$ + GFP mice (**Figure 3, Reviewer #3).** Thus, it seems unlikely that the GFP construct is toxic and activates Caspase 3 in spinal cord (**Note**: Our quantification of un-injected $SOD1^{G93A}$ mice compared with the $SOD1^{G93A}$ + GFP injected mice suggests a non-significant trend towards protection with GFP expression). Furthermore, other groups (i.e. Guo *et al*, 2016) administered AAV-GFP construct into WT mice by intrathecal injections, as we did, and reported no adverse effects on astrogliosis or cell death in the spinal cord.

We agree with the reviewer that the cleanest and most straightforward way to test the toxicity of AAV-GFP is to simply inject WT mice with this construct. Unfortunately, however, due to Covid-19 limitations and regulations we could not perform this specific experiment. Nonetheless, we infected primary spinal cord neurons with our AAV-GFP construct *in vitro* and monitored their viability. Consistent with our previous data, these results demonstrate no apparent toxicity to primary spinal cord neurons in this system (**Figure 4, Reviewer #3).** Altogether, it seems unlikely that our AAV-GFP construct is toxic to spinal cord neurons *in vivo*.

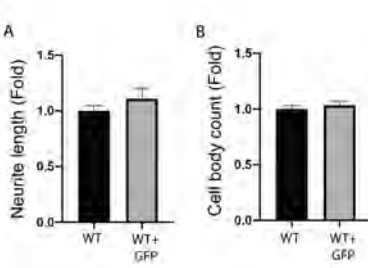

Figure 4, Reviewer #3: Quantification of the neurite length (A) and cell body number (B) of wild type murine motor neuron cultures at 14 days post GFP infection compare with uninfected control.

Furthermore, it is not shown that GFP and GFP50aa overexpression levels are similar (especially because the two images provided show completely different saturation levels), nor do they provide results on CRMP4 accumulation in G93A motor neurons (which is expected to decrease upon intervention).

Following reviewer #3 suggestion we measured the percent of GFP infected cells in P90 spinal cord neurons in AAV-GFP and AAV-GFP-50aa conditions. We found no difference between these two groups **(Figure 5, Reviewer #3).** We also measured the GFP intensity in GFP positive cells in both groups. Here too, we observed no difference in GFP intensity

(Figure 5, Reviewer #3). Thus, it seems that the expression of each construct is similar. This important point has now been incorporated into the revised

manuscript.

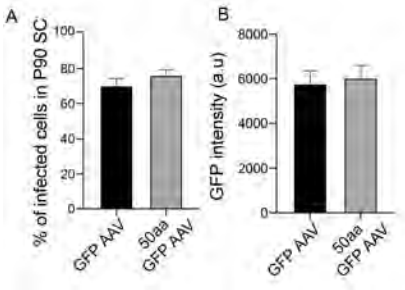

Figure 5, Reviewer #3 (Supplementary Figure 9F in the revised manuscript) Quantification of %infected cells in P90 spinal cord in GFP-AAV/50a.a GFP AAV conditions (B) Quantification of GFP intensity in spinal cord neurons positive cells in GFP-AAV/50a.a GFP AAV conditions.

We also monitored CRMP4 levels in the spinal cord of AAV-treated mice. We immunoassayed CRMP4 in both GFP and GFP-50aa injected mice and measured CRMP4 intensity levels in spinal cord neurons of these mice one-month post injection. Assessing the distributions of CRMP4 expression in spinal cord neurons of both groups suggested a complex result **(Figure 6, Reviewer #3)**. While injection of GFP-50aa reduced CRMP4 levels in neurons expressing low amounts of CRMP4, we did not observe a similar shift in neurons expressing high levels of CRMP4. Instead, we found an increase in the number of cells express high levels of CRMP4 in the GFP-50aa injected group compared with GFP. These unexpected results might be due to: I) Recognition of the 50aa by our CRMP4 antibody, even though the antibody was not raised against this epitope; II) time-dependent CRMP4 mis-localization. In this case, measuring CRMP4 expression at earlier time points post injection might result in higher shift; III) a CRMP4 homeostasis loop that results in overproduction of CRMP4 due to low levels of CRMP4 protein in the soma. As discussed in the text, since CRMP4 is not a transcription factor we assume that the CRMP4-Dynein complex is enriched with other toxic transcription factors that induce cell death, thus CRMP4 elevation in the cell body will not result in cell death. We believe that the right measurement for the function of 50aa would involve monitoring CRMP4-Dynein complexes post treatment rather than measurement of overall CRMP4 expression. Following this idea, we performed proximity ligation assays for CRMP4 and Dynein *in vivo* (similar to what was done before *in vitro)* with the ultimate goal of measuring CRMP4-Dynein interactions post AAV injections. Unfortunately, the PLA experiments were technically unsuccessful *in vivo.* However, our data demonstrated the ability of overexpressed GFP-50aa to reduce CRMP4 and Dynein complexes *in vitro*. Furthermore, we observed a beneficial effect of overexpressing GFP-50aa on neuronal number and Caspase3 activation, compared to the GFP control, suggesting an active role of this construct.

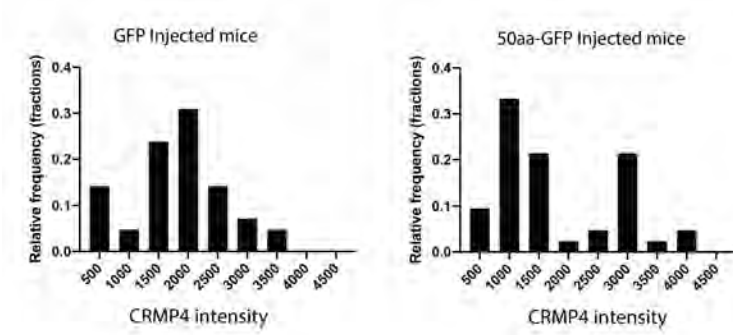

Figure 6, Reviewer #3 (Supplementary Figure 9G in the revised manuscript): Quantification of the distribution of CRMP4 intensity in spinal cord neurons of GFP injected mice (left) and 50aa-GFP injected mice (right)

4. Specificity of the antibodies used: the authors observe a strong expression of CRMP4 in Schwann cells (Figure 1), yet this is at odds with publications of other groups ((eNeuro. 2020 Mar-Apr; 7(2): ENEURO.0479-19.2020.). Could the authors provide evidence for the specificity of their immunostaining for CRMP4?

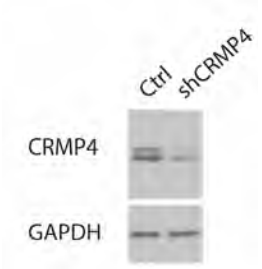

Figure 7, Reviewer #3: Western blot of murine cortical neurons neuronal cultures expressing either GFP or GFP-shCRMP4. GAPDH used as endogenous control.

In order to validate the specificity of our CRMP4 antibody we performed western blot **(Figure 7, Reviewer #3)** and immunostaining for CRMP4 **(Figure 8, Reviewer #3)** in the presence or absence of shCRMP4 to reduce CRMP4 expression. Our analysis demonstrates that the antibody used in this study is specific and capable of identifying CRMP4 reductions clearly in both assays. We understand reviewer #3's point, however, since in contrast to (Girouard *et al*, 2020) we identified CRMP4 also in Schwann cells. Nonetheless, the homemade CRMP4 antibody that this group used in their study is different than the commercial antibody we used. Different antibodies recognize different motifs in the protein which can explain this difference. It could be that the structure of CRMP4 in non-neuronal cells is different and different antibodies will not react the same. Future studies could explore this interesting observation.

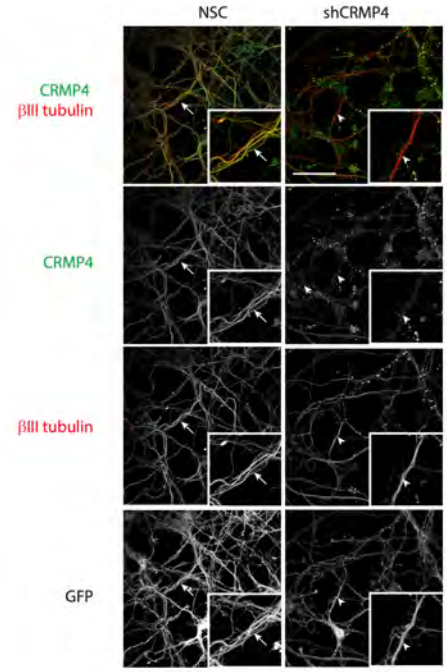

Figure 8, Reviewer #3: Primary cortical neurons infected with CRMP4-silencing lentiviral vector (shCRMP4, GFP-positive) show reduced CRMP4 signal (arrowheads) using CRMP4 antibodies, but not reduced βIII tubulinsignal (neuronal marker). Non-silencing control lentivirus-infected neurons (NSC) were used as a negative control (arrow, GFP-positive, βIII tubulin-positive). Scale bar: 50 µm.

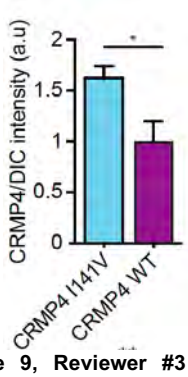

Figure 9, Reviewer #3 (Main Figure 5D in the main manuscript): Quantification of four repeated pull-down experiments with anti-DIC. The CRMP4 intensity band was normalized to the DIC intensity band for each repeat. (Ratio Paired t-test, n = 4, p = 0.0383).

5. Overinterpretation of conclusions in Figure 4D: the authors claim that the interaction between CRMP4 and DIC is increased by the mutant I141V, yet, the p-value obtained is of 0.0532. While this is a clear trend, the authors should repeat this experiment to demonstrate a truly significant effect.

We agree with the reviewer that in the first submitted manuscript we overstated the finding that the CRMP4 mutation increases the interaction of dynein with CRMP4. In order to validate this result, we repeated the pull-down assay that we originally used to demonstrate enhanced interaction mutant CRMP4 with Dynein. The fourth repeat of this assay showed a similar effect, confirming the stronger interaction of mutant CRMP4 with dynein. These data are incorporated into the main

text and presented in Figure 5D of the revised manuscript (**Figure 9, Reviewer #3**).

6. Selectivity of the mechanism regarding ALS: it has been documented in a number of experimental models that CRMP4 is increased by neuronal injury (see eg Injury-induced CRMP4 expression in adult sensory neurons; a possible target gene for ciliary neurotrophic factor, Jang SY, Shin YK, Jung J, Lee SH, Seo SY, Suh DJ, Park HT; Neurosci Lett. 2010 Nov 12; 485(1):37-42.). It is thus entirely possible that the results of the authors are reflecting a response of moitor neurons to stress rather than an ALS-specific mechanism. While this does not necessarily decrease interest, this should be clearly stated and discussed.

Very interesting point. We added this idea to the revised discussion section:

…"it has been documented in a number of experimental models that CRMP4 is increased by neuronal injury. It is thus entirely possible that the results in this manuscript reflecting a response of motor neurons to stress rather than an ALS-specific mechanism (Jang et al, 2010)".

7. The authors also do not discuss the possibility that part of their results could be explained by proteolytic degradation of CRMP4 at the injured site of the neuron, as has been shown by others (eNeuro. 2020 Mar-Apr; 7(2): ENEURO.0479-19.2020.)

We also added this point to the revised discussion section:

…"Another possibility for CRMP4 alterations in ALS disease is a proteolytic degradation of CRMP4 at the injured site of the neuron, as has been shown before (Jang et al, 2010). Further experiments are need to test these ideas".

8. The authors should appropriately discuss the novelty of their findings as compared to the work of Duplan and collaborators, which previously showed the toxicity of CRMP4 to motor neurons. As such, the current discussion does not provide such critical discussion of novelty.

We agree with Reviewer #3 that discussion of the novelty of our manuscript is important:

…"Duplan el at; have reported that CRMP4 elevations are key in SOD^{G93A} MNs both in vivo and in vitro and lead to their loss. By reducing CRMP4 levels the group demonstrated protective effect on MN health (Duplan et al, 2010). ***However, the mechanism by which CRMP4 mediate MN toxicity, its involvement in other ALS models and its relevant to human ALS disease were unknown****. In this work we discover subcellular alterations in CRMP4 levels in in sALS human patients, C9orf72 human derived MNs and in the SOD^{G93A} mice CRMP4 alterations dependent on dynein activity. Specifically, CRMP4-dynein interactions are mediated by amino acid 100-150 in the CRMP4 protein, a region that a mutation correlated with ALS was identified (Blasco et al, 2013). Importantly, CRMP4-dynein complexes are enriched in ALS diseased MNs, and lead to ~25% cell death observed in ALS diseased spinal cord. Finally, we show that blocking the CRMP4-dynein interaction rescued this MN population, both in vitro and in vivo (Figure 7F)".*

9. The western blot of Figure 4H is of poor quality, and the GFP-IgG well seems to show unspecific signal. Could another experiment be shown here?.

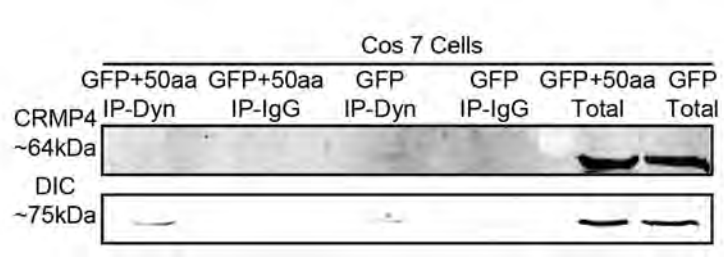

Figure 10, Reviewer #3 (Figure 5Jin the revised manuscript) Immunoprecipitation of DIC followed by Western blot analysis of CRMP4 in COS7 cells that were transfected with CRMP4 and AAV9-50aa or its control. IgG antibody was used as a control.

We thank reviewer #3 for his/her observation and agree that a blot with no IgG signal is more appropriate for publication. We now changed the blot and incorporated it to the revised manuscript **(Figure 10, Reviewer #3)**

Also, all uncropped western blots should be provided in supplementary figures with molecular weight markers

We added all our raw data to the supplementary material of the revised manuscript, including blots, images, and quantifications. Here is one example of raw blot **(Figure 11, Reviewer #3):**

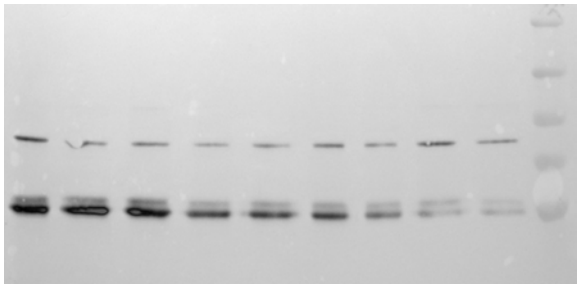

Figure 11, Reviewer #3: Example of raw blot added to the supplementary material.

References for the rebuttal:

Blasco H, Bernard-Marissal N, Vourc'h P, Guettard YO, Sunyach C, Augereau O, Khederchah J, Mouzat K, Antar C, Gordon PH, *et al* (2013) A Rare Motor Neuron Deleterious Missense Mutation in the *DPYSL3* (*CRMP4*) Gene is Associated with ALS. *Hum Mutat* 34: 953–960

Cagnetta R, Frese CK, Shigeoka T, Krijgsveld J & Holt CE (2018) Rapid Cue-Specific Remodeling of the Nascent Axonal Proteome. *Neuron* 99: 29-46.e4

Cagnetta R, Wong HH-W, Frese CK, Mallucci GR, Krijgsveld J & Holt CE (2019) Noncanonical Modulation of the eIF2 Pathway Controls an Increase in Local Translation during Neural Wiring. *Mol Cell* 73: 474-489.e5

Campbell DS & Holt CE (2001) Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* 32: 1013–26

Castellani V, Falk J & Rougon G (2004) Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM. *Mol Cell Neurosci* 26: 89–100

Costa CJ & Willis DE (2018) To the end of the line: Axonal mRNA transport and local translation in health and neurodegenerative disease. *Dev Neurobiol* 78: 209–220

Duplan L, Bernard N, Casseron W, Dudley K, Thouvenot E, Honnorat J, Rogemond V, De Bovis B, Aebischer P, Marin P, *et al* (2010) Collapsin response mediator protein 4a (CRMP4a) is upregulated in motoneurons of mutant SOD1 mice and can trigger

motoneuron axonal degeneration and cell death. *J Neurosci* 30: 785–96

Fournier AE, Nakamura F, Kawamoto S, Goshima Y, Kalb RG & Strittmatter SM (2000) Semaphorin3a Enhances Endocytosis at Sites of Receptor–F-Actin Colocalization during Growth Cone Collapse. *J Cell Biol* 149: 411–422

Girouard MP, Simas T, Hua L, Morquette B, Khazaei MR, Unsain N, Johnstone AD, Rambaldi I, Sanz RL, Raddo ME Di, *et al* (2020) Collapsin response mediator protein 4 (Crmp4) facilitates wallerian degeneration and axon regeneration following sciatic nerve injury. *eNeuro* 7

Guo Y, Wang D, Qiao T, Yang C, Su Q, Gao G & Xu Z (2016) A Single Injection of Recombinant Adeno-Associated Virus into the Lumbar Cistern Delivers Transgene Expression Throughout the Whole Spinal Cord. *Mol Neurobiol* 53: 3235–3248

Haramati S, Chapnik E, Sztainberg Y, Eilam R, Zwang R, Gershoni N, McGlinn E, Heiser PW, Wills A-M, Wirguin I, *et al* (2010) miRNA malfunction causes spinal motor neuron disease. *Proc Natl Acad Sci U S A* 107: 13111–6

Jang SY, Shin YK, Jung J, Lee SH, Seo SY, Suh DJ & Park HT (2010) Injury-induced CRMP4 expression in adult sensory neurons; a possible target gene for ciliary neurotrophic factor. *Neurosci Lett* 485: 37–42

Kenchappa RS, Tep C, Korade Z, Urra S, Bronfman FC, Yoon SO & Carter BD (2010) p75 neurotrophin receptor-mediated apoptosis in sympathetic neurons involves a biphasic activation of JNK and up-regulation of tumor necrosis factor-alpha-converting enzyme/ADAM17. *J Biol Chem* 285: 20358–68

Krach F, Batra R, Wheeler EC, Vu AQ, Wang R, Hutt K, Rabin SJ, Baughn MW, Libby RT, Diaz-Garcia S, *et al* (2018) Transcriptome–pathology correlation identifies interplay between TDP-43 and the expression of its kinase CK1E in sporadic ALS. *Acta Neuropathol* 136: 405–423

Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C & Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell* 10: 839–50

Maimon R, Ionescu A, Bonnie A, Sweetat S, Wald-Altman S, Inbar S, Gradus T, Trotti D, Weil M, Behar O, *et al* (2018) miR126-5p Downregulation Facilitates Axon Degeneration and NMJ Disruption via a Non-Cell-Autonomous Mechanism in ALS. *J Neurosci* 38: 5478–5494

Manns RPC, Cook GMW, Holt CE & Keynes RJ (2012) Differing semaphorin 3A concentrations trigger distinct signaling mechanisms in growth cone collapse. *J Neurosci* 32: 8554–9

Rotem N, Magen I, Ionescu A, Gershoni-Emek N, Altman T, Costa CJ, Gradus T, Pasmanik-Chor M, Willis DE, Ben-Dov IZ, *et al* (2017) ALS Along the Axons – Expression of Coding and Noncoding RNA Differs in Axons of ALS models. *Sci Rep* 7: 44500

Söderberg O, Leuchowius K-J, Gullberg M, Jarvius M, Weibrecht I, Larsson L-G & Landegren U (2008) Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. *Methods* 45: 227–232

Wu KY, Hengst U, Cox LJ, Macosko EZ, Jeromin A, Urquhart ER & Jaffrey SR (2005) Local translation of RhoA regulates growth cone collapse. *Nature* 436: 1020–1024

1st Revision - Editorial Decision 5th May 2021

Dear Eran,

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been seen by the three referees. As you can see below, they all appreciated the introduced changes and support publication here. They have only a few minor points to address. I am therefore very pleased to let you know that we will accept the manuscript for publication here. In addition to addressing the points raised by the referees there are also some editorial points that we need to sort out. You can use the link below to upload the revised version.

- Please add a Data Availability section instead the Availability of data and material section that you do have. The Data availability section is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

- "Competing Interests" Should be listed as Conflict of Interest

- Author contributions are missing for Ariel Ionescu, Gayster Alexandra, Natalia Shelestovich, Yarden Opatosky, Amir Dori, Sami Barmada and Martin Balastik.

- The Arimura et al. reference is listed twice in the reference list. Please also remove the html link

- The funding information should also be listed in the online submission system and included in the Acknowlegements section.

- Figures need to be supplied as separate files

- Figure callouts are missing to Supp. Fig. 1B and to the individual panels in Supp. Figs. 2,5,6 panels

- The appendix needs a ToC and the figures should be named 'Appendix Figure S#'. Please also correct callout in text. Supp. Fig 4 does not need the A panel label.

- Regarding the source data file provided: The source data needs to be split and uploaded as one PDF file per figure. Please also remove the statistic from the source data and add that to the figure legends.

- From looking at the source data I think that you need to add a splice mark to Figure 4D and 5C to indicate the removal of lane(s). Please also double check the other figures for this.

- For panels where you show replicate data for the experiment in the source data (like fig 4H and 5C) please indicate which blot was used in the figure.

- I think the source data blot is missing for Figure 5A

- Matthew Patrick Nelson is not listed as an author in the online submission system

- "Methods" needs correcting to Materials and Methods.

- I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Can you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- I also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

That should be all - Let me now if we need to discuss anything further. When you resubmit please also provided a point-by-point response also to the editorial points.

Congratulations on a nice paper!!

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 3rd Aug 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have done a great job at responding to the extensive list of comments from the three reviewers. Their rebuttal is thorough and results in increased confidence in the reported data. Although I still have reservations about using single axons/neurons as the experimental unit, I believe that the cohesion of the results across models is sufficient to believe the major findings of the paper. I am therefore happy to accept.

Referee #2:

The authors have thoroughly addressed all of the previous concerns and significantly revised their manuscript. I recommend acceptance for publication. There are just 2 minor corrections that need to be made:
1. lines 239-241 on p.9 mention that peptide 4 blocked the CRMP4-dynein interactionm, but not dynactin. However, there is no data shown for the effects of peptide 4 alone on the CRMP4-dynein interaction.
2. Could the authors use a darker exposure for the blot in Fig. 4H? It is very difficult to see the coIP bands.

Referee #3:

The authors did an outstanding amount of work to answer the many comments of all reviewers, and should be congratulated for this important effort.
The new data, especially the new controls added, as well as some of the new experiments performed in response to reviewers 1 and 2 now provide a very convincing body of evidence that CRMP4 could mediate a retrograde toxic signal.

I have noticed a typo (line 153: C9otf72), and would advise that the authors indicate the molecular weights markers on their western blot according to the EMBO guidelines.

Response to Editor and Reviewers and of Maimon et al; (EMBOJ-2020-107586)

General responses

We were pleased to see that the editor as well as each of the three reviewers found the work appropriate for publication in *EMBO Journal.* We want thank the editor and the reviewers for practical and quick process. Please find our further corrections appended below (editors'/reviewers' comments are in blue, our responses in black):

Editorial comments:

Please add a Data Availability section instead the Availability of data and material section that you do have. The Data availability section is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements.

We added it:

This study includes no data deposited in external repositories. All data generated or analyzed during this study are included in this published article.

"Competing Interests" Should be listed as Conflict of Interest

Done.

Author contributions are missing for Ariel Ionescu, Gayster Alexandra, Natalia Shelestovich, Yarden Opatosky, Amir Dori, Sami Barmada and Martin Balastik.

We added author contributions for missing authors

The Arimura et al. reference is listed twice in the reference list. Please also remove the html link

We deleted it.

The funding information should also be listed in the online submission system and included in the Acknowlegements section.

We changed it:
This work was supported by IsrALS Foundation, the Israel Science Foundation (735/19), and the European Research Council (grant number 309377) to E.P, Czech Health Research Council

grant no. NV18-04-00085 to MB, Czech Science Foundation grant no. 21-24571S to MB and RW, and Grant Agency of the Charles University grants no. 524218 to RW.

Figures need to be supplied as separate files

We separated it.

Figure callouts are missing to Supp. Fig. 1B and to the individual panels in Supp. Figs. 2,5,6 panels

We added.

The appendix needs a ToC and the figures should be named 'Appendix Figure S#'.

We changed the name.

Please also correct callout in text. Supp. Fig 4 does not need the A panel label.

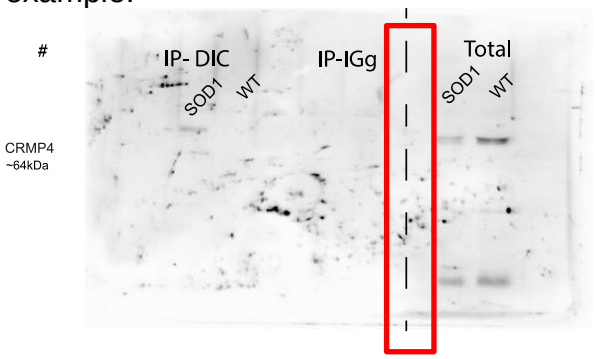
We changed it.

Regarding the source data file provided: The source data needs to be split and uploaded as one PDF file per figure. Please also remove the statistic from the source data and add that to the figure legends.

Done.

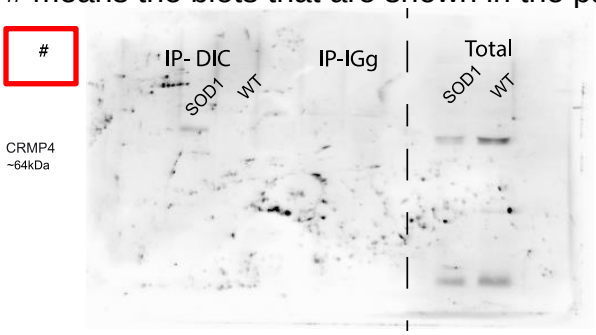
From looking at the source data I think that you need to add a splice mark to Figure 4D and 5C to indicate the removal of lane(s). Please also double check the other figures for this.

We added a splice mark that indicate the removal of lanes for these blots, see as example:



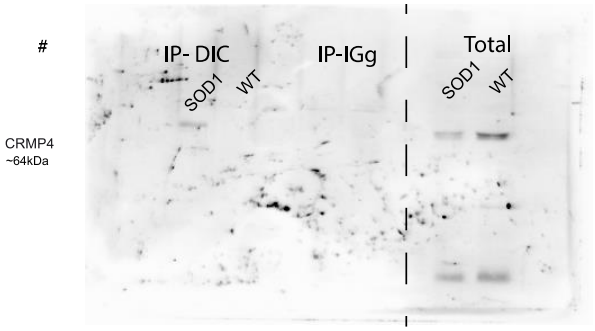
For panels where you show replicate data for the experiment in the source data (like fig 4H and 5C) please indicate which blot was used in the figure.

means the blots that are shown in the paper, see as example:



I think the source data blot is missing for Figure 5A

We added it:



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We changed it.

"Methods" needs correcting to Materials and Methods.

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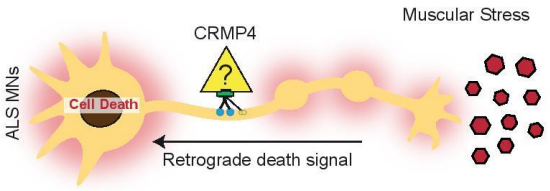
I have asked our publisher to do their checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Can you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- CRMP4 protein level is altered along ALS diseased motor unit
- Dynein mediates CRMP4 mis localization in motor neurons via specific CRMP4 motif.
- CRMP4-dynein complexes are enhanced in ALS Diseased MNs.
- CRMP4-dynein complex formation facilitates selective neuronal loss in ALS.

I also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

Identification of an intracellular mechanism that mediates motor neuron death in ALS. CRMP4 binds the motor protein dynein and transports from distal axons to the soma where it prompts motor neuron death. Blocking CRMP4-dynein interaction profoundly reduces motor neuron death both *in vitro* in human-derived MNs (C9orf72), and *in vivo* in ALS mice.



That should be all - Let me now if we need to discuss anything further. When you resubmit please also provided a point-by-point response also to the editorial points.

Congratulations on a nice paper!!

Reviewers comments:

Referee #1:

The authors have done a great job at responding to the extensive list of comments from the three reviewers. Their rebuttal is thorough and results in increased confidence in the reported data. Although I still have reservations about using single axons/neurons as the experimental unit, I believe that the cohesion of the results across models is sufficient to believe the major findings of the paper. I am therefore happy to accept.

Thanks you.

Referee #2:

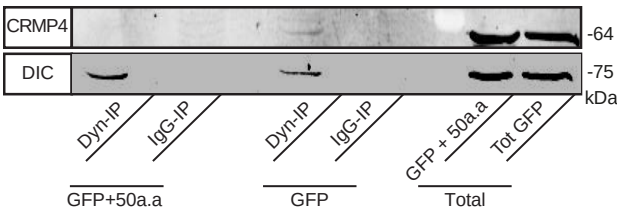
The authors have thoroughly addressed all of the previous concerns and significantly revised their manuscript. I recommend acceptance for publication. There are just 2 minor corrections that need to be made:

1. lines 239-241 on p.9 mention that peptide 4 blocked the CRMP4-dynein interactionm, but not dynactin. However, there is no data shown for the effects of peptide 4 alone on the CRMP4-dynein interaction.

We changed it to Dynactin.

2. Could the authors use a darker exposure for the blot in Fig. 4H? It is very difficult to see the coIP bands.

H



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The new data, especially the new controls added, as well as some of the new experiments performed in response to reviewers 1 and 2 now provide a very convincing body of evidence that CRMP4 could mediate a retrograde toxic signal.

I have noticed a typo (line 153: C9otf72), and would advise that the authors indicate the molecular weights markers on their western blot according to the EMBO guidelines.

We changed it:

H

2nd Revision - Editorial Decision 28th May 2021

Dear Eran,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Prof. Eran Perlson
Journal Submitted to: EMBO Journal
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs 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 and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 $\chi 2$ 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For in vitro experiments at least 3 microfluidic chambers in each condition with at least 10 cell bodies/ axons from 3 independent biological repeats.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For in vivo experiment we used at least three mice from each group. In each experiment we used at least 10 cell bodies/ axons / NMJs/ sections from 3 independent biological repeats.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	no oulier-identification was perfomed.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	no applicable.
For animal studies, include a statement about randomization even if no randomization was used.	Samples (mice) were predetermined by their genotype. Samples from human participants were predetermined by the clinical diagnosis.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Blinding was only applicable in the analysis of CRMP4 in the spinal in ALS and non ALS patients (Figure 1A). In the rest, blinding was not applicable due to insufficient manpower. For every experiment, analysis was perfomed by the same person who obtained the data.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The mice were taken for experiment after preforming genotype and were not assesed blindly.
5. For every figure, are statistical tests justified as appropriate?	Yes, all statistical were preformed by Prism Graphpad V.8.0, Perseus V.1.6.2.3.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, all statistical were preformed by Prism Graphpad V.8.0, Perseus V.1.6.2.3 and checked all the assumptions for the statistical test by that software.
Is there an estimate of variation within each group of data?	ROUT method; Q<1%.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	yes, most of the time the variance in each group was similar in the statistical tests.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	CRMP4 (Millipore- AB5454), DIC (Millipore- MAB1618) , p150 (BD Bioscience- 611003), Flag (Sigma-Alderich F3165), Tubulin (Abcam- ab7291), IgG (Santa Cruz- SC-2025), tERK (Sigma-Alderich M5670), NFH (Abcam ab72996/ Covance smi31p/ Covance smi32p), GFP (Abcam ab13970), NeuN (Milipore- MAB377), Tau (abcam- ab80579), PlexinA1 (Alomone lab- APR-081-F), GAPDH (abcam-ab9484), HB9 (IMGENEX, IMG-6549A). All antibodies were prevalidated by manufacturer, as stated in the supplied antibody data sheet and QA certificate.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	COS-7 cells from ATCC : COS-7 (ATCC® CRL-1651). Cell lines are routinly tested for mycoplasma, and were found to be negative. HEK Pro-AAV from Mike Fainzilber's lab from Weizmann instetute, originally from Takara-Clontech, catalog number is: 632273. Cell lines are routinly tested for mycoplasma, and were found to be negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	SOD1G93A (Stock No. 002726) mice were originally obtained from Jackson Laboratories, and maintained by breeding with C57BL/6J mice. SOD1 mice were injected at p60 ans dacrrefied at p90. B6;129S6-ChATtm2(cre)Lowl/J (Stock No. 006410) and B6;129S6Gt(ROSA)26 Sortm14(CAG−tdTomato)Hze/J (Stock No. 007908) mice were originally obtained from Jackson Laboratories. Animals were cross-bred in the Tel-Aviv SPF animal unit to yield homozygous ChAT::RosatdTomato mice. The ChAT::RosatdTomato colony was maintained by in-breeding males and females from the colony. The ChAT::RosatdTomato colony was cross-bred with SOD1G93A to yield SOD1G93A/ChAT::tdTomato mice. C57BL/6 J mice were used as a WT mouse strain. Mice were genotyped using the PCR reaction (KAPA Bio Systems - Wilmington, MA, USA). DNA samples were generated from mouse ear or tail.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were performed under the supervision and approval of the Tel-Aviv University Committee for Animal Ethics.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	no applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All clinical and muscle biopsy materials used in this study were obtained with written informed consent during 2016-2020 for diagnostic purposes followed by research application, approved by the institutional review board.
12. 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.	All clinical and muscle biopsy materials used in this study were obtained with written informed consent during 2016-2020 for diagnostic purposes followed by research application, approved by the institutional review board.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	no applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	no applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	no applicable
16. 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.	no applicable
17. 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.	no applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	no applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	no applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	no applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	no applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no applicable