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ENGRAILED-1 transcription factor has a paracrine neurotrophic activity on adult spinal α-motoneurons

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The present work focuses on Engrailed 1 (En1), a homeoprotein that is expressed in spinal V1 interneurons that connect to α-motoneurons (MNs). The authors studied its role in neuromuscular strength and MN retention and loss with the aid of different approaches. First, they studied its expression in spinal cord with RNAscope, a novel ISH method that makes possible to detect biomarkers that would be otherwise difficult to study with traditional ISH techniques. They then delivered 86/8 and LSBio anti-En1 antibodies, that catch En1 in the cleft and prevent it from being captured by MNs; moreover, they used a heterozygotic En1 mouse model to reduce En1 levels. The behavioral assessment, studied with grip strength, inverted grip test and hindlimb extensor reflex, showed motor alterations, paralleled by α-MNs loss and with an even stronger phenotype in the heterozygotic mice. This phenotype, however, appeared weeks before the MN loss, so they used NMJ assessment to determine what it seems to be a retrograde degeneration. En1 administered intrathecally was effectively internalized by the MNs and led to a long-term amelioration of the motor impairments and renervation of the NMJ, that needed to be boosted after 12 weeks for a stable therapeutic effect. Finally, heterozygotes revealed also a degeneration in dopaminergic neurons within midbrain similar to the one observed in spinal MNs, along with an upregulation of SQTSM1/p62 gene/protein, a factor in MN ageing linked to the classical genes implicated in familial forms of ALS (SOD1, TDP-43, FUS, and C9ORF72). They authors did not observe degeneration in V1 interneurons. They conclude that En1 might have a role in regulating MN ageing in degenerative motor disorders.

2. Significance:

Significance (Required)

Overall, the manuscript is well written however, some of the data appears too preliminary for publication. While the potential beneficial effect of En1 intrathecal administration looks promising and worth of publication, it is difficult to understand the mechanism of action. Some of the results are puzzling and require further investigations.

Major comments:

It is unclear why levels of intensity for RNAscope were not quantified, and qPCR was preferred for quantifications in Figure 1b. RNAscope is a technique that allows for spatial distribution analysis of the markers and their level of the expression. This data can be easily quantified utilizing the QuPath software which is open access. Same concerns apply to Figure 2a.

Antibodies should be validated utilizing a reporter mouse. En1cre mice are

commercially available and can be crossed with reporters (TdTomato or YFP mice). Utilizing this tissue En1 antibodies can be easily validated. The EN1 antibody shown in Figure 1c seems unspecific, staining several neuronal populations in the spinal cord.

Investigations of En1 expression in motor neurons from already available omics data sets would support the idea that En1 is expressed in motor neurons.

Differentiation between Gamma and Alpha motor neurons should be performed using specific markers as Err3, Wnt7a or NeuN.

How can the authors explain the lack of loss of En1 interneurons in the En1-Het mice? Do spinal En1 interneurons show any signs of apoptosis (e.g., cleaved caspase 3 marker)? Which levels of the spinal cord were used for interneuron quantifications? Segments between L1 and L3 would be preferable.

The set of experiments reported in Figure 4 is of difficult interpretation without showing the actual presence of extracellular En1, that could be assessed with protein detection or RNAscope.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Engrailed-1 does not act only in a cell-autonomous way in neural development, but also has non-cell-autonomous functions. These functions depend on the release of this homeoprotein which has been characterized in much detail by previous work of this group. In this paper, they show that EN-1 is expressed in spinal V1 interneurons, both on the RNA and on the protein level. In spinal motoneurons, EN-1 protein but not RNA is detected. Neutralization of extracellular EN-1 with a secreted antibody apparently blocks transfer from these interneurons to motoneurons and causes motoneuron disease symptoms. A similar phenotype is also observed in EN-1 +/- mice. Most importantly, the authors also demonstrate that intrathecal injection of EN-1 into EN-1 +/- mice restores loss of muscle strength and prevents motoneuron death. The authors also show that the autophagy modulator SQTSM1/p62 is expressed at elevated levels in EN-1 +/- mice and in mice after injection of the EN-neutralizing antibody. Since p62 expression also seems to be increased in general during aging in motoneurons, the authors conclude that EN-1 from spinal V1 interneurons is a regulator of motoneuron aging. In general, most of the experiments shown in this study are well done and convincing. However, the data on p62 upregulation appear correlative and do not allow any conclusions about the mechanism and function how EN-1 modulates motoneuron survival and function. In addition, this study is not very precise on the mechanisms how motoneurons degenerate in this model so that there are only limited insights into the way how EN-1 acts on motoneurons in a physiological manner and under pathophysiological conditions.

Specific points of criticism:

1. In Fig. 2a, the authors show that EN-1-positive interneurons are not reduced at 4.5 months in the spinal cord. No data are shown for later time points such as 9 months, the corresponding stage when motoneuron loss is observed, or at 16 months which corresponds to the data shown in Fig.1. The argument that there is no reduction of V1 interneurons between 4.5 months and 16 months because there is no decrease of EN-1 expression between 4.5 and 16 months, as shown in Fig. 1b is not convincing. EN-1 expression could change in individual cells, thus compensating for the loss. Data on numbers of EN-1-positive cells at 9 and 16 months should be included, and a potential autocrine effect of EN-1 on V1 interneurons, as observed in midbrain dopaminergic neurons, characterized in more detail.
2. In Fig. 2e, the authors present data on loss of muscle strength between 4.5 and 15.5 months. They conclude that this reflects gradual neuromuscular strength loss. Since neuromuscular endplates have a very high safety factor, they can maintain full function even if transmitter release is reduced by more than 80%. Therefore, the loss of muscle strength seems to reflect the progressive loss of presynaptic terminals at neuromuscular endplates, rather than a gradual loss of neuromuscular strength.
3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced

localization of p62 at neuromuscular endplates?
4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.
5. The authors present evidence for a glycosaminoglycan (GAG) binding domain that appears responsible for uptake of EN-1 into motoneurons. However, it is unclear into which cellular compartment EN-1 is taken up after GAG binding on motoneurons. The authors propose this could be an alternative pathway to conventional endosomal uptake. How can the EN-1 that is taken up into cells exert transcriptional effects in motoneurons? As a minimum, more data on the subcellular distribution of endocytosed EN-1 should be included to support current hypotheses and to close the gap from cellular uptake to transcriptional regulation.
6. The differences in p62 expression with age in WT and EN-1 +/- mice as shown in Fig. 8c are not convincing. First, the $p = 0.0499$ and $p = 0.0536$ values for differences at 3-4 months of age appear borderline, and it is unclear what the dispersion analysis that is shown really means. Moreover, the question remains how a potential dysregulation of p62 then affects NMJ morphology and function. Is this change in p62 also detectable in presynaptic compartments?
7. Is there any molecular evidence that EN-1 modulates the p62 gene promoter directly? What is the argument to assume that increase in SQTSM1/p62 expression and dispersion is an indicator of aging? The mean intensity, if I understand Fig. 8c correctly, does not significantly increase, it is only the dispersion that changes. In general, the data shown in Fig. 8c are hard to read and interpret. For example, in the right panel, the difference between the dispersion in 4.5 and 9 month old EN +/- mice is indicated as $p = 0.06$, but marked with 4 stars. The presentation of these data should be changed to make them clearer.

Referees cross-commenting

I agree with all comments from the other reviewers

2. Significance:

Significance (Required)

This study expands previous work of the authors, in particular work that has been performed and published on the effects of EN-1 on mesencephalic dopaminergic neurons. If adequately revised, it could make an interesting contribution to the general understanding how spinal V1 interneurons act on funcitonality and survival of spinal motoneurons.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This is an interesting and provocative manuscript reporting non-cell autonomous trophic activities of a homeobox protein, a concept pioneered by Dr. Prochiantz since many years ago. The study involves a significant amount of experimental work and the authors are to be congratulated by the scope and ambition of their study. Given previous studies by this laboratory on EN-1 functions in midbrain dopaminergic neurons, the concept advanced in the present paper is not entirely novel, although it is indeed interesting to find EN-1 activities in motoneurons; these were unexpected. Given that this is a non-cell-autonomous effect (EN-1 is made and released by neurons adjacent to MNs), it would have been interesting to explore the conditions under which EN-1 synthesis, release and effects are regulated, whether by lesion, degeneration, etc. But that may be something the authors wish to leave for a future report. It is welcome that an effort was put into trying to mechanistically understand how these trophic effects are mediated. This reviewer understands that this is a major undertaking. Nevertheless, the connection between EN-1 and p62 is not well developed by the data

presented and future readers may be left with many questions regarding how EN-1 and p62 are related (e.g. direct interaction? transcriptional regulation?), whether p62 is indeed the mediator of EN-1 trophic effects, or the significance of the increased levels of p62 for motoneuron disease. In its present form, this paper will be welcome, if nothing else by the provocative ideas that it advances. For this, it clearly deserves to be published in a good journal (whatever that means these days). Here below are a few questions and suggestions which the authors may want to take into consideration.

Figure 1C: There appears to be EN1 immunoreactivity (green) in several areas of the spinal cord, including dorsal regions. Can the authors clarify what that labeling could be representing?

Figure 1D: These immunoprecipitation results lack a negative control with irrelevant antibody to confirm that the band shown it's being recognized specifically by the antibodies reacting with the blot.

Figure 1E: The intensity of the EN1 labeling in MNs, much stronger than in V1 interneurons, is intriguing, given that MNs do not express engrailed-1 mRNA. One would have expected the opposite. It may help here if it was possible to show that immunoreactivity in MNs is diminished in the het mutant mouse.

Figure 2D: There are a few possible problems with these data and their interpretation. First, this reviewer feels that 5 neurons (y-axis) is a rather small number. Are these 5 neurons per what area? From how many mice? I did not find that information in the figure legend. A larger area should be quantified so that we get numbers that are more robust. Second, such differences could also be due to hypotrophy of the MNs, namely, that MN number is the same but they are smaller.

Figure 3A: It would be useful that the authors explain how these AChR clusters were defined, visualized and counted. I could not find this information in the Methods. Perhaps this could be done by showing an alpha-BTX image illustrating the clusters.

Figure 3B: As each adult endplate is only innervated by one MN, one would have expected fewer clusters and/or endplates, if indeed MNs are missing in this mouse, rather than endplates that are partially occupied. This could be clarified a bit more explicitly.

Figure 6B: Would not be better to do this with a virus, like in the case of the antibody? A more robust effect on MN survival may be attainable and thus strengthen the concept.

Figure 7A: The protein seems to be mainly in the cytoplasm of those cells (nuclei are dark and unlabeled), which is also unusual for a transcription factor that functions in the nucleus. Also surprising that the protein is gone in 3 days, but has effects over 24 weeks. Any explanation for that?
Figure 7B: It's not clear what the blue and red bars mean, as this is not explained in the legend. Also, the y-axis says "%Chat+" suggesting they are counting MNs, but in the text they talk about EN-1 capture. If the latter, the y-axes should indicate % EN-1 over Chat, or something like that. In general, better figure legends would improve the experience

of the reader.

Statistical analyses: In principle, comparisons of data obtained in studies that involved two variable parameters (such as time and genotype/treatment) should be weighted by a 2-way ANOVA test, which is more stringent since more conditions are being tested simultaneously. Usually a t-test is reserved for a pairwise comparison in an experiment involving only two conditions of the same variable.

2. Significance:

Significance (Required)

see above

3. How much time do you estimate the authors will need to complete the suggested revisions:

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Revision Plan

Review COMMONS

Manuscript number: RC-2022-01632
Corresponding author(s): Alain Prochiantz and Kenneth Moya

1. General Statements [optional]

Goal of the study

Similar to several homeoproteins (HPs), EN1 has both cell-autonomous and non-cell-autonomous activities. All presently known *in vivo* non-cell-autonomous ENGRAILED functions are exclusively developmental. In the adult, we have reported that EN1 cell-autonomous expression by mesencephalic dopaminergic (mDA) neurons supports their survival as illustrated by their retrograde degeneration in the *En1* heterozygote (*En1*-Het) mouse. Accordingly, recombinant EN1 protein addressed to mDA neurons protects them in mouse and macaque Parkinson Disease models.

En1 is also expressed in adult spinal cord V1 interneurons that synapse onto ventral horn αMNs that retrogradely degenerate in Amyotrophic Lateral Sclerosis (ALS). Because V1 interneurons are among the various cell types implicated in ALS, we were eager to determine if they degenerate in the *En1*-Het mouse, as mDA neurons do.

We were surprised to find that this is not the case but, in contrast, that αMNs degenerate in this mutant. Because αMNs do not express EN1 and in view of its transduction properties found during development, we examined if EN1 was transported between V1 interneurons and αMNs, thus acting as a neurotrophic factor in the adult. Indeed, reducing EN1 import into MNs, via its extracellular neutralization, induces αMN retrograde degeneration. We conclude that EN1 in the adult mouse is a direct non-cell-autonomous survival factor for αMNs.

This led us to envisage that EN1 injected intrathecally could rescue the *En1*-Het phenotype. When such injections were done, successfully rescuing αMNs, we found a specific EN1 accumulation in MNs, suggesting the existence of EN1 binding sites at the MN surface. For 3 other transferring HPs (EN2, VAX1 and OTX2), the specificity of transfer to target neurons involves an interaction with glycosaminoglycans (GAGs). By homology, we identified within EN1 a sequence with predictable GAG-binding activity and found that a synthetic peptide harboring the same sequence interferes with the transfer specificity of EN1 for MNs.

In the long-term objective to use some homeoproteins as therapeutic agents, a line of research initiated with EN1 in rodent and Non-Human Primate models of Parkinson Disease, we needed to know the duration of the effect of EN1 in the spinal cord and observed that one intrathecal injection (1µg of protein) prolonged *En1*-Het αMN “health” for 12 weeks, a rather long period that could be renewed by a second injection. Although this suggested, as in the case of mDA neurons, an effect at the level of epigenetic marks, we decided to keep the exploration of this “epigenetic hypothesis” for future studies on MNs derived from human iPSCs with or without, specific fALS mutations.

We were, however, intrigued by the similarities in the response of mDA neurons and αMNs to *En1* hypomorphism and the rescuing effect of EN1 internalization. This led us to a bioinformatic study that allowed us to identify putative EN1 targets, among which *p62/SQTSM1* has important autophagosome/proteasome activities and is mutated in certain ALS patients. Our first analyses suggest that p62/SQTSM1 level in αMNs is an age marker and that EN1 rescuing activities is possibly associated with anti-aging properties.

Finally, we wish to thank the 3 referees for their careful evaluation of the manuscript. We see many issues that they have raised as legitimate and have tried to provide experimental or editorial answers. In contrast, some issues are presently addressed in the context of a future manuscript and we had rather not introduce these studies in the revised manuscript.

Below, please find the description of the revisions already introduced in response to the points raised by the referees (questions are recalled in *italics*). In addition to changes in the Figures, text modifications in the revised manuscript are in red.

2. Description of the planned revisions

In addition to changes in the Figures, text modifications in the revised manuscript are in red.

Referee # 1

Evidence, reproducibility and clarity

They then delivered 86/8 and LSBio anti-En1 antibodies, that catch En1 in the cleft and prevent it from being captured by MNs.

Perhaps we were not clear. We did not deliver the antibodies 86/8 and LSBio, we used them for western blots and immunohistochemistry (IHC) to identify EN1 and localize it. We delivered the third antibody, a single-chain anti EN1 antibody (scFvEN1), that captures extracellular EN1 and prevents it from being captured by MNs on the basis of the LSBio staining (Figure 4A-C).

Finally, heterozygotes revealed also a degeneration in dopaminergic neurons within midbrain similar to the one observed in spinal MNs, along with an upregulation of SQTSM1/p62 gene/protein, a factor in MN ageing linked to the classical genes implicated in familial forms of ALS (SOD1, TDP-43, FUS, and C9ORF72).

This is a fair comment/work description, that does not require answers.

Significance

Major comments:

It is unclear why levels of intensity for RNAscope were not quantified, and qPCR was preferred for quantifications in Figure 1b. RNAscope is a technique that allows for spatial distribution analysis of the markers and their level of the expression. This data can be easily quantified utilizing the QuPath software which is open access. Same concerns apply to Figure 2a.

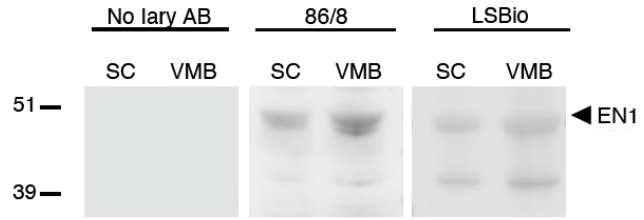
Quantitative RT-PCR provides a quantitative measure of gene expression. Since only V1 interneurons (including, Renshaw cells) express EN1, we infer the spatial distribution, although not expression level cell by cell. Figure 2A is an actual counting at 4.5 months of *En1+* cells and of *Calbindin+* cells (Renshaw cells), both identified by RNAscope. Thus, it is clear that the number of *En1*-expressing cells (V1 interneurons) is not modified at 4.5 months when muscle weakness and death of αMNs are well advanced (around 70% of the αMNs that will eventually die, are already gone). Long-term survival of V1 interneurons is further demonstrated in Figure 2D (left panel) until 15.5 months, (see also below) whereas total *En1* expression is reduced by half. Quantification neuron by neuron of the amount of *En1* transcribed (RNAscope) would indicate the variation, among interneurons, of *En1* transcription in WT and mutant mice. This is interesting *per se* but would not modify the main information that these neurons do not die in the heterozygote and that *En1* transcription does not decrease with time in both WT and mutant genotypes (at least until 15.5 months).

Antibodies should be validated utilizing a reporter mouse. En1cre mice are commercially available and can be crossed with reporters (TdTomato or YFP mice). Utilizing this tissue En1 antibodies can be easily validated. The EN1 antibody shown in Figure 1c seems unspecific, staining several neuronal populations in the spinal cord.

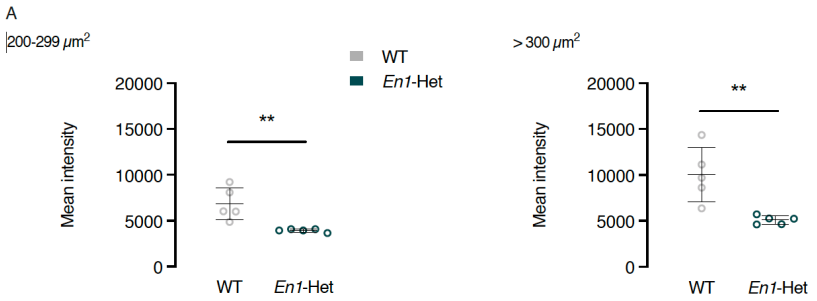
Indeed, antibody validation is extremely important. LSBio is commercial (CliniSciences), 86/8 was developed in the laboratory and fully characterized and used in previous studies (e.g. Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J*. **37**: e97374, 2018), scFv against EN1 was prepared from the 4G11 hybridoma (Developmental Hybridoma Bank, Iowa City, USA) and validated in previous studies (e.g. Wizenmann et al. *Neuron* **64**: 355-366, 2009). In the present study, the two polyclonal were further validated in several ways.

In the WBs we compared ventral midbrain (VMB) and spinal cord (SC) tissues and found similar patterns. Strong evidence for antibody specificity is immunostaining extinction with the antigen and with absence of first antibody, which we carried out.

We have now used LSBio and 86/8 to perform a WB on spinal cord (SC) and ventral midbrain (VMB) extracts with or without the first antibody and we find that the absence of first antibody fully eliminates band staining. The western blot is shown below and has been introduced in the revised manuscript in place of the cross immunoprecipitation.



Finally, we have quantified EN1 in the αMNs of the heterozygote at 3 months (before cell death), showing that EN1 content is decreased by approximately 2-fold (LSBio antibody) in both α and γMNs with no change in neuron number. This result demonstrating that EN1 is diluted by approximately twofold (concentration per neuron when all neurons are still present), in addition to further validating the antibody, is itself interesting and has been introduced in the revised manuscript as Supp. Fig. 1A. Supp. Fig. 1A:



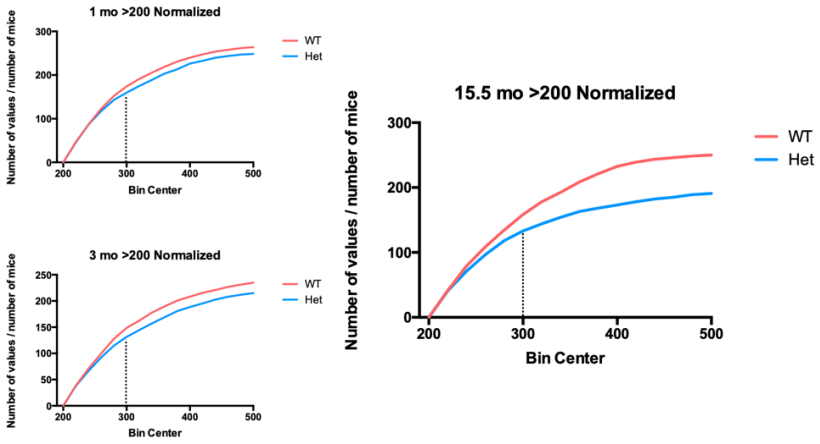
Regarding the staining in other neuronal populations, there is always some background, in particular in the tissue treatment conditions used for RNAscope. Furthermore, given the large number and wide distribution of V1 interneurons (Fig. 1A), we cannot preclude that EN1 is present at a low concentration in the extracellular space and in several cell types (discussed in Fig. 9 of the manuscript). This does not weaken the main conclusion that it primarily accumulates in MNs which do not express *En1* (RNAscope).

Investigations of En1 expression in motor neurons from already available omics data sets would support the idea that En1 is expressed in motor neurons.

The *En1* locus is silent in MNs. Microdissection of MNs and proteomic analysis would not be definitive since the interneurons that produce EN1 are in close vicinity of the MNs and since some protein is necessarily present in the extracellular space (where it is trapped by scFvEN1), making contamination unavoidable.

Differentiation between Gamma and Alpha motor neurons should be performed using specific markers as Err3, Wnt7a or NeuN.

This is a possible way to do the distinction, but size criterion in Cresyl violet is supported in the literature (Wu et al. *Journal of Biological Chemistry*, **287**: 27335-27344, 2012; Dutta et al. *Experimental Neurology*, **309**: 193-204, 2018). In our study, it is further validated by the demonstration that, in 9-month-old animals, the results obtained (cell number and specific death of large neurons >300μm^2, but not of intermediate size ones 200-299μm^2) are replicated by counting ChAT-stained neuron (Figure 2C). It is of particular interest that the number of medium size neurons (also ChAT-positive medium size MNs) does not increase when the number of large size (Cresyl and ChAT-positive) neurons decreases, thus precluding a "shrinkage effect". Most importantly, the size criterion (Cresyl violet) allows us not to be mistaken by a possible down-regulation of markers in the mutant, independently of cell survival. We provide for the reviewer but not for publication, the evolution with time of the number of neurons based on size (above 200 μm^2) showing clearly that at 15.5 months the large population (>300 μm^2) is decreased in the *En1*-Het, with very little change for neurons between 200 and 300 μm2, and certainly not an increase which would be expected if shrinkage occurred.



How can the authors explain the lack of loss of En1 interneurons in the En1-Het mice? Do spinal En1 interneurons show any signs of apoptosis (e.g., cleaved caspase 3 marker)? Which levels of the spinal cord were used for interneuron quantifications? Segments between L1 and L3 would be preferable.

We were indeed surprised by this finding and a plausible explanation is that a lower metabolic activity makes interneurons less sensitive to stress than αMNs which have to "fuel" long axons and high firing rates (not the case for γMNs). We propose this explanation in the discussion and make it clearer in our revised version. We agree that it is speculative and that the point raised by the reviewer is very interesting. We hope to address this in the future and have discussed this point.

Since the cells do not die, we did not look for signs of apoptosis.

We analyze lumbar sections from L1 to L5 as now indicated in the methods section in the manuscript

The set of experiments reported in Figure 4 is of difficult interpretation without showing the actual presence of extracellular En1, that could be assessed with protein detection or RNAscope.

This is another interesting suggestion, but we think that it will be difficult to distinguish low extracellular staining due to EN1 diffusion from some unspecific background. Since the scFvEN1 is secreted by astrocytes, it necessarily neutralizes extracellular EN1, resulting in a decrease in the MN content of the protein. This is an experiment with high specificity since the same scFv harboring a Cysteine to Serine point mutation that prevents EN1 recognition (no disulfide bound formation between the light and heavy chains) does not block EN1 capture by MNs (Fig. 4C for IHC and quantifications).

As for extracellular EN1 mRNA identified by RNAscope, we hesitate to embark on the idea as mRNAs are likely secreted in insufficient amounts to be identified, even by RNAscope. The results that we have (no *En1* visible by RNAscope in MNs, loss of EN1 in MNs following extracellular scFvEN1 activity, and preferential addressing of injected EN1 to MNs) demonstrate EN1 capture by MNs. Indeed, we cannot completely preclude the transfer of tiny amounts (escaping RNAscope detection in MNs) of *En1* mRNA (for example, through extracellular vesicles), but we plead for not considering this

hypothesis in the present paper. However, if the reviewer wishes, the possibility can be introduced in the discussion.

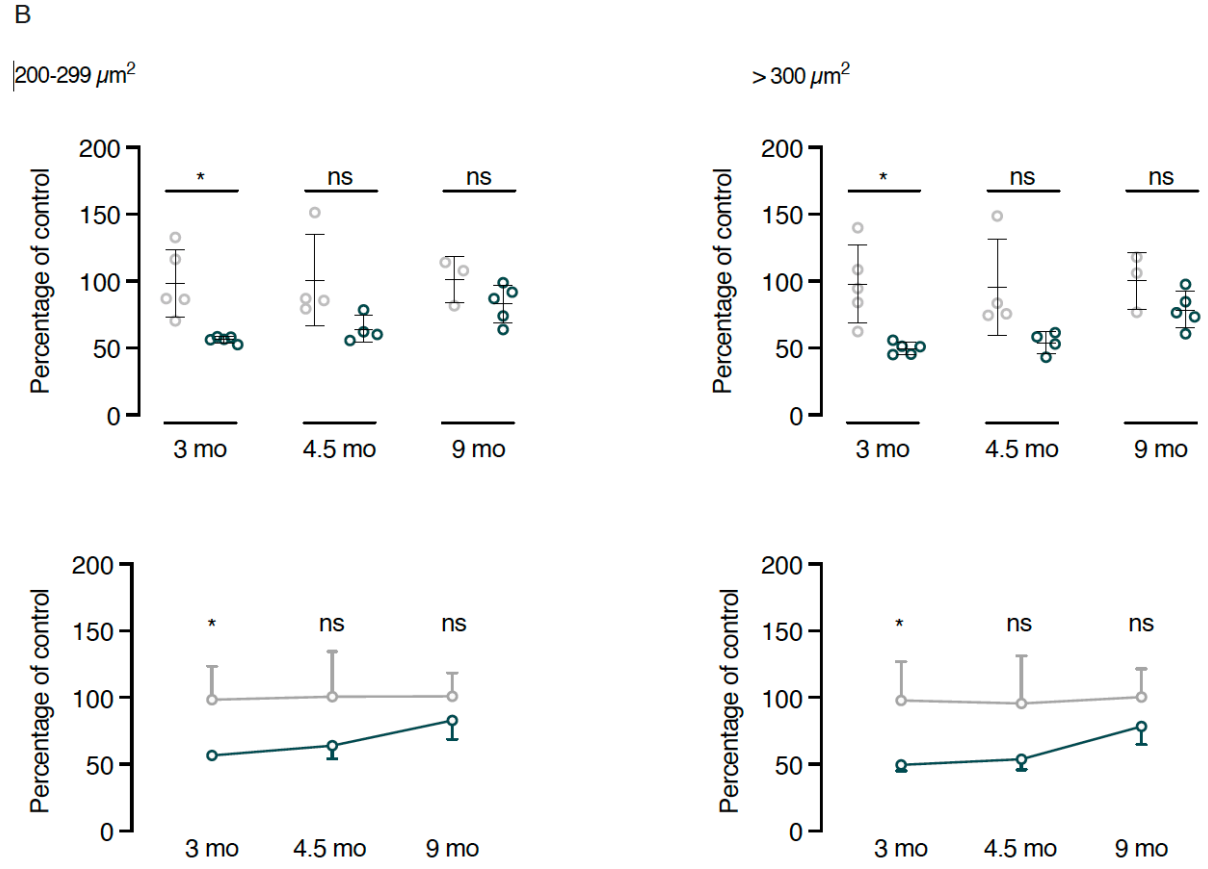
Referee 2

Evidence, reproducibility and clarity

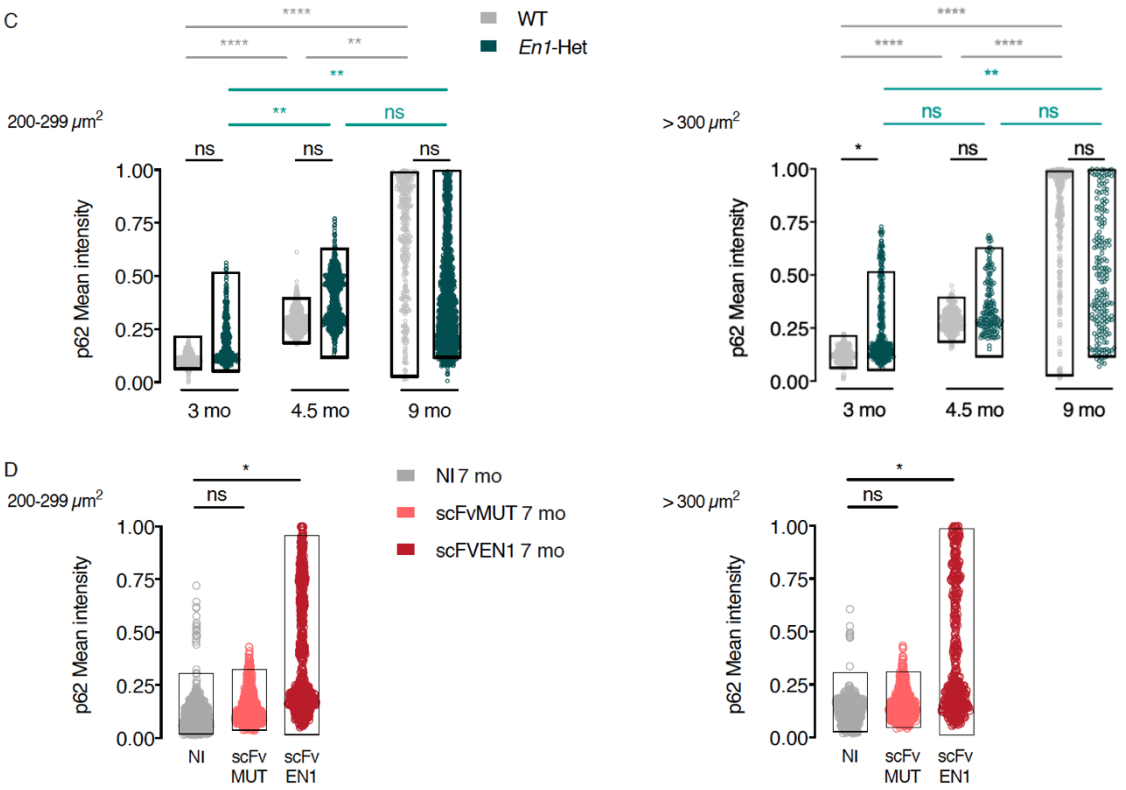
In general, most of the experiments shown in this study are well done and convincing. However, the data on p62 upregulation appear correlative and do not allow any conclusions about the mechanism and function how EN-1 modulates motoneuron survival and function. In addition, this study is not very precise on the mechanisms how motoneurons degenerate in this model so that there are only limited insights into the way how EN-1 acts on motoneurons in a physiological manner and under pathophysiological conditions.

This criticism is justified, at least in part, as we agree that p62 upregulation is correlative. However, the fact that the neutralization of extracellular EN1 by the scFv increases p62 expression, is in favor of a causative link. The increase is also seen at 3 months in the *En1*-Het when all αMNs are still present but not after, which is interesting because, due to αMNs death, surviving MNs receive more EN1, information provided below and now introduced and discussed in the revised manuscript (Supp. Fig. 1B).

Supp. Fig. 1.B:



As for p62, and as also mentioned by referee 3, Fig. 8 is very hard to follow and we propose to simplify it to make the message clearer:
Below is the revised Fig. 8C, D in which we focus exclusively on SQTSM1/p62 mean expression:
Revised Fig. 8C, D:



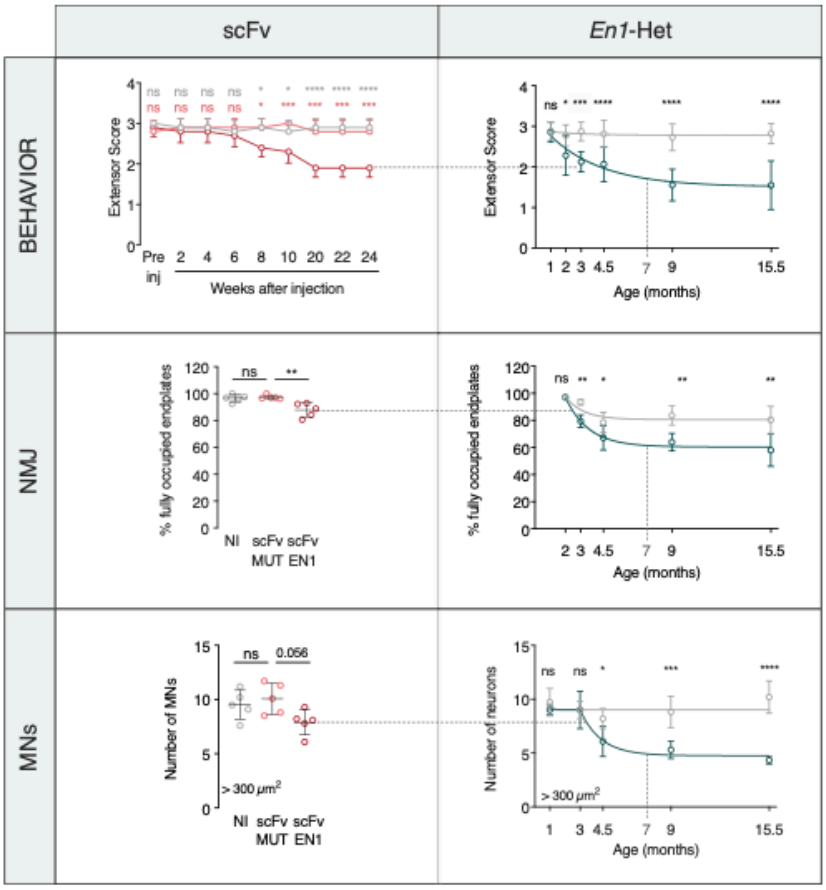
In this new figure, the main information is that mean p62 expression increases with time in WT α and γMNs, and can be seen as an aging marker.

A second information is that a difference in mean p62 expression between WT and Het is seen only at 3 months in αMNs. For αMNs, we propose that this is due to the fact that they are very sensitive to EN1 dosage (in contrast with γMNs which do not die in the *En1*-Het). At 3 months, αMNs have only half of their normal EN1 content. Later, at 4.5 months 75% of the αMNs bound to die are already dead (Fig. 2D) and the remaining neurons receive more EN1 (even more so at 9 months), as could be measured (see above Supp. Fig. 1B). We thus can propose an accelerated aging of αMNs at 3 months due to both EN1 decrease and high metabolic activity (higher than in γMNs).

In the case of the scFv, scFvEN1, but not the mutated version induces enhanced mean p62 expression in the 80% surviving αMNs and in γMNs at 7 months (low αMN death in this model, see Fig. 4F). As can be seen also in a newly added figure (Supp. Fig. 2) that has been introduced in the revised manuscript and is shown below, 7-month-old scFv animals and 3- to 3.5-month-old *En1*-Het have similar phenotypes. This mild scFv phenotype (α-MN death and muscle strength loss) in 7-month-old mice in spite of a huge loss in the EN1 content of MNs (Fig. 4C) suggests that the *En1*-Het phenotype

is not entirely due to the decrease in EN1 transport from V1 interneurons to MNs (see discussion and Fig. 9).

Supp. Fig. 2 (new) comparing the phenotypes of *En1*-Het and of WT mice expressing scFv-EN1:



It remains true that we have voluntarily decided not to examine in depth the molecular mechanisms allowing EN1 to exert its protective activity, a decision that we would like to defend and maintain.

A first reason is that in previous papers on mesencephalic dopaminergic (mDA) neurons (Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J*. **37**: e97374, 2018), we evaluated several mechanisms involved in EN1 neurotrophic activity and we did not want this study to be a duplication of studies done on a different neuronal population, even if mechanisms might differ in part, between αMNs and mDA neurons. What has interested us more is that, in the two cases, age is an important factor in the unveiling of the degeneration phenotype (mDA neurons start dying at 1.5 months and αMNs at 3 months). It is because of this similarity that we performed the bioinformatic study that has led us to SQTSM1/p62. In this context, it is of interest that mean SQTSM1/p62 expression (variability of expression between neurons is not discussed in the revised version) increases with age in the wild type, thus can be seen as an age marker. It allows us to propose that EN1 extracellular neutralization and the loss of one *En1* allele, that increases mean SQTSM1/p62 expression accelerate aging.

A second reason is that the study is oriented toward a possible use of EN1 as a therapeutic protein. This orientation also has to do with the focus on SQTSM1/p62. Indeed, there are probably many pathways downstream of EN1, but in the bioinformatic analysis of genes differentially regulated in WT and *En1*-Het mDA neurons and also expressed in MNs, *SQTSM1*/*p62* is the only one that interacts with the 4 genes mutated in the major ALS familial forms. In addition, *SQTSM1/p62* mutations have been observed in ALS patients (References 41 to 45 in the manuscript).

Finally, the most important point is that the main message of this paper is the discovery of a non-cell autonomous EN1 activity in the spinal cord and of its ability to travel between V1 interneurons and MNs. This specificity best explained by a targeting signal that we have identified is at the basis of the specific addressing to MNs of EN1 intrathecally injected, which also has implications for its potential therapeutic use.

Specific points of criticism

1. In Fig. 2a, the authors show that EN-1-positive interneurons are not reduced at 4.5 months in the spinal cord. No data are shown for later time points such as 9 months, the corresponding stage when motoneuron loss is observed, or at 16 months which corresponds to the data shown in Fig.1. The argument that there is no reduction of V1 interneurons between 4.5 months and 16 months because there is no decrease of EN-1 expression between 4.5 and 16 months, as shown in Fig. 1b is not convincing. EN-1 expression could change in individual cells, thus compensating for the loss. Data on numbers of EN-1-positive cells at 9 and 16 months should be included, and a potential autocrine effect of EN-1 on V1 interneurons, as observed in midbrain dopaminergic neurons, characterized in more detail.

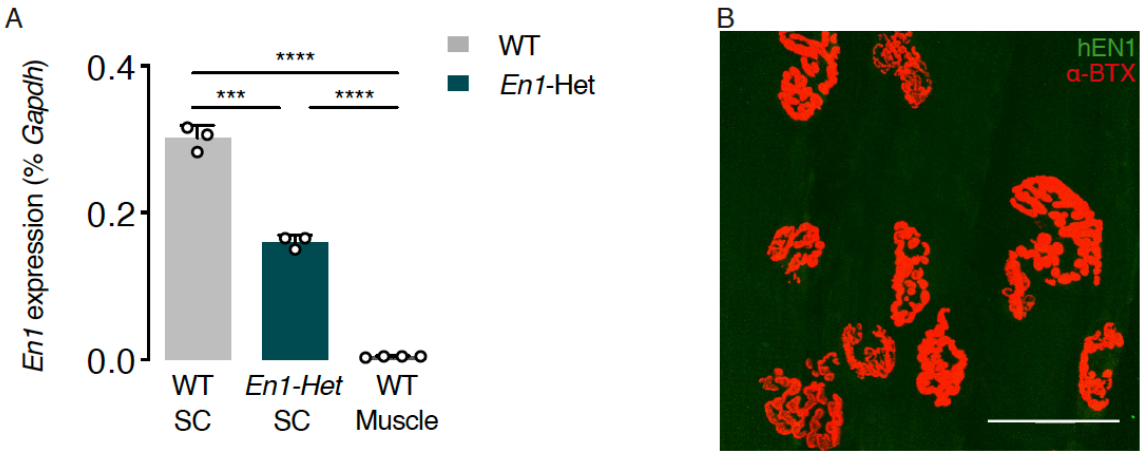
Fig. 2A illustrates the absence of interneuron loss at 4.5 months, but this set of data is completed by those of Fig. 2D that demonstrate the maintenance of V1 interneuron number until 15.5 months, at least. It can be noted that, in contrast with interneurons, αMNs at 4.5 months have experienced massive cell death (70% approx. of total αMN death at 15.5 months). As a whole, data of Fig. 2 demonstrate that the number of small neurons (100-199 μm^2) and intermediate size neurons (200-299 μm^2) does not change with age, at least through 15.5 months. This is in strong contrast with large αMNs (>300 μm^2). As already explained in our answers to referee 1, size is an excellent marker for the identification of neuronal subtypes and the analysis of survival (See answers to referee 1, justifying the use of neuron size).

2. In Fig. 2e, the authors present data on loss of muscle strength between 4.5 and 15.5 months. They conclude that this reflects gradual neuromuscular strength loss. Since neuromuscular endplates have a very high safety factor, they can maintain full function even if transmitter release is reduced by more than 80%. Therefore, the loss of muscle strength seems to reflect the progressive loss of presynaptic terminals at neuromuscular endplates, rather than a gradual loss of neuromuscular strength.

We apologize for the semantic confusion. What is measured is a progressive loss of muscle strength due to the progressive loss of presynaptic terminals and not a gradual loss of neuromuscular strength. This is now modified throughout the revised text.

3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced localization of p62 at neuromuscular endplates?

We do not see expression of *En1* mRNA or the presence of EN1 protein at the level of the endplate (Supp. Fig. 3).



4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.

SV2a and NF staining (it is not only SV2a) at the level of endplates identified by α-Bungarotoxin labeling has been used in a large number of studies (Wahlin et al. J. Comp. Neurol. 506: 822-837, 2008; Hasting et al. *Scientific Reports* **10**: 1-13, 2020; Yahata et al. *J. Neurosci*. **29**: 6276-6284, 2009 ; Jones et al. *Cell Reports* **21**: 2348-2356, 2017) Our goal was not to document the loss of synaptic activity through the use of the three suggested markers, Bassoon, Piccolo and Munc-13. Doing it would force us to initiate experiments taking several months to prepare the material and do a quantitative analysis in the models of EN1 loss of function (*En1*-Het) and neutralization (scFv), plus rescue by EN1. Nor do we wish to initiate a novel collaboration to produce a quantitative ultrastructural

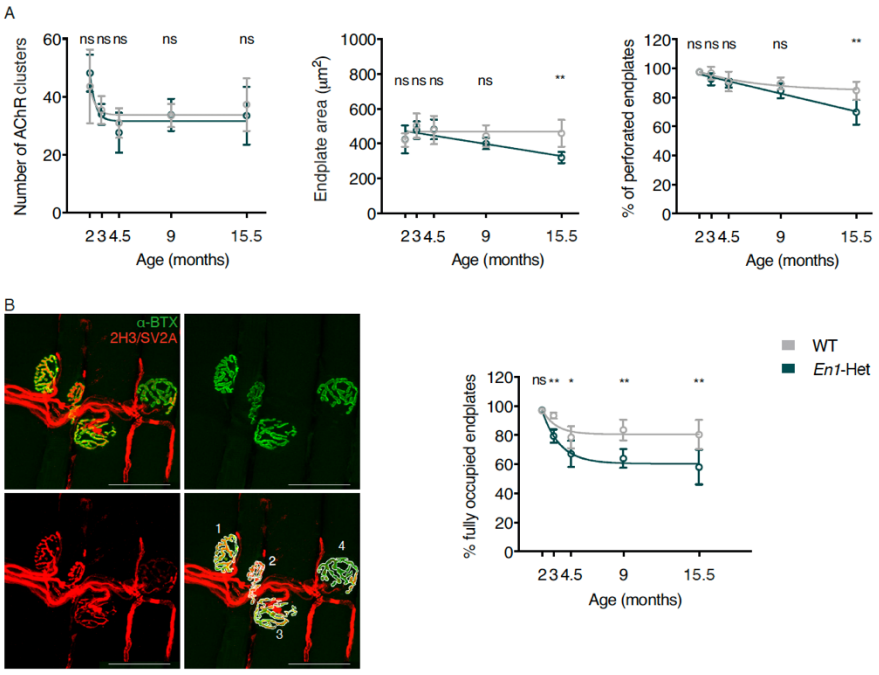
study. We see the latter morpho-functional studies beyond the scope of the manuscript and wish to be given the possibility to present them in a separate study (see below in "Description of the experiments that the authors prefer not to carry out").
The distinction between fully occupied, partially occupied and denervated endplates is not arbitrary and we apologize for not having sufficiently described the methodology. As illustrated in modified Fig. 3 and explained in Material and Methods, a fully innervated endplate is defined as an endplate in which 80% or more of the green pixels (α-BGT) are covered by a red pixel (SV2a), a partially one is between 20 and 80% and a denervated one below 20% coverage. Thus at 9 months and later ages, close to 30% of the endplates are either partially innervated or denervated. In fact, it is more likely that they are partially innervated since the number of AChR clusters does not change (totally denervated clusters normally dissolve). The 80% threshold for fully innervated was selected to give a margin of security, and it is likely that the percentage of 25 to 30% of partially innervated endplates is an underestimation.

Below is the mean calculations for WT and *En1*-Het mice at 3, 4.5, 9 and 15.5 months 9 months on the basis of the criteria explained above:

Grouped	Fully	Partially	Dennervated
3 mo WT	93.51000000	5.05300000	1.439000000
3 mo Het	79.39000000	17.64000000	2.964000000
4,5 mo WT	90.07000000	8.29100000	1.639000000
4,5 mo Het	73.82000000	16.71000000	8.898000000
9 mo WT	95.33000000	4.21100000	0.799400000
9 mo Het	75.47000000	16.74000000	7.794000000
15,5 mo WT	83.58000000	11.54000000	4.879000000
15,5 mo Het	63.95000000	25.08000000	10.970000000

From top to bottom in the table, we used 4, 4, 7, 6, 5, 5, 6 and 5 mice per condition.

Modified Fig.3:



We agree that we were not clear enough in our description and that it may have given the impression that the differences were relatively small. We think that retrograde degeneration is strongly supported by a loss of muscle strength that parallels the decrease in fully occupied endplates (α-BGT, NF, SV2a) and precedes αMN loss by more than 1 month. We have recently contacted an electrophysiology group to establish a collaboration that will allow us to follow functional changes at the level of the spinal cord and of the neuromuscular junction and we see the experiments proposed by the reviewer as complementary to these physiological approaches. Yet, we do not want to ignore the opinion of the reviewer and mention it in the conclusion, on the basis of his/her comment.

5. The authors present evidence for a glycosaminoglycan (GAG) binding domain that appears responsible for uptake of EN-1 into motoneurons. However, it is unclear into which cellular compartment EN-1 is taken up after GAG binding on motoneurons. The authors propose this could be an alternative pathway to conventional endosomal uptake. How can the EN-1 that is taken up into cells exert transcriptional effects in motoneurons? As a minimum, more data on the subcellular distribution of endocytosed EN-1 should be included to support current hypotheses and to close the gap from cellular uptake to transcriptional regulation.

The question is justified since we did not recall until page 12 of the Discussion that EN1 is, as most tested homeoprotein transcription factors, captured by a mechanism distinct from endocytosis. While not yet fully understood, the process involves the formation of inverted micelles that allow for direct targeting to the cytoplasm and from there to the nucleus thanks to the NLS. We now mention in the introduction that EN1 transfer and HP transfer is based on unconventional secretion and internalization processes.

6. The differences in p62 expression with age in WT and EN-1 +/- mice as shown in Fig. 8c are not convincing. First, the p = 0.0499 and p = 0.0536 values for differences at 3-4 months of age appear borderline, and it is unclear what the dispersion analysis that is shown really means. Moreover, the question remains how a potential dysregulation of p62 then affects NMJ morphology and function. Is this change in p62 also detectable in presynaptic compartments?

We agree that p values in the range of 0.05 are not extremely high and this is due to the heterogeneity in SQTSM1/p62 expression, that reflects that of MN populations, and induces a high variance. We also agree that this figure is too complicated and a simplified version has been proposed above (see answers to reviewer 1). To summarize, Fig. 8C shows that in WT animals, with no αMN death (grey) the level of SQTSM1/p62 expression in αMNs and γMNs increases between 3 and 4.5 months and between 4.5 months and 9 months, with significances varying between $p<0.01$ (**) and $p<0.0001$ (****). In *En1*-Het mice, the situation is more complex and we have to consider that 50% αMNs die (see above). However, expression increases between 3 and 9 months with $p<0.01$ (**) for WT and *En1*-Het neurons. SQTSM1/p62 can thus be taken as an age marker. Dispersion is a poor word for population heterogeneity for SQTSM1/p62 expression and it is clear that αMNs and γMNs do not constitute homogeneous populations and do not evolve similarly with time. As this is obvious, we have decided to focus only on mean levels of expression. Yet, the analysis of heterogeneity shows that it also increases between 3 and 9 months for WT and *En1*-Het mice with $p<0.0001$ (****) and this information can be reintroduced if requested.

The new Fig. 8 panel D (please see above, answers to referee 1) now includes the results obtained with the scFvs. A phenotype comparison between the two models (En1-Het and scFvEN1) has been introduced in Supp. Fig. 2 (see above)

7. Is there any molecular evidence that EN-1 modulates the p62 gene promoter directly? What is the argument to assume that increase in SQTSM1/p62 expression and dispersion is an indicator of aging? The mean intensity, if I understand Fig. 8c correctly, does not significantly increase, it is only the dispersion that changes. In general, the data shown in Fig. 8c are hard to read and interpret. For example, in the right panel, the difference between the dispersion in 4.5 and 9 month old EN +/- mice is indicated as p = 0.06, but marked with 4 stars. The presentation of these data should be changed to make them clearer.

We have no evidence that EN1 modulates the *SQTSM1/p62* promoter directly. The identification of this gene as a target (not necessarily a direct target) of EN1 comes from the bioinformatic analysis described in the manuscript and we were intrigued by the interaction with the 4 main familial ALS mutations and the existence of families with *SQTSM1/p62* mutations. This is what led us to analyze its expression in our two models of EN1 loss of function. Although the *En1*-Het mouse is not an ALS model, the results support the idea that EN1 could be used as a therapeutic protein in several familial and even sporadic forms of the disease. The latter hypothesis is now being tested on MNs derived from iPSCs (sporadic patients, fALS and isogenic variants, and healthy controls). If the data lend weight to our hypothesis, as collaborative and in-house preliminary data suggest, then a complete

analysis of EN1 targets in human MNs will be undertaken. Again, we really think that this is out of the scope of this study.

For Fig. 8, we fully agree that it can give headaches and we apologize. Moreover, it induces wrong interpretations (mean intensity increases with age and dispersion between 4.5 and 9 months has a calculated $p<0.0001$). We have now simplified it as suggested by the reviewer (see above answers to referee 1).

Referee #3

Evidence, reproducibility and clarity

Nevertheless, the connection between EN-1 and p62 is not well developed by the data presented and future readers may be left with many questions regarding how EN-1 and p62 are related (e.g. direct interaction? transcriptional regulation?), whether p62 is indeed the mediator of EN-1 trophic effects, or the significance of the increased levels of p62 for motoneuron disease

The reviewer is right and we have tried to better explain and to simplify. Please see responses to referees 1 and 2.

Figure 1C: There appears to be EN1 immunoreactivity (green) in several areas of the spinal cord, including dorsal regions. Can the authors clarify what that labeling could be representing?

Unfortunately, there is always some background staining, in particular in the tissue treatment conditions appropriate for RNAscope. Furthermore, given the large number and wide distribution of V1 interneurons (Fig. 1A), we cannot preclude that EN1 is present at a low concentration in the extracellular space and in several cell types (now represented in Fig. 9). This does not weaken the main conclusion that it primarily accumulates in MNs which do not express *En1* (RNAscope).

Figure 1D: These immunoprecipitation results lack a negative control with irrelevant antibody to confirm that the band shown it's being recognized specifically by the antibodies reacting with the blot.

Please see the response to reviewer 1 above with the Western blot and the absence of staining on a WB in absence of first antibody (86/8 or LSBio).

Figure 1E: The intensity of the EN1 labeling in MNs, much stronger than in V1 interneurons, is intriguing, given that MNs do not express engrailed-1 mRNA. One would have expected the opposite. It may help here if it was possible to show that immunoreactivity in MNs is diminished in the het mutant mouse.

We also were surprised by this intensity higher in MNs than in V1 interneurons, as if the protein was exported rapidly towards the target neurons. We have done the experiment proposed by the referee, found a twofold (approx.) immunoreactivity reduction in *En1*-Het MNs (see above Supp. Fig. 2A in answers to referee 2). This supplemental figure has been introduced in the revised version. The experiment was done at 3 months when no MN death has yet occurred. Later the neurons "replenish" with EN1, probably because they do not have to share the limited supply with the dead ones (see above answers to referee 2 and Supp. Fig. 2B).

Figure 2D: There are a few possible problems with these data and their interpretation. First, this reviewer feels that 5 neurons (y-axis) is a rather small number. Are these 5 neurons per what area? From how many mice? I did not find that information in the figure legend. A larger area should be

quantified so that we get numbers that are more robust. Second, such differences could also be due to hypotrophy of the MNs, namely, that MN number is the same but they are smaller.

At least 10 ventral horns (five lumbar spinal cord sections through L1-L5) separated by ≥ 900 µm were analyzed for each animal. As indicated in the legend of Fig. 2D, 5 to 6 mice were analyzed per condition. For each mouse, hundreds of cells were counted. For example, in a WT mouse P2464 4.5 months of age 558, 158 and 112 cells were counted in the 100-199, 200-299 and >300µm^2 classes, respectively (5 sections). In 4.5-month-old *En1*-Het mouse P2458, the values were 562, 149, and 66, respectively. The data in the graphs are the average number of each cell category in one ventral horn. So, the WT mouse had an average of 11.2 large MNs and the En1-*Het* mouse had an average of 6.6 large MNs. The methodology has been better described in Material and Methods and in the legends.

The differences cannot be attributed to hypotrophy. A first reason is that, at 9 months, the Cresyl violet and ChAT staining give the same results for medium size and large neurons (Fig. 2C). Furthermore, when one counts the cells throughout 15.5 months, the decrease in the number of large neurons is not compensated by an increase in the number of medium size or small ones. The reasoning and a graph, not intended for publication can be found in answers to referee 1.

Figure 3A: It would be useful that the authors explain how these AChR clusters were defined, visualized and counted. I could not find this information in the Methods. Perhaps this could be done by showing an alpha-BTX image illustrating the clusters.

We fully agree that the procedure was not well explained and we have introduced a correction in the Material and Methods section. For more details, please see answers to referee 2.

Figure 3B: As each adult endplate is only innervated by one MN, one would have expected fewer clusters and/or endplates, if indeed MNs are missing in this mouse, rather than endplates that are partially occupied. This could be clarified a bit more explicitly.

This is true and the ambiguity takes its origin in insufficient explanation of how fully innervated, partially innervated and denervated endplates were defined. Please see above and also in answers to reviewer 2. Modifications have been introduced in the text and in Fig. 3. The referee is right, the absence of change in the number of AChR clusters suggests that there are very few fully denervated endplates and that what is defined as such in the analysis corresponds to partially innervated endplates (see above). This is now discussed in the text.

Figure 6B: Would not be better to do this with a virus, like in the case of the antibody? A more robust effect on MN survival may be attainable and thus strengthen the concept.

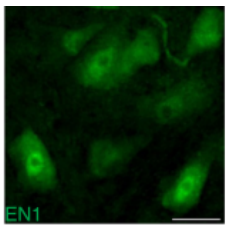
This would be another interesting experiment and we are presently exploring this possibility (with preliminary results). The choice of the virus and of the promoters is very important. We are comparing

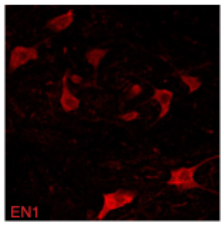
several AAVs, including AAV2, AAV2-TT (which diffuses better) and AAV8. For the promoter, we do not want to express within MNs as the imported protein might have special properties, associated with import. V1 interneurons would be best, but we have to verify if this does not modify V1 physiology. Astrocyte is another option, but with a similar pitfall. This means that we have a long way to go before proposing a "gene therapy" approach.

In addition, in the context of future clinical studies, we were eager, on the basis of the long-lasting activity of the protein already observed in the mesencephalic dopaminergic neurons (Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J*. **37**: e97374, 2018), to try a protein therapy in the spinal cord. Interestingly, the effects are also long-lasting in the spinal cord, (12 weeks in the mouse before a second injection is needed) and, according to contacted physicians, intrathecal injections, every second month or even more frequently, could be envisaged in the human. In that case, protein injection is possibly advantageous for the following reasons:
(i) viral particles can travel far and we do not know what would be the side effects.
(ii) the protein is short-lived but specifically addressed to MNs (thanks to the presence of EN1 binding sites at their surface), thus minimizing the issues associated with permanent expression and side effects.
(iii) EN1 is a natural protein normally secreted and the immune system might not be solicited as much as with viral approaches.

Figure 7A: The protein seems to be mainly in the cytoplasm of those cells (nuclei are dark and unlabeled), which is also unusual for a transcription factor that functions in the nucleus. Also surprising that the protein is gone in 3 days, but has effects over 24 weeks. Any explanation for that?

The protein is imported and is thus both in the cytoplasm where it exerts an effect on protein translation (Brunet et al. *Nature* **438**: 94-98, 2005; Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Yoon et al. *Cell* **148**: 752-764, 2012) and in the nucleus where it exerts its transcriptional and "epigenetic activity (see below for the latter). In fact, different antibodies and fixation procedures can favor cytoplasmic or nuclear staining. When nuclear, the dark point at the center, probably the nucleolus is less stained.
The two images below taken from Fig. 1 C (RNAscope fixation) and 1E ("normal" fixation) illustrate this point:





For the second part of the question, three days are sufficient for a long-lasting activity. This was also observed in the midbrain where the protein restores the epigenetic marks jeopardized by an acute oxidative stress (Rekaik et al. *Cell Reports* **13**: 242-250). This has led to the hypothesis that EN1 has

an important action at the level of the structure of the heterochromatin, thus a long-lasting "epigenetic" activity. We are presently working on the latter effects on the chromatin structure using human MNs derived from iPSCs (patients and control).

Figure 7B: It's not clear what the blue and red bars mean, as this is not explained in the legend. Also, the y-axis says "%Chat+" suggesting they are counting MNs, but in the text they talk about EN-1 capture. If the latter, the y-axes should indicate % EN-1 over Chat, or something like that. In general, better figure legends would improve the experience of the reader.

In this experiment, we wanted to test the presence of a GAG-binding domain in EN1. To test its potential role in EN1 internalization and localization, we co-injected or not the RK-EN1 with hEN1 protein. Then, we counted the percentage of MNs (%ChAT+) which contain, or not, the hEN1 protein (hEN1+ in red or hEN1- in blue), allowing us to verify if the RK-EN1 alters the internalization of the hEN1 protein. So yes, we are looking at the capture of EN1 by the MNs with or without the RK-peptide (or control peptides). We have modified the text to make the point clearer.

Statistical analyses: In principle, comparisons of data obtained in studies that involved two variable parameters (such as time and genotype/treatment) should be weighted by a 2-way ANOVA test, which is more stringent since more conditions are being tested simultaneously. Usually a t-test is reserved for a pairwise comparison in an experiment involving only two conditions of the same variable.

The reviewer is correct. The two-way ANOVA is explained in the Statistical analyses section of the Methods. The analyses were carried out and the results listed in the legends for Figs 2, 3, 4, 6 and Supp. Fig. 1.

3. Description of the revisions that have already been incorporated in the transferred manuscript

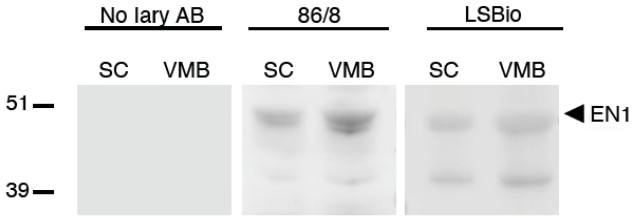
Referee 1

Antibodies should be validated utilizing a reporter mouse. En1cre mice are commercially available and can be crossed with reporters (TdTomato or YFP mice). Utilizing this tissue En1 antibodies can be easily validated. The EN1 antibody shown in Figure 1c seems unspecific, staining several neuronal populations in the spinal cord.

Indeed, antibody validation is extremely important. LSBio is commercial (CliniSciences), 86/8 was developed in the laboratory and fully characterized and used in previous studies (e.g. Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J*. **37**: e97374, 2018), scFv against EN1 was prepared from the 4G11 hybridoma (Developmental Hybridoma Bank, Iowa City, USA) and validated in previous studies (Wizenmann et al. *Neuron* **64**: 355-366, 2009). In the present study, the two polyclonal were further validated in several ways, including IHC and WBs.

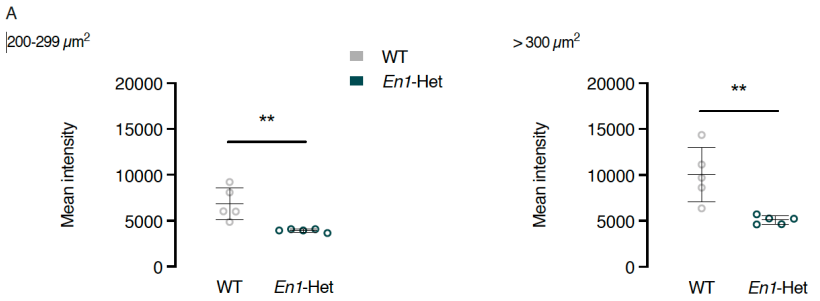
In the WBs we compared ventral midbrain (VMB) and spinal cord (SC) tissues and found similar patterns. Strong evidence for antibody specificity is extinction with the antigen and loss of staining in absence of the first antibody, both of which were performed in immunohistochemistry and WB.

We have now used LSBio and 86/8 to perform a WB on spinal cord (SC) and ventral midbrain (VMB) extracts with or without the first antibody and we find that the absence of first antibody fully eliminates band staining. The western blot is shown below and has been introduced in the revised manuscript.



Finally, we have quantified EN1 in the αMNs of the heterozygote at 3 months (before cell death), showing that EN1 content is decreased by 2-fold (LSBio antibody) in both α and γMNs (see below) with no change in neuron number. This result demonstrates that, before cell death, the amount of EN1 par MN is diluted by approximately 2fold in the mutant, further validates the antibody, and is interesting per se. It has been introduced in the revised manuscript as Supp. Fig. 1A.

Supp. Fig. 1A:



How can the authors explain the lack of loss of En1 interneurons in the En1-Het mice? Do spinal En1 interneurons show any signs of apoptosis (e.g., cleaved caspase 3 marker)? Which levels of the spinal cord were used for interneuron quantifications? Segments between L1 and L3 would be preferable.

We were indeed surprised by this finding and a plausible explanation is that a lower metabolic activity makes interneurons less sensitive to stress than αMNs which have to "fuel" long axons and high firing rates (not the case for γMNs). We propose this explanation in the discussion and will make it clearer in our revised version. We agree that it is speculative and that the point raised by the reviewer is very interesting. We hope to address this in the future and have discussed this point in the manuscript.

Since the cells do not die, we did not look for signs of apoptosis.

We analyze lumbar sections from L1 to L5 as now indicated in the methods section in the manuscript.

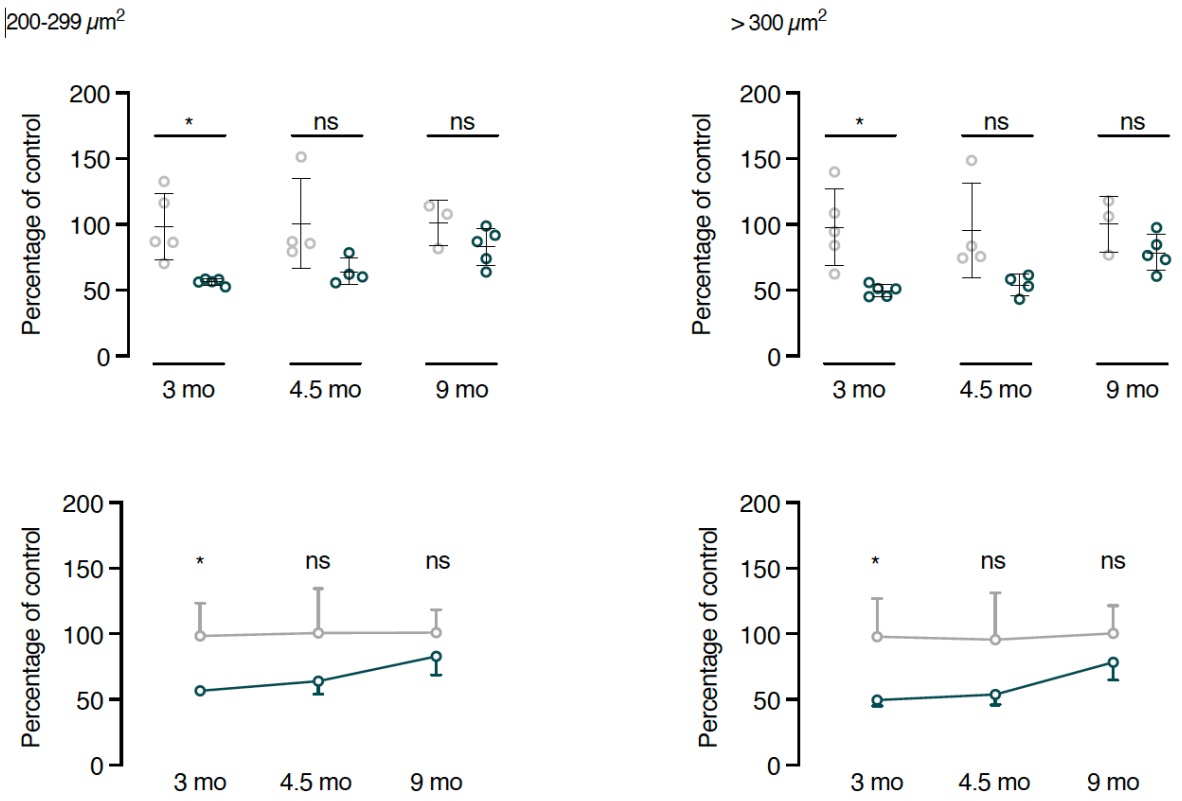
Referee 2

Evidence, reproducibility and clarity

In general, most of the experiments shown in this study are well done and convincing. However, the data on p62 upregulation appear correlative and do not allow any conclusions about the mechanism and function how EN-1 modulates motoneuron survival and function. In addition, this study is not very precise on the mechanisms how motoneurons degenerate in this model so that there are only limited insights into the way how EN-1 acts on motoneurons in a physiological manner and under pathophysiological conditions.

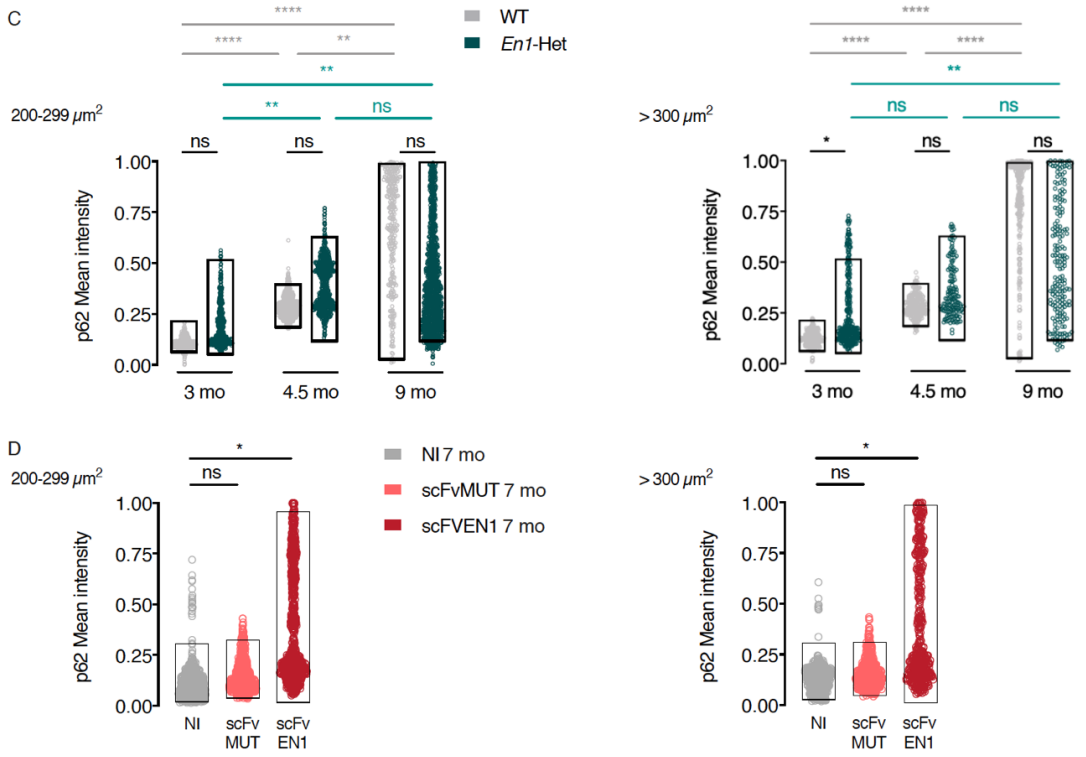
This criticism is justified, at least in part, as we agree that p62 upregulation is correlative. However, the fact that the neutralization of extracellular EN1 by the scFv increases p62 expression, is in favor of a causative link. The increase is also seen at 3 months in the *En1*-Het when all αMNs are still present but not after, which is interesting because, due to αMNs death, surviving MNs receive more EN1, an information provided below and now introduced and discussed in the revised manuscript (Supp. Fig. 1B).

Supp. Fig. 1.B:



As for p62, and as also mentioned by referee 3, Fig. 8 is very hard to follow and we propose to simplify it to make the message clearer:
Below is the revised Fig. 8C, D in which we focus exclusively on SQTSM1/p62 mean expression:

Revised Fig. 8C, D:

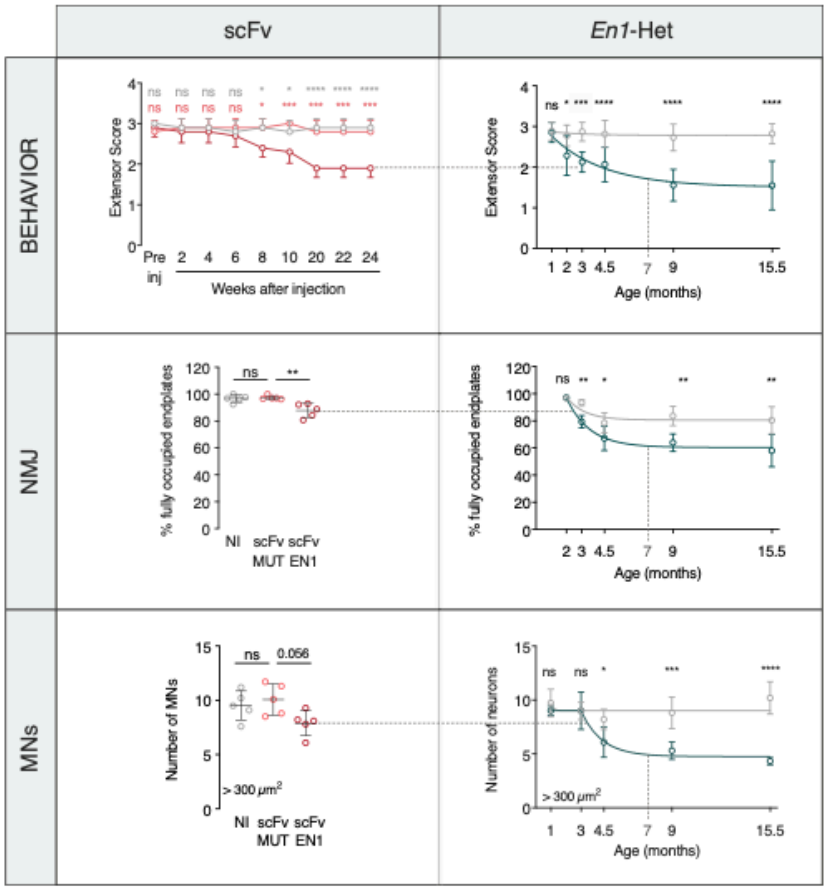


In this new figure, the main information is that mean p62 expression increases with time in WT α and γMNs, and can be seen as an aging marker.

A second information is that a difference in mean p62 expression between WT and *En1*-Het is seen only at 3 months in αMNs. For αMNs, we propose that this is due to the fact that they are very sensitive to EN1 dosage (in contrast with γMNs which do not die in the *En1*-Het). At 3 months, αMNs have only half of their normal EN1 content. Later, at 4.5 months 75% of the αMNs bound to die are already dead (Fig. 2D) and the remaining neurons receive more EN1 (even more so at 9 months), as could be measured (see above Supp. Fig. 1B). We thus can propose an accelerated aging of αMNs at 3 months due to both EN1 decrease and high metabolic activity (higher than in γMNs).

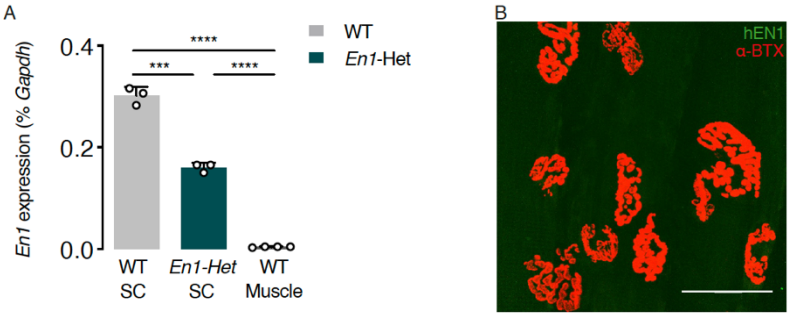
In the case of the scFv, scFvEN1, but not its mutated version induces enhanced mean p62 expression in the 80% surviving αMNs and in all γMNs at 7 months (low αMN death in this model, see Fig. 4F). As can be seen also in a novel figure (Supp. Fig. 2) that has been introduced in the revised manuscript and is shown below, 7-month-old scFv animals and 3- to 3.5-month-old *En1*-Het have similar phenotypes. This mild scFv phenotype (αMN death and muscle strength loss) in 7-month-old mice in spite of a huge loss in the EN1 content of MNs (Fig. 4C) suggests that the *En1*-Het phenotype is not entirely due to the decrease in EN1 transport from V1 interneurons to MNs (see discussion and Fig. 9).

Supp. Fig. 2 (new figure) comparing the phenotypes of *En1*-Het and of WT mice expressing scFv-EN1:



3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced localization of p62 at neuromuscular endplates?

We do not see the expression of *En1* mRNA or the presence of EN1 protein at the level of the endplate (Supp. Fig. 3).



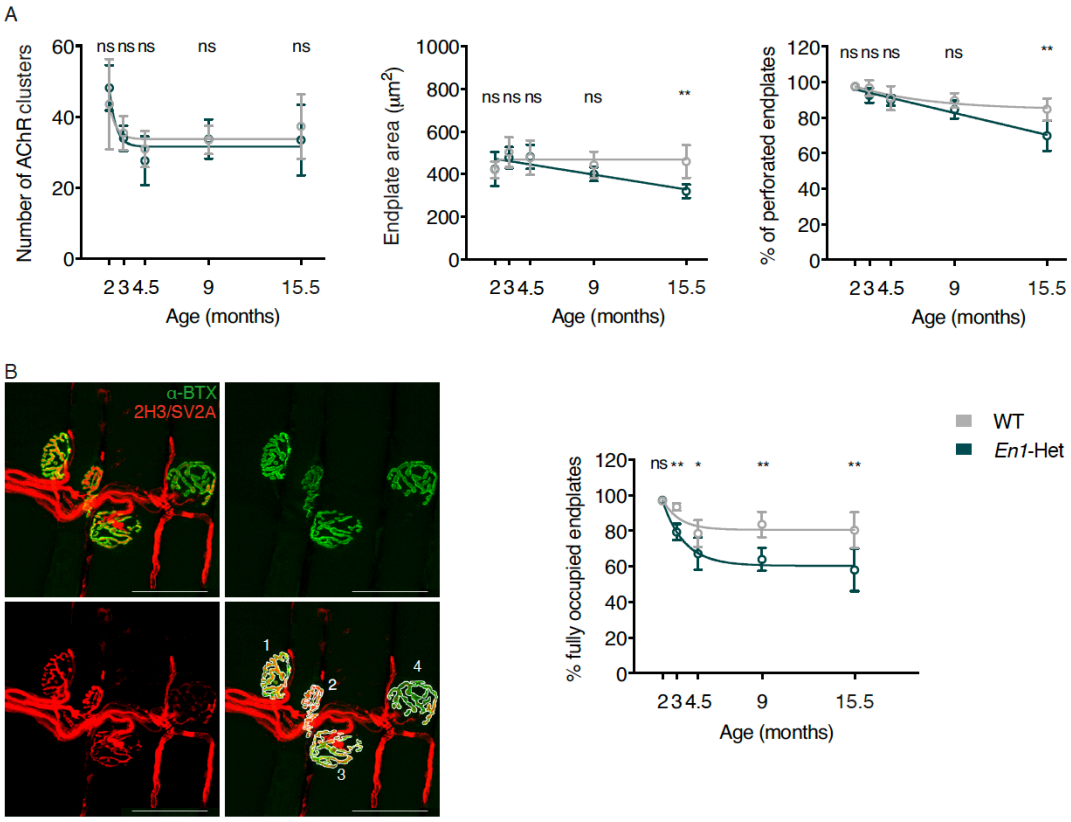
4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.

The distinction between fully occupied, partially occupied and denervated endplates is not arbitrary and we apologize for not having sufficiently described the methodology. As illustrated in modified Fig. 3 and explained in Material and Methods, a fully innervated endplate is defined as an endplate in which 80% or more of the green pixels (α-BGT) are covered by a red pixel (SV2a), a partially one is between 20 and 80% and a denervated one below 20% coverage. Thus at 9 months and after, close to 30% of the endplates are either partially innervated or denervated. In fact, it is more likely that they are partially innervated since the number of AChR clusters does not change (totally denervated clusters normally dissolve). The 80% threshold for fully innervated was selected to give a margin of security, and it is likely that the percentage of 25 to 30% of partially innervated endplates is an underestimation.
Below is the mean calculations for WT and *En1*-Het mice at 3, 4.5, 9 and 15.5 months 9 months on the basis of the criteria explained above:

Grouped	Fully	Partially	Dennervated
3 mo WT	93.51000000	5.05300000	1.439000000
3 mo Het	79.39000000	17.64000000	2.964000000
4,5 mo WT	90.07000000	8.29100000	1.639000000
4,5 mo Het	73.82000000	16.71000000	8.898000000
9 mo WT	95.33000000	4.21100000	0.799400000
9 mo Het	75.47000000	16.74000000	7.794000000
15,5 mo WT	83.58000000	11.54000000	4.879000000
15,5 mo Het	63.95000000	25.08000000	10.970000000

From top to bottom, the numbers of mice used per condition are: 4, 4, 7, 6, 5, 5, 6, 5

Modified Fig. 3:



We agree that we were not precise enough in our description and that it may have given the impression that the differences were relatively small. We think that retrograde degeneration is strongly supported by a loss muscle strength that parallels the decrease in fully occupied endplates (α-BGT, NF, SV2a) and precedes αMN loss by more than 1 month. We have recently contacted an electrophysiology group to establish a collaboration that will allow us to follow functional changes at the level of the spinal cord and of the neuromuscular junction and we see the experiments proposed by the reviewer as complementary to these physiological approaches. Yet, we do not want to ignore the opinion of the reviewer and propose to mention it in the conclusion, on the basis of his/her comment.

Referee # 3

Figure 1D: These immunoprecipitation results lack a negative control with irrelevant antibody to confirm that the band shown it's being recognized specifically by the antibodies reacting with the blot.

Please see the response to reviewer 1 above with the Western blot.

Figure 1E: The intensity of the EN1 labeling in MNs, much stronger than in V1 interneurons, is intriguing, given that MNs do not express engrailed-1 mRNA. One would have expected the opposite.

It may help here if it was possible to show that immunoreactivity in MNs is diminished in the het mutant mouse.

We also were surprised by this intensity higher in MNs that in V1 interneurons, as if the protein was exported rapidly towards the target neurons. We have done the experiment proposed by the referee, found a twofold (approx.) immunoreactivity reduction in *En1*-Het MNs (see above Supp. Fig. 2A in answers to Referee 2). This supplemental figure has been introduced in the revised version.

The experiment was done at 3 months when no MN death has yet occurred. Later the neurons "replenish" with EN1, probably because they do not have to share the limited supply with the dead ones (see above answers to referee 2 and Supp. Fig. 2B).

Figure 2D: There are a few possible problems with these data and their interpretation. First, this reviewer feels that 5 neurons (y-axis) is a rather small number. Are these 5 neurons per what area? From how many mice? I did not find that information in the figure legend. A larger area should be quantified so that we get numbers that are more robust. Second, such differences could also be due to hypotrophy of the MNs, namely, that MN number is the same but they are smaller.

At least 10 ventral horns (five spinal cord sections) separated by $\geq$ 900µm were analyzed for each animal. The number on the y-axis is small because it corresponds to the mean of the number of MNs present in one ventral horn, for 5 to 6 mice as indicated in the legend of Fig. 2D. The methodology has been better described in Material and Methods and in the legends.

Figure 3A: It would be useful that the authors explain how these AChR clusters were defined, visualized and counted. I could not find this information in the Methods. Perhaps this could be done by showing an alpha-BTX image illustrating the clusters.

As illustrated in modified Fig. 3 and now better explained in Material and Methods, a fully innervated endplate is defined as an endplate in which 80% or more of the green pixels (α-BGT) are covered by a red pixel (SV2a), a partially one is between 20 and 80% and a denervated one below 20% coverage. Thus at 9 months and after, close to 30% of the endplates are either partially innervated or denervated. In fact, it is more likely that they are partially denervated since the number of AChR clusters does not change (totally denervated clusters normally dissolve). The 80% threshold for fully innervated was selected to give a margin of security, and it is likely that the percentage of 25 to 30% of partially innervated endplates is an underestimation.

Figure 3B: As each adult endplate is only innervated by one MN, one would have expected fewer clusters and/or endplates, if indeed MNs are missing in this mouse, rather than endplates that are partially occupied. This could be clarified a bit more explicitly.

This is true and the ambiguity take its origin in insufficient explanation of how fully innervated, partially innervated and denervated endplates were defined. Please see above and also in answers to reviewer 2. Modifications have been introduced in the text (an in Fig. 3. The referee is right, the absence of change in the number of AChR clusters suggests that there are very few fully denervated endplates and that what is defined as such in the analysis corresponds to partially innervated endplates (see above). This is now discussed in the text.

Statistical analyses: In principle, comparisons of data obtained in studies that involved two variable parameters (such as time and genotype/treatment) should be weighted by a 2-way ANOVA test, which is more stringent since more conditions are being tested simultaneously. Usually a t-test is reserved for a pairwise comparison in an experiment involving only two conditions of the same variable.

The reviewer is correct. The two-way ANOVA is explained in the Statistical analyses section of the Methods. Analyses were carried out and their results listed in the legends for Figs. 2, 3, 4, 6 and Supp. Fig. 1.

4. Description of analyses that authors prefer not to carry out

We have tried to address most points raised by the three referees, either at an experimental level or through editorial changes and hope that the answers will be found satisfactory. The main series of experiments that we wish to postpone for another study was proposed by referee 2 in his/her points 3 and 4.

3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced localization of p62 at neuromuscular endplates?

4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.

Both points focus on the neuromuscular endplate. We have only done the EN1 immunostaining and *En1* RT-PCR, demonstrating the absence of mRNA and protein. We plead for not doing the other experiments, in particular the follow up of markers associated with presynaptic activities and quantitative ultrastructural studies. The reasons are as follows:
1. The post-docs who have conducted the work have left or will do so in the near future. Therefore, the proposed experiments necessitate to hire of new post-docs and to identify collaborators interested in the project and expert in electron-microscopy. The experiments might thus be difficult to achieve in a reasonable time.

2. We really think, that although interesting, the proposed experiments mostly aimed at evaluating presynaptic activity are not in the scope of the study. Our main discovery is that EN1 is transferred from V1 interneurons to MNs and that reducing EN1 synthesis or transfer induces αMN retrograde degeneration and death. It is the first time that an adult function is described for EN1 transfer and, in the context of this finding, we have done several experiments that provide a story which we see as self-sufficient.

3. This does not mean that we do not appreciate the suggestions of the referee but that we would like to consider them in the context of novel experiments that we have undertaken and include the following steps:

We have contacted an electrophysiology group that works *in vivo* on the activity of spinal cord neurons and will investigate, in collaboration with us, the activity of V1 interneurons, MNs, and the synaptic activity at the neuromuscular junction, in our models (WT, En1-Het, scFvEN1, rescue by EN1 injected in L5).

We have initiated microfluidic experiments with human iPSC-derived MNs from patients with familial and sporadic ALS forms (plus healthy or isogenic controls) and shown that EN1 “cures” the ALS phenotype. We will now co-culture the MNs with fused myoblasts in the distal compartment to follow the effect of EN1 added at the level of the cell body or of the terminals on electrical activity, local protein synthesis (with identification of the changes in local translation), quantification of presynaptic and post-synaptic markers. This will allow us to better define *in vivo* studies.

Using the same human MNs (sick, healthy, sick and complemented with EN1) we will investigate the status of the nuclei (ATAC-seq, Cut and Run, ChIP with EN1 antibodies…) to better understand the molecular basis for long-lasting effects of short-term exposure to EN1.

These studies have been initiated and will be part of a future manuscript distinct from the one presently under review.

1st Editorial Decision **24th Nov 2022**

Dear Prof. Prochiantz,

Thank you for the transfer of your manuscript and proposed point-by-point response to EMBO reports.

I have discussed your study with my colleagues here, and we agree that it could be a good contribution to our scientific article section. We also agree with your proposed revision plan.

I would therefore like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (24th Feb 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data

Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n < 3 please use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

EMBOR-2022-56525-T

ENGRAILED-1 transcription factor exerts a paracrine neurotrophic activity on adult spinal cord α-motoneurons.
Lebœuf, Vargas et al

Revisions highlighted in red in the text. The abstract was modified to respect the 175 words limit

Answers to referees (Referees' comments and questions are in blue, answers in black)
First of all, we wish to thank the 3 referees for their careful evaluation of the manuscript. We see many issues that they have raised as legitimate, to which we now provide experimental or editorial answers.

Reviewer #1
Evidence, reproducibility and clarity:
The present work focuses on Engrailed 1 (En1), a homeoprotein that is expressed in spinal V1 interneurons that connect to α-motoneurons (MNs). The authors studied its role in neuromuscular strength and MN retention and loss with the aid of different approaches. First, they studied its expression in spinal cord with RNAscope, a novel ISH method that makes possible to detect biomarkers that would be otherwise difficult to study with traditional ISH techniques. They then delivered 86/8 and LSBio anti-En1 antibodies, that catch En1 in the cleft and prevent it from being captured by MNs; moreover, they used a heterozygotic En1 mouse model to reduce En1 levels. The behavioral assessment, studied with grip strength, inverted grip test and hindlimb extensor reflex, showed motor alterations, paralleled by α-MNs loss and with an even stronger phenotype in the heterozygotic mice. This phenotype, however, appeared weeks before the MN loss, so they used NMJ assessment to determine what it seems to be a retrograde degeneration. En1 administered intrathecally was effectively internalized by the MNs and led to a long-term amelioration of the motor impairments and renervation of the NMJ, that needed to be boosted after 12 weeks for a stable therapeutic effect. Finally, heterozygotes revealed also a degeneration in dopaminergic neurons within midbrain similar to the one observed in spinal MNs, along with an upregulation of SQTSM1/p62 gene/protein, a factor in MN ageing linked to the classical genes implicated in familial forms of ALS (SOD1, TDP-43, FUS, and C9ORF72). They authors did not observe degeneration in V1 interneurons. They conclude that En1 might have a role in regulating MN ageing in degenerative motor disorders.

This is a fair description of the study that, however, contains the following error:
They then delivered 86/8 and LSBio anti-En1 antibodies, that catch En1 in the cleft and prevent it from being captured by MNs.

Perhaps we were not clear but we did not deliver the antibodies 86/8 and LSBio. We used them for western blots and immunohistochemistry (IHC) to identify EN1 and localize it. We delivered the third antibody, a single-chain anti EN1 antibody (scFvEN1), that captures extracellular EN1 and prevents it from being captured by MNs on the basis of the LSBio staining (Figure 4A-C).

Significance
Overall, the manuscript is well written however, some of the data appears too preliminary for publication. While the potential beneficial effect of En1 intrathecal administration looks promising and worth of publication, it is difficult to understand the mechanism of action. Some of the results are puzzling and require further investigations.

Major comments

It is unclear why levels of intensity for RNAscope were not quantified, and qPCR was preferred for quantifications in Figure 1b. RNAscope is a technique that allows for spatial distribution analysis of the markers and their level of the expression. This data can be easily quantified utilizing the QuPath software which is open access. Same concerns apply to Figure 2a.

Quantitative RT-PCR provides a quantitative measure of gene expression. Since only V1 interneurons (including, Renshaw cells) express EN1, we infer the spatial distribution, although not expression level cell by cell. Figure 2A is an actual counting at 4.5 months of *En1+* cells and of *Calbindin+* cells (Renshaw cells), both identified by RNAscope. Thus, it is clear that the number of *En1*-expressing cells (V1 interneurons) is not modified at 4.5 months when muscle weakness and death of αMNs are well advanced (around 70% of the αMNs that will eventually die, are already gone). Long-term survival of V1 interneurons is further demonstrated in Figure 2D (left panel) until 15.5 months, (see also below) whereas total *En1* expression is reduced by half. Quantification neuron by neuron of the amount of *En1* transcribed (RNAscope) would indicate the variation, among interneurons, of *En1* transcription in WT and mutant mice. This is interesting *per se* but would not modify the main information that these neurons do not die in the heterozygote and that *En1* transcription does not decrease with time in both WT and mutant genotypes (at least until 15.5 months).

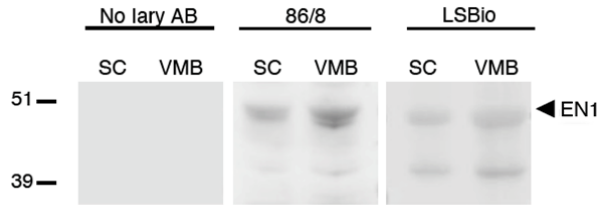
Antibodies should be validated utilizing a reporter mouse. En1cre mice are commercially available and can be crossed with reporters (TdTomato or YFP mice). Utilizing this tissue En1 antibodies can be easily validated. The EN1 antibody shown in Figure 1c seems unspecific, staining several neuronal populations in the spinal cord.

Indeed, antibody validation is extremely important. LSBio is commercial (CliniSciences), 86/8 was developed in the laboratory and fully characterized and used in previous studies (e.g., Alvarez-Fischer et al. *Nature Neurosci.* **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J.* **37**: e97374, 2018), scFv against EN1 was prepared from the 4G11 hybridoma (Developmental Hybridoma Bank, Iowa City, USA) and validated in previous studies (e.g., Wizenmann et al. *Neuron* **64**: 355-366, 2009). In the present study, the two polyclonal were further validated in several ways.

In the WBs we compared ventral midbrain (VMB) and spinal cord (SC) tissues and found similar patterns. Strong evidence for antibody specificity is immunostaining extinction with the antigen and with absence of first antibody, which we carried out.

We have now used LSBio and 86/8 to perform a WB on spinal cord (SC) and ventral midbrain (VMB) extracts with or without the first antibody and we find that the absence of first antibody fully eliminates band staining. The western has been introduced in Figure 1D of the revised manuscript in place of the cross immunoprecipitation.

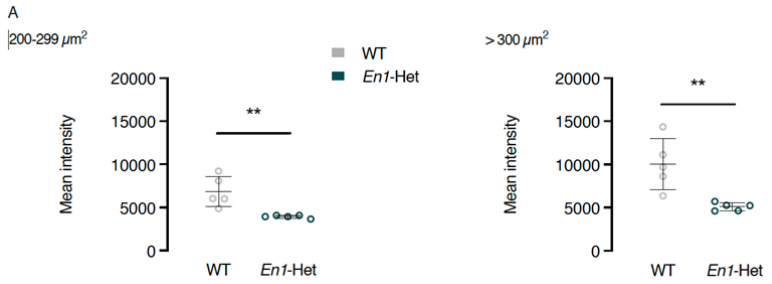
Figure 1D (modified in the revised manuscript)



Finally, we have quantified EN1 in the αMNs of the heterozygote at 3 months (before cell death), showing that EN1 content is decreased by approximately 2-fold (LSBio antibody) in both α and γMNs with no change in neuron number. This result demonstrating that EN1 is diluted by approximately

twofold (concentration per neuron when all neurons are still present), in addition to further validating the antibody, is itself interesting and has been introduced in Figure EV1 of the revised manuscript

Figure EV1A (added to the revised manuscript)



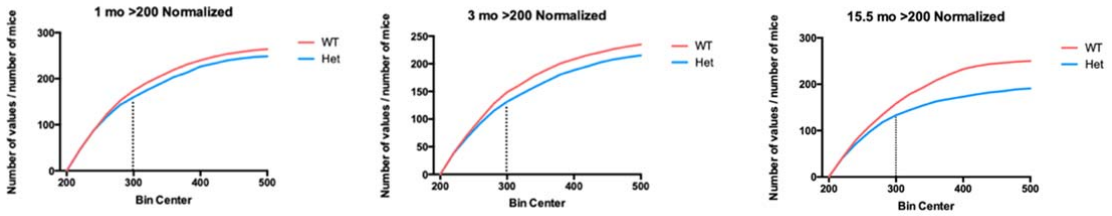
Regarding the staining in other neuronal populations, there is always some background, in particular in the tissue treatment conditions used for RNAscope. Furthermore, given the large number and wide distribution of V1 interneurons (Figure 1A), we cannot preclude that EN1 is present at a low concentration in the extracellular space and in several cell types (discussed in Figure 9 of the manuscript). This does not weaken the main conclusion that it primarily accumulates in MNs which do not express *En1* (RNAscope).

Investigations of En1 expression in motor neurons from already available omics data sets would support the idea that En1 is expressed in motor neurons.

The *En1* locus is silent in MNs and only the protein is present, a consequence of its internalization. Microdissection of MNs and proteomic analysis would not be definitive since the interneurons that produce EN1 are in close vicinity of the MNs and since some protein is necessarily present in the extracellular space (where it is trapped by scFvEN1), making contamination unavoidable.

Differentiation between Gamma and Alpha motor neurons should be performed using specific markers as Err3, Wnt7a or NeuN.

This is a possible way to do the distinction, but size criterion in Cresyl violet is supported in the literature (Wu et al. *Journal of Biological Chemistry*, **287**: 27335-27344, 2012; Dutta et al. *Experimental Neurology*, **309**: 193-204, 2018). In our study, it is further validated by the demonstration that, in 9-month-old animals, the results obtained (cell number and specific death of large neurons >300µm^2, but not of intermediate size ones 200-299µm^2) are replicated by counting ChAT-stained neuron (Figure 2C). It is of particular interest that the number of medium size neurons (also ChAT-positive medium size MNs) does not increase when the number of large size (Cresyl and ChAT-positive) neurons decreases, thus precluding a “shrinkage effect”. Most importantly, the size criterion (Cresyl violet) allows us not to be mistaken by a possible down-regulation of markers in the mutant, independently of cell survival. We provide for the reviewer, but not for publication, the evolution with time of the number of neurons based on size (above 200 µm^2) showing clearly that at 15.5 months the large population (>300 µm^2) is decreased in the *En1*-Het, with very little change for neurons between 200 and 300 µm2, and certainly not an increase which would be expected if shrinkage occurred.



How can the authors explain the lack of loss of En1 interneurons in the En1-Het mice? Do spinal En1 interneurons show any signs of apoptosis (e.g., cleaved caspase 3 marker)? Which levels of the spinal cord were used for interneuron quantifications? Segments between L1 and L3 would be preferable.

We were also surprised by this finding and a plausible explanation is that a lower metabolic activity makes interneurons less sensitive to stress than αMNs which have to "fuel" long axons and high firing rates (not the case for γMNs). We propose this explanation in the discussion and make it clearer in our revised version. We agree that it is speculative and that the point raised by the reviewer is very interesting. We hope to address this in the future and have discussed this point.

Since the cells do not die, we did not look for signs of apoptosis.

We analyze lumbar sections from L1 to L5 as now indicated in the methods section in the manuscript

The set of experiments reported in Figure 4 is of difficult interpretation without showing the actual presence of extracellular En1, that could be assessed with protein detection or RNAscope.

This is another interesting suggestion, but we think that it will be difficult to distinguish low extracellular staining due to EN1 diffusion from some unspecific background. Since the scFvEN1 is secreted by astrocytes, it necessarily neutralizes extracellular EN1, resulting in a decrease in the MN content of the protein. This is an experiment with high specificity since the same scFv harboring a Cysteine to Serine point mutation that prevents EN1 recognition (no disulfide bound formation between the light and heavy chains) does not block EN1 capture by MNs (Figure 4C for IHC and quantifications).

As for extracellular EN1 mRNA identified by RNAscope, we hesitate to embark on the idea as mRNAs are likely secreted in insufficient amounts to be identified, even by RNAscope. The results presented (no *En1* visible by RNAscope in MNs, loss of EN1 in MNs following extracellular scFvEN1 activity, and preferential addressing of injected EN1 to MNs) demonstrate EN1 capture by MNs. Indeed, we cannot completely preclude the transfer of tiny amounts (escaping RNAscope detection in MNs) of *En1* mRNA (for example, through extracellular vesicles), but we plead for not considering this hypothesis in the present paper. However, if the reviewer wishes, the possibility can be introduced in the discussion.

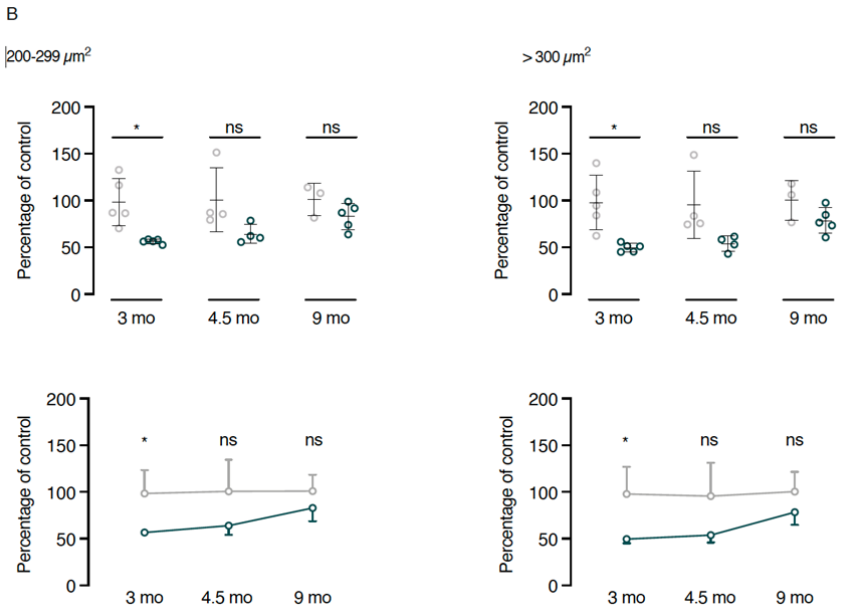
Reviewer # 2

Evidence, reproducibility and clarity

Engrailed-1 does not act only in a cell-autonomous way in neural development, but also has non-cell-autonomous functions. These functions depend on the release of this homeoprotein which has been characterized in much detail by previous work of this group. In this paper, they show that EN-1 is expressed in spinal V1 interneurons, both on the RNA and on the protein level. In spinal motoneurons, EN-1 protein but not RNA is detected. Neutralization of extracellular EN-1 with a secreted antibody apparently blocks transfer from these interneurons to motoneurons and causes motoneuron disease symptoms. A similar phenotype is also observed in EN-1 +/- mice. Most importantly, the authors also demonstrate that intrathecal injection of EN-1 into EN-1 +/- mice restores loss of muscle strength and prevents motoneuron death. The authors also show that the autophagy modulator SQTSM1/p62 is expressed at elevated levels in EN-1 +/- mice and in mice after injection of the EN-neutralizing antibody. Since p62 expression also seems to be increased in general during aging in motoneurons, the authors conclude that EN-1 from spinal V1 interneurons is a regulator of motoneuron aging. In general, most of the experiments shown in this study are well done and convincing. However, the data on p62 upregulation appear correlative and do not allow any conclusions about the mechanism and function how EN-1 modulates motoneuron survival and function. In addition, this study is not very precise on the mechanisms how motoneurons degenerate in this model so that there are only limited insights into the way how EN-1 acts on motoneurons in a physiological manner and under pathophysiological conditions.

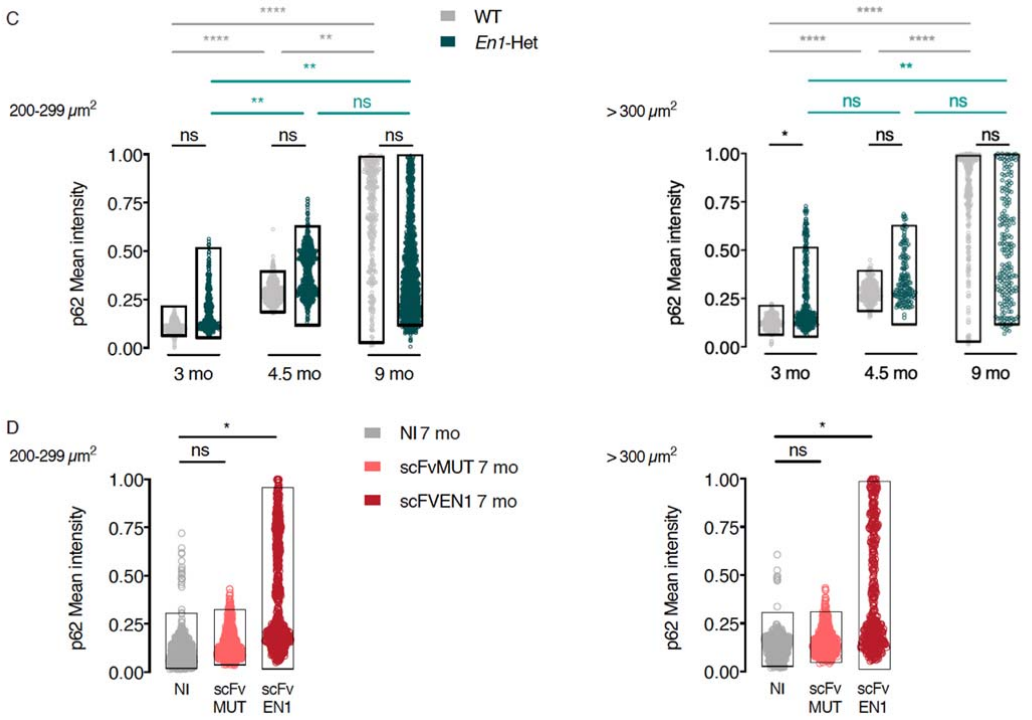
We thank the reviewer for her/his general positive assessment of the study. For the criticism on p62, we agree that p62 upregulation is correlative. However, the fact that the neutralization of extracellular EN1 by the scFv increases p62 expression, is in favor of a causative link. The increase is also seen at 3 months in the *En1*-Het when all αMNs are still present with half of their normal EN1 content (Figure EV1B) but not after, which is interesting because, due to αMNs death, surviving MNs receive more EN1, information provided below and now introduced and discussed in the revised manuscript (Figure EV1B).

Figure EV1B (added to the revised manuscript)



As for p62, and as also mentioned by referee 3, Figure 8 is very hard to follow and we propose to simplify it to make the message clearer:
We have revised Figure 8C, D in which we focus exclusively on SQTSM1/p62 mean expression

Figure 8C, D (modified in the revised manuscript)

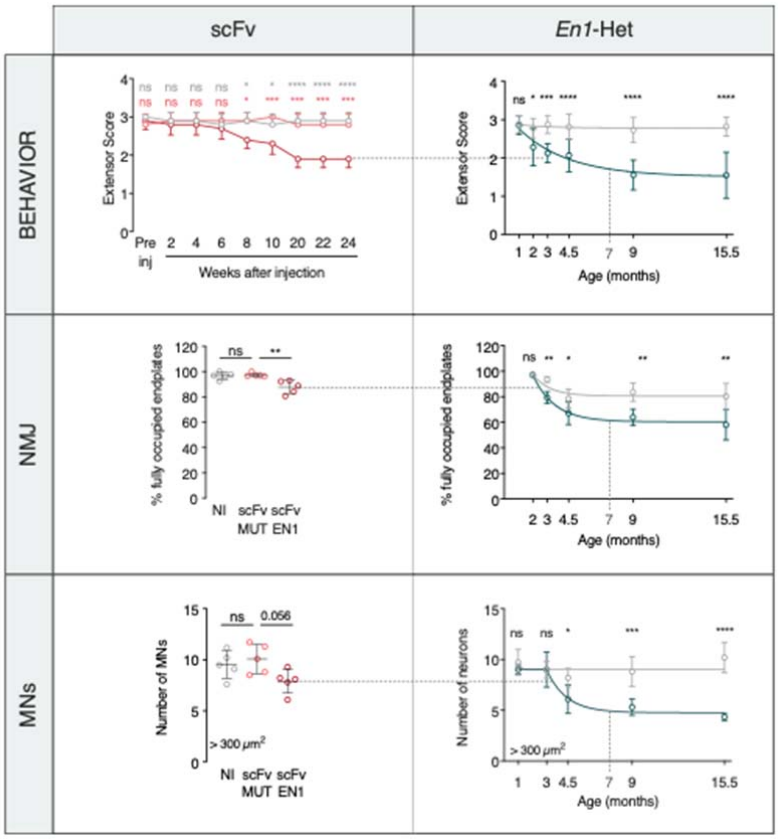


In this new version of Figure 8C,D, the main information is that mean p62 expression increases with time in WT α and γMNs, and can thus be seen as an aging marker.

A second information is that a difference in mean p62 expression between WT and Het is seen only at 3 months in αMNs. For αMNs, we propose that this is due to the fact that they are very sensitive to EN1 dosage (in contrast with γMNs which do not die in the *En1*-Het). At 3 months, αMNs have only half of their normal EN1 content (see above Figure EV1B). Later, at 4.5 months 75% of the αMNs bound to die are already dead (Figure 2D) and the remaining neurons receive more EN1 (even more so at 9 months), as could be measured (see above Figure EV1B). We thus can propose an accelerated aging of αMNs at 3 months due to both EN1 decrease and high metabolic activity (higher than in γMNs).

In the case of the scFv, scFvEN1, but not the mutated version induces enhanced mean p62 expression in the 80% surviving αMNs and in γMNs at 7 months (low αMN death in this model, see Figure 4F). As can be seen also in a newly added figure (Figure EV2) that has been introduced in the revised manuscript and is shown below, 7-month-old scFv animals and 3- to 3.5-month-old *En1*-Het have similar phenotypes. This mild scFv phenotype (αMN death and muscle strength loss) in 7-month-old mice in spite of a huge loss in the EN1 content of MNs (Fig. 4C) suggests that the *En1*-Het phenotype is not entirely due to the decrease in EN1 transport from V1 interneurons to MNs (see Discussion and Fig. 9).

Figure EV2 (added in the revised version) comparing the phenotypes of *En1*-Het and of WT mice expressing scFv-EN1:



It remains true that we have voluntarily decided not to examine in depth the molecular mechanisms allowing EN1 to exert its protective activity, a decision that we would like to defend and maintain.

A first reason is that in previous papers on mesencephalic dopaminergic (mDA) neurons (Alvarez-Fischer et al. *Nature Neurosci.* **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J.* **37**: e97374, 2018), we evaluated several mechanisms involved in EN1 neurotrophic activity and we did not want this study to be a duplication of studies done on a different neuronal population, even if mechanisms might differ in part, between αMNs and mDA neurons. What has interested us more is that, in the two cases, age is an important factor in the unveiling of the retrograde degeneration phenotype (mDA neurons start dying at 1.5 months and αMNs at 3 months). It is because of this similarity that we performed the bioinformatic study that has led us to SQTSM1/p62. In this context, it is of interest that mean SQTSM1/p62 expression (variability of expression between neurons is not discussed in the revised version) increases with age in the wild type, thus can be seen as an age marker. It allows us to propose that EN1 extracellular neutralization and the loss of one *En1* allele, that increases mean SQTSM1/p62 expression both accelerate aging.

A second reason is that the study is oriented toward a possible use of EN1 as a therapeutic protein. This orientation also has to do with the focus on SQTSM1/p62. Indeed, there are probably many pathways downstream of EN1, but in the bioinformatic analysis of genes differentially regulated in WT and *En1*-Het mDA neurons and also expressed in MNs, *SQTSM1/p62* is the only one that interacts with the 4 genes mutated in the major ALS familial forms. In addition, *SQTSM1/p62* mutations have been observed in ALS patients (References 41 to 45 in the manuscript).

Finally, the most important point is that the main message of this paper is the discovery of a non-cell autonomous EN1 activity in the spinal cord and of its ability to travel between V1 interneurons and MNs. This specificity best explained by a targeting signal that we have identified is at the basis of the specific addressing to MNs of EN1 intrathecally injected, which also has implications for its potential therapeutic use.

Specific points of criticism

1. In Fig. 2a, the authors show that EN-1-positive interneurons are not reduced at 4.5 months in the spinal cord. No data are shown for later time points such as 9 months, the corresponding stage when motoneuron loss is observed, or at 16 months which corresponds to the data shown in Fig.1. The argument that there is no reduction of V1 interneurons between 4.5 months and 16 months because there is no decrease of EN-1 expression between 4.5 and 16 months, as shown in Fig. 1b is not convincing. EN-1 expression could change in individual cells, thus compensating for the loss. Data on numbers of EN-1-positive cells at 9 and 16 months should be included, and a potential autocrine effect of EN-1 on V1 interneurons, as observed in midbrain dopaminergic neurons, characterized in more detail.

Figure 2A illustrates the absence of interneuron loss at 4.5 months, but this set of data is completed by those of Figure 2D that demonstrate the maintenance of V1 interneuron number until 15.5 months, at least. It can be noted that, in contrast with interneurons, αMNs at 4.5 months have experienced massive cell death (70% approx. of total αMN death at 15.5 months). As a whole, data of Figure 2 demonstrate that the number of small neurons (100-199 μm^2) and intermediate size neurons (200-299 μm^2) does not change with age, at least through 15.5 months. This is in strong contrast with large αMNs (>300 μm^2). As already explained in our answers to referee 1, size is an excellent marker for the identification of neuronal subtypes and the analysis of survival (see answers to referee 1, justifying the use of neuron size).

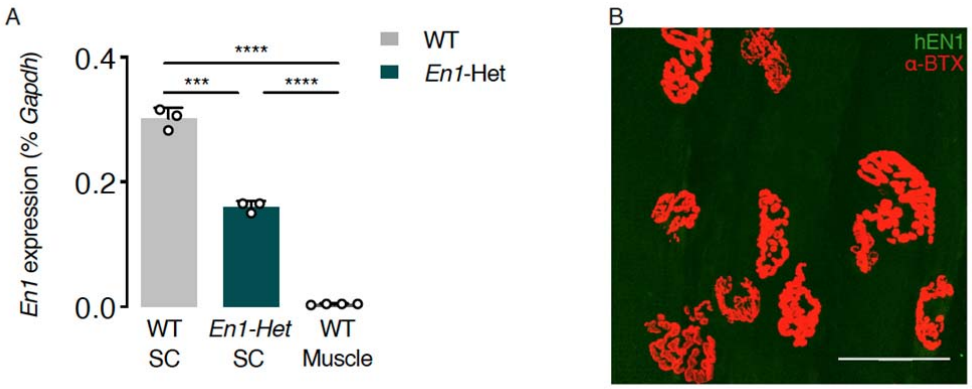
2. In Fig. 2e, the authors present data on loss of muscle strength between 4.5 and 15.5 months. They conclude that this reflects gradual neuromuscular strength loss. Since neuromuscular endplates have a very high safety factor, they can maintain full function even if transmitter release is reduced by more than 80%. Therefore, the loss of muscle strength seems to reflect the progressive loss of presynaptic terminals at neuromuscular endplates, rather than a gradual loss of neuromuscular strength.

We apologize for the semantic confusion. What is measured is a progressive loss of muscle strength due to the progressive loss of presynaptic terminals and not a gradual loss of neuromuscular strength. This is now modified throughout the revised text.

3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced localization of p62 at neuromuscular endplates?

We do not see expression of *En1* mRNA or the presence of EN1 protein at the level of the endplate. This information is now provided in Figure EV3.

Figure EV3 (introduced in the revised version)



4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.

SV2a and NF (2H3) staining (it is not only SV2a) at the level of endplates identified by α-Bungarotoxin labeling has been used in a large number of studies (Wahlin et al. J. Comp. Neurol. 506: 822-837, 2008; Hasting et al. *Scientific Reports* **10**: 1-13, 2020; Yahata et al. *J. Neurosci*. **29**: 6276-6284, 2009; Jones et al. *Cell Reports* **21**: 2348-2356, 2017) Our goal was not to document the loss of synaptic activity through the use of the three suggested markers, Bassoon, Piccolo and Munc-13. Doing it would force us to initiate experiments taking several months to prepare the material and do a quantitative analysis in the models of EN1 loss of function (*En1*-Het) and neutralization (scFv), plus rescue by EN1. Nor do we wish to initiate a novel collaboration to produce a quantitative ultrastructural study. We see the latter morpho-functional studies beyond the scope of the manuscript and wish to be given the possibility to present them in a separate study (see at the end of this document in "Description of the experiments that the authors prefer not to carry out").

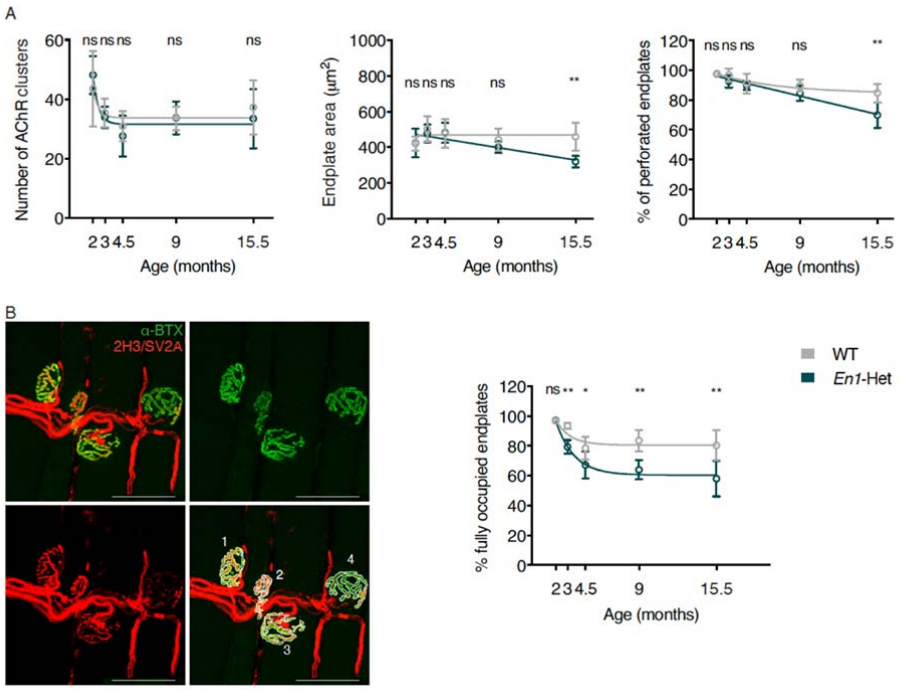
The distinction between fully occupied, partially occupied and denervated endplates is not arbitrary and we apologize for not having sufficiently described the methodology. As illustrated in modified Figure 3 and explained in Material and Methods, a fully innervated endplate is defined as an endplate in which 80% or more of the green pixels (α-BGT) are covered by a red pixel (SV2a/2H3), a partially one is between 20 and 80% and a denervated one below 20% coverage. Thus at 9 months and later ages, close to 30% of the endplates are either partially innervated or denervated. In fact, it is more likely that they are partially innervated since the number of AChR clusters does not change (totally denervated clusters normally dissolve). The 80% threshold for fully innervated was selected to give a margin of security, and it is likely that the percentage of 25 to 30% of partially innervated endplates is an underestimation.

Below is the mean calculations for WT and *En1*-Het mice at 3, 4.5, 9 and 15.5 months 9 months on the basis of the criteria explained above:

Grouped	Fully	Partially	Dennervated
3 mo WT	93.51000000	5.05300000	1.439000000
3 mo Het	79.39000000	17.64000000	2.964000000
4,5 mo WT	90.07000000	8.29100000	1.639000000
4,5 mo Het	73.82000000	16.71000000	8.898000000
9 mo WT	95.33000000	4.21100000	0.799400000
9 mo Het	75.47000000	16.74000000	7.794000000
15,5 mo WT	83.58000000	11.54000000	4.879000000
15,5 mo Het	63.95000000	25.08000000	10.970000000

From top to bottom in the table, we used 4, 4, 7, 6, 5, 5, 6 and 5 mice per condition.

New Figure 3 (modified in the revised version)



We agree that we were not clear enough in our description and that it may have given the impression that the differences were relatively small. We think that retrograde degeneration is strongly supported by a loss of muscle strength that parallels the decrease in fully occupied endplates (α-BGT, NF, SV2a) and precedes αMN loss by more than 1 month. We have recently contacted an electrophysiology group to establish a collaboration that will allow us to follow functional changes at the level of the spinal cord and of the neuromuscular junction and we see the experiments proposed by the reviewer as complementary to these physiological approaches. Yet, we do not want to ignore the opinion of the reviewer and mention it in the conclusion, on the basis of his/her comment.

5. The authors present evidence for a glycosaminoglycan (GAG) binding domain that appears responsible for uptake of EN-1 into motoneurons. However, it is unclear into which cellular compartment EN-1 is taken up after GAG binding on motoneurons. The authors propose this could be an alternative pathway to conventional endosomal uptake. How can the EN-1 that is taken up into cells exert transcriptional effects in motoneurons? As a minimum, more data on the subcellular distribution of endocytosed EN-1 should be included to support current hypotheses and to close the gap from cellular uptake to transcriptional regulation.

The question is justified since we did not recall until page 12 of the Discussion that EN1 is, as most tested homeoprotein transcription factors, captured by a mechanism distinct from endocytosis. While not yet fully understood, the process involves the formation of inverted micelles that allow for direct targeting to the cytoplasm and from there to the nucleus thanks to the NLS. We now mention in the introduction that EN1 transfer and HP transfer is based on unconventional secretion and internalization processes.

6. The differences in p62 expression with age in WT and EN-1 +/- mice as shown in Fig. 8c are not convincing. First, the p = 0.0499 and p = 0.0536 values for differences at 3-4 months of age appear borderline, and it is unclear what the dispersion analysis that is shown really means. Moreover, the question remains how a potential dysregulation of p62 then affects NMJ morphology and function. Is this change in p62 also detectable in presynaptic compartments?

We agree that p values in the range of 0.05 are not extremely high and this is due to the heterogeneity in SQTSM1/p62 expression, that reflects that of MN populations, and induces a high variance. We also agree that this figure is too complicated and a simplified version has been proposed above (see answers to reviewer 1). To summarize, Figure 8C shows that in WT animals, with no αMN death (grey) the level of SQTSM1/p62 expression in αMNs and γMNs increases between 3 and 4.5 months and between 4.5 months and 9 months, with significances varying between p<0.01 (**) and p<0.0001 (****). In *En1*-Het mice, the situation is more complex and we have to consider that 50% αMNs die (see above). However, expression increases between 3 and 9 months with p<0.01 (**) for WT and *En1*-Het neurons. SQTSM1/p62 can thus be taken as an age marker. Dispersion is a poor word for population heterogeneity for SQTSM1/p62 expression and it is clear that αMNs and γMNs do not constitute homogeneous populations and do not evolve similarly with time. As this is obvious, we have decided to focus only on mean levels of expression. Yet, the analysis of heterogeneity shows that it also increases between 3 and 9 months for WT and *En1*-Het mice with p<0.0001 (****) and this information can be reintroduced if requested.

The new Figure 8 panel D (please see above, answers to referee 1) now includes the results obtained with the scFvs. A phenotype comparison between the two models (*En1*-Het and scFvEN1) has been introduced in Figure EV2 (new Figure introduced in the revised version and shown above in answers to referee 1).

7. Is there any molecular evidence that EN-1 modulates the p62 gene promoter directly? What is the argument to assume that increase in SQTSM1/p62 expression and dispersion is an indicator of aging? The mean intensity, if I understand Fig. 8c correctly, does not significantly increase, it is only the dispersion that changes. In general, the data shown in Fig. 8c are hard to read and interpret. For example, in the right panel, the difference between the dispersion in 4.5 and 9 month old EN +/- mice is indicated as p = 0.06, but marked with 4 stars. The presentation of these data should be changed to make them clearer.

We have no evidence that EN1 modulates the *SQTSM1/p62* promoter directly. The identification of this gene as a target (not necessarily a direct target) of EN1 comes from the bioinformatic analysis described in the manuscript and we were intrigued by the interaction with the 4 main familial ALS mutations and the existence of families with *SQTSM1/p62* mutations. This is what led us to analyze its expression in our two models of EN1 loss of function. Although the *En1*-Het mouse is not an ALS model, the results support the idea that EN1 could be used as a therapeutic protein in several familial and even sporadic forms of the disease. The latter hypothesis is now being tested on MNs derived from iPSCs (sporadic patients, fALS and isogenic variants, and healthy controls). If the data lend weight to our hypothesis, as collaborative and in-house preliminary data suggest, then a complete analysis of EN1 targets in human MNs will be undertaken. Again, we really think that this is out of the scope of this study.

For Figure 8, we fully agree that it can give headaches and we apologize. Moreover, it induces wrong interpretations (mean intensity increases with age and dispersion between 4.5 and 9 months has a calculated $p<0.0001$). We have now simplified it as suggested by the reviewer (see above answers to referee 1).

CROSS-CONSULTATION COMMENTS
I agree with all comments from the other reviewers

Reviewer #2 (Significance (Required)):

This study expands previous work of the authors, in particular work that has been performed and published on the effects of EN-1 on mesencephalic dopaminergic neurons. If adequately revised, it could make an interesting contribution to the general understanding how spinal V1 interneurons act on funcitonality and survival of spinal motoneurons.

Referee #3

Evidence, reproducibility and clarity

This is an interesting and provocative manuscript reporting non-cell autonomous trophic activities of a homeobox protein, a concept pioneered by Dr. Prochiantz since many years ago. The study involves a significant amount of experimental work and the authors are to be congratulated by the scope and ambition of their study. Given previous studies by this laboratory on EN-1 functions in midbrain dopaminergic neurons, the concept advanced in the present paper is not entirely novel, although it is indeed interesting to find EN-1 activities in motoneurons; these were unexpected. Given that this is a non-cell-autonomous effect (EN-1 is made and released by neurons adjacent to MNs), it would have been interesting to explore the conditions under which EN-1 synthesis, release and effects are regulated, whether by lesion, degeneration, etc. But that may be something the authors wish to leave for a future report. It is welcome that an effort was put into trying to mechanistically understand how these trophic effects are mediated. This reviewer understands that this is a major undertaking. Nevertheless, the connection between EN-1 and p62 is not well developed by the data presented and future readers may be left with many questions regarding how EN-1 and p62 are related (e.g. direct interaction? transcriptional regulation?), whether p62 is indeed the mediator of EN-1 trophic effects, or the significance of the increased levels of p62 for motoneuron disease. In its present form, this paper will be welcome, if nothing else by the provocative ideas that it advances. For this, it clearly deserves to be published in a good journal (whatever that means these days). Here below are a few questions and suggestions which the authors may want to take into consideration

We thank the referee for his/her positive assessment of the study. We agree that "the connection between EN-1 and p62 is not well developed and that many questions remain regarding how EN-1 and p62 are related remain. We have therefore tried to better explain and to simplify. Please see responses to referees 1 and 2.

Figure 1C: There appears to be EN1 immunoreactivity (green) in several areas of the spinal cord, including dorsal regions. Can the authors clarify what that labeling could be representing?

Unfortunately, there is always some background staining, in particular in the tissue treatment conditions appropriate for RNAscope. Furthermore, given the large number and wide distribution of V1 interneurons (Figure 1A), we cannot preclude that EN1 is present at a low concentration in the extracellular space and in several cell types (now represented in revised Figure 9). This does not weaken the main conclusion that it primarily accumulates in MNs which do not express *En1* (RNAscope).

Figure 1D: These immunoprecipitation results lack a negative control with irrelevant antibody to confirm that the band shown it's being recognized specifically by the antibodies reacting with the blot.

Please see the response to reviewer 1 above with the Western blot and the absence of staining on a WB in absence of first antibody (86/8 or LSBio).

Figure 1E: The intensity of the EN1 labeling in MNs, much stronger than in V1 interneurons, is intriguing, given that MNs do not express engrailed-1 mRNA. One would have expected the opposite. It may help here if it was possible to show that immunoreactivity in MNs is diminished in the het mutant mouse.

We also were surprised by this intensity higher in MNs than in V1 interneurons, as if the protein was exported rapidly towards the target neurons. We have done the experiment proposed by the referee, found a twofold (approx.) immunoreactivity reduction in *En1*-Het MNs (see above Figure

EV2A in answers to referee 2). This novel figure has been introduced in the revised version. The experiment was done at 3 months when no MN death has yet occurred. Later the neurons "replenish" with EN1, probably because they do not have to share the limited supply with the dead ones (see above answers to referee 2 and Figure EV2B).

Figure 2D: There are a few possible problems with these data and their interpretation. First, this reviewer feels that 5 neurons (y-axis) is a rather small number. Are these 5 neurons per what area? From how many mice? I did not find that information in the figure legend. A larger area should be quantified so that we get numbers that are more robust. Second, such differences could also be due to hypotrophy of the MNs, namely, that MN number is the same but they are smaller.

At least 10 ventral horns (five lumbar spinal cord sections through L1-L5) separated by $\geq$ 900 µm were analyzed for each animal. As indicated in the legend of Figure 2D, 5 to 6 mice were analyzed per condition. For each mouse, hundreds of cells were counted. For example, in a WT mouse P2464 4.5 months of age 558, 158 and 112 cells were counted in the 100-199, 200-299 and >300µm^2 classes, respectively (5 sections). In 4.5-month-old *En1*-Het mouse P2458, the values were 562, 149, and 66, respectively. The data in the graphs are the average number of each cell category in one ventral horn. So, the WT mouse had an average of 11.2 large MNs and the En1-*Het* mouse had an average of 6.6 large MNs. The methodology has been better described in Material and Methods and in the legends.

The differences cannot be attributed to hypotrophy. A first reason is that, at 9 months, the Cresyl violet and ChAT staining give the same results for medium size and large neurons (Figure 2C). Furthermore, when one counts the cells throughout 15.5 months, the decrease in the number of large neurons is not compensated by an increase in the number of medium size or small ones. The reasoning and a graph, not intended for publication can be found in answers to referee 1.

Figure 3A: It would be useful that the authors explain how these AChR clusters were defined, visualized and counted. I could not find this information in the Methods. Perhaps this could be done by showing an alpha-BTX image illustrating the clusters.

We fully agree that the procedure was not well explained and we have introduced a correction in the Material and Methods section. For more details, please see answers to referee 2.

Figure 3B: As each adult endplate is only innervated by one MN, one would have expected fewer clusters and/or endplates, if indeed MNs are missing in this mouse, rather than endplates that are partially occupied. This could be clarified a bit more explicitly.

This is true and the ambiguity takes its origin in insufficient explanation of how fully innervated, partially innervated and denervated endplates were defined. Please see above and also in answers to reviewer 2. Modifications have been introduced in the text and in Figure 3. The referee is right, the absence of change in the number of AChR clusters suggests that there are very few fully denervated endplates and that what is defined as such in the analysis corresponds to partially innervated endplates (see above). This is now discussed in the text.

Figure 6B: Would not be better to do this with a virus, like in the case of the antibody? A more robust effect on MN survival may be attainable and thus strengthen the concept.

This would be another interesting experiment and we are presently exploring this possibility (with preliminary results). The choice of the virus and of the promoters is very important. We are comparing several AAVs, including AAV2, AAV2-TT (which diffuses better) and AAV8. For the promoter, we do not want to express within MNs as the imported protein might have special

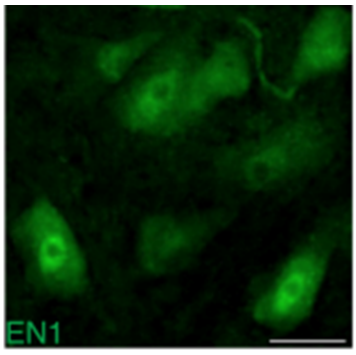
properties, associated with import. V1 interneurons would be best, but we have to verify if this does not modify V1 physiology. Astrocyte is another option, but with a similar pitfall. This means that we have a long way to go before proposing a "gene therapy" approach.

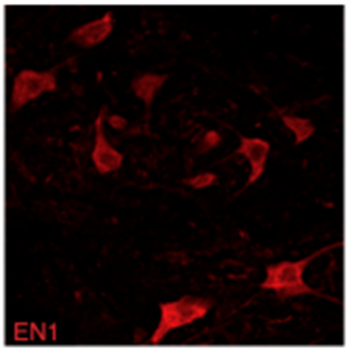
In addition, in the context of future clinical studies, we were eager, on the basis of the long-lasting activity of the protein already observed in the mesencephalic dopaminergic neurons (Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Rekaik et al. *Cell Reports* **13**: 242-250, 2015; Blaudin de Thé et al. *EMBO J*. **37**: e97374, 2018), to try a protein therapy in the spinal cord. Interestingly, the effects are also long-lasting in the spinal cord, (12 weeks in the mouse before a second injection is needed) and, according to contacted physicians, intrathecal injections, every second month or even more frequently, could be envisaged in the human. In that case, protein injection is possibly advantageous for the following reasons:
(i) viral particles can travel far and we do not know what would be the side effects.
(ii) the protein is short-lived but specifically addressed to MNs (thanks to the presence of EN1 binding sites at their surface), thus minimizing the issues associated with permanent expression and side effects.
(iii) EN1 is a natural protein normally secreted and the immune system might not be solicited as much as with viral approaches.

Figure 7A: The protein seems to be mainly in the cytoplasm of those cells (nuclei are dark and unlabeled), which is also unusual for a transcription factor that functions in the nucleus. Also surprising that the protein is gone in 3 days, but has effects over 24 weeks. Any explanation for that?

The protein is imported and is thus both in the cytoplasm where it exerts an effect on protein translation (Brunet et al. *Nature* **438**: 94-98, 2005; Alvarez-Fischer et al. *Nature Neurosci*. **14**: 1260-1266, 2011; Yoon et al. *Cell* **148**: 752-764, 2012) and in the nucleus where it exerts its transcriptional and "epigenetic" activity (see below for the latter). In fact, different antibodies and fixation procedures can favor cytoplasmic or nuclear staining. When nuclear, the dark point at the center, probably the nucleolus is less stained.

The two images below taken from Figure 1 C (RNAscope fixation) and 1E ("normal" fixation) illustrate this point:





For the second part of the question, three days are sufficient for a long-lasting activity. This was also observed in the midbrain where the protein restores the epigenetic marks jeopardized by an acute oxidative stress (Rekaik et al. *Cell Reports* **13**: 242-250). This has led to the hypothesis that EN1 has an important action at the level of the structure of the heterochromatin, thus a long-lasting "epigenetic" activity. We are presently working on the latter effects on the chromatin structure using human MNs derived from iPSCs (patients and isogenic controls).

Figure 7B: It's not clear what the blue and red bars mean, as this is not explained in the legend. Also, the y-axis says "%Chat+" suggesting they are counting MNs, but in the text they talk about EN-1 capture. If the latter, the y-axes should indicate % EN-1 over Chat, or something like that. In general, better figure legends would improve the experience of the reader.

In this experiment, we wanted to test the presence of a GAG-binding domain in EN1. To test its potential role in EN1 internalization and localization, we co-injected or not the RK-EN1 with hEN1 protein. Then, we counted the percentage of MNs (%ChAT+) which contain, or not, the hEN1 protein (hEN1+ in red or hEN1- in blue), allowing us to verify if the RK-EN1 alters the internalization of the hEN1 protein. So yes, we are looking at the capture of EN1 by the MNs with or without the RK-peptide (or control peptides). We have modified the text to make the point clearer.

Statistical analyses: In principle, comparisons of data obtained in studies that involved two variable parameters (such as time and genotype/treatment) should be weighted by a 2-way ANOVA test, which is more stringent since more conditions are being tested simultaneously. Usually a t-test is reserved for a pairwise comparison in an experiment involving only two conditions of the same variable.

The reviewer is correct. The two-way ANOVA is explained in the Statistical analyses section of the Methods. The analyses were carried out and the results listed in the legends for Figures 2, 3, 4, 6 and Figure EV1.

Description of analyses that authors prefer not to carry out

As can be seen above, we have tried to address most points raised by the three referees, either at an experimental level, with revised or novel figures, or through editorial changes and we hope that the answers will be found satisfactory. The main series of experiments that we wish to postpone for another study was proposed by referee 2 in his/her points 3 and 4.

3. More detailed data on NMJ morphology should be included. How does EN-1 modulate neuromuscular endplates? Is EN-1 located at neuromuscular endplates after being taken up from motoneurons? Even if the mechanism is indirect, via upregulation of p62 under conditions when EN-1 signaling is reduced, does this situation lead to enhanced localization of p62 at neuromuscular endplates?

4. The data shown in Fig. 3 on changes in NJM morphology appear incomplete and not convincing. As SV2a is not a good marker for changes in presynaptic compartments since it does not allow conclusions on how many synaptic vesicles are released, additional markers for presynaptic active zones such as Bassoon, Piccolo, Munc-13 should be studied. The analysis of fully occupied endplates appears arbitrary, and the differences are relatively small. Additional EM pictures and quantitative analyses of active zone proteins in the presynaptic compartment would help to support the argument of the authors that presynaptic compartments degenerate before cell bodies are lost in EN-1 +/- mice.

Both points focus on the neuromuscular endplate. We have only done the EN1 immunostaining and *En1* RT-PCR, demonstrating the absence of mRNA and protein (Figure EV3 introduced in the revised manuscript). We plead for not doing the other experiments, in particular the follow up of markers associated with presynaptic activities and quantitative ultrastructural studies. The reasons are as follows:

1. The post-docs who have conducted the work have left or will do so in the near future. Therefore, the proposed experiments necessitate to hire new post-docs and to identify collaborators interested in the project and expert in electron-microscopy. The experiments might thus be difficult to achieve in a reasonable time.

2. We really think, that although interesting, the proposed experiments mostly aimed at evaluating presynaptic activity are not in the scope of the study. Our main discovery is that EN1 is transferred from V1 interneurons to MNs and that reducing EN1 synthesis or transfer induces αMN retrograde degeneration and death. It is the first time that an adult function is described for EN1 transfer and, in the context of this finding, we have done several experiments that provide a story which we see as self-sufficient.

3. This does not mean that we do not appreciate the suggestions of the referee but that we would like to consider them in the context of novel experiments that we have undertaken and include the following steps:

We have contacted an electrophysiology group that works *in vivo* on the activity of spinal cord neurons and will investigate, in collaboration with us, the activity of V1 interneurons, MNs, and the synaptic activity at the neuromuscular junction, in our models (WT, *En1*-Het, scFvEN1, rescue by EN1 injected in L5).

We have initiated microfluidic experiments with human iPSC-derived MNs from patients with familial and sporadic ALS forms (plus healthy or isogenic controls) and shown that EN1 “cures” the ALS phenotype. We will now co-culture the MNs with fused myoblasts in the distal compartment to

follow the effect of EN1 added at the level of the cell body or of the terminals on electrical activity, local protein synthesis (with identification of the changes in local translation), quantification of presynaptic and post-synaptic markers. This will allow us to better define *in vivo* studies.

Using the same human MNs (sick, healthy, sick and complemented with EN1) we will investigate the status of the nuclei (ATAC-seq, Cut and Run, ChIP with EN1 antibodies…) to better understand the molecular basis for long-lasting effects of short-term exposure to EN1.

These studies have been initiated and will be part of a future manuscript distinct from the one presently under review.

Dear Alain,

Thank you for the submission of your revised manuscript, and I am sorry for the delay in getting back to you. We have only received the third referee report now.

I am sorry to say that the evaluation of your manuscript is not a positive one.
As you will see, while referee 1 is positive, and while all referees agree that the study has been improved, both referees 2 and 3 do not find the current set of data sufficiently convincing to support the main conclusions.

Given these comments from 2 experts in the field, and the fact that EMBO reports can only proceed with papers that receive enthusiastic support from the referees, I am afraid that we cannot offer to publish your manuscript.

I am sorry to disappoint you on this occasion, and hope that the referee comments will be helpful in your continued work in this area.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

I am satisfied with the responses provided by the authors.

Referee #2:

The authors have revised their manuscript. Several points that could give rise to misunderstandings have now been made clearer, but many central points of my initial criticism are still not adequately addressed.

1. The authors show in their paper that EN1 is expressed in V1 interneurons, both on protein and mRNA level. It is then apparently released and taken up by motoneurons, and levels in motoneurons are then higher than in the V1 interneurons that originally produced it. This raises questions on the mechanisms for this enrichment. The only explanation given is on page 3 is the new sentence: "This novel signaling pathway, involving unconventional secretion and internalization with direct access to the cytoplasm, ...". To my mind, this is not concrete enough, and the mechanism how EN1 is taken up by motoneurons and released into the cytoplasm should be investigated in the context of this study in more detail.

2. The authors provide evidence in Fig. 2E that muscle strength is lost over time in EN1 +/- mice. This precedes the loss of motoneurons, indicating that functional defects at NMJs are responsible. However, the nature of this synaptic dysfunction and the mechanisms how EN1 leads to defective neurotransmission at NMJs remains obscure. The sentence "These results suggest that strength loss is a consequence of αMN retrograde degeneration" is not a satisfying answer to this point.

3. The authors observe that expression of SQTSM1/p62 increases in EN1 +/- mice. Treatment with EN1 normalizes this expression, but the mechanism how this takes place, either on a translational level, on a transcriptional level or via transcriptional control of a master regulator for autophagy, remains obscure. I would also disagree with labeling SQTSM1/p62 as an "age marker". Regulation of autophagy is important for many dynamic processes, not only for aging. Conditions such as lesion, regeneration, functional adaption, etc. also need to be considered, and it is very likely that p62 expression is highly regulated under such conditions.

4. The new data added in Fig. EV1B are difficult to understand. They show increased EN1 immune fluorescence levels both in the 200-299 µm group of neurons and in the > 300 µm size group. A control for the 100-200 µm group is missing, and given that many V1 interneurons are also be included in the 200-300 µm group, this points to an increase of EN1 expression in individual interneurons. To my mind, it is hard to distinguish interneurons from gamma-motoneurons on the basis of size, and the recommendation of the other reviewers to identify gamma motoneurons by specific antibodies makes a lot of sense to increase precision of this study. With respect to mean p62 expression in alpha- and gamma motoneurons, the authors propose that these two types of motoneurons differ in their sensitivity to EN1 dosage. How can this be explained? Is there a difference in uptake between these two cell types? This does not seem to be the case on the basis of the quantitative analyses of immune staining. In addition, why should remaining neurons after the period of death of alpha-motoneurons receive more EN1? This would require plastic regrowth of axons from EN1 interneurons towards the surviving motoneurons. However, there is no evidence for

such a mechanism in this manuscript. At this point, this proposed mechanism remains highly speculative and other possibilities such as altered cellular uptake, altered stability or altered subcellular distribution of EN1 should also be considered.

Referee #3:

Upon revision, the manuscript is somewhat improved, however, several of the concerns previously raised remain unaddressed and, overall, the molecular mechanisms are still unclear.

Antibody staining shown in Figure 1C and 1E is puzzling and further characterization should have been performed. The papers cited for the 86/8 antibody validation do not seem to report immunofluorescence applications and they do not refer to spinal cord tissue. En1 expression in the adult spinal cord has been previously reported by several studies (Salamantina A et al 2020, Bikoff J et al 2016, Allodi I et al 2021) based on cre-dependent expression and RNAscope detection. In En1cre mice, V1 interneuron death can be detected by decrease of fluorescently tagged cells (Salamantina A et al 2020).
Engrailed 1 is a low abundance transcript within the spinal cord, cell by cell quantifications should have been performed.
In the Answer to the Referees, the authors state that "The En1 locus is silent in MNs and only the protein is present, a consequence of its internalization." However, they do not report literature supporting this claim.
Overall, the molecular mechanisms behind motor neuron degeneration and rescue remain unclear and the authors decided to not investigate this aspect further.

Referee #1:

I am satisfied with the responses provided by the authors.
We are satisfied with this reviewer

Referee #2:

The authors have revised their manuscript. Several points that could give rise to misunderstandings have now been made clearer, but many central points of my initial criticism are still not adequately addressed.

1. The authors show in their paper that EN1 is expressed in V1 interneurons, both on protein and mRNA level. It is then apparently released and taken up by motoneurons, and levels in motoneurons are then higher than in the V1 interneurons that originally produced it. This raises questions on the mechanisms for this enrichment. The only explanation given is on page 3 is the new sentence: "This novel signaling pathway, involving unconventional secretion and internalization with direct access to the cytoplasm, ...". To my mind, this is not concrete enough, and the mechanism how EN1 is taken up by motoneurons and released into the cytoplasm should be investigated in the context of this study in more detail.

We do not understand this criticism. Homeoprotein transduction has been demonstrated for more than 150 members of the family (Lee et al. *Cell Rep* **28**:712-722, 2019) and we have shown in the past that two highly conserved regions in the homeodomain are responsible for secretion and internalization. These transfer sequences have allowed for mechanistic studies by physicists, chemist and biologists. There is a huge list of references and, if interested, one can start by looking at the thee most recent reviews and references therein: Prochiantz & Di Nardo *Neuron* **85**: 911-925, 2015; Di Nardo et al. *Physiological Reviews* **98**: 1943-1982, 2018; Di Nardo et al. *Science Advance* **6**:eabc6374, 2020. A third domain that binds glycosaminoglycans in the extracellular matrix is responsible for the specific targeting to receiving cells and that of EN1 (allowing for MN recognition) has been characterized in the present study. It remains that our interest is in the demonstration and physiological significance of EN1 transfer in the spinal cord, not in the mechanisms which, except for the specific addressing to MNs, are similar for all homeoproteins (due to the conservation of the transfer sequences).

Several groups using either *En1*-Cre driven reporter genes or RNAscope ISH have reported that the *En1* locus is active in V1 interneurons during development and throughout adulthood and NOT in MNs (Bikoff et al. *Cell* **165**: 207-219, 2016, Salamatina et al. *Neuroscience* **450**: 81-95, 2020; Allodi et al. *Nature Com*. https://doi.org/10.1038/s41467-021-23224-7, 2021). Our own data (*En1*/*ChAT* RNA-scope) confirm it. This being established, it is exact that IHC is stronger in MNs that receive and accumulate EN1 than in interneurons that export it. We have no other explanation at this stage and we do not see why it should really matter.

2. The authors provide evidence in Fig. 2E that muscle strength is lost over time in EN1 +/- mice. This precedes the loss of motoneurons, indicating that functional defects at NMJs are responsible. However, the nature of this synaptic dysfunction and the mechanisms how EN1

leads to defective neurotransmission at NMJs remains obscure. The sentence "These results suggest that strength loss is a consequence of αMN retrograde degeneration" is not a satisfying answer to this point.

The deafferentation at the NMJ level and the loss of strength precedes cell body loss. This is the definition of retrograde degeneration, in contrast with Wallerian degeneration. The reviewer wants to know the mechanisms through which EN1 regulates this deafferentation. To partially satisfy him we showed that EN1 is not present at the synapse. EN1 activity is thus likely the consequence of transcription or translation regulation taking place at the nuclear or cell body levels. Identifying these mechanisms is not the focus of our study. Its focus is on the discovery of a non-cell autonomous EN1 activity with effects on MN physiology/survival and on the possibility of rescuing MNs by intrathecal injections and specific addressing of EN1 to MNs thanks to an addressing sequence that we have identified. The mechanisms of action of EN1 in MNs have not been studied in detail. This is the reason why *EMBO J*. transferred the manuscript to *EMBO Reports* after talking, with Esther Schnapp. I copy the mail that we have received:

That said, given the general interest in this topic, we still found this work potentially suitable for our sister journal EMBO reports, in light of their focus on interesting key observations that do not necessarily need to be fully mechanistically followed up. I therefore briefly discussed the work with my EMBO reports colleague, Dr. Esther Schnapp, who considered the study interesting and would be happy to consider a revised version of the manuscript in case you transfer it to EMBO reports.

3. The authors observe that expression of SQTSM1/p62 increases in EN1 +/- mice. Treatment with EN1 normalizes this expression, but the mechanism how this takes place, either on a translational level, on a transcriptional level or via transcriptional control of a master regulator for autophagy, remains obscure. I would also disagree with labeling SQTSM1/p62 as an "age marker". Regulation of autophagy is important for many dynamic processes, not only for aging. Conditions such as lesion, regeneration, functional adaption, etc. also need to be considered, and it is very likely that p62 expression is highly regulated under such conditions.

Recall (it is explained in the manuscript) that SQTSM1/p62 was not found by chance. In a previous study on mesencephalic dopaminergic neurons that express *En1* and experience retrograde degeneration and death in the *En1*-Het (just like alpha-MNs do) we performed RNA-seq on the neurons (before they start dying) and identified differentially expressed genes (DEGs). The DEG library was blasted on a MN library allowing for the identification of 400 shared genes. We then looked at the interaction of these 400 genes with the 4 main mutations found in human familial ALS (fALS) allowing for a narrowing to 20 genes and SQTSM1/p62 is the only one in interaction with the 4 fALS genes, individually. In addition, it is mutated in familial forms of the disease. This agnostic finding led us to study the expression of SQTSM1/p62 in MNs from WT and *En1*-Het mouse. Expression, very strong in MNs, increases with age in the WT (why we called it a marker of age, not a cause of aging), increases at 3 months in the *En1*-Het and also increases when EN1 import to MN is blocked *in vivo*. These are the conditions that we have tried, because they correspond to the study. We see no imperative reason to add lesion, regeneration and functional adaptation studies.

The new data added in Fig. EV1B are difficult to understand. They show increased EN1 immune fluorescence levels both in the 200-299 µm group of neurons and in the > 300 µm size group. A control for the 100-200 µm group is missing, and given that many V1 interneurons are also be included in the 200-300 µm group, this points to an increase of EN1 expression in individual interneurons. To my mind, it is hard to distinguish interneurons from gamma-motoneurons on the basis of size, and the recommendation of the other reviewers to identify gamma motoneurons by specific antibodies makes a lot of sense to increase precision of this study.

The data from EV1B have been added to satisfy one of the reviewers and are extremely useful. They show that the amount of EN1 accumulated by MNs increases with time in the *En1*-Het to reach at 4.5 months levels similar to those found in WT MNs. This is best explained by the fact the *En1* expression by V1 interneurons being constant (Figure 1B), each surviving MN captures more EN1 (the dead MNs, no longer present, do not capture EN1). This may explain why we see a difference in SQTSM1/p62 expression between WT and *En1*-Het only at 3 months (and not at 4.5 or 9 months when many MNs are already dead) and why death is limited to 50% of the alpha-MNs. We had no reason to look at V1 interneurons that make the protein. More impotantly, intensities were measured in ChAT positive cells, thus only in MNs precluding that we measured an increase in interneurons as proposed by the reviewer. We separated MNs (ChAT-positive cells) on the basis of size to distinguish alpha- from gamma-MNs and both populations gave the same result. We could have pooled these MNs but we thought it more interesting and complete to provide separate data.

With respect to mean p62 expression in alpha- and gamma motoneurons, the authors propose that these two types of motoneurons differ in their sensitivity to EN1 dosage. How can this be explained? Is there a difference in uptake between these two cell types? This does not seem to be the case on the basis of the quantitative analyses of immune staining. In addition, why should remaining neurons after the period of death of alpha-motoneurons receive more EN1? This would require plastic regrowth of axons from EN1 interneurons towards the surviving motoneurons. However, there is no evidence for such a mechanism in this manuscript. At this point, this proposed mechanism remains highly speculative and other possibilities such as altered cellular uptake, altered stability or altered subcellular distribution of EN1 should also be considered.

The difference of sensitivity between categories of MNs is a well-known fact and we discuss the possible reasons ("Discussion"). As for why should remaining neurons accumulate more EN1, please see above (less MNs capture the same amount of EN1, there is thus more EN1 per MN). The expression of the antibody by ASTROCYTES (GFAP promoter) that blocks EN1 capture by MNs and the targeting to MNs of intrathecally injected EN1 support the idea that internalization does not necessarily require synaptic contacts, and thus any type of plastic regrowth, between V1 interneurons and MNs.

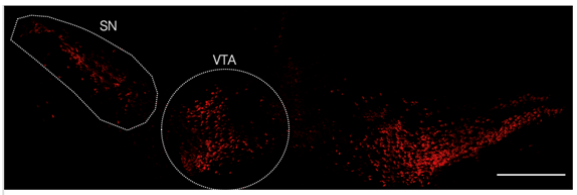
Referee #3:

Upon revision, the manuscript is somewhat improved, however, several of the concerns

previously raised remain unaddressed and, overall, the molecular mechanisms are still unclear.

Antibody staining shown in Figure 1C and 1E is puzzling and further characterization should have been performed. The papers cited for the 86/8 antibody validation do not seem to report immunofluorescence applications and they do not refer to spinal cord tissue.

The papers cited use peroxidase staining in the SNpc but we also have immunofluorescence. Initially it was in the supplemental data but we removed it.



As for the absence of reference to SC studies, nobody has ever provided results on protein expression, this is one of the contributions of this study.

En1 expression in the adult spinal cord has been previously reported by several studies (Salamantina A et al 2020, Bikoff J et al 2016, Allodi I et al 2021) based on cre-dependent expression and RNAscope detection. In En1cre mice, V1 interneuron death can be detected by decrease of fluorescently tagged cells (Salamantina A et al 2020).

Yes, by Cre-dependent reporter gene expression (LacZ, GFP, TdT), one can see the locus active in V1 interneurons at least up to p21 for lacZ and GFP and in the adult for TdT. Neither Bikoff et al, nor Salamatina show *En1* expression in MNs, confirming that the *En1* locus is silent in MNs (Bikoff et al. *Cell* **165**: 207-219, 2016, Salamatina et al. *Neuroscience* **450**: 81-95, 2020; Allodi et al. *Nature Com*. https://doi.org/10.1038/s41467-021-23224-7, 2021). Here we show the same thing by *ChAT*/*En1* RNAScope double-labelling (what is wrong with that? In fact, we think that it is better to follow *En1* mRNA than a reporter). We do not want to use the En1-Cre mouse which is *En1*-Het (*Cre* is knocked into *En1*), even if we agree that C57BL6 genetic background is less sensitive to *En1* hypomorphism.
It might also be useful to recall that Allodi et al refer to our study uploaded on *bioRxiv*:

Moreover, the recent finding that loss of the En1 transcription factor expressed in V1 neurons may lead to motor neuron degeneration[58] may be another mechanism that contributes to the ALS disease progression when the inhibitory synapses are retracted. The actual cause of the retraction is not known but the study underscores that ALS may start as an interneuron affection.
58. Vargas Abonce, S. E., Lebœuf, M., Moya, K. L. & Prochiantz, A. Homeoprotein ENGRAILED-1 promotes motoneuron survival and motor functions. Preprint at https://doi.org/10.1101/734020 (2020).

Finally, the death of V1 interneurons that precedes MN death, if this is what troubles the reviewer, was seen by Allodi et al. and Salamatina et al in the *SOD1*^{G93A} mouse, which is a very severe ALS model on an *En1*-het (*En1*-Cre) background and has nothing to do with our own study where V1 interneurons are preserved.

Engrailed 1 is a low abundance transcript within the spinal cord, cell by cell quantifications should have been performed.

We do not see why. The amount of *En1* transcribed is stable over 16 months (Figure 1B) and the number of cells that express *En1* (V1 interneurons) is not modified (Figure 2A). To our understanding, cell by cell quantification at the RNAscope level, would not bring any useful information in the context of this study. We can also add that we observed alpha-MN degeneration and p62 upregulation by blocking extracellular EN1 in a wild-type context (Figures 4 and 8).

In the Answer to the Referees, the authors state that "The En1 locus is silent in MNs and only the protein is present, a consequence of its internalization." However, they do not report literature supporting this claim.

As mentioned above, no reporter gene driven by *En1*-Cre is transcribed in the MNs. We, as well as Allodi et al. (see above), confirm it by RNAscope for *En1* (which might be better than a reporter gene).

Overall, the molecular mechanisms behind motor neuron degeneration and rescue remain unclear and the authors decided to not investigate this aspect further.

There is a limited amount of data that one can generate and publish in a given time. Look above at the email from *EMBO J* that we received in which we are incited to submit to *EMBO Reports*. As a prolongation of this study we presently explore the mechanism by combining in vivo studies with studies using MNs derived from human iPSCs.

Dear Alain,

Thank you for your email asking us to reconsider our decision on your manuscript. I sent your point-by-point response back to the 2 critical referees, and also re-discussed your study with the EMBO reports team, including our chief editor Bernd Pulverer. I am sorry to say that the outcome of these discussions is that we stand by our decision that we cannot offer to publish your manuscript as it stands now.

I talked to referee 3 on the phone and while s/he acknowledges that the descriptions of the EN1 Het mice are fine, her/his major concern regards the use of your antibody, which has not been validated in vivo. Given that the main findings of your manuscript rely on this antibody detecting EN1 protein in vivo, this is a crucial point. The referee suggests in vivo validation of your antibody using for example the EN1-Cre mouse, or the use of antibodies that others have validated. Both referees 2 and 3 also point out that it remains unclear why there is much more EN1 in motorneurons than in interneurons. This surprising/unexplained observation enforces the need for a thorough antibody validation.

Referee 2 also sent us more comments that I paste below for your information.

I would like to underline that we are not asking for insight into the mechanism of EN1 uptake and MN rescue. Our main concern is that the data as they stand now are not sufficiently convincing according to 2 experts in the field. IF the current data can be sufficiently strengthened to a satisfying level, we would be interested in a revised study for EMBO reports.

I am sorry that I cannot be more positive this time, and hope that the referee comments will be helpful in your continued work in this area.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee 2 latest comments:

I am still worried by the relatively high accumulation of En1 in motoneurons. This is so much higher than what one finds in the V1 interneurons that produce this factor, and to my mind needs to be explained. Alain Prochiantz has some hypotheses about this point which sound interesting and could be true, but there is no experimental evidence.

Also the effect on SQTMS1/p62 expression is to my mind not well explained. In the former publications about En1 effects on mesencephalic dopaminergic neurons, this group focused on the transcriptional activation of Ndufs1 and Ndufs3 subunits of complex I in mitochondria. Now, in the motoneurons, it is autophagy regulation, without going in much depth. No word why they did not look into Ndufs1 in motoneurons, but instead on SQTMS1.

There seems to be a synaptic dysfunction in the En1 +/- mice at a relatively early stage, but the reason for this dysfunction is unclear. The new suppl. Fig. EV2 lacks a positive control, it is hard to say from these data that En1 is not present at these synapses. If it is not present and does not play a role in translational control, as shown in the previous study with dopaminergic neurons and as originally suggested by the authors as a possible mechanism for this phenotype , what is the reason why the presynaptic apparatus becomes dysfunctional when En1 is not taken up in motoneurons from V1 interneurons ? When looking at Fig. 3B, in the left panel, the right upper picture suggests that all NMjs are unoccupied by presynaptic terminals. I would expect this is not compatible with survival, this is probably a selection bias.

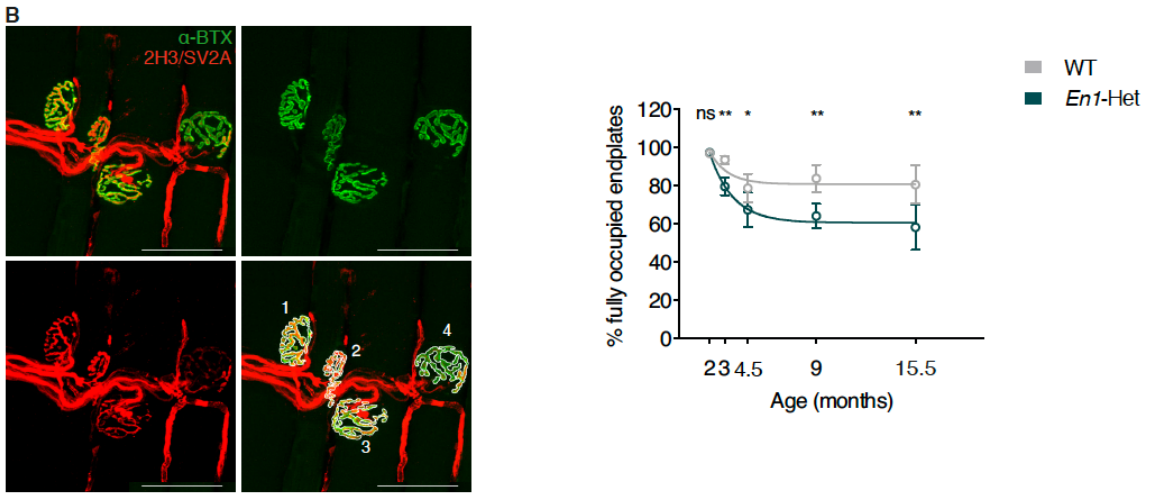
There are many other points like that, for example the virtually complete absence of En1 staining in Fig. 4c, right lower panel, and all of this kind of indirect data in Fig. 5 and 6. These points add up to make me feel unhappy with this paper.

Answers to Referee 2 latest comments

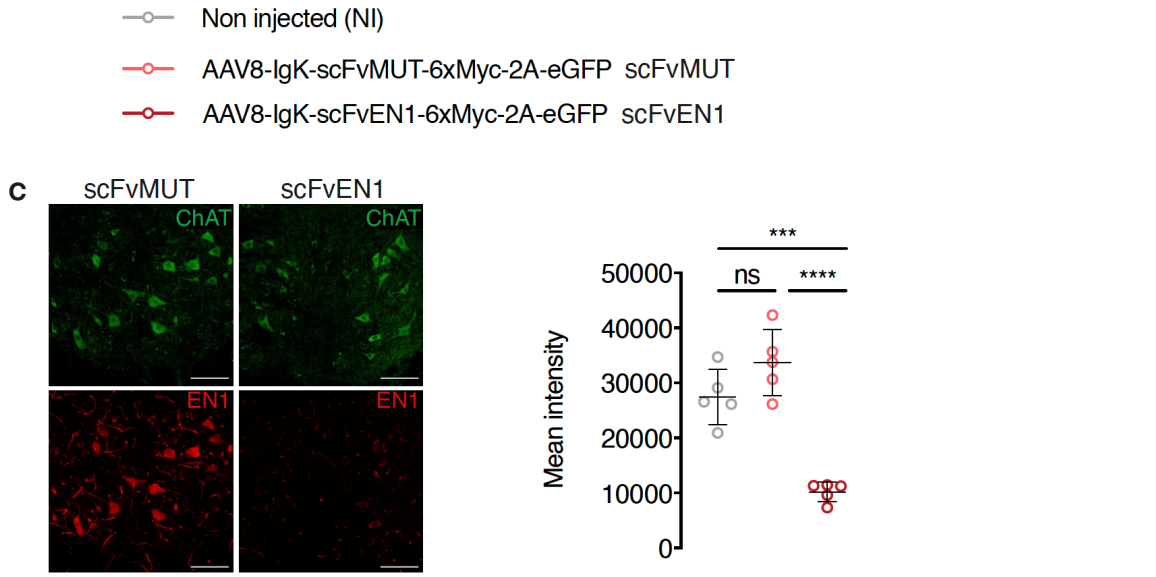
1. In the former publications we did not focus on transcriptional activation of NdufS1 and NdufS1, but on translation. In this study we used transcriptomic analyses is the *En1*-Het and Motoneurons and SQTSM1/p62 came up in an unbiased bioinformatic analysis.

2. Positive controls for *En1* transcription and for EN1 protein are in almost all figures in the paper

3. As can be seen in the copy of the Figure below, In Figure 3B left panel right upper photograph shows no terminals because it is a single staining with alpha-BGT, but indeed there are terminals as seen in the single staining with 2H3/SV2A in the lower left panel and the merging of the two images in the upper left and lower rights panels



4. In Figure 4C, there is no staining in the right lower panel because the wild-type anti-EN1 extracellular antibody (but not its mutated form, left lower panel) blocks EN internalization. This is exactly what we wanted to show and what was quantified (right panel). This demonstrates that EN1 is captured from the outside as a basis for its paracrine function. We do not see how this could be taken against the study.



Point by point response to the “advisor notes” as per email dated Feb 23, 2023.

Advisor notes:
'I took a look at the manuscript, reviews, and the response to reviewers. It certainly seems unreasonable to expect a mechanistic study of how En1 exactly becomes enriched in motor neurons. I'm in agreement that this should not be a factor in the ultimate decision.

Regarding the data on En1 immunohistochemical staining in the adult mouse spinal cord, this does seem like an important point [....]. I'm certainly convinced the motor neurons don't express En1 themselves. But in my experience, motor neurons in older tissue sometimes exhibit non-specific immunostaining. They do some validation (e.g. Figure 1E) for the LSBio antibody, which is great. However, the most rigorous way to demonstrate specificity is to use a knockout mouse lacking En1 (null allele) and to show the immunoreactivity goes away. As far as I can tell, the authors don't do this. But they could.

The En1-Cre allele (from reference #30, I think) is a null allele. If they make this allele homozygous, the animal will not produce any En1 protein. It therefore seems reasonable to ask for a single experiment using homozygous En1 KO to show En1 immunoreactivity with the relevant antibody goes away, especially since they have this animal in hand. En1 null animals generally die around birth, so they won't be able to assess this in the adult, but they can still do it in neonatal animals. This seems like a key piece of data that would strengthen the paper.'

We have performed the suggested experiment with *En1* KO embryos (the mutation is embryonic lethal and adult animals cannot be obtained). In the E15 WT, we observe robust EN1 signal in ventral spinal cord, weaker signal in the *En1*-Het spinal cord and no signal in the KO embryo. Most importantly, EN1 signal colocalizes with ChAT establishing that, also at this early age, EN1 is captured by MNs. The genotyping and the immunostaining results are now added to a reorganized Figure 1 and discussed in the text.

In addition, we performed a dilution experiment on adult spinal cord sections. We observe that the EN1 signal on WT spinal cord diminishes when the antibody is serially diluted from 1/200 to 1/800, but can still be detected at 1/800. In the *En1*-Het spinal cord, the EN1 signal is greatly reduced at 1/200 compared to WT and is barely detectable at 1/600. These results provide additional evidence for the specificity of the antibody. This is now presented in Figure EV2.

In Figure 9 we present new results on EN1 and p62/SQTSM1. In this experiment, we injected hEN1 at 1 month of age in *En1*-Het mice and analyzed ventral spinal cord p62/SQTSM1 expression and mouse behavior 2 months later (3 months of age). Compared to WT siblings, non-injected *En1*-Het mice show strength weakness and enhanced p62/SQTSM1 expression, as anticipated. In contrast, *En1*-Het mice injected with hEN1 are indistinguishable from WT siblings both for strength and p62/SQTSM1 expression in αMNs and γMNs. These results reinforce the idea that EN1 has anti-ageing properties.

We have added a schema to Figure EV2 that illustrate that, after 4.5 months, surviving MNs in the *En1*-Het mice may receive sufficient EN1 from the V1 interneurons to prevent their degeneration.

3rd Revision - Editorial Decision **23rd May 2023**

Dear Alain,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from referee 3 and the advisor consulted (referee 4). Referee 3 only has 2 more minor comments that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few editorial requests will also need to be addressed:

- Please add a Data Availability Section (DAS) to the end of the materials and methods. You can list the Biostudies link that is in the authors checklist in the DAS, and if you have deposited any data in other public databases please also list links for these in the DAS.

- Please update the conflict of interest subheading to "Disclosure and Competing Interest Statement"

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- Fig 4A is called out before 2A. Fig 9A, EV2A&C, EV3A&B callouts are missing.
Fig EV2B is called out after EV4. Please correct.

- Please upload the source data (SD) as one file or folder per figure.

- The manuscript sections are in the wrong order, please correct. AC, COI and funding info need to be removed from the title page.

- Please add the subheading 'Expanded View Figure legends'.

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I would like to suggest some minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Several homeoprotein transcription factors transfer between cells and regulate gene expression, protein translation, and chromatin organization in recipient cells. ENGRAILED-1 is one such homeoprotein expressed in spinal V1 interneurons synapsing α-motoneurons. Neutralizing extracellular ENGRAILED-1 by expressing a secreted single-chain antibody blocks its capture by spinal motoneurons resulting in α-motoneurons loss and limb weakness. A similar but stronger phenotype is observed in the Engrailed-1 heterozygote mouse, confirming that ENGRAILED-1 exerts a paracrine neurotrophic activity on spinal cord α-motoneurons. Intrathecal injection of ENGRAILED-1 leads to its specific internalization by spinal motoneurons and has long-lasting protective effects against neurodegeneration and weakness. Midbrain dopaminergic neurons express Engrailed-1 and, similarly to spinal cord α-motoneurons, degenerate in the heterozygote. We identify genes expressed in spinal cord motoneurons and whose expression changes in mouse Engrailed-1 heterozygote midbrain neurons. Among these, p62/SQTSM1 shows increased expression during aging in spinal cord motoneurons in the Engrailed-1 heterozygote and upon extracellular ENGRAILED-1 neutralization. We conclude that ENGRAILED-1 might regulate motoneuron ageing and has non-cell autonomous neurotrophic activity.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #3:

The revised manuscript is considerably improved, and the previous concerns were properly addressed. In the discussion, at "deficits in inhibitory interneurons in the spinal cord of motorneuron disease models have been reported" the study McGown A et al 2013, Annals of Neurology should be cited as well.
Moreover, the same sentence includes 2 imprecisions, both Chang & Martin 2009 and Allodi et al 2021 observed glycinergic interneuron pathology before MN loss and not after. Here, only Chang & Martin 2009 analysed Renshaw cells, while the Allodi et al 2021 study did not. These changes should be included in the final manuscript.

Referee #4:

I took a look at the data. In my opinion, the data in the new Figure 1E using En1 KO mice is convincing evidence at least in embryonic animals that the En1 staining in motor neurons is specific. The supplemental dilution data, while perhaps less convincing, is still a nice addition.

Point by point responses in **blue**.

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from referee 3 and the advisor consulted (referee 4). Referee 3 only has 2 more minor comments that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. The McGown reference has been incorporated and the imprecisions corrected as suggested. Referee #4 was satisfied with the last version.

- Please add a Data Availability Section (DAS) to the end of the materials and methods. You can list the Biostudies link that is in the authors checklist in the DAS, and if you have deposited any data in other public databases please also list links for these in the DAS. Now included at the end of the Materials and Methods.

- Please update the conflict of interest subheading to "Disclosure and Competing Interest Statement" Updated.

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I would like to suggest some minor changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

We agree and this version is not incorporated into the manuscript.

Several homeoprotein transcription factors transfer between cells and regulate gene expression, protein translation, and chromatin organization in recipient cells. ENGRAILED-1 is one such homeoprotein expressed in spinal V1 interneurons synapsing α-motoneurons. Neutralizing extracellular ENGRAILED-1 by expressing a secreted single-chain antibody blocks its capture by spinal motoneurons resulting in α-motoneurons loss and limb weakness. A similar but stronger phenotype is observed in the Engrailed-1 heterozygote mouse, confirming that ENGRAILED-1 exerts a paracrine neurotrophic activity on spinal cord α-motoneurons. Intrathecal injection of ENGRAILED-1 leads to its specific internalization by spinal motoneurons and has long-lasting protective effects against neurodegeneration and weakness. Midbrain dopaminergic neurons express Engrailed-1 and, similarly to spinal cord α-motoneurons, degenerate in the heterozygote. We identify genes expressed in spinal cord motoneurons and whose expression changes in mouse Engrailed-1 heterozygote midbrain neurons. Among these, p62/SQTSM1 shows increased expression during aging in spinal cord motoneurons in the Engrailed-1 heterozygote and upon extracellular ENGRAILED-1 neutralization. We conclude that ENGRAILED-1 might regulate motoneuron ageing and has non-cell autonomous neurotrophic activity.

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The data shown in figures should satisfy the following conditions:

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- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Endpoint criteria for removal and euthansia is indicated in the Animal management section. In a very few cases a mouse refused to perform the behvioral test and this is noted in the Figure Source Data File.
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
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