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Expression of ALS-linked TDP-43 Mutant in Astrocytes Causes Non-cell-autonomous Motor Neuron Death in Rats

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

Editor:

1st Editorial Decision 22 January 2013

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

Both referees find the study interesting, but they also indicate that the analysis would have to be significantly extended in order to consider publication here. The transgenic rat model needs to be much better characterized in terms of mutant TDP-43 expression and motor neuron loss. Referee #2 also finds that expression of wt TDP-43 is needed as a control. Should you be able to address the concerns raised below and significantly extend the in vivo characterization of the rat TDP-43 model then we would consider a revised version. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. I can extend the revision time to 6 months should that

be helpful.

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

REFEREE REPORTS

Referee #1 This paper strives to address an important issue in the ALS field -whether astrocytes are involved in disease course in the case of ALS caused by TDP43 mutations. To address this question, the authors create a rat model where human mutant TDP43 is expressed from the GFAP promoter with the goal to express mutant TDP43 only in astrocytes.

Overall, this is a really interesting manuscript that could be an important piece of a puzzle allowing us to dissect mutant TDP-related astrocyte toxicity to the development of ALS as well as an interesting tool for further investigation.

The way in which the manuscript is written attempts to touch on a variety of hypotheses with a little data in each (for example ubiquitin inclusion formation in astrocytes, aberrant astrocytes, lower motor neuron loss, cortical motor neuron loss, in vitro assessment of toxic factors, anterograde motor neuron degeneration) making the story a little difficult to read and less cohesive.
Their approach is reasonable; however, the fast disease course of 15-20 days between disease onset and death triggers questions about how relevant this model is to ALS. Although the rats seem to develop a disease reminiscent of ALS, there are major questions as to whether expression of hTDP43 is restricted to astrocytes, what level of expression of hTDP43 is achieved compared to endogenous TDP43, and whether this potentially high level of hTDP43 expression is causing death of astrocytes which may result in the pathology observed.

Major issues:

1.) In this report, the authors create a new transgenic rat expressing tTA driven by an "undefined" 21 kb fragment of the human GFAP promoter from a BAC clone. The authors provide no characterization for this rat and the pattern of tTA expression. They cross this rat to the TRE-TDP43M337V (previously characterized) and show by IHC that the human TDP43 is expressed in GFAP+ astrocytes, but not microglia, oligodendrocytes, or neurons. However, further characterization is needed including:

-Is the human mutant TDP43 expressed in peripheral organs (ex. Muscle, gut, schwann cells?). This is particularly important because this model is the first attempt at expression of TDP43 in astrocytes and may serve as the basis for future TDP43 modeling.

-A more high power image showing human TDP43 (with a nuclear stain such as DAPI) in GFAP+ astrocytes would be helpful. This is particularly important as seen in Fig 1 f where it appears that GFAP expression runs right through the nucleus of TDP43 staining whereas in other cells in this image one doesn't appreciate GFAP expression around these TDP43+ nuclei.

-Because NG2 cells also seem to proliferate quite robustly in SOD1 mice (and because they have a glial lineage), it is probably also worthwhile to assess whether there is any hTDP43 in NG2 cells as well to make certain this is not contributing to the model.

-What is the level of expression of human TDP43 in this rat compared to endogenous rat TDP43 expression? Western blot analysis of both whole spinal cord homogenate as well as homogenate from isolated, purified astrocytes cultures in vitro would be helpful.

-In figure 3 the authors note quite dramatic loss of both motor neurons and axons in the ventral root and sciatic nerve. However, in GFAP-tTA line #1 (figure S2) they note motor neuron loss without ventral root loss. This is quite surprising unless this were a very acute finding but I think less likely that at endstage one would see motor neuron loss without axon loss. Rather than suggesting that this loss is anterograde, it may be more likely that the animals at endstage are dying from something else

(i.e. seizures, upper motor neuron loss?). Maybe an explanation here as to their hypothesis would be helpful.

-A figure about cortical (or brain) expression of hTDP43 as far as distribution would be really helpful. This would make a more complete story. The focus of the manuscript can still be related to spinal cord motor neuron loss but the authors seem to indicate at least some degeneration in Fig. 3J and K but do not elaborate.

2.) A major point in the paper is that the astrocytes expressing human mutant TDP43 become "transformed" and lose expression of GFAP over the disease course. However, in Fig. 4b1-5 and Supp. Fig. 3, it appears that the total number of hTDP43-expressing cells is decreasing dramatically throughout disease, suggesting either that astrocytes are losing expression of the transgene or astrocytes expressing these high levels of TDP43 are dying over time.

-The authors should quantify the number of hTDP43-expressing cells over the disease course to address these points. Assessing whether astrocytes are dying because of hTDP43 expression could also be tested in vitro over a period of time to assess whether this is evident.

-The use of another astrocyte marker besides s100B (a marker for immature glia) would help determine whether astrocytes are dying or losing expression of GFAP. Perhaps, the authors could stain for GLAST to show astrocytes are still present (and not dying) in the spinal cord at end stage. This would also help in Fig. 5 to determine whether astrocytes are losing expression of all glutamate transporters, or specifically GLT1. The specific loss of GLT1, but not GLAST occurs in the G93A SOD1 mouse model and human ALS. This is important be cause s100B can also be expressed by NG2 cells (Vives et al 2003) which definitely proliferate in ALS.

The loss of GLT1 is interesting as is the pattern of GLT1 loss (notably in the dorsal horn as well as the ventral horn). This is an important point as it would suggest that GLT1 loss from hTDP43 astrocytes is cell autonomous (we do not think of dorsal horn neuron loss as part of an ALS phenotype) and is either related to downregulation of GLT1 in astrocytes or possibly astrocyte death?

It is not clear how one would colocalize s100B staining with hTDP43 expression based on the images provided as the s100B looks quite diffuse.

In figure 5 it is noted that there are some ubiquitin inclusions in astrocytes (as nicely illustrated in fig. 5 T-V). One wonders whether the important point here is not that ubiquitin is not seen in neurons but do the authors think that ubiquitin in astrocytes means that astrocytes themselves are degenerating?

3.) For the microarray analysis of the astrocytes in vitro, there are no methods to explain how the list of genes in Table S1 was compiled. It should be described how the data were analyzed and by what parameters (ex. Fold change of >2) for analysis. Once this is established, the entire list of genes which meet these criteria should be provided.

-Given that the majority of the manuscript is a description of alpha motor neuron death in the spinal cord, why did the authors choose to examine the toxicity of Chi3L1 in primary cortical neuron culture? The authors note on page 7 that "motor neurons are the preferred targets of neurodegeneration caused by astrocytic TDP43 expression". Previous studies examining astrocyte toxicity (Nagai et al) have made note that astrocyte toxicity (at least with relationship to SOD1 toxicity) seems to be motor neuron specific. While it is certainly interesting (in fact not surprising given what is known about TDP43 in humans and mouse models) that cortical motor neurons are susceptible, it seems inconsistent with the rest of the manuscript which focuses on spinal cord motor neurons.

Minor issues:
1.) Quantification of the axons in dorsal roots (as is done for the ventral roots) is needed to conclude that these axons were preserved.
2.) In Fig. 5n, it appears that the hTDP43 expression is not restricted to the nucleus. Does the mutant TDP43 mislocalize to the cytoplasm or form aggregates during the course of disease?

3.) The authors should provide more information on the secretory proteins Chi3L1 and Lcn2 and what their normal biological functions are in astrocytes. Also, are these proteins altered in the G93A SOD1 mouse model?
4.) The authors need to identify the stain in Fig. 7m-o as well as in Fig. 8.
5) In the discussion section, the authors note that the independent Tg rat lines developed progressive paralysis indicating that astrocytic TDP43 expression causes reproducible phenotypes but I think this is overstated particularly because one line causes seizures which is not seen in the other line nor is it really part of an ALS spectrum.
6) The statement in the manuscript regarding GLT1 mRNA (page 11) not being changed in astrocytes is not surprising as it is known that very little GLT1 is expressed in cultured astrocytes in the absence of neurons. That is why the in vivo analysis that the authors performed is so important.

Taken together, this is a very worthwhile model and lends evidence to suggest that astrocytes may contribute to motor neuron toxicity and progressive paralysis. That is the strength of the manuscript. However, the manuscript would be much better if a more complete analysis of the model itself (with regard to brain expression, cortical motor neuron loss, other cell type expression, etc) rather than broad-ranging statements without more extensive data. The in vitro analyses are interesting but rather distracting to the theme of the manuscript. These concerns I suspect could be addressed with regard to model characterization and the subtraction of some of the in vitro data until more mature. The in vitro data, if examined in more detail, could stand on their own.

Referee #2

This new manuscript, co-authored by Tong et al., reports on the contributive role of mutant astrocytes in the degeneration of motor neurons in transgenic rats expressing the ALS-linked mutant TDP-43 M337V. After providing a series of information about the development of this new line of engineered rats that expressed, in a conditional manner, mutant TDP-43 specifically in GFAP-positive cells, the authors show behavioral and morphological data supporting the idea that mutant TDP-43 expressing astrocytes exert age-dependent deleterious effects on spinal cord motor neurons. They also demonstrate that, over time, starting with the emergence of the ALS-like manifestations, the expression of the glutamate transporter GLT1 in astrocytes decreases. Then, the authors determine the gene profile of mutant TDP-43 expressing astrocytes and show a list of deregulated gene families. Among these and given their role in neurodegeneration, the authors elected to focus on two specific target genes, namely Chi3L1 and Lcn2. The authors provide evidence that these two genes are upregulated in mutant TDP-43 expressing astrocytes and show that, upon in vitro incubation of cortical neurons with exogenous Chi3L1, neurodegeneration occurs. Finally, the authors show evidence of microglial activation that coincides with the emergence of the ALS-like manifestations. In light of these results, the authors concluded that mutant TDP-43 expressing astrocytes can cause a non-cell autonomous neurodegenerative phenotype that is mediated by a combination of a loss of function (i.e. reduction of GLT1) and a gain of function (overexpression of Chi3L1 and Lcn2).

This is quite an interesting and timely study. However, as is, it is still quite shallow and many of the key conclusions lack the necessary experimental evidence to assure their veracity. Moreover, some of the phraseology used is misleading and/or confusing and some of the experiments aimed at defining the specificity and the extent of the damage are missing.

1. Contrary to the study the authors have published previously in PloS Genetics (2010), probably one of the most unfortunate aspects of this study resides in the fact that they do not include this time, as control, engineered rats expressing wild-type TDP-43.

2. The introduction is unfortunately convoluted and it would be preferable that the authors get to the goal of the study more directly without calling on data regarding mutant SOD1, for example, which is a setting that they do not test here.

3. The authors refer to an inducible construct with a tetracycline-responsive element, but, unless overlooked, failed to indicate that the construct is a Tet-off (i.e. turn on of TDP-43 following Doxycycline withdrawal). This fact can be surmised from the text, but is not clearly stated and may be quite confusing for non-expert readers. Also, the authors refer to, throughout the manuscript,

"disease onset", but this terminology is erroneous. What the authors are referring to is merely the emergence of disease-related manifestations such as motor and/or morphological abnormalities. Thus, perhaps a more correct term to use would be ALS-like manifestations or onset of weakness, or motor problems, but not disease onset. Similarly, it is not "end-stage disease", but severe paralysis or something like this. On page 6, the authors refer to spasticity, which is a clinical term? I am not too sure of how the authors have determined that the affected animals had spasticity? So, please address and correct these points throughout to avoid misleading the readers.

4. Since at least one line seems to develop upper motor neuron problems, it would be essential to assess the status of the corticospinal track morphologically. Perhaps, some similar kind of investigations, as done by Macklis and colleagues (J Neurosci 31:4166-77, 2011), could be done here.

5. While the demonstration that the posterior root is unaffected is a valubale piece of information, the authors are urged to also assess a population of anterior horn neurons other than motor neurons such as GABAergic neurons, as well, to provide more compelling evidence of the specificity of the deleterious effect mediated by mutant TDP-43 astrocytes.

6. The authors indicate that while the neurodegenerative process developed, GFAP expression, in astrocytes, declined. Although this is an interesting observation, does this not raise the question as to how will the reduction of GFAP expression affect TDP-43 expression given the fact that the conditional construct relies on the GFAP promoter?

7. The observation of the time-dependent decline of GLT1 expression is interesting, but in absence of additional mechanistic investigations, these data are purely descriptive and do not allow for any pathogenic conclusions and absolutely cannot be taken as evidence that mutant TDP-43 expressing astrocytes exert their deleterious effect by a loss of function. Such a conclusion is highly misleading and must be deleted throughout, unless the authors provide new data showing that inhibiting glutamate or overexpressing GLT1 can affect the natural course of the pathological phenotype seen in the engineered rats. Please note also that as soon as motor neurons die, microglia and astrocytes will become activated, and once astrocytes become reactive, GLT1 downregulates. So, since the reduction in GLT1 expression is seen when the abnormal manifestations emerge, it means that neurodegeneration has already occurred and thus, the downregulation of GLT1 is a consequence and not a cause. Even as a consequence, this molecular alteration could have had some pathogenic role, but since the authors fail to present any mechanistic data, one cannot conclude anything mechanistic about this finding. Accordingly, if these data remain in the final version of the paper, they can only be reported as descriptive findings. Incidentally, a similar comment applies to the microglial data. Since microglial cells become quickly activated in response to even modest change in the cellular microenvironment, it is not clear what the authors are trying to show or say with the results of Fig. 8 that is more than obvious?

8. The gene array data are interesting, but, unless overlooked, there is a lack of technical details about how the arrays were performed, no information about the number of technical and biological repeats, little or no information on how the time points were selected, or how the effect of DOX and human TDP-43 expression (not the effect of the mutation but simply of the human protein) were corrected for. Also, again unless overlooked, is the entire list of changes posted somewhere for the community to be able to review it?

9. The reason of the selection of Chi3L1 and Lcn2 is interesting but poorly justified and the testing of the significance of Chi3L1 toxicity, as done herein, is irrelevant to the present study. Indeed, the question asked is not whether an extra-dose of exogenous Chi3L1 is toxic, but whether Chi3L1 up-regulation in mutant TDP-43 astrocytes drives the toxic phenotype. Thus, here, what is required is to silence Chi3L1 in astrocytes and see if this eliminates mutant TDP-43 astrocytes' deleterious effects on motor neurons. Also, the selection of cortical neurons for this investigation is an unfortunate decision since such a culture is a mixture of neurons with only a small fraction being motor neurons (if at all). So, not only is this primary culture system selected here inappropriate to test the question under investigation, but since the authors show that exogenous Chi3L1 kill cortical neurons it also raises questions about the claim that the observed Chi2L1 toxicity is specific to ALS-susceptible neuronal subpopulations.

1st Revision - authors' response 14 April 2013

Author's response to reviewer's comments

Referee #1

Comment-1: *Overall, this is a really interesting manuscript that could be an important piece of a puzzle allowing us to dissect mutant TDP-related astrocyte toxicity to the development of ALS as well as an interesting tool for further investigation…… Taken together, this is a very worthwhile model and lends evidence to suggest that astrocytes may contribute to motor neuron toxicity and progressive paralysis. That is the strength of the manuscript. However, the manuscript would be much better if a more complete analysis of the model itself (with regard to brain expression, cortical motor neuron loss, other cell type expression, etc) rather than broad-ranging statements without more extensive data. The in vitro analyses are interesting but rather distracting to the theme of the manuscript. These concerns I suspect could be addressed with regard to model characterization and the subtraction of some of the in vitro data until more mature. The in vitro data, if examined in more detail, could stand on its own*.
Response: We agree with the reviewers on that our manuscript comprises a wide range of *in vivo* and *in vitro* data with limited cohesiveness. Following reviewer's suggestions, we will focus our study on new TDP-43 transgenic rats to characterize the model in greater details and will remove *in vitro* data to avoid distraction from the main topic on non-cell-autonomous motor neuron death. Therefore, microarray analysis of gene expression in cultured astrocytes is removed and will be published in a follow-up study. Accordingly, the title of the manuscript is revised to reflect the main findings in this study and is changed to "*Mutant TDP-43 in Astrocytes Causes Non-cell-autonomous Motor Neuron Death in Rats*."

Comment-2: *In this report, the authors create a new transgenic rat expressing tTA driven by an "undefined" 21 kb fragment of the human GFAP promoter from a BAC clone. The authors provide no characterization for this rat and the pattern of tTA expression. They cross this rat to the TRE-TDP43M337V (previously characterized) and show by IHC that the human TDP43 is expressed in GFAP+ astrocytes, but not microglia, oligodendrocytes, or neurons. However, further characterization is needed including: Is the human mutant TDP43 expressed in peripheral organs (ex. Muscle, gut, Schwann cells?). This is particularly important because this model is the first attempt at expression of TDP43 in astrocytes and may serve as the basis for future TDP43 modeling. A more high power image showing human TDP43 (with a nuclear stain such as DAPI) in GFAP$^+$ astrocytes would be helpful. This is particularly important as seen in Fig 1 f where it appears that GFAP expression runs right through the nucleus of TDP43 staining whereas in other cells in this image one doesn't appreciate GFAP expression around these TDP43+ nuclei. Because NG2 cells also seem to proliferate quite robustly in SOD1 mice (and because they have a glial lineage), it is probably also worthwhile to assess whether there is any hTDP43 in NG2 cells as well to make certain this is not contributing to the model.*
Response: New experiments are added to demonstrate the profile of human TDP-43 expression in transgenic rats by Immunoblotting (Figure S2). Human TDP-43 is barely detectable in the sciatic nerve and skeletal muscle of the transgenic rats. Our data show that the GFAP promoter drives transgene expression in the astrocytes, but not in the neurons, of rat's spinal cords. Figure-1 shows the colocalization of GFAP and human TDP-43 by fluorescence staining. The image (Figure 1: G-I) is a composite of a series of scanned planes from confocal microscopy, which resulted in the appearance that GFAP-stained astrocytic processes pass through the nucleus of some cells. In reality, GFAP staining did not colocalize with human TDP-43, but circled the nucleus. We now have added images from individual plains to show this point. We also did new immunostaining to examine NG2-positive cells in the rat models and the result does not indicate the presence of human TDP-43 in the NG2+ cells (Figure S4).

Comment-3: *What is the level of expression of human TDP43 in this rat compared to endogenous rat TDP43 expression? Western blot analysis of both whole spinal cord homogenate as well as homogenate from isolated, purified astrocytes cultures in vitro would be helpful. In figure 3 the authors note quite dramatic loss of both motor neurons and axons in the ventral root and sciatic nerve. However, in GFAP-tTA line #1 (figure S2) they note motor neuron loss without ventral root loss. This is quite surprising unless this was a very acute finding but I think less likely that at*

endstage one would see motor neuron loss without axon loss. Rather than suggesting that this loss is anterograde, it may be more likely that the animals at endstage are dying from something else (i.e. seizures, upper motor neuron loss?). An explanation here as to their hypothesis would be helpful. A figure about cortical (or brain) expression of hTDP43 as far as distribution would be really helpful. This would make a more complete story. The focus of the manuscript can still be related to spinal cord motor neuron loss but the authors seem to indicate at least some degeneration in Fig. 3J and K but do not elaborate.

Response: Relative levels of human TDP-43 protein are examined in the spinal cord of transgenic rats (Figure 1: B, C). Line$^{\#}$1 indeed displayed the phenotypes of both cortical neurons and spinal motor neurons and the compound phenotypes were discussed in Results. Seizure and motor neuron loss both likely contributed to animal death. New text is added to further discuss this point. New data are added to show the expression of human TDP-43 in the cortex and to show the measurement of cortical neurons by stereological cell counting (Figure 4).

Comment-4: *A major point in the paper is that the astrocytes expressing human mutant TDP43 become "transformed" and lose expression of GFAP over the disease course. However, in Fig. 4b1-5 and Supp. Fig. 3, it appears that the total number of hTDP43-expressing cells is decreasing dramatically throughout disease, suggesting either that astrocytes are losing expression of the transgene or astrocytes expressing these high levels of TDP43 are dying over time. The authors should quantify the number of hTDP43-expressing cells over the disease course to address these points. Assessing whether astrocytes are dying because of hTDP43 expression could also be tested in vitro over a period of time to assess whether this is evident. The use of another astrocyte marker besides s100B (a marker for immature glia) would help determine whether astrocytes are dying or losing expression of GFAP. Perhaps, the authors could stain for GLAST to show astrocytes are still present (and not dying) in the spinal cord at end stage. This would also help in Fig. 5 to determine whether astrocytes are losing expression of all glutamate transporters, or specifically GLT1. The specific loss of GLT1, but not GLAST occurs in the G93A SOD1 mouse model and human ALS. This is important because s100B can also be expressed by NG2 cells (Vives et al 2003) which definitely proliferate in ALS. The loss of GLT1 is interesting as is the pattern of GLT1 loss (notably in the dorsal horn as well as the ventral horn). This is an important point as it would suggest that GLT1 loss from hTDP43 astrocytes is cell autonomous (we do not think of dorsal horn neuron loss as part of an ALS phenotype) and is either related to downregulation of GLT1 in astrocytes or possibly astrocyte death? It is not clear how one would colocalize s100B staining with hTDP43 expression based on the images provided as the s100B looks quite diffuse. In figure 5 it is noted that there are some ubiquitin inclusions in astrocytes (as nicely illustrated in fig. 5 T-V). One wonders whether the important point here is not that ubiquitin is not seen in neurons but do the authors think that ubiquitin in astrocytes means that astrocytes themselves are degenerating?*

Response: New experiments assessed the expression of GLAST in transgenic rats over disease courses (Figure 5). The colocalization of S100B with hTDP43 was determined on the images of high magnification (Figure 6). Ubiquitin aggregation is a feature of ALS and is observed in neurons and astrocytes in ALS patients. Ubiquitin-positive inclusion is not detected in degenerating neurons, suggesting that ubiquitin aggregation is not required for the non-cell-autonomous neurodegeneration. Expression of mutant TDP-43 indeed induced ubiquitin aggregation, recapitulating a feature of glial pathology in the disease.

Comment-5: *For the microarray analysis of the astrocytes in vitro, there are no methods to explain how the list of genes in Table S1 was compiled. It should be described how the data were analyzed and by what parameters (ex. Fold change of >2) for analysis. Once this is established, the entire list of genes which meet these criteria should be provided. Given that the majority of the manuscript is a description of alpha motor neuron death in the spinal cord, why did the authors choose to examine the toxicity of Chi3L1 in primary cortical neuron culture? The authors note on page 7 that "motor neurons are the preferred targets of neurodegeneration caused by astrocytic TDP43 expression". Previous studies examining astrocyte toxicity (Nagai et al) have made note that astrocyte toxicity (at least with relationship to SOD1 toxicity) seems to be motor neuron specific. While it is certainly interesting (in fact not surprising given what is known about TDP43 in humans and mouse models) that cortical motor neurons are susceptible, it seems inconsistent with the rest of the manuscript which focuses on spinal cord motor neurons.*

Response: As suggested by the reviewer, we will focus our study on characterizing new transgenic rats and have removed the *in vitro* data including microarray assay.

Comment-6: *Minor issues: 1.) Quantification of the axons in dorsal roots (as is done for the ventral roots) is needed to conclude that these axons were preserved. 2.) In Fig. 5n, it appears that the hTDP43 expression is not restricted to the nucleus. Does the mutant TDP43 mislocalize to the cytoplasm or form aggregates during the course of disease? 3.) The authors should provide more information on the secretory proteins Chi3L1 and Lcn2 and what their normal biological functions are in astrocytes. Also, are these proteins altered in the G93A SOD1 mouse model? 4.) The authors need to identify the stain in Fig. 7m-o as well as in Fig. 8. 5) In the discussion section, the authors note that the independent Tg rat lines developed progressive paralysis indicating that astrocytic TDP43 expression causes reproducible phenotypes, but I think this is overstated particularly because one line causes seizures which is not seen in the other line nor is it really part of an ALS spectrum. 6) The statement in the manuscript regarding GLT1 mRNA (page 11) not being changed in astrocytes is not surprising as it is known that very little GLT1 is expressed in cultured astrocytes in the absence of neurons. That is why the in vivo analysis that the authors performed is so important.*
Response: Axons in the dorsal roots are quantified and shown in Figure S6. At very late disease stages, moderate TDP-43 mislocalization is detected in the transgenic rats expressing mutant TDP-43 in the astrocytes (Figure S7: E). Following the suggestions by the reviewers, we have removed *in vitro* data regarding Chi3L1 neurotoxicity. In addition, we have recently published in PNAS a paper characterizing Lcn2 neurotoxicity (http://www.pnas.org/content/110/10/4069.long). We have revised figures to label immunostaining and revised the discussion to explain the data in greater details.

Referee #2

Comment-1: *Contrary to the study the authors have published previously in PloS Genetics (2010), probably one of the most unfortunate aspects of this study resides in the fact that they do not include this time, as control, engineered rats expressing wild-type TDP-43.*
Response: We agree with the reviewer on that wildtype form of a disease gene is an ideal control for transgene overexpression in animal models. Unlike *Sod1*, both wildtype and mutant *TDP-43* causes similar disease phenotypes in rodents though the wildtype appears less toxic. Consistent with several studies in transgenic mice, our findings in rats showed that mutant TDP-43 causes more severe phenotypes compared to the wildtype form. In our previous study (PLOS Genetics 2010), we tried to express TDP-43 transgene in its intrinsic spatial and temporal patterns by using mini TDP-43 gene isolated from a human BAC clone. While we have established transgenic lines carrying the wildtype mini TDP-43 transgene, we failed to establish a mutant line because all of the mutant transgenic founders developed severe phenotypes in postnatal ages. To overcome this difficulty in breeding, we then developed Tet-off inducible transgenic rats carrying mutant TDP-43, but we did not create inducible wildtype TDP-43 transgenic rats as it appears to add limited knowledge to existing findings in transgenic mice and rats. We could not use the existing wildtype mini TDP-43 transgenic rats for testing non-cell-autonomous motor neuron death (i.e. restricted overexpression of wildtype TDP-43 in astrocytes). In the present study, we attempt to examine a mechanism—non-cell-autonomous motor neuron death caused by astrocytic overexpression of TDP-43 and our conclusion can be drawn from the data without a wildtype TDP-43 transgenic rat.

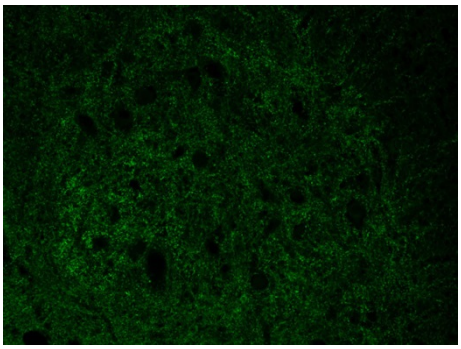
Comment-2: *The introduction is unfortunately convoluted and it would be preferable that the authors get to the goal of the study more directly without calling on data regarding mutant SOD1, for example, which is a setting that they do not test here.*
Response: The introduction is revised to present the information closely relevant to our current study.

Comment-3: *The authors refer to an inducible construct with a tetracycline-responsive element, but, unless overlooked, failed to indicate that the construct is a Tet-off (i.e. turn on of TDP-43 following Doxycycline withdrawal). This fact can be surmised from the text, but is not clearly stated and may be quite confusing for non-expert readers. Also, the authors refer to, throughout the manuscript, "disease onset", but this terminology is erroneous. What the authors are referring to is merely the emergence of disease-related manifestations such as motor and/or morphological abnormalities. Thus, perhaps a more correct term to use would be ALS-like manifestations or onset of weakness, or motor problems, but not disease onset. Similarly, it is not "end-stage disease", but severe paralysis or something like this. On page 6, the authors refer to spasticity, which is a clinical*

term? I am not too sure of how the authors have determined that the affected animals had spasticity? So, please address and correct these points throughout to avoid misleading the readers.
Response: This is a good point, reminding us of rethinking appropriate terms for describing disease phenotypes in model animals. Transgenic studies commonly use "disease-onset and disease stages" to describe the occurrence and progression of phenotypes observed in transgenic animals. In revision, we have chosen the onset of weakness instead of disease onset and have used advanced paralysis to describe the phenotypes. Spastic paralysis and spasticity are commonly used to describe the phenotypes observed in ALS models and the other neurological disease models. We will use spastic paralysis to describe the phenotype observed in line#1 transgenic rats. Indeed, we used Tel-off system to reversibly express mutant TDP-43 transgene in rats and have revised the text to present the information clearly.

Comment-4: *Since at least one line seems to develop upper motor neuron problems, it would be essential to assess the status of the corticospinal track morphologically. Perhaps, some similar kind of investigations, as done by Macklis and colleagues (J Neurosci 31:4166-77, 2011), could be done here.*
Response: GFAP-tTA/TRE-TDP43^{M337V} rats displayed the phenotypes of both upper and lower motor neurons. We examined the degeneration of upper motor neurons by silver staining and detected degenerating neurons in the cortex of transgenic rats (Figure 4: Q). Upper motor neurons are indeed affected in the transgenic rats. Retrograde FluoroGold labeling suggested by the reviewer is an elegant technique to trace the upper motor neurons and the axons (J Neuroscience 2011, 31:4166-77); however, this experiment is technically challenging and we do not have experience in retrograde labeling. FluoroGold labeling may display upper motor neuron degeneration and confirm our findings in silver staining, but the evidence from silver staining is sufficient to show the degeneration of upper motor neurons in the transgenic rats.

Comment-5: *While the demonstration that the posterior root is unaffected is a valuable piece of information, the authors are urged to also assess a population of anterior horn neurons other than motor neurons such as GABAergic neurons, as well, to provide more compelling evidence of the specificity of the deleterious effect mediated by mutant TDP-43 astrocytes*.
Response: GABAergic neurons in the ventral spinal cords mainly express the enzyme GAD-67. Unfortunately, GAD-67 staining in rat's spinal cord is quite diffusing (see image). It is unlikely to accurately count neurons stained with GAD-67 antibody. Alternatively, we have quantified neurons in the frontal cortex by stereological cell counting and used brain neurons as a control for the selectivity of neuronal death in response to astrocytic mutant TDP-43 expression. As shown in the new figure (Figure 6), human TDP-43 was substantially expressed in rat's brain, but no significant loss of brain neurons was detected by stereological cell counting though sliver staining detected scattered degenerating neurons. Compared to the brain neurons, spinal motor neurons appear more sensitive to astrocytic overexpression of mutant TDP-43.



Comment-6: *The authors indicate that while the neurodegenerative process developed, GFAP expression, in astrocytes, declined. Although this is an interesting observation, does this not raise the question as to how will the reduction of GFAP expression affect TDP-43 expression given the fact that the conditional construct relies on the GFAP promoter?*
Response: This question is common for cell-specific transgene expression that is accomplished by using cell-specific promoter (i.e. GFAP). Expression of transgene may decrease when targeted cells become abnormal (i.e., GFAP reduction in astrocytes). Unfortunately, this complication is unavoidable for cell-specific gene expression. In our case, a decline in transgene expression will not prevent us from testing non-cell-autonomous neuron death, since the expression of mutant human TDP-43 is still restricted in astrocytes.

Comment-7: *The observation of the time-dependent decline of GLT1 expression is interesting, but in absence of additional mechanistic investigations, these data are purely descriptive and do not allow for any pathogenic conclusions and absolutely cannot be taken as evidence that mutant TDP-*

43 expressing astrocytes exert their deleterious effect by a loss of function. Such a conclusion is highly misleading and must be deleted throughout, unless the authors provide new data showing that inhibiting glutamate or overexpressing GLT1 can affect the natural course of the pathological phenotype seen in the engineered rats. Please note also that as soon as motor neurons die, microglia and astrocytes will become activated, and once astrocytes become reactive, GLT1 downregulates. So, since the reduction in GLT1 expression is seen when the abnormal manifestations emerge, it means that neurodegeneration has already occurred and thus, the downregulation of GLT1 is a consequence and not a cause. Even as a consequence, this molecular alteration could have had some pathogenic role, but since the authors fail to present any mechanistic data, one cannot conclude anything mechanistic about this finding. Accordingly, if these data remain in the final version of the paper, they can only be reported as descriptive findings. Incidentally, a similar comment applies to the microglial data. Since microglial cells become quickly activated in response to even modest change in the cellular microenvironment, it is not clear what the authors are trying to show or say with the results of Fig. 8 that is more than obvious?
Response: As suggested by reviewers, revised paper will be focused on characterizing transgenic rats that express mutant TDP-43 selectively in astrocytes and develop non-cell-autonomous neuron death. The involvement of GLT1 in ALS is well studied and our findings in TDP-43 transgenic rats further confirm GLT1 downregulation as a common phenomenon in ALS pathology. Microglia often becomes reactive in response to neurodegeneration in ALS. Figure 8 showed that microglia severely reacted to non-cell-autonomous motor neuron death and is likely involved in the pathology. Following reviewer's suggestions, we have removed *in vitro* data and restricted the study within the capacity for a single paper. The title of the manuscript along with the text has been revised accordingly.

Comment-8: *The gene array data are interesting, but, unless overlooked, there is a lack of technical details about how the arrays were performed, no information about the number of technical and biological repeats, little or no information on how the time points were selected, or how the effect of DOX and human TDP-43 expression (not the effect of the mutation but simply of the human protein) were corrected for. Also, again unless overlooked, is the entire list of changes posted somewhere for the community to be able to review it?*
Response: As suggested by the reviewers, the microarray data has been removed in order to focus the study on *in vivo* analyses and will be published in a separate study after interested genes are further analyzed for functional involvement. The array data was deposited to a public resource before the manuscript submission and now is secured until these data are ready for publication.

Comment-9: *The reason of the selection of Chi3L1 and Lcn2 is interesting but poorly justified and the testing of the significance of Chi3L1 toxicity, as done herein, is irrelevant to the present study. Indeed, the question asked is not whether an extra-dose of exogenous Chi3L1 is toxic, but whether Chi3L1 up-regulation in mutant TDP-43 astrocytes drives the toxic phenotype. Thus, here, what is required is to silence Chi3L1 in astrocytes and see if this eliminates mutant TDP-43 astrocytes' deleterious effects on motor neurons. Also, the selection of cortical neurons for this investigation is an unfortunate decision since such a culture is a mixture of neurons with only a small fraction being motor neurons (if at all). So, not only is this primary culture system selected here inappropriate to test the question under investigation, but since the authors show that exogenous Chi3L1 kill cortical neurons it also raises questions about the claim that the observed Chi2L1 toxicity is specific to ALS-susceptible neuronal subpopulations.*
Response: The in vitro data on Chi3L1 and Lcn2 toxicity has been removed at revision. One attempt of this study is to examine whether abnormal astrocytes impose toxicity on neurons by secreting toxic factors such as lcn2. We have recently published in PNAS a paper characterizing the neurotoxicity of astrocytic Lcn2 (http://www.pnas.org/content/110/10/4069.longng).

2nd Editorial Decision 28 April 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by referee #1. As you can see below, referee #1 appreciates the introduced changes. However, this referee also has some remaining issues on the newly added data that should be resolved before publication here. I would therefore like to ask you to respond to these last points in a final revision. Maybe it would be helpful if we discuss how to address these last issues. We can do so by email.

REFEREE REPORTS

Referee #1

The exclusion of the in vitro data make this paper much easier to read and more concise. That represents a substantial improvement of the manuscript by subtraction of these data. The focus on the rat model itself makes it of continued interest. There are some key issues which I think need to be addressed based on the inclusion of new data and some questions about the interpretation of the data.

Comment 1
It is interesting that the data in figure S2 show that TDP-43 is expressed in a variety of tissues that appear to be equal if not greater than expression in the spinal cord and brain. Does this suggest that the expression is not GFAP specific or are there other cells in the heart, spleen, that express GFAP? The authors note that this is an "interesting(ly)" observation. It probably requires some explanation and analysis.

It is reassuring to see that there is little expression in muscle and nerve which could contribute to some of the phenotype observed.

Comment 2
It is not clear how the data in figures 5 and 6 should be interpreted. It appears in figure 5 that the glutamate transporters EAAT1 and EAAT2 are both reduced quite dramatically. In figure 6, it also appears that GFAP expression is reduced, S100B is reduced, and TDP43 expression is also reduced to the point at which in Fig 6 B5 there is little TDP43 expression. Given that all of these astrocyte markers are reduced (or absent?) one wonders if this represents astrocyte death related to TDP43 expression. This may be relevant given the recent publication by the Serio PNAS 2013 where mutant TDP-43 expression in human astrocytes appears to cause some degree of astrocyte death.

It is not clear why if GFAP promoter drives TDP43 expression, that there is still TDP43 immunostaining (Figure 6, B5 arrowheads) when there is no GFAP colocalization. I would expect loss of GFAP expression to coincide with TDP43 expression in this image.

If the authors don't think that astrocytes may be dying, then quantification of the number of hTDP-43-expressing cells in the SC over the course of disease may be helpful to establish that that hTDP43 nuclei are constant.

Comment 3

The text on page 7 says that neuronal loss was not detected by stereological counting but Figure 4 figure legend title says that "Astrocytic TDP43 overexpression causes cortical neuron degeneration in rats." These statements would appear to be contradictory.

In figure 4, it is not clear that silver staining in 4Q shows degeneration nor does this image show that this is a neuron. The authors note that "compared to spinal motor neurons, the brain neurons appear less sensitive to astrocytic TDP-43 expression." It is not clear how to interpret this statement. Based on Supplemental figure S2, it appears that expression of the human transgene in brain is less than that in spinal cord. Furthermore, the other line (line #1) would appear to have some cortical involvement based on the observation that these animals have seizures.

This is important for the field because unlike other models where there is cortical pathology when TDP43 is expressed under different promoters, these data would imply that astrocyte TDP43 overexpression is selective to spinal cord motor neuron pathology and not cortical neuron pathology.

There is no description of how cortical neurons were counted for Fig. 4V. What stain was used for counting? What part of the cortex was counted? Were all layers included in the count? These data are not included in the Materials and methods section.

Although the figure is supposed to address cortical neuron degeneration, half of the images in the

figure are of the dentate gyrus. Why are these included?

Comment 4
Perhaps one of the most interesting observations is that the 2 lines created (line #1 and line #2) appear to have very different behavioral phenotypes, one wonders whether this is related to differences in the expression pattern of TDP43. The authors found little/no cortical neuron loss in line #2 but the expression of TDP43 in brain appears to be less than in the spinal cord. Does line #1, which has seizures and possible spasticity, have a different expression pattern (higher in brain than in spinal cord as can be determined by western blot)? This is important as I don't think models of "ALS" develop seizures. Does the cortical pathology in line #1 look different?

Comment 5
The authors state it may be difficult to use GAD67 for analysis of other neurons in the spinal cord. To address the issue of whether other neurons besides motor neurons are dying, one could use a pan neuronal marker (i.e. NeuN) to see if there is a greater loss of neurons besides what one would expect by loss of motor neurons alone.

Minor comment
1. C9ORF72 is now considered a major genetic etiology for ALS development and should be included (briefly) in the introduction about SOD1, TDP and the 10% with mutations in individual genes.
2. Figure 4 A, D, and G it is very difficult to see the TDP43 expression. It also appears that A-O are hippocampus and not cortex? This should be made more clear.
3. In figure S3, one cannot see the DAPI staining.
4. There are still some difficulties with grammar that sometimes make reading difficult.

2nd Revision - authors' response 02 May 2013

Author's response to reviewer's comments

Comment: *The exclusion of the in vitro data make this paper much easier to read and more concise. That represents a substantial improvement of the manuscript by subtraction of these data. The focus on the rat model itself makes it of continued interest. There are some key issues which I think need to be addressed based on the inclusion of new data and some questions about the interpretation of the data.*
Response: These constructive comments are highly appreciated. The remaining concerns have been addressed below.

Comment: *It is interesting that the data in figure S2 show that TDP-43 is expressed in a variety of tissues that appear to be equal if not greater than expression in the spinal cord and brain. Does this suggest that the expression is not GFAP specific or are there other cells in the heart, spleen, that express GFAP? The authors note that this is an "interesting(ly)" observation. It probably requires some explanation and analysis. It is reassuring to see that there is little expression in muscle and nerve which could contribute to some of the phenotype observed.*
Response: While glial fibrillary acidic protein (GFAP) is selectively expressed in astrocytes in the central nervous system (CNS), GFAP is known to be expressed in some cell types outside the CNS. For example, GFAP is expressed in the stellate cells of the liver and the GFAP promoter is widely used to drive transgene expression in the liver cells ***(1, 2)***. Hepatic stellate-shaped cells are observed in the lung, heart, kidney, and intestine ***(3, 4)***. It is not surprising to detect transgene expression in our GFAP-tTA transgenic rats. We have added text to explain the data in more detail.

Comment: *It is not clear how the data in figures 5 and 6 should be interpreted. It appears in figure 5 that the glutamate transporters EAAT1 and EAAT2 are both reduced quite dramatically. In figure 6, it also appears that GFAP expression is reduced, S100B is reduced, and TDP43 expression is also reduced to the point at which in Fig 6 B5 there is little TDP43 expression. Given that all of these astrocyte markers are reduced (or absent?) one wonders if this represents astrocyte death related to TDP43 expression. This may be relevant given the recent publication by the Serio PNAS 2013 where mutant TDP-43 expression in human astrocytes appears to cause some degree of astrocyte death. It is not clear why if GFAP promoter drives TDP43 expression, that there is still*

TDP43 immunostaining (Figure 6, B5 arrowheads) when there is no GFAP colocalization. I would expect loss of GFAP expression to coincide with TDP43 expression in this image. If the authors don't think that astrocytes may be dying, then quantification of the number of hTDP-43-expressing cells in the SC over the course of disease may be helpful to establish that that hTDP43 nuclei are constant.

Response: We agree with the reviewer on that overexpression of mutant TDP-43 in astrocytes is likely to induce astrocyte death. We realize that a recent paper published in the PNAS showed mutant TDP-43 toxicity to astrocytes in culture. We have added text to discuss this point in more detail. As GFAP is mainly expressed in the processes of astrocytes, a reduction in GFAP expression may cause difficulty in localizing the astrocyte. In fact, residual GFAP was weakly stained around human TDP-43 in Figure 6 (B4 and B5). This point has been explained at revision.

Comment: *The text on page 7 says that neuronal loss was not detected by stereological counting but Figure 4 figure legend title says that "Astrocytic TDP43 overexpression causes cortical neuron degeneration in rats." These statements would appear to be contradictory. In figure 4, it is not clear that silver staining in 4Q shows degeneration nor does this image show that this is a neuron. The authors note that "compared to spinal motor neurons, the brain neurons appear less sensitive to astrocytic TDP-43 expression." It is not clear how to interpret this statement. Based on Supplemental figure S2, it appears that expression of the human transgene in brain is less than that in spinal cord. Furthermore, the other line (line #1) would appear to have some cortical involvement based on the observation that these animals have seizures. This is important for the field because unlike other models where there is cortical pathology when TDP43 is expressed under different promoters, these data would imply that astrocyte TDP43 overexpression is selective to spinal cord motor neuron pathology and not cortical neuron pathology.*

Response: Thanks for pointing out the confusing description in the manuscript. Although stereological cell counting revealed that neuronal loss in the brain is not statistically significant, scattered degenerating neurons can be detected by silver staining that is a classical method for detecting degenerating neurons. We realize that it is difficult to make a conclusion on the sensitivity of cortical and spinal neurons to astrocytic TDP-43^{M337V} overexpression. As pointed out by the reviewer, transgene expression may vary among different tissues. Mutant TDP-43 expression might be higher in the spinal cord than that in the brain. We have removed the uncertain statement and revised the manuscript to present the data more precisely.

Comment: *There is no description of how cortical neurons were counted for Fig. 4V. What stain was used for counting? What part of the cortex was counted? Were all layers included in the count? These data are not included in the Materials and methods section.*

Response: The method of stereological cell counting for frontal cortex has been added at revision. The detail of the method has been described in our publication and is cited in Method **(*5*)**. Cresyl violet-stained neurons have been counted on the coronal sections of the frontal cortex for all the layers as described **(*5*)**.

Comment: *Although the figure (Figure 4) is supposed to address cortical neuron degeneration, half of the images in the figure are of the dentate gyrus. Why are these included?*

Response: In TDP-43 transgenic mice and rats reported, hippocampal neurons appear sensitive to TDP-43 toxicity. Compared to the cortex, the hippocampus has a distinct anatomy that makes it easier to demonstrate the selective expression of mutant TDP-43 in the astrocytes rather than the neurons. We intend to show the selective expression of mutant TDP-43 in astrocytes in the brain including the cortex and the hippocampus. We have revised the text to present the data precisely.

Comment: *Perhaps one of the most interesting observations is that the 2 lines created (line #1 and line #2) appear to have very different behavioral phenotypes, one wonders whether this is related to differences in the expression pattern of TDP43. The authors found little/no cortical neuron loss in line #2 but the expression of TDP43 in brain appears to be less than in the spinal cord. Does line #1, which has seizures and possible spasticity, have a different expression pattern (higher in brain than in spinal cord as can be determined by western blot)? This is important as I don't think models of "ALS" develop seizures. Does the cortical pathology in line #1 look different?*

Response: While the line $^{\#}$2 develops a predominant phenotype of paralysis, the line $^{\#}$1 also develops seizure and spasticity. The overall expression of mutant TDP-43 in the brain is lower in the line $^{\#}$1 than in the line $^{\#}$2, but the focal expression of mutant TDP-43 in the medial septum is higher

in the line #1 than in the line #2. Different patterns of transgene expression may account for the varied phenotypes. We have included these immunoblotting data in Figure S5.

Comment: *The authors state it may be difficult to use GAD67 for analysis of other neurons in the spinal cord. To address the issue of whether other neurons besides motor neurons are dying, one could use a pan neuronal marker (i.e. NeuN) to see if there is a greater loss of neurons besides what one would expect by loss of motor neurons alone.*
Response: In fact, we used Cresyl violet staining to reveal all cell types in the spinal cord and quantitated the neurons with a diameter larger than 25μm (Figure 3). As the predominant phenotype in our transgenic rats is paralysis, the motor neurons appear sensitive to astrocytic TDP-43^{M337V} expression. Our goal of this study is to examine non-autonomous motor neuron death caused by selectively expressing mutant TDP-43 in the astrocytes. Indeed, NeuN immunostaining can reveal certain types of neurons (not all types of neurons) and can provide additional input on astrocytic TDP-43^{M337V} expression. For our purpose, however, it is not necessary to quantify the selectivity of spinal neurons to astrocytic TDP-43^{M337V} overexpression.

Comment: ***1).*** *C9ORF72 is now considered a major genetic etiology for ALS development and should be included (briefly) in the introduction about SOD1, TDP and the 10% with mutations in individual genes.* ***2)****. Figure 4 A, D, and G it is very difficult to see the TDP43 expression. It also appears that A-O are hippocampus and not cortex? This should be made more clear.* ***3)****. In figure S3, one cannot see the DAPI staining.* ***4)****. There are still some difficulties with grammar that sometimes make reading difficult.*
Response: The information about C9ORF72 mutation is added to Introduction. Figure 4 is revised such that the data are presented more precisely. Unfortunately, it is indeed difficult to show the nucleus of spinal cells by DAPI. The language of the manuscript has been edited for clarity.

Literatures cited in author's response:

1. A. L. Gard, F. P. White, G. R. Dutton, *J Neuroimmunol* **8**, 359 (Jun, 1985).
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4. G. H. Buniatian *et al.*, *Anat Rec* **267**, 296 (Aug 1, 2002).
5. C. Huang *et al.*, *PLOS Genetics* **7**, e1002011 (2011).

