Prevention of intestinal obstruction reveals progressive neurodegeneration in mutant TDP-43 (A315T) mice

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Abstract

Background: Intraneuronal inclusions of TAR DNA-binding protein 43 (TDP-43) have been found in the majority of Amyotrophic Lateral Sclerosis (ALS) patients. Mutations in the gene encoding TDP-43 cause familial ALS. Transgenic mice expressing mutant TDP-43 with one such mutation (TDP-43 (A315T)) under control of the murine prion promoter develop motor symptoms, but their use is currently hampered by sudden death. We aimed to understand and overcome the cause of sudden death in TDP-43 (A315T) mice. Since intestinal obstruction was suspected to be the cause, intestinal motility of TDP-43 (A315T) mice was studied in an ex-vivo pellet propulsion assay. The effect on the enteric and motor phenotype was assessed, both in animals on normal chow or on a jellified fiber deprived diet, aimed at preventing intestinal obstruction.

Results: The frequency of the propulsive motor complexes was significantly reduced in the colon of TDP-43 (A315T) compared to non transgenic (NTG) mice. Immunohistochemistry revealed significant enlargement in size and reduction in number of the nitric oxide synthase (NOS) neurons in the myenteric plexus of TDP-43 (A315T) mice. Prevention of intestinal obstruction by jellified food abolished sudden death, allowing the motor phenotype to develop and slowly progress with a more pronounced degeneration of upper and lower motor axons. A downregulation of endogenous TDP-43 mRNA and protein levels was observed prior to neurodegeneration.

Conclusion: TDP-43 (A315T) mice suffer from intestinal dysmotility due to degeneration of NOS neurons in the myenteric plexus. Feeding the mice jellified food prevents sudden death and allows the motor phenotype to progress.

Keywords: ALS, TDP43, Gastro-intestinal tract, Neurodegeneration, Enteric nervous system
overexpression of mutant TDP-43 (A315T) under control of the mouse prion promoter, leading to a threefold overexpression of TDP-43 in spinal cord and brain. These mice were reported to develop gait abnormalities from 3 to 4 months on, a swimming gait accompanied by weight loss at 4.5 months and to survive 154 ± 19 days. At the pathological level, there was a 20% loss of lower motor neurons and a 50% loss of upper motor axons in the spinal cord. However, we and others have observed a sudden death in these mice, prior to the development of full neurological symptoms [19-21].

The aim of this study was to identify the pathological mechanism behind this sudden death and provide a solution to allow the neurodegeneration to develop further and render this transgenic mouse a useful model to study TDP-43 induced neurodegeneration.

Results
Sudden death in TDP-43 (A315T) mice is due to intestinal dysfunction
We observed a sexual dimorphism in disease onset and survival of TDP-43 (A315T) mice, as reported previously [19]. Male mice had a median survival of 84 days, whereas females survived for 126 days (Figure 1A). This difference could not be explained by a difference in TDP-43 expression levels as assessed by Western blot analysis of spinal cord lysates. Although the disease onset was quite variable, most mice died within a week after onset of swimming gait abnormalities (Figure 1B). Contrary to mutant SOD1 mice, TDP-43 (A315T) mice died suddenly before the development of end stage (ES) motor symptoms. Sometimes, mice even died before the onset of neurologic abnormalities. These animals looked lethargic, a phenotype that is not directly compatible with ALS. Upon inspection, these mice appeared to have an extremely rigid abdomen indicative of intestinal obstruction, which was confirmed at autopsy. Even before the onset of motor symptoms, intestinal abnormalities with signs of pseudo-obstruction, thinned colon, enlarged caecum and distension of the small intestines could be observed (Figure 1C). The intestinal distension seemed most pronounced at the ileocaecal junction and was found to be progressive with age.

To understand the mechanisms underlying the intestinal dysmotility, we performed video experiments to measure the intestinal propulsion (Figure 1D) and also examined the myenteric plexus by immunohistochemistry. The number of contractions induced by the presence of an artificial pellet inserted into the isolated colon was significantly reduced in TDP-43 (A315T) mice, compared to NTG littermates (Figure 1D and E, upper graph). The force of muscle contractions, as measured by the displacement of the pellet per contraction, was not affected in TDP-43 (A315T) mice compared to NTG (Figure 1D and E, lower graph), indicating that the peristaltic malfunction was of pure intrinsic neuronal origin. Immunostaining with a specific human TDP-43 antibody [22] confirmed the selective expression of the transgene in the nuclei of myenteric neurons (and glial cells), which is in line with a prion promoter (Prnp) pattern (Figure 2A and A, 2B and B). Although no major loss of enteric neurons was detected (HuCD positive neurons per ganglion: 37.1 ± 3.4 vs. 33.9 ± 2.5 for TDP-43 (A315T) and NTG mice, respectively), one specific population, the nitric-oxide synthase (NOS) expressing neurons, was severely affected in terminal ileum and colon. Their number was significantly reduced (8.4 ± 1.7 vs. 15.7 ± 1.9 per ganglion), and an enlarged swollen appearance of NOS neurons was noted (Figure 2C and C'), especially as mice got older. On the other hand, a staining for excitatory neurons, using a choline acetyltransferase (ChAT) antibody did not reveal any obvious differences between TDP-43 (A315T) and NTG mice (Figure 2D and D'). Furthermore, the enteric glial cell network that normally surrounds individual nerve cells was also severely distorted as shown by GFAP immunostaining (Figure 2A and A, 2B and B').

Downregulation of endogenous TDP-43 at mRNA and protein level
Immunostainings using an antibody that recognizes the C-terminal part of both mouse and human TDP-43 (12892-1-AP) in NTG (Figure 3A, upper panels) and TDP-43 (A315T) mice (Figure 3A, middle panels), revealed that most neurons from TDP-43 (A315T) mice retain the nuclear TDP-43 expression, even at advanced disease stage. A (partial) loss of nuclear TDP-43 (Figure 3A, middle panels) and (intranuclear) TDP-43 inclusions (Figure 3A, lower panels) were only rarely seen in the TDP-43 (A315T) mice.

We could also detect a clear downregulation at the mRNA level of endogenous TDP-43 due to the overexpression of human mutant TDP-43 [10,23,24]. Using qPCR, we measured a 20% reduction of endogenous TDP-43 mRNA in the spinal cord of TDP-43 (A315T) mice compared to NTG littermates (Figure 3B) and a 31% reduction in the brain (Figure 3C). This downregulation of endogenous TDP-43 was also confirmed at the protein level by Western blot, where we detected a reduction of 48% in the spinal cord (Figure 3D and E).

To assess whether this downregulation of endogenous TDP-43 was associated with downstream splicing defects, we measured sortilin exon 17 b expression. In line with previous studies [25,26], we found a decrease in the levels of the splice variant of sortilin 1 with exon 17b inclusion (Sort1 + Ex17b) in the TDP-43 (A315T) mice (Figure 3F). Since it has been shown [25,26] that down-regulation of TDP-43 in mouse N2a cells, leads to an
Figure 1 (See legend on next page.)
increase in Sort1 + Ex17b levels, this suggests that the human TDP-43(A315T) overexpression can compensate for the downregulation of mouse TDP-43.

**Effect of gel food on disease progression and neurodegeneration in TDP-43 (A315T) mice**

We sought to prevent the intestinal obstruction by removing non-digestible elements from the food and feeding the mice a nutrient gel (referred to as "gel" food). This diet was initiated before the development of gait abnormalities (median = 7 days for the males and median = 11 days for the females, n = 22 male and 15 female mice, p = 0.29).

C. Comparison of intestines between NTG and TDP-43 (A315T) mice. Left: TDP-43 (A315T) mice displayed enlarged ileum (red arrowhead) and caecum (white arrow), which are easily detectable as compared to the normal-size ileum and colon from NTG mice. Right: Picture of the entire colon revealed no shortening in TDP-43 (A315T) compared to NTG mice. Note the absence of inflammatory patches or bleeding in the colon of TDP-43 (A315T) mice.

D. Propulsion in colon segments from TDP-43 (A315T) and NTG mice. Recordings were made after the pellet was introduced in the colon (upper panels). Spatiotemporal map depicting the diameter of the colon segment in TDP-43 (A315T) (right) and NTG (left) mice (bar in x: 10 mm; bar in y: 100 s).

E. The graphs show a significant reduction (p = 0.006) of frequency of contractions (upper graph) but not of force of muscle contraction (lower graph) between TDP-43 (A315T) (red bar) and NTG (grey bar) mice. n values indicate the number of mice from which the colon segments were analyzed.

Discussion

To unravel the disease mechanisms of TDP-43 induced neurodegeneration, several transgenic mouse models have been generated. Unfortunately, so far none of them fulfills the expectations. The TDP-43 (A315T) mice (designed by Wegorzewska et al. [15]) develop motor symptoms, but the sudden death, due to intestinal distension as reported previously [19-21] limits their use.

In this study we unraveled the nature of the intestinal problems in an ex-vivo pellet study, which revealed a significant reduction in the ability to generate propulsive contractions in these mice. Using immunohistochemical analysis, we observed a degeneration of NOS neurons in the ENS, which is responsible for the coordination of peristaltic movements. NOS neurons are responsible for inhibitory signaling within the ENS and together with excitatory neurons finely regulate intestinal peristalsis. Both excitation and inhibition are quintessential for luminal...
Figure 2 (See legend on next page.)
Herdewyn TDP-43 in spinal cord and brain, both at mRNA and protein level, supporting a role for autoregulation of TDP-43 expression. TDP-43 can bind the 3' UTR of its own transcript. This leads to decreased expression of endogenous TDP-43 due to the 3-fold overexpression of exogenous TDP-43 [15]. Since we and others [25,26] measured a decrease of Sort1 + Ex17b in the brain of human TDP-43 (A315T) overexpressing mice, in contrast to the upregulation seen in mouse N2a cells with TDP-43 knock-down, this suggests that the reduction of endogenous TDP-43 is overcompensated by the expression of human mutant TDP-43 (A315T). As suggested previously [10,23,24], the downregulation of endogenous TDP-43 can contribute to the pathogenesis of neurodegeneration, but so does overcompensation, since a physiological range of expression, below or above which pathology develops, appears to be important. In line with previous studies [15,19], we could detect some loss of nuclear TDP-43 and (nuclear) TDP-43 inclusions in TDP-43 (A315T) mice at ES, but both were rare phenomena, suggesting that cytoplasmic mislocalization and inclusions are not the major cause of toxicity in this model.

Interestingly, a similar phenomenon of pseudo-obstruction was described in transgenic mice expressing CAG-repeats under the control of the Prnp [31]. Severely enlarged and loss of NOs neurons, together with intestinal pseudo-obstruction was seen in mice overexpressing the polyglutamine expansion responsible for SCA7. This suggests that the ENS is vulnerable to neurodegeneration as well, and may be a readily accessible part of the nervous system to study mechanisms of neurodegeneration, even in intestinal biopsies obtained from patients [32]. In addition, TDP-43 (A315T) mice could become a useful model to study intestinal motility disorders caused by degeneration, in addition to the developmental models of intestinal dysfunction.

In this study we succeeded to overcome the sudden death by feeding the mice gel food and thereby preventing intestinal constipation. This intervention significantly extended survival and allowed a progressive motor phenotype to develop. Disease progression was associated with a more pronounced loss of upper and lower motor axons.

content to be propelled. Lack of excitation would obviously fail to produce any contractile force, but also lack of inhibition prevents peristalsis as gut muscle relax-
Figure 3 (See legend on next page.)
An important limitation of this model remains the lack of lower motor neuron degeneration in the ventral horn of the spinal cord. Compared to mutant SOD1 mice, TDP-43 (A315T) mice have limited loss of NMJs. A dysphenotype observed can most likely be attributed to loss weakness observed. However, the progressive motor in the current study and may contribute to the muscle weakness observed. However, the progressive motor phenotype observed can most likely be attributed to loss of upper motor neuron axons. Therefore, TDP-43 (A315T) mice can be of value in studying the upper motor neuron degeneration, that is an essential hallmark of ALS (with and without TDP-43 mutations), but is less well studied so far. The large variation in disease onset makes this model less suited for cross breeding experiments, although a similar heterogeneity in disease onset is also apparent in ALS patients.

Conclusions
In summary, feeding the TDP-43 (A315T) mice jellified fiber deprived food, abolished sudden death due to intestinal dysmotility and allowed the slowly progressive upper and lower motor axon phenotype to develop further, thus rendering this mouse a suitable model for TDP-43 induced (upper motor) neurodegeneration.

Materials and methods
Feeding and follow-up of TDP-43 (A315T) mice
The TDP-43 (A315T) mice designed by Wegorzewska et al. [15] contain the mutant human TDP-43 gene, preceded by the Flag-tag, under control of the mouse Prnp, leading to the highest expression in spinal cord and brain, but also in other tissues. Originally, these mice were on a mixed C57BL/6 J and CBA background. We obtained these mice from the Jackson Laboratory, stock number 010700 (The Jackson Laboratory, Bar Harbor, Maine, USA). The mice were already backcrossed for 10 generations into a C57BL/6 J background, leading to a pure C57BL/6 J background. For genotyping, we used the primers and protocol as previously described [20]. Mice were kept in a conventional facility and fed ad libitum with dry pellet food (Ssniff® R/M-H, Ssniff Spezialdiäten GmbH, Soest, Germany) for the non-transgenic (NTG) mice and the TDP-43 (A315T) mice with normal food. The gel fed mice got DietGel® boost (ClearH20, Maine, USA) from 30 days on for male and 80 days on for female mice. This gel food contains all necessary nutrients, but is a soft, high calorie, easily digestible paste with hardly any fibers.

The mice on gel food were put in cages, without bedding to prevent that wood fibers would stick to their food. These cages have a grid on the bottom, which allows the stool and urine to pass, but does not cause difficulties walking. Because of the high grade of humidity of the gel food, the fur of the mice looked wet. In some cages mice with the earliest disease onset, lost (part) of their fur (and whiskers) because of grazing by the others. This phenomenon does not affect their well-being and was reversible within 2 weeks after they were put alone in a cage.

NTG C57BL/6 J mice of both genders were compared to TDP-43 (A315T) mice with normal food and gel food for motor performance, body appearance and survival once a week till onset, than twice a week. Onset of gait abnormalities was defined as the moment at which a clear swimming gait appeared, with the paws wide-based and the animal being unable to hold its lower body from the ground. End stage (ES) for the TDP-43 (A315T) mice with normal food was the moment at which the animal appeared immobile (even after being gently pushed) and lethargic, since we experienced that in this stage the death would occur within 24 h. For the gel fed mice, ES was the moment at which the mouse could not turn from a side anymore within 30 s. At this point the mice were euthanized using a lethal dose of 10% Nembutal and tissues were collected.

Ex-vivo pellet propulsion assay and video analysis of colonic function
TDP-43 (A315T) female mice of around 90 days were compared to NTG littermates of the same age (n = 5). After killing the mice by cervical dislocation, the entire distal colon was removed and suspended in an organ bath (±30 min after death) filled with Krebs solution (in mM: 120.9 NaCl, 5.9 KCl, 1.2 MgCl₂, 2.5
Figure 4 (See legend on next page.)
CaCl$_2$, 1.2 Na$_2$HPO$_4$, 14.4 NaHCO$_3$, 11.5 glucose) kept at 37°C and continuously bubbled with 95% O$_2$–5% CO$_2$ (pH 7.4). The intestine was allowed to equilibrate for 30 min, after which an artificial pellet was gently introduced into the proximal colon. Due to the intrinsic peristaltic reflex, the pellet was pushed along the intestine in a consecutive series of individual contraction, which can be monitored using a video setup. All images were recorded using custom written routines in Igor pro (WaveMetrics, Eugene, OR) [33]. The position of the pellet can be monitored by the dilated diameter (top images) that is shown as a gray scale line in the spatio-temporal maps (white arrows) [34]. Frequency and propulsive force of the contraction can be deduced from the generated maps.

Histopathologic analyses

Scoring of the NMJ was done as previously described [35]. Eight mice were analyzed in each group at 100–150 NMJs/mouse.

For the measurement of creatine kinase (CK) levels, blood was taken from the right atrium after euthanizing, but before perfusion of the mice (at ES for the TDP-43 (A315T) mice with normal or gel food). The sample was centrifuged for 10 minutes at 14000 rpm. The supernatant was pipetted into another eppendorf tube, which was again centrifuged for 10 minutes at 14000 rpm. This supernatant was stored at −80°C until all the samples were obtained by using a microtome (Slee cryostat, Mainz, Germany). For immunostainings, they were blocked with 10% normal donkey serum (Sigma Aldrich, St Louis, Missouri, USA) at room temperature, incubated overnight at 4°C with the C-terminal total (mouse and human) TDP-43 (polyclonal rabbit-anti-TDP43-Ab 12892-1-AP, Proteintech, 1/200), followed by incubation with NeuN-Ab (MAB377, Millipore, Billerica, Massachusetts, USA, 1/200 for 1 h at room temperature) or incubated for 2 hours at room temperature with GFAP-Ab (polyclonal rabbit-anti-GFAP-Ab, DAKO, Glostrup, Denmark, 1/500) followed by 3 washes with PBS-T and incubation with the secondary Ab for 1 h at room temperature (1/500, Alexa Fluor 555 anti-rabbit (for TDP-43-Ab and GFAP-Ab) or Alexa Fluor 488 anti-mouse (for NeuN), Invitrogen Life Technologies, Carlsbad, CA, USA). After 3 washes with PBS-T, slides were mounted with Vectashield with DAPI and analyzed using a Zeiss Imager M1 microscope (Zeiss, Oberkochen, Germany).

To analyze the intestinal tract by immunostainings, segments of intestine (ileum and colon) were collected, opened along the mesenteric border and pinned flat in a sylgard lined dissection dish. Using fine forceps the mucosal and submucosal layers were removed prior to fixation in 4% paraformaldehyde (30 min). After rinsing in PBS, circular muscle layers were peeled in case of the small intestine; while for the colon the longitudinal muscle was removed. Tissues were treated in permeabilizing (0.5% triton-x) and 4% goat/donkey serum, prior to a 24 h (4°C) incubation in primary antibodies: specific human TDP-43 antibody (monoclonal mouse anti-hTDP-43-Ab 60019-2-Ig, Proteintech, Chicago, USA, 1/200), total TDP-43 (polyclonal rabbit-anti-TDP43-Ab 12892-1-AP, Proteintech, 1/200), GFAP (Abcam, Cambridge, UK, 1/5000), NO-synthetase (Santa Cruz biotechnologies, Santa Cruz, USA, 1/400), ChAT (Chemicon International, 1/500) and HuCD (Invitrogen Life Technologies, Carlsbad, CA, USA) and frozen at −80°C. Slices of 20 μm were obtained by using a microtome (Slee cryostat, Mainz, Germany). For immunostainings, they were blocked with 10% normal donkey serum (Sigma Aldrich, St Louis, Missouri, USA) at room temperature, incubated overnight at 4°C with the C-terminal total (mouse and human) TDP-43 (polyclonal rabbit-anti-TDP43-Ab 12892-1-AP, Proteintech, 1/200), followed by incubation with NeuN-Ab (MAB377, Millipore, Billerica, Massachusetts, USA, 1/200 for 1 h at room temperature) or incubated for 2 hours at room temperature with GFAP-Ab (polyclonal rabbit-anti-GFAP-Ab, DAKO, Glostrup, Denmark, 1/500) followed by 3 washes with PBS-T and incubation with the secondary Ab for 1 h at room temperature (1/500, Alexa Fluor 555 anti-rabbit (for TDP-43-Ab and GFAP-Ab) or Alexa Fluor 488 anti-mouse (for NeuN), Invitrogen Life Technologies, Carlsbad, CA, USA). After 3 washes with PBS-T, slides were mounted with Vectashield with DAPI and analyzed using a Zeiss Imager M1 microscope (Zeiss, Oberkochen, Germany).

We performed immunostainings for total (mouse and human) TDP-43 and glial fibrillary acid protein (GFAP) on spinal cord of ES (normal or gel fed) TDP-43 (A315T) mice compared to NTG mice. To this end, mice were euthanized with 10% Nembutal and perfused with PBS and 4% PFA. Lumbar spinal cord was dissected and dehydrated overnight in 30% sucrose in PBS. The tissue was embedded in Tissue Tek (OCT Compound, 361603E, VWR International, Randor, Pennsylvania, USA) and frozen at −80°C. Slices of 20 μm were obtained by using a microtome (Slee cryostat, Mainz, Germany). For immunostainings, they were blocked with 10% normal donkey serum (Sigma Aldrich, St Louis, Missouri, USA) at room temperature, incubated overnight at 4°C with the C-terminal total (mouse and human) TDP-43 (polyclonal rabbit-anti-TDP43-Ab 12892-1-AP, Proteintech, 1/200), followed by incubation with NeuN-Ab (MAB377, Millipore, Billerica, Massachusetts, USA, 1/200 for 1 h at room temperature) or incubated for 2 hours at room temperature with GFAP-Ab (polyclonal rabbit-anti-GFAP-Ab, DAKO, Glostrup, Denmark, 1/500) followed by 3 washes with PBS-T and incubation with the secondary Ab for 1 h at room temperature (1/500, Alexa Fluor 555 anti-rabbit (for TDP-43-Ab and GFAP-Ab) or Alexa Fluor 488 anti-mouse (for NeuN), Invitrogen Life Technologies, Carlsbad, CA, USA). After 3 washes with PBS-T, slides were mounted with Vectashield with DAPI and analyzed using a Zeiss Imager M1 microscope (Zeiss, Oberkochen, Germany).
Figure 5 (See legend on next page.)
Figure 5 Progressive loss of NOS neurons in TDP-43 (A315T) mice with gel food. A. (left) The graph shows the NOS/HuCD neurons ratio in NTG (color code: blue; n = 7), TDP-43 (A315T) on normal food (color code: red; n = 4) and TDP-43 (A315T) on gel food (color code: green; n = 3) males and females was 1/500. After rinsing, fluorescently labeled appropriate secondary antibodies were added for 2 h. Immunohistochemical staining was visualized under an epifluorescence microscope (BX 41 Olympus, Belgium) with specific filter cubes (EX/DM/EM in nm) for blue (325-375/400/435-485), green (460-495/505/510-550) and red fluorescent probes (570-590/595/660-660). Images were recorded using Cell^F software on an XM10 (Olympus) camera.

For counting of motor neurons, the lumbar spinal cord was cut in 20 μm thick slices, with each 6th slice placed on a slide (10 in total along the whole lumbar region). These were stained by Cresyl violet (Sigma). After taking photos of the ventral horns at 40x (10/mouse) with a Zeiss Imager M1 microscope (Zeiss), manual counting and analyses of the size of motor neurons was done with use of AxioVision (Zeiss). Six NTG mice, 5 ES TDP-43 (A315T) mice with normal food and 5 ES TDP-43 (A315T) mice with gel food were analyzed.

Staining and quantification of the dorsolateral corticospinal tract axons in the distal thoracic spinal cord (of 4 NTG, 4 ES TDP-43 (A315T) mice on normal food and 4 ES mice on gel food) was done as described by Wegrzewska et al. [15].

Western blot and qPCR
Spinal cord and brain from presymptomatic TDP-43 (A315T) or NTG mice (n = 3 for each genotype) were collected in Tripure isolation reagent (Roche Diagnostics, IN, USA) or tissue protein extraction reagents (Thermo Scientific, Rockford, IL, USA) with Complete (Complete EDTA-free, Roche Diagnostics) for qPCR and Western blot respectively.

RNA extraction was performed as described previously [36]. qPCR assays were run in triplicate with n = 3 for each condition. The mRNA expression in spinal cord and brain of mouse TDP-43 (Mm.PT.51.5553804 Tardp exon 2–3, IDT, Coralville, Iowa, USA) was compared to the expression of several housekeeping genes, using taqman assays: beta-actin (VIC-MGB 4352341E-0905010, Applied Biosystems, Life Technologies, CA, USA), mouse hypoxanthine-guanine phosphoribosyltransferase (Mm01545399_m1 Hprt, Applied Biosystems) and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm.PT.39a.1 GAPDH exon 2–3, IDT, Leuven, Belgium). Expression of mouse Sort1 + Ex17b mRNA in the brain of TDP-43 (A315T) and NTG mice was determined using Cyber green. The primers were: mouse Sort1 + Ex17b: 5′-AAAT CCCAGGAGACAAATGC-3′ and 5′-GAGCTGAGATT TGGGACAAG-3′; mouse GAPDH: 5′-TTGGCCTTTCCG TGTTCTTAC-3′ and 5′-GAGTTGCTGTGAAGTG CGCA-3′.

For Western blot, 40 μg of spinal cord of TDP-43 (A315T) or NTG mice (n = 3 for each condition) were blotted using a precast gel (NuPAGE® Bis-Tris gel IM-8042, Novex, Life technologies, Carlsbad, California, USA) and following the protocol described in Van Hoecke et al. [37], allowing separation of the mouse TDP-43 (of 43 kDa) and the exogenous human TDP-43, with the Flag-tag running at a slightly higher molecular weight as described in Wegrzewska et al. [15]. The membranes were probed with polyclonal rabbit-anti-TDP43-Ab (12892-1-AP, Proteintech, 1/500). The mouse TDP-43 levels were compared between TDP-43 (A315T) mice and NTG mice. Alfa-tubulin (mouse anti-alfa-tubulin, T6199, Sigma, 1/5000) was used as a loading control. Also, the total TDP-43 expression between 90 days old TDP-43 (A315T) males and females was compared by using the polyclonal rabbit-anti-TDP43-Ab (12892-1-AP, Proteintech, 1/500) and normalized against mouse GAPDH (mouse anti-GAPDH AM4300, Ambion, Life Technologies, 1/5000).

Statistics
Data are presented as mean ± standard error of the mean, except for Figure 3E and F in which the standard deviations are shown. All statistics were performed using Graph Path Prism or Stats Direct software. In case of not normally distributed values, a non parametric test was used to compare 2 groups. For more than 2 groups, an Anova or Kruskal-Wallis (in case of not normally distributed data) was used. Survival data were analyzed using the log-rank test.

Additional file
Additional file 1: Movies 1–4: Motor phenotype of symptomatic and ES TDP-43 (A315T) mice. Difference in gait abnormalities between the TDP-43 (A315T) mouse on normal food (additional movie 1.vc), compared to one on gel food (additional movie 2.vc). The swimming gait

http://www.molecularneurodegeneration.com/content/9/1/24
of the gel fed mouse is much more pronounced and slowly proceeds till an ES situation, as it is visible in movie 4 (additional movie 3.MOV). The mouse is not able to turn from its side anymore due to the severe neurologic impairment, whereas the TDP-43 (A315T) mouse on normal food in movie 3 (additional movie 3.MOV) is about to die due to intestinal pseudo-obstruction.

Abbreviations
TDP-43: TAR DNA-binding protein 43; ALS: Amyotrophic lateral sclerosis; FTLD: Frontotemporal lobar degeneration; NTG: Non-transgenic; CK: Creatine kinase; ES: Endstage; GFAP: Gliarial fibrillary acid protein; Pmp: Pion promotor; NOS: Nitric-oxide synthase; CHAT: Choline acetyltransferase; NMJ: Neuromuscular junctions; ENS: Enteric nervous system; Sort1 + ex17b: Sortilin 1 including exon 17b.

Competing interests
The authors declare that they have no competing interests. All experiments on rodents were approved by the local ethical committee of the University of Leuven, Belgium.

Authors’ contributions
SH carried out the breeding, genotyping, perfusions, qPCRs, Western blots, motor assessment, immunoassays and histopathology of the spinal cord and NMJs of the mice, as well as the quantifications and statistical analysis of these results. SH drafted the manuscript. CC has helped substantially to acquisition, analysis and interpretation of data on intestinal assays and in drafting the manuscript. PVD and PWB participated in the design and coordination of the studies and helped to analysis and interpretation of data and in drafting the manuscript. LVDB and WR have been involved in revising the manuscript. All authors read and approved the manuscript.

Authors’ information
Pieter Vanden Berge and Philip Van Damme share last senior authorship.

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