21 Brooks BR, Miller RG, Swash M, *et al*. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2000;1:293–9.
22 Ohashi Y, Tashiro K, Itoyama Y, *et al*. Study of functional rating scale for amyotrophic lateral sclerosis: revised ALSFRS(ALSFRS-R) Japanese version. *No To Shinkei* 2001;53:346–55.
23 Atsuta N, Watanabe H, Ito M, *et al*. Development of a telephone survey system for patients with amyotrophic lateral sclerosis using the ALSFRS-R (Japanese version) and application of this system in a longitudinal multicenter study. *Brain Nerve* 2011;63:491–6.
24 Kasarskis EJ, Dempsey-Hall L, Thompson MM, *et al*. Rating the severity of ALS by caregivers over the telephone using the ALSFRS-R. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2005;6:50–4.
25 Kaufmann P, Levy G, Montes J, *et al*. Excellent inter-rater, intra-rater, and telephone-administered reliability of the ALSFRS-R in a multicenter clinical trial. *Amyotroph Lateral Scler* 2007;8:42–6.
26 Mannino M, Cellura E, Grimaldi G, *et al*. Telephone follow-up for patients with amyotrophic lateral sclerosis. *Eur J Neurol* 2007;14:79–84.
27 Chancellor AM, Slattery JM, Fraser H, *et al*. The prognosis of adult-onset motor neuron disease: a prospective study based on the Scottish Motor Neuron Disease Register. *J Neurol* 1993;240:339–46.
28 Miller RG, Mitchell JD, Moore DH. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev* 2012;3:CD001447.
29 Brendler SJ. Human cervical myotomes—functional anatomy studied at operation. *J Neurosurg* 1968;28:105–11.
30 Parent A. *Carpenter's human neuroanatomy*. Quebec, Canada: Williams & Wilkins, 1996.
31 Pinto S, de Carvalho M. Motor responses of the sternocleidomastoid muscle in patients with amyotrophic lateral sclerosis. *Muscle Nerve* 2008;38:1312–17.
32 Pinto S, Pinto A, de Carvalho M. Phrenic nerve studies predict survival in amyotrophic lateral sclerosis. *Clin Neurophysiol* 2012;123:2454–9.
33 Ravits JM, La Spada AR. ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration. *Neurology* 2009;73:805–11.
34 Fujimura-Kiyono C, Kimura F, Ishida S, *et al*. Onset and spreading patterns of lower motor neuron involvements predict survival in sporadic amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry* 2011;82:1244–9.
35 Kanouchi T, Ohkubo T, Yokota T. Can regional spreading of amyotrophic lateral sclerosis motor symptoms be explained by prion-like propagation? *J Neurol Neurosurg Psychiatry* 2012;83:739–45.
36 Brettschneider J, Tredici KD, Toledo JB, *et al*. Stages of pTDP-43 pathology in amyotrophic lateral sclerosis. *Ann Neurol* Published Online First: 20 May 2013. doi:10.1002/ana.23937.
37 Kavanagh J, Barrett R, Morrison S. The role of the neck and trunk in facilitating head stability during walking. *Exp Brain Res* 2006;172:454–63.
38 Ballesteros ML, Buchthal F, Rosenfalck P. The pattern of muscular activity during the arm swing of natural walking. *Acta Physiol Scand* 1965;63:296–310.
39 Richter RR, VanSant AF, Newton RA. Description of adult rolling movements and hypothesis of developmental sequences. *Phys Ther* 1989;69:63–71.
40 Cudkowicz ME, Katz J, Moore DH, *et al*. Toward more efficient clinical trials for amyotrophic lateral sclerosis. *Amyotroph Lateral Scler* 2010;11:259–65.
41 Sorenson EJ. What is next in ALS clinical trials? *Neurology* 2007;69:719–20.
42 Gourie-Devi M, Nalini A, Sandhya S. Early or late appearance of "dropped head syndrome" in amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry* 2003;74:683–6.
43 Uemura M, Kosaka T, Shimohata T, *et al*. *et al*. Dropped head syndrome in amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Frontotemporal Degeneration* 2013;14:232–3.
44 Katz JS, Wolfe GI, Burns DK, *et al*. Isolated neck extensor myopathy: a common cause of dropped head syndrome. *Neurology* 1996;46:917–21.

Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, 2013; 14: 116–123

ORIGINAL ARTICLE

Tracheostomy with invasive ventilation for ALS patients: Neurologists' roles in the US and Japan

JUDITH RABKIN[1,2], MIEKO OGINO[3], RAYMOND GOETZ[1], MARTIN McELHINEY[1,2], ALLISON MARZILIANO[4], TAKASHI IMAI[5], NAOKI ATSUTA[6], MITSUYA MORITA[7], TAKAHISA TATEISHI[8], TSUYOSHI MATSUMURA[9] & HIROSHI MITSUMOTO[10]

[1]*New York State Psychiatric Institute,* [2]*College of Physicians and Surgeons, Department of Psychiatry, Columbia University, USA,* [3]*Department of Neurology, Kitasato University School of Medicine, Japan,* [4]*Memorial Sloan Kettering Cancer Center, New York, USA,* [5]*Miyagi National Hospital,* [6]*Nagoya University,* [7]*Jichi Medical University,* [8]*Kyushu University Graduate School of Medicine,* [9]*Toneyama National Hospital, Japan, and* [10]*College of Physicians and Surgeons, Department of Neurology, Columbia University, USA*

Abstract

Our objective was to determine whether substantial differences in rates of TIV utilization in the U.S. and Japan are associated with the role of the treating neurologist. Questionnaires in English and Japanese were sent to neurologists who treated ALS patients in both countries. Questions included queries about rates of TIV use in their practices, level of encouragement of TIV use, the role of the neurologist in TIV decision making, management of patient/family requests to discontinue TIV once initiated, and personal choices if neurologists themselves had ALS. Results showed that 84% of American neurologists reported fewer than 10% of their patients had TIV, compared to 32% of Japanese. Americans less often encouraged TIV use (79% of American and 36% of Japanese seldom or never suggested or encouraged TIV). Finally, neurologists were asked whether they would choose TIV for themselves in the hypothetical scenario where they had ALS: over 70% of both groups declined TIV for themselves. In conclusion, consistent with past findings, Japanese neurologists were more likely to recommend TIV and more of their patients received TIV. Both groups believed their recommendations influence patient decisions. While Americans seldom recommended TIV to patients and most would not choose TIV for themselves, Japanese neurologists' recommendations and personal choices diverged.

Key words: *TIV, tracheostomy, ALS, end-of-life decisions*

Introduction

ALS is a progressive, incurable, irreversible neurodegenerative disease that inexorably progresses to a point where respiratory capacity fails, even with non-invasive ventilatory support. At this point, a choice must be made between tracheostomy with invasive (long-term) mechanical ventilation (TIV or LTMV, we use here TIV), and palliative care with subsequent respiratory failure in the short-term future. With TIV, ALS continues to progress to the point where some patients may reach a 'locked-in' state in which they cannot communicate effectively, but breathing is maintained indefinitely. Survival after TIV varies widely, from months (1) to several years (2).

Substantial disparities in rates of TIV utilization by ALS patients have been noted for some time (3). Rates vary widely between countries, regions within countries, and even within institutions (3,4). Among American studies, TIV rates of 2% to 6% have been reported (5,6). In European studies, TIV use rates have ranged from 0% to 10.6% (1,7). In contrast, Japanese studies report rates of TIV use ranging from 25% to 46% (8–10).

Several explanations have been offered to account for this variation in TIV use, including different conceptualizations of the physician's role in medical decision-making, attitudinal differences of health care professionals, national variations in health insurance coverage and availability of institutional facilities, varying degrees of commitment to a life-prolonging approach (8), and availability of alternatives such as hospice.

Correspondence: J. G. Rabkin, New York State: Psychiatric Institute, Unit 51 1051 Riverside Drive, New York, NY 10032, USA. Fax: 212 543 5326. E-mail: jgr1@columbia.edu

(Received 11 June 2012; accepted 28 August 2012)

ISSN 2167-8421 print/ISSN 2167-9223 online © 2013 Informa Healthcare
DOI: 10.3109/17482968.2012.726226

We conducted a survey of American and Japanese neurologists who treat ALS patients in order to determine whether these differences in rate of TIV utilization still persist, and to identify possible contributing factors.

Methods

Sample

In the United States, we (HM) sent e-mails to 324 neurologists who were directors of ALS clinics and adult MDA clinics and their affiliates. Recipients could respond on the internet (using Survey Monkey) or by returning questionnaires as e-mail attachments. In Japan, questionnaires were mailed to 210 neurologists known to see ALS patients (selected by Ogino et al.).

Questionnaire

The questionnaire was written by the New York team (HM, JR with input from colleagues), with 24 fully structured multiple-choice items, several with subscales. Areas covered are shown in Table I. Questions were based on previous findings as well as clinical observations and published suggestions of other investigators (1,8). In order to preserve anonymity, minimal personal information was sought. The instrument was translated into Japanese and then reviewed and edited by MO and the Japanese TIV Study Group members. Questions about withdrawal of tracheostomy were modified to be appropriate in Japan. The protocol was approved by the New York State Psychiatric Institute – Columbia University Department of Psychiatry IRB. Data were collected during 2011.

Table I. Components of TIV questionnaire.

Domain	Number of items
1. Neurologists' experience in general practice, and experience with ALS patients	3
2. Doctor-patient communications: initial diagnosis; discussions of advance directives; assisted suicide	10
3. Beliefs about the doctor's role in TIV decision-making; percentage of patients with TIV in own practice. How often they suggest and encourage TIV	4
4. Attributes of patients and perceived patient preferences that influence their decision-making about TIV; practices regarding TIV discontinuation	9*
5. Personal choices if the neurologist him/herself had ALS (PEG, NIV, TIV)	3

*Includes subscale queries about 16 patient/situational attributes and 12 reasons patients may or may not want TIV.

Statistical analysis

Comparisons of responses of American and Japanese neurologists were first analyzed using χ^2 tests or *t*-tests. Logistic and multiple regression analyses were also performed. For items with skewed response distributions, Mann-Whitney *U*-tests were used. For those with a 1–10 response format with anchors, ranging from 1 = 'almost never' to 10: 'almost always', responses of 1–3 were classified as 'low' or 'seldom/never'; scores of 4–7 = 'medium' or 'some of the time'; scores of 8–10 as 'high' or 'very often/almost always'. In those analyses regarding neurologists' degree of enthusiasm in recommending TIV to their patients, the 1–10 scale was dichotomized to reflect less (1–5) and more (6–10) enthusiasm. Because of the multiple comparisons examined, trend differences are not reported. Bonferroni corrections were not applied because this is an exploratory study. Alpha = .05, two-tailed.

Results

Samples

One hundred American neurologists in 44 states returned questionnaires, including 258 comments about various issues, a response rate of 31%. In Japan, 120 neurologists (57% response rate) living in all eight provinces, responded to mailed surveys, including 163 written comments. Respondents were highly experienced: over 85% had practiced neurology for >10 years (87% of U.S. and 90% of Japanese respondents), and over half of each group had practiced neurology 20+ years ($\chi^2 = 0.18$, 1df, $p = .672$). However, Americans were far more specialized regarding ALS treatment: 71% had seen more than 20 new patients in the past year, compared to 10% of Japanese ($\chi^2 = 87.79$, 1df, $p < .0001$). Similarly, 77% of American neurologists were currently treating >20 patients/year vs. 11% of Japanese neurologists ($\chi^2 = 100.43$, 1df, $p < .0001$).

Question 1. Do diagnostic styles (communication of initial diagnosis, discussion of advance directives, and frequency of requests for assisted suicide) vary by country?

There were more similarities than differences in diagnostic styles and management of advance directives among American and Japanese neurologists. Most said they "tell the diagnosis as clearly as possible", although more Japanese neurologists said they "tell in different words, somewhat vaguely", and 6% of Japanese and no Americans said they defer telling at all (2×3 cross-tabulation analysis, FET = .017). Thus we focus on the data more directly associated with TIV. Regarding when neurologists raise the issue of future preferences, there

were no major differences in timing. However, while advance directives as independent documents are widely used in the U.S., they are not in Japan. Rather, Japanese patients who express preferences have their wishes noted in their charts.

Assisted suicide. Neurologists were asked whether any of their patients raised the issue of physician assisted suicide. Keeping in mind that Americans saw far more ALS patients than Japanese respondents did, and thus were more likely to hear such requests, 51% of their patients vs. 25% of Japanese patients had made such requests ($\chi^2 = 15.25$, 1df, $p < .0001$).

Question 2. Do rates of TIV use in neurologists' practices vary by country? Do they differ in the extent to which they suggest and encourage use of TIV?

Respondents were asked "in your own practice, about what % of patients get TIV?" Response options were "1−2%, 3−9%, 10−25%, 26−49%, 50+%". Eighty-four percent of American neurologists reported fewer than 10% of their patients had TIV, compared to 32% of Japanese. In contrast, 38% of Japanese but only 5% of Americans reported that more than 25% of their patients had TIV ($\chi^2 = 62.08$, 5df, $p < .0001$). Overall, TIV rates continue to show substantial differences between countries.

We then asked "While there are probably always exceptions, in general do you suggest and encourage the use of TIV?" Similarly large differences were seen between samples, with substantially higher rates of encouragement among Japanese neurologists. Classifying the 1−10 rating scale into never/seldom, moderately often, and very often/almost always, 79% of American and 36% of Japanese neurologists seldom or never encouraged and suggested TIV, 20% of American and 58% of Japanese said sometimes, and 1% of U.S. and 6% of Japanese said almost always ($\chi^2 = 39.36$, 2df, $p < .0001$).

Question 3. The role of the neurologist in TIV decision making

Three possible roles for physicians in medical decision-making were offered: 1) It is my responsibility to make decisions for the patient and inform them what will be done; 2) My role is to present treatment options along with my recommendations; 3) I present the options and let them decide.

American neurologists never endorsed the first (doctor decides) and overwhelmingly (70%) chose the second − present options and their recommendations, while Japanese neurologists either agreed that it is their responsibility to make decisions (18%) or present options without a recommendation to let the patient decide (58%). The overall $\chi^2 = 67.19$, 2df, $p < .0001$.

Is there a relationship between size of ALS caseload and level of enthusiasm for TIV? We examined number of patients currently followed and likelihood of recommending TIV. We used a cut-off of 1−10 patients vs. 11+ patients, and dichotomized the 1−10 rating scale, asking "In general do you suggest and encourage use of TIV?" (1−5 = less encouragement, 6−10 = more encouragement). Among neurologists seeing < 10 patients, TIV was not encouraged by 71% of American and 81% of Japanese neurologists ($\chi^2 = 0.21$, 1df, $p = .647$, NS). There was, however, a difference among those who saw 11+ patients: 95% of American neurologists did not recommend TIV compared to 68% of Japanese ($\chi^2 = 15.06$, 1df, $p = .0001$).

Is neurologists' level of encouragement of TIV related to percent of their patients with TIV? Our data show such a relationship. As shown in Table II, patients were more likely to have TIV if their doctors encouraged its use in both Japanese ($\chi^2 = 12.94$, 2df, $p = .002$) and American ($\chi^2 = 18.26$, 2df, $p < .0001$) samples.

Table II. Relationship between neurologist's level of encouragement in recommending TIV[1] and the percent of their patients who receive TIV.

	Patients in practice receiving TIV			
Encouragement for TIV	0–9 %	10 – 25 %	26+%	
U.S.				
Less	75 (87%)	9 (10%)	2 (2%)	$\chi^2 = 18.26$, df = 2, $p < .0001$
More	4 (50%)	1 (13%)	3 (38%)	
Japan				
Less	33 (38%)	28 (33%)	25 (29%)	$\chi^2 = 12.94$, df = 2, $p = .002$
More	3 (12%)	5 (20%)	17 (68%)	

[1]Neurologists were asked "In general do you suggest and encourage the use of TIV rather than comfort care?" rated on a 1 (= almost never) to 10 (= almost always) scale which we dichotomized here.

Note: Based upon dichotomizing Encouragement for TIV, only eight of 94 (8.5%) U.S. neurologists were more encouraging of TIV compared to 25 of 111 (22.5%) of the Japanese neurologists. The 2 × 2 cross-tabulation for samples and the dichotomized encouragement exhibited a significant $\chi^2 = 7.40$, 1 df, $p < .01$.

Question 4. Is neurologists' length of experience with ALS or their perception of patient characteristics related to their encouragement/advice about TIV?

Neurologists' experience and encouragement of TIV. The dependent variable was percent of neurologists' own patients who have TIV. Predictor variables included years practicing neurology, number of new ALS patients seen in past year, and number of ALS patients currently followed. In both samples, only years of practice of neurology was positively associated with higher percent of patients with TIV (U.S.: Beta = .244, $t = 2.44$, $p = .017$; Japanese sample Beta = .234, $t = 2.52$, $p = .013$). Direct experience with ALS patients (new patients seen in past year, number currently followed) was unrelated to percent of own patients with TIV.

Perceived patient attributes that influence neurologists' decision-making about TIV. Neurologists were asked to rate the importance of each of 14 patient attributes in their decision-making and recommendations for TIV, on a 1−10 scale with higher numbers signifying greater importance. Scores on virtually all scales were skewed in the 'important' direction, but there were group differences. We performed a principal component analysis using varimax rotation with Kaiser normalization and identified four factors, as shown in Table III. The samples differed on three of the four factors: Japanese respondents considered Factor 1 (hope for the future) and Factor 3 (younger, young children, believe in cure) as more important than Americans, while U.S. respondents weighed Factor 4, patient functioning, as more important than Japanese respondents did. Regarding the influence of financial considerations on their recommendations, the majority of both American and Japanese neurologists agreed that they were highly important (55% vs. 63%, $\chi^2 = 1.59$, 1df, p = .451).

Perceived reasons that patients decide against TIV. Respondents were asked "When patients choose not to have TIV, what are their reasons?" Seven reasons were listed. As shown in Table IV, responses to five of the seven options differed by country. More American neurologists endorsed the reasons "patient so disabled that he/she is ready to go", "financial burden", and "can't be cared for at home and doesn't want to go to facility". More Japanese neurologists endorsed the items "because it is illegal to discontinue TIV once started", and "patient doesn't want to live any longer".

Table III. Patient attribute factors that may influence neurologists' decision-making about TIV (1−10 scale: higher = more important).

Factor	U.S. ($n = 93$) Mean (SD)	Japan ($n = 120$) Mean (SD)	t-test, df, p
1. Hope	7.38 (2.00)	8.23 (1.45)	3.42, 161, .001
2. Support	7.74 (1.99)	7.57 (1.71)	0.66, 211, .509
3. Cure/Age	5.29 (1.85)	6.47 (1.97)	4.43, 211, <.001
4. Function	8.12 (2.27)	7.28 (2.16)	2.74, 211, .007

Factor 1: patient characteristics of hope for the future and capacity for enjoyment in the absence of depression and cognitive impairment.
Factor 2: external support (financial and familial).
Factor 3: having young children, being younger, and believing that an ALS cure is imminent.
Factor 4: poor patient functioning and medical comorbidities.

Perceived reasons that patients decide in favor of TIV. Neurologists were asked "What factors do you think influence the patient's decision to choose TIV?" Five options were listed. Most neurologists (63% and 69%) agreed that "the way the doctor explains TIV" is influential ($\chi^2 = 0.93$, 1df, p = .335). Japanese neurologists were far more likely to think that patients are influenced by availability of facilities for ALS patients (72% vs. 36%, $\chi^2 = 24.36$, 1df, $p < .0001$). Questions about frequency of communication with patients who have TIV, and financial factors did not differ by country: about half of all neurologists thought communication with patients who had TIV was an influence ($\chi^2 = .021$, 1df, p = .647), and the majority thought financial factors were influential (67% vs. 55%, $\chi^2 = 2.59$, 1df, p = .108).

Percent of patients with TIV having emergency intubation. The range of reported rates was zero to 100%. We classified responses as 0−9%, 10−19%, and 20+%, based on skew in response distributions. Overall, about one-third of neurologists in both countries reported that more than 20% of their patients received TIV on an emergent basis without an advance directive requesting it ($\chi^2 = 5.71$, 2df, p = .058).

TIV discontinuation. Neurologists were queried whether they asked patients starting TIV to specify circumstances in which they would want it discontinued. Seventy-one percent of American and 8% of Japanese neurologists reported such conversations ($\chi^2 = 24.18$, 1df, $p < .0001$). They were asked whether patients or family had requested discontinuation of TIV, and if yes, whether they had done so. Seventy-one percent of American and 49% of Japanese neurologists reported being asked to discontinue TIV ($\chi^2 = 8.39$, 1df, $p = .004$). Of the 63 Americans asked, 78% had agreed to do so, usually after consulting their hospital ethics committee or conferring with their palliative care team. Others referred the family to hospice. Most Japanese told their patients that it is not supported legally to discontinue TIV, while 12% just said it should not be removed. Finally, the proportion of patients who actually had discontinued TIV differed substantially: 39% of Americans reported that more than a quarter of their patients had done so, compared to none in Japan (FET <.0001).

Table IV. Perceived determinants of patients' decisions regarding TIV; number (%) of neurologists who endorse each reason.

	U.S. Yes (%)	Japanese Yes (%)	χ^2	p
Reasons patients do not want TIV				
Patient is so disabled that he/she is ready to go	80 (87%)	45 (38%)	50.62	<.0001
Burden on family	84 (91%)	105 (88%)	0.44	.507
No caregivers	53 (58%)	76 (63%)	0.50	.480
Financial burden	67 (73%)	66 (55%)	6.34	.012
Not able to discontinue TIV once initiated	8 (9%)	58 (48%)	36.34	<.0001
Patient doesn't want to live any longer (including psychological factors)	65 (71%)	103 (86%)	6.40	.012
Cannot be cared for at home and does not want institutional care	70 (76%)	48 (40%)	26.04	<.0001
Influences on patient's decision in favor of TIV				
The way the doctor explains TIV	63 (72%)	83 (69%)	0.14	.706
Communication with patients who already have TIV	44 (50%)	65 (54%)	0.21	.647
Home care availability 24/7	67 (76%)	83 (69%)	0.90	.343
Available facilities for TIV patients	32 (36%)	86 (72%)	24.36	<.0001
Financial factors	59 (67%)	66 (55%)	2.59	.108

Question 5. Would neurologists choose for themselves (in the hypothetical scenario where they have ALS) what they recommend to their patients?

Respondents were asked whether they would choose to have three interventional procedures, if medically indicated, as shown on Table V. In this hypothetical situation, although the majority of both groups did agree to have either percutaneous endoscopy (PEG) or non-invasive ventilation (NIV), Japanese neurologists were less likely to want these procedures than American neurologists (p= .001 and <.0001, respectively). The groups did not differ with respect to TIV: over 70% of both groups would not choose TIV for themselves, and the remainder was divided between answering affirmatively and saying that they "can't tell now".

We then examined the correspondence between neurologists' choices for themselves and their degree of encouragement of TIV for their patients. If neurologists would not accept TIV for themselves, would they usually recommend it to their patients? Among American neurologists, 80% seldom or never encouraged TIV for their patients and 76% would decline it for themselves. In contrast, only 36% of Japanese neurologists seldom or never encouraged TIV for their patients while 72% declined to have it for themselves (see Table VI). We then examined level of encouragement for TIV for their own patients (using the dichotomous scale "Do you generally encourage TIV for your patients?") by Japanese neurologists who did not want TIV for themselves compared to those who did want TIV for themselves. We found that 89% of those who did not want TIV for themselves were less likely to recommend it to their patients, compared to only 33% who were less enthusiastic about TIV for their patients among neurologists who did personally want TIV (89% (75/84) vs. 33% (6/18), $\chi^2 = 28.38$, 1df, p<.0001).

Discussion

While Japanese and American neurologists share many beliefs and practices about management of ALS, several consistent differences emerge. These must be considered in view of differences between countries in practice structure, government roles and laws. It is legal to discontinue TIV in the United States. In Japan, the situation is ambiguous. There is no specific law regarding TIV discontinuation, and there are Ministry of Health guidelines for doing so, but discontinuation is rarely considered. Even when a neurologist proposes this, hospital ethics committees and hospital administrators typically refuse, as there is no legal protection from prosecution.

Most American neurologists specialized in treating ALS patients saw them in major clinics with extensive staff. Government insurance (Medicare) is available for all ALS patients. Palliative care is the standard of care for patients approaching death, and

Table V. Neurologists' choices for themselves regarding interventional procedures.

	PEG	NIV	TIV		
Procedure	Yes (%)	Yes (%)	Yes (%)	No (%)	Cannot tell now (%)
U.S.	80 (94%)	82 (94%)	7 (8%)	68 (76%)	14 (16%)
Japan	85 (76%)	74 (67%)	18 (15%)	84 (72%)	15 (13%)
	$\chi^2 = 10.50$ df = 1 p = .001	$\chi^2 = 19.85$ df = 1 p = <.0001		$\chi^2 = 2.80$ df = 2 p = .247	

Table VI. Relationship between encouraging TIV for patients and personal choice of TIV.

U.S.		
Encourage TIV		
Seldom/Never	Sometimes	Often/Always
69 (80%)	16 (19%)	1 (1%)
Choose TIV for self		
No	Uncertain	Yes
65 (76%)	14 (16%)	7 (8%)
Japan		
Encourage TIV		
Seldom/Never	Sometimes	Often/Always
42 (36%)	68 (58%)	7 (6%)
Choose TIV for self		
No	Uncertain	Yes
84 (72%)	15 (13%)	18 (15%)

Query 1: "In general, do you suggest and encourage the use of TIV rather than comfort care?".
Score on 1–10 scale: 1–3 = seldom/never, 4–7 = sometimes, 8–10 = often/always.
Query 2: "Should you be diagnosed with ALS, and had progressive dyspnea, would you accept TIV?".
Score: choice of yes/probably yes; cannot answer until I find myself in that position (= uncertain); no/probably no.
Note: Within the national samples, there was 65% agreement/consistency among the U.S. neurologists compared to 44% among the Japanese neurologists. The U.S. neurologists exhibited significantly higher agreement for encouraging and self-choosing TIV compared to the Japanese neurologists ($\chi^2 = 9.21$, df = 1, $p < .003$).

according to a large database from the 1990s, most ALS patients in the United States receive palliative care, hospice, and die at home (5). Long-term facilities are limited and generally not geared to ALS patients (11).

Japanese neurologists typically have individual practices, are less specialized and personally treat far fewer patients with ALS. They are, however, informed and knowledgeable about the disease and its prognosis. Patients with 'intractable diseases' including ALS receive all necessary medical care including hospitalization without charge, as well as long-term home care visits by personnel including physicians under the Long-term care Insurance Act law (12). A Japanese ALS patient voluntary organization vigorously promotes TIV (13), while American organizations do not take an advocacy position. Consistent with the literature and national policies, in our study Japanese neurologists were more likely to recommend TIV and more of their patients received TIV, while Americans seldom did so and few of their patients received TIV. Most (75%) Americans presented treatment options to patients along with their recommendations. Among Japanese neurologists, 58% said they presented options without recommendations (even though two-thirds said in response to another query that they moderately often encourage/suggest TIV). Both groups believed that their recommendations influence patient decisions, and our data support this. This may be one of the first studies to show a direct correspondence between neurologists' level of enthusiasm for TIV and the percentage of their patients who actually receive TIV.

In addition, American and Japanese neurologists largely agreed that most of the patient and family characteristics listed in the questionnaire did influence their decision-making, especially familial support and adequacy of financing. There were, however, some differences: Japanese neurologists weighed more heavily the patient's degree of hope for the future, their having young children and believing in an imminent cure, while Americans emphasized current patient functioning including medical comorbidities.

Our finding that two-thirds of Japanese neurologists moderately often encourage TIV for ALS patients is not universally seen among Japanese physicians. Aita et al. (14) queried 27 internists and surgeons about TIV use in older adults with stroke-caused profound impairment and no hope for recovery. Most discouraged TIV use, although artificial nutrition and hydration were considered essential. The difference between Aita's findings and ours may be due to the fact that most ALS patients retain substantial cognitive capacity, an attribute considered 'highly important' in recommending TIV by 87% of the Japanese neurologists we surveyed. Cognitive capacity was the most widely endorsed of the 14 attributes we asked about (listed in Table III). Thus, differences in level of consciousness and cognitive capacity, which are generally present in ALS patients and essentially absent in severely impaired stroke patients, likely account for these disparate attitudes.

An interesting finding concerns the association between what doctors recommend for their patients and what they would choose for themselves, should they have ALS. While Americans were largely consistent in not recommending TIV for patients and not wanting it for themselves, Japanese neurologists were far more likely to recommend TIV to their patients than choose it for themselves. This disparity is consistent with research showing that physicians are more likely to recommend invasive procedures with attendant risks of serious adverse sequelae to their patients than they would choose for themselves, given death as the alternative. For example, Ubel et al. (15) presented to physicians a colon cancer scenario with two options, varying in terms of survival rate with lower survival accompanied by fewer serious adverse events (i.e. length of life versus quality of life). Given two options, 38% of 242 internists chose for themselves the option with better quality of life despite a higher death rate, while only 25% chose this option to recommend to a hypothetical patient. In an invited commentary, Shaban et al. (16) observed that these data suggest physicians more often favor prolongation of life for their patients but place more emphasis on quality of life concerns when making decisions for themselves. Other investigators similarly have observed in differing

contexts that choosing for others differs from choosing for oneself in decision-making (17–19), and this has been widely discussed after a 2012 publication of an article in The Wall Street Journal (U.S.) entitled 'Why doctors die differently'. In our survey, the Japanese neurologists behaved more like participants in these studies than Americans, who seldom recommended TIV either to patients or chose it for themselves. An alternative explanation might be that Japanese neurologists express a general societal option to their patients, that TIV is recommended, which is the message of the influential ALS association in Japan.

We note several limitations. First, although the response rates are equivalent to those expected of e-mail surveys (20) and postal surveys (21) and are regionally distributed, respondents represent only a portion of the population of neurologists treating patients with ALS. Secondly, we collected little information about respondents to protect anonymity, and kept the survey brief to encourage participation. Thirdly, the neurologist's philosophy and approach regarding TIV for their patients with ALS may be only one component of the story. We are currently surveying patients and caregivers with similar questionnaires. Ultimately, the combined input from neurologists, patients and caregivers is expected to increase our understanding of TIV use in our two countries.

Despite these limitations, to our knowledge this is the first systematic survey concerning neurologists' roles in TIV decision-making. Responses of 220 neurologists in almost all regions of the two countries do provide a glimpse of our practice philosophy and approach to patients in the terminal stages of this disease, and we are beginning to understand the observed variations in TIV use. A better understanding of the neurologist's role in this process can improve patient care during this most precarious phase of the disease.

Acknowledgements

We thank Ezgi Tiraki, Paul Barkus, Greg Carter, Daragh Heitzman and Donald Tamulonis, as well as the other neurologists in both the United States and Japan who responded to our survey. We thank the MDA and ALS associations for providing the names of affiliated ALS clinic directors in the U.S., and Wings Over Wall Street for a grant to support the project in the United States. M. Ogino received a Health and Labour Sciences Research Grant on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan to support the project in Japan.

Declaration of interest: J. Rabkin: Cephalon-Teva currently provides armodafinil for an NIH-funded study, as well as funds and product to complete an NIH-supported placebo controlled randomized clinical trial of armodafinil in 2010.

H. Mitsumoto received grants from Avanir, Knopp, Biogen Idec., and Cytokinetics for clinical trials and honoraria for participating in advisory board meetings from Avanir, Sanofi-Aventis, Shionogi, and Biogen Idec. He is now a member of the Data Safety Monitoring Board for the Neural-Stem clinical trial. He received honoraria from Sanofi-Aventis Japan for giving seminars at the annual meetings of the Japanese Neurological Society in 2009 and 2010 in Japan. He received a conference grant to Columbia University for the 2011 International ALS Conference from NIDNS, NIH ORDR, MDA, ALS Association, ALS Society of Canada, Adams Foundation, Ride for Life, ALS Hope Foundation, Les Turner Foundation, Sanofi-Aventis, Biogen Idec, Knopp, and Avania.

The authors alone are responsible for the content and writing of the paper.

References

1. Chio A, Calvo A, Ghiglione P, Mazzini L, Mutani R, Mora G for the PARALS. Tracheostomy in amyotrophic lateral sclerosis: a 10-yeaer population based study in Italy. J Neurol Neurosurg Psychiatry. 2010;81:1141–3.
2. Hayashi H. The long-term communication strategy in patients with TPPV throughout the total course of ALS. Amyotroph Lateral Scler. 2006;7(Suppl 1):25–6.
3. Smyth A, Riedl M, Kimura R. Olick R, Siegler M. End-of-life decisions in amyotrophic lateral sclerosis: a cross-cultural perspective. J Neurolog Sci. 1997;152(Suppl 1): S93–6.
4. Moss AH, Casey P, Stocking CB, Roos R, Brooks BR, Siegler M. Home ventilation for amyotrophic lateral sclerosis patients: outcomes, costs and physician attitudes. Neurology. 1993;43:438–43.
5. Miller RG, Anderson FA, Bradley W, Brooks B, Mitsumoto H, Munsat T, Ringel S. The ALS patient care database: goals, design and early results. ALS C.A.R.E. Study Group. Neurology. 2000;54:53–7.
6. Vender RL, Mauger D, Walsh S, Alam S, Simmons Z. Respiratory systems abnormalities and clinical milestones for patients with amyotrophic lateral sclerosis with emphasis on survival. Amyotroph Lateral Scler. 2007;8:36–41.
7. Neudert C, Oliver D, Wasner M, Borasio G. The course of the terminal phase in patients with amyotrophic lateral sclerosis. J Neurol. 2001;248:612–6.
8. Borasio G, Gelinas D, Yanagisawa N. Mechanical ventilation in amyotrophic lateral sclerosis: a cross-cultural perspective. J Neurol. 1998;245(Suppl 2):S7–12.
9. Atsuka N, Watanabe H, Ito M, Aoki M, Nakano I, Yuasa T, et al. Clinical features of ALS in Japan from the registration system of intractable diseases. Amyotroph Lateral Scler. 2006;7(Suppl 1):100.
10. Kimura I. Patient support in Japan. Amyotroph Lateral Scler. 2006;7(Suppl 1):7.
11. Moss AH, Casey P, Stocking CB, Roos RP, Brooks BR, Siegler M. Home ventilation for amyotrophic lateral sclerosis patients: outcomes, costs, and patient, family and physician attitudes. Neurology. 1993;43:438–43.
12. Mitsumoto HM, Borasio G, Genge A, Hardiman O, Leigh P, Meininger V, et al. The multidisciplinary case clinic: the principles and an international perspective. In: Mitsumoto H, Przedblorski S, Gordon P, editors. Amyotrophic Lateral Sclerosis. New York: Taylor & Francis; 2006. pp 605–31.

13. Takahashi H. Pathological aspects of long-term survival of ALS patients on respirators. Amyotroph Lateral Scler. 2006;7(Suppl 1):25.
14. Aita K, Miyata H, Takahashi M, Kai I. Japanese physicians' practice of withholding and withdrawing mechanical ventilation and artificial nutrition and hydration from older adults with very severe stroke. Arch Gerontol Geriatr. 2008;46: 263–72.
15. Ubel PA, Angott A, Zikmund-Fisher B. Physicians recommend different treatments for patients than they would choose for themselves. Arch Int Med. 2011;171:630–4.
16. Shaban E, Guerry R, Quill T. Reconciling physician bias and recommendations. Arch Int Med. 2011;171;634–5.
17. Zigmund-Fisher B, Sarr B, Fagerlin A, Ubel P. A matter of perspective: choosing for others differs from choosing for yourself in making treatment decisions. J Gen Int Med. 2006;21:618–22.
18. Kray L, Gonzalez R. Differential weighing in choice versus advice: 'I'll do this, you do that'. J Behav Dec Making. 1999;12:207–17.
19. Polman E. Information distortion in self/other decision-making. J Exp Soc Psychology. 2010;46:432–5.
20. Sheehan K. E-mail survey response rates: a review. J Computer Mediated Communication 6 (2). Retrieved January 18, 2012, from http://jcmc.indiana.edu/vol6/issue2/sheehan.html
21. Cook J, Dickinson H, Eccles M. Response rates in postal surveys of health care professionals between 1996 and 2005: an observational study. BMC Hlth Serv Res. 2009; 9:160–8.

doi:10.1093/brain/awt029 Brain 2013: 136; 1371–1382 | 1371

BRAIN
A JOURNAL OF NEUROLOGY

Loss of TDP-43 causes age-dependent progressive motor neuron degeneration

Yohei Iguchi,[1] Masahisa Katsuno,[1] Jun-ichi Niwa,[2] Shinnosuke Takagi,[1] Shinsuke Ishigaki,[1] Kensuke Ikenaka,[1] Kaori Kawai,[1] Hirohisa Watanabe,[1] Koji Yamanaka,[3,4] Ryosuke Takahashi,[5] Hidemi Misawa,[6] Shoichi Sasaki,[7] Fumiaki Tanaka[1,8] and Gen Sobue[1,4]

1 Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan
2 Stroke Centre, Aichi Medical University, Aichi 480-1195, Japan
3 Laboratory for Motor Neuron Disease, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan
4 CREST, Japan Science and Technology Agency, 4-1-8, Honcho, Kawaguchi, Saitama 332-0012, Japan
5 Department of Neurology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan
6 Department of Pharmacology, Keio University Faculty of Pharmacy, Tokyo 105-8512, Japan
7 Department of Neurology, Neurological Institute, Tokyo Women's Medical University, Tokyo 162-8666, Japan
8 Department of Neurology and Stroke Medicine, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan

Correspondence to: Gen Sobue
Showa-ku, Nagoya 466-8550,
Japan
E-mail: sobueg@med.nagoya-u.ac.jp

Amyotrophic lateral sclerosis is a devastating, progressive neurodegenerative disease that affects upper and lower motor neurons. Although several genes are identified as the cause of familial cases, the pathogeneses of sporadic forms, which account for 90% of amyotrophic lateral sclerosis, have not been elucidated. Transactive response DNA-binding protein 43 a nuclear protein regulating RNA processing, redistributes to the cytoplasm and forms aggregates, which are the histopathological hallmark of sporadic amyotrophic lateral sclerosis, in affected motor neurons, suggesting that loss-of-function of transactive response DNA-binding protein 43 is one of the causes of the neurodegeneration. To test this hypothesis, we assessed the effects of knockout of transactive response DNA-binding protein 43 in mouse postnatal motor neurons using Cre/loxp system. These mice developed progressive weight loss and motor impairment around the age of 60 weeks, and exhibited degeneration of large motor axon, grouped atrophy of the skeletal muscle, and denervation in the neuromuscular junction. The spinal motor neurons lacking transactive response DNA-binding protein 43 were not affected for 1 year, but exhibited atrophy at the age of 100 weeks; whereas, extraocular motor neurons, that are essentially resistant in amyotrophic lateral sclerosis, remained preserved even at the age of 100 weeks. Additionally, ultra structural analysis revealed autolysosomes and autophagosomes in the cell bodies and axons of motor neurons of the 100-week-old knockout mice. In summary, the mice in which transactive response DNA-binding protein 43 was knocked-out specifically in postnatal motor neurons exhibited an age-dependent progressive motor dysfunction accompanied by neuropathological alterations, which are common to sporadic amyotrophic lateral sclerosis. These findings suggest that transactive response DNA-binding protein 43 plays an essential role in the long term maintenance of motor neurons and that loss-of-function of this protein seems to contribute to the pathogenesis of amyotrophic lateral sclerosis.

Keywords: transactive response DNA-binding protein 43; amyotrophic lateral sclerosis; axonal degeneration; autophagosome

Abbreviations: ALS = amyotrophic lateral sclerosis; FTLD = frontotemporal lobar degeneration; TDP CKO = motor neuron-specific TDP-43 knockout; TDP hCKO = TDP heterozygous CKO

Received July 20, 2012. Revised December 17, 2012. Accepted December 28, 2012. Advance Access publication February 28, 2013

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disease that affects upper and lower motor neurons in the brain stem and spinal cord. Although previous studies using animal models of ALS have focused mainly on the toxicity of mutant SOD1, one of the causative genes of familial ALS (ALS1), there are pathophysiological differences between ALS1 and sporadic ALS that accounts for ~90% of ALS. The most striking recent discovery regarding ALS is that transactive response DNA-binding protein 43 (TDP-43) was identified as a major component of ubiquitinated neuronal cytoplasmic inclusions in both sporadic ALS and frontotemporal lobar degeneration (FTLD) (Arai *et al.*, 2006; Neumann *et al.*, 2006). In addition, TDP-43 is a causative gene of familial ALS (ALS10) (Gitcho *et al.*, 2008; Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008; Yokoseki *et al.*, 2008). Taken together, these data suggest that TDP-43 plays a key role in the pathogenesis of sporadic ALS. Although TDP-43 is a nuclear protein, it redistributes to the cytoplasm and forms aggregates in affected neurons of patients with sporadic ALS (Arai *et al.*, 2006; Neumann *et al.*, 2006), suggesting that loss of TDP-43 function underlies sporadic ALS pathogenesis. TDP-43 is known to regulate gene transcription, stability of messenger RNA, and exon splicing through interactions with RNA, heterogeneous nuclear ribonucleoproteins and nuclear bodies (Wang *et al.*, 2004; Ayala *et al.*, 2005; Buratti *et al.*, 2005, 2010; Strong *et al.*, 2007; Polymenidou *et al.*, 2011; Sephton *et al.*, 2011; Tollervey *et al.*, 2011). Knockdown of TDP-43 in neuronal cells inhibits neurite outgrowth and diminishes cell viability (Iguchi *et al.*, 2009), whereas TDP-43 depletion induces apoptosis in HeLa or U2OS cells (Ayala *et al.*, 2008). In addition, *Drosophila* without TDP-43 present deficient locomotive behaviours, reduced life span and anatomical defects at neuromuscular junctions (Feiguin *et al.*, 2009). TDP-43-depleted zebrafish exhibit swimming deficits along with excessive, premature branching and shortened motor axons (Kabashi *et al.*, 2011). Furthermore, TDP-43 knockout mice are embryonic lethal (Kraemer *et al.*, 2010; Sephton *et al.*, 2010; Wu *et al.*, 2010), and postnatal deletion of TDP-43 leads to rapid death with loss of body fat (Chiang *et al.*, 2010). Although these findings indicate that TDP-43 is essential for survival of mice at both embryonic and post-natal stages, the effects of TDP-43 depletion in postnatal mammalian neurons have not been fully elucidated. In the present study, we generated motor neuron-specific TDP-43 knockout (TDP CKO) mice using the Cre/loxP recombination system to investigate the effects of TDP-43 loss on postnatal motor neurons in mice.

Materials and methods

Generation and maintenance of TDP-43 conditional knockout mouse

The targeting construct was designed to insert an Frt-flanked neomycin cassette and a loxP site upstream, and a loxP site downstream of the second exon of the *Tardbp* gene. This construct was electroporated into iTL1 BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem cells. Correctly targeted embryonic stem cells were injected into recipient blastocysts and chimeric mice were bred with C57BL/6J mice. The resulting En1flox-neo mice were then bred to C57BL/6J mice constitutively expressing Flp recombinase to remove the Frt-flanked neo cassette, generating En1flox offspring. En1flox mutant mice were backcrossed with C57BL/6J mice for at least five generations, and then crossed with VAChT-Cre.Fast mice, which are the most validated mice that specifically express Cre in motor neurons (Misawa *et al.*, 2003). To generate TDP-43 conditional knockout mice, we crossed TDP-43$^{flox/flox}$ mice with TDP-43$^{flox/+}$/VAChT-Cre mice. Finally, we obtained TDP-43$^{flox/flox}$/VAChT-Cre (motor neuron-specific TDP-43 knockout: TDP CKO), TDP-43$^{flox/+}$/VAChT-Cre (TDP heterozygous CKO: TDP hCKO), TDP-43$^{flox/flox}$ and TDP-43$^{flox/+}$ mice. The TDP-43$^{flox/flox}$ mice were then used as control littermates in the present analyses. Mice were kept on a 12-h light/12-h dark cycle, with food and water provided *ad libitum*. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Nagoya University Animal Experiment Committee (Nagoya, Japan).

Neurological and behavioural testing

The control ($n = 21$) and TDP CKO ($n = 20$) mice were subjected to the Rotarod task (Economex Rotarod; Columbus Instruments) weekly as described previously (Katsuno *et al.*, 2002). Grip strength was measured weekly by a grip strength meter (MK-380M, Muromachi kikai Co. LTD). During this test, the mice griped the mesh with four limbs and their tail was pulled backwards. Gait stride was measured from 50 cm of footsteps, and the average value was recorded for each mouse.

Immunofluorescent analysis and immunohistochemistry

For immunofluorescent analysis, we perfused 20 ml of a 4% paraformaldehyde fixative in phosphate buffer (WAKO Corp.) through the left cardiac ventricle of mice deeply anaesthetized with medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol (5 mg/kg), interperitoneally. Tissues postfixed overnight in 10% phosphate-buffered formalin were then processed for paraffin embedding. We then deparaffinized 3 -μm thick tissue sections and dehydrated them with alcohol. Sections were first microwaved for 20 min in 50 mM citrate buffer (pH 6.0), treated with TNB blocking buffer (PerkinElmer) and incubated overnight with anti-TDP-43 rabbit polyclonal antibody (1:1000, ProteinTech), anti-choline-acetyltransferase (ChAT) goat polyclonal antibody (1:100, Milipore), anti-microtubule-associated protein 1 light chain 3 (LC3) mouse monoclonal antibody (1:1000, MBL), phosphorylated neurofilament-H (pNF-H) mouse monoclonal antibody (SMI31) (1:1000, Covance), or anti-neuronal nuclei (NeuN) mouse monoclonal antibody (1:100, Millipore). After washing, for the ChAT staining, sections were incubated with biotinylated donkey anti-goat IgG (1:300, Vector Lab.) for 30 min, washed and incubated with streptavidin conjugated with Alexa Fluor® 546 (1:1000, Invitrogen) for 30 min. For the other antigens, sections were incubated with the indicated secondary antibody and TO-PRO®3 (Invitrogen), a nuclear marker, for 30 min, mounted with ProLong® Gold Antifade reagent (Invitrogen), and then imaged with a laser confocal microscope (LSM710, Carl Zeiss). For immunohistochemistry, sections were incubated overnight with anti-glial fibrillary acidic protein (GFAP) mouse

monoclonal antibody (1:1000, Sigma-Aldrich), stained using the DAKO EnVision™+ HRP system (Dako Corp.) and photographed with an optical microscope (Axio Imager M1). The immunoreactive area in the ventral horn of TDP CKO mice and control littermates at the indicated ages ($n = 3$ for each age) was analysed with WinROOF (Mitani). The binary treatment included application of a staining intensity threshold and size exclusion criteria to distinguish the significant structures from the background signals. All sections analysed were treated with the same threshold and exclusion criteria.

Retrograde FluoroGold neurotracer labelling

Retrograde labelling of motor neurons was performed as described previously (Katsuno *et al.*, 2006). Briefly, a total volume of 4.5 µl of 2.5% FluoroGold solution (Biotium) was injected into the gastrocnemius muscle of anaesthetized mice. Lumbar spinal cords were removed 46 h after FluoroGold administration. The frozen optimal cutting temperature (OCT) compound-embedded samples were sectioned longitudinally on a cryostat at 10 µm and mounted on silane-coated slides. After the FluoroGold labelled motor neurons in the L5 segment was photographed with Zeiss Axio Imager M1 (Carl Zeiss), the sections were fixed with 4% paraformaldehyde, stained with anti-TDP-43 and anti-ChAT antibody, and photographed with LSM710. For the quantification of retrograde labelling, we measured every third section (a total of five sections in L5 ventral horn), and counted the degree of FluoroGold labelling in motor neurons of two control mice, and TDP-43-positive or -negative motor neurons of two TDP CKO mice.

Electron microscopy

Under the deep anaesthesia, 2-year-old TDP CKO mice and control littermates were transcardially perfused with 3% paraformaldehyde and 1% glutaraldehyde in PBS. The spinal cords and sciatic nerves were removed, and postfixed overnight in the perfusing solution. After fixation, the spinal cords were immersed in the solution (0.1 M cacodylic acid, 2% paraformaldehyde, 2.5% glutaraldehyde) for 12 h. The anterior half of lumbar spinal cord was sectioned transversely, postfixed in 1% osmium tetroxide for several hours, dehydrated and embedded in epoxy resin. Each block was cut into serial semithin sections (~1-µm thick). These sections were stained with toluidine blue. Appropriate portions of the sections were cut into ultrathin sections, which were then stained with uranyl acetate and lead citrate. Two-year-old TDP CKO mice and control littermates were analysed. Electron microscopic photographs were obtained under an original magnification of ×5000 and printed at a final magnification of ×9500.

Analysis of muscle, neuromuscular junction and motor axon

To investigate the presence of muscle atrophy, gastrocnemius muscles were dissected free, quickly frozen by immersion in cooled acetone and powdered CO_2. Ten-micrometre thick transverse frozen sections were stained with haematoxylin and eosin. For analysis of neuromuscular junctions, 30-µm thick frozen longitudinal sections of the tibialis anterior muscle were incubated overnight with alpha bungarotoxin conjugated with biotin-XX (1:80, Invitrogen), anti-pNF-H mouse monoclonal antibody (SMI31, 1:100) and anti-synaptophysin rabbit polyclonal antibody (1:100, Cell Signaling Technologies). After washing, sections were incubated with goat anti-rabbit and anti-mouse IgG conjugated with Alexa Fluor® 488 (1:1000 for each, Invitrogen) and streptavidin conjugated with Alexa Fluor® 564 (1:1000, Invitrogen) and mounted with ProLong® gold (Invitrogen). The stained sections were imaged with a laser scanning confocal microscope (LSM710, Carl Zeiss). More than 100 neuromuscular junctions from TDP CKO mice aged 20, 50, 80 and 100 weeks were analysed ($n = 3$ mice for each group). For morphological analyses, epoxy resin embedded transverse sections of L5 ventral roots were stained with toluidine blue. L5 ventral roots of 20, 50 and 100 week-old mice ($n = 6$ axons for each group) were assessed. The diameter of myelinated fibres was automatically measured using a computer-assisted image analyser (Luzex FS), as described previously (Katsuno *et al.*, 2002). Paraffin embedded transverse sections of L4 ventral roots of 100-week-old mice were stained with an antibody against ChAT and photographed by Zeiss Axio Imager M1.

Quantification and morphological analysis of motor neurons

For the quantifications and morphological analyses of motor neurons, we performed the immunofluorescent analyses of the paraffin-embedded sections stained with anti-TDP-43 and anti-ChAT antibodies of L5 spinal cord ($n = 5$ for each) and brain stem ($n = 3$ for each) of control and TDP CKO mice. All the neurons within the every fifth sections from the 50 consecutive sections of lumbar spinal cord, or every third sections from all consecutive sections of brain stem including the each cranial motor nucleus were assessed using AxioVision softwear (Carl Zeiss), after samples were photographed by Zeiss Axio Imager M1 (Carl Zeiss). The ChAT-positive neurons in the ventral horns or cranial nuclei were regarded as motor neurons. We examined the presence of nuclear immunoreactivity for TDP-43 in ventral horns and brainstems, and calculated the TDP-43-knockout efficiencies, the number of remaining motor neurons, and the size of motor neurons in each group. To evaluate the involvement of gamma-motor neuron, we measured the number of large ($>250\,\mu m^2$) or small ($<250\,\mu m^2$) lumbar motor neurons.

Statistical analyses

Statistical differences were analysed by Kaplan–Meier and logrank test for survival rate, ANOVA and Bonferroni *post hoc* analyses for multiple group comparisons and the unpaired Student's *t*-test for two group comparisons (SPSS version 15.0, SPSS Inc.).

Results

Generation of TDP CKO mice

We constructed a TDP-43flox allele by flanking the second exon of the mouse TDP-43 gene (*Tardbp*) with loxP sites (Supplementary Fig. 1A and B). Because the second exon contains the *Tardbp* start codon, Cre-mediated deletion of this exon inhibits mouse TDP-43 translation. To delete TDP-43 expression specifically in motor neurons, TDP-43$^{flox/flox}$ mice were crossed with VAChT-Cre.Fast mice, in which Cre expression is mostly restricted in the postnatal somatomotor neurons (Misawa *et al.*, 2003). The immunofluorescent analysis of the ventral horn from TDP CKO mice at post-natal Day 2 showed that all the assessed motor neurons were positive for TDP-43 (Supplementary Fig. 1C). On

the other hand, the quantitative analysis of the lumbar ventral horn and hypoglossal nucleus from 10-week-old TDP CKO mice showed that TDP-43 was knocked-out in 48% of motor neurons in the lumbar ventral horn and 45% in the hypoglossal nucleus (Fig. 1A and B). In addition, reverse transcriptase PCR analysis of total RNA from motor neurons isolated by laser microdissection, revealed that exon 2 of *Tarbdp* was partially skipped under the Cre expression (Supplementary Fig. 1D and E). Immunoblot analysis showed that the TDP-43 protein expressions in liver, kidney, heart, skeletal muscle and cerebral cortex of TDP CKO mice were not altered compared with their control littermates (Supplementary Fig. 1F). Immunofluorescent analysis of the

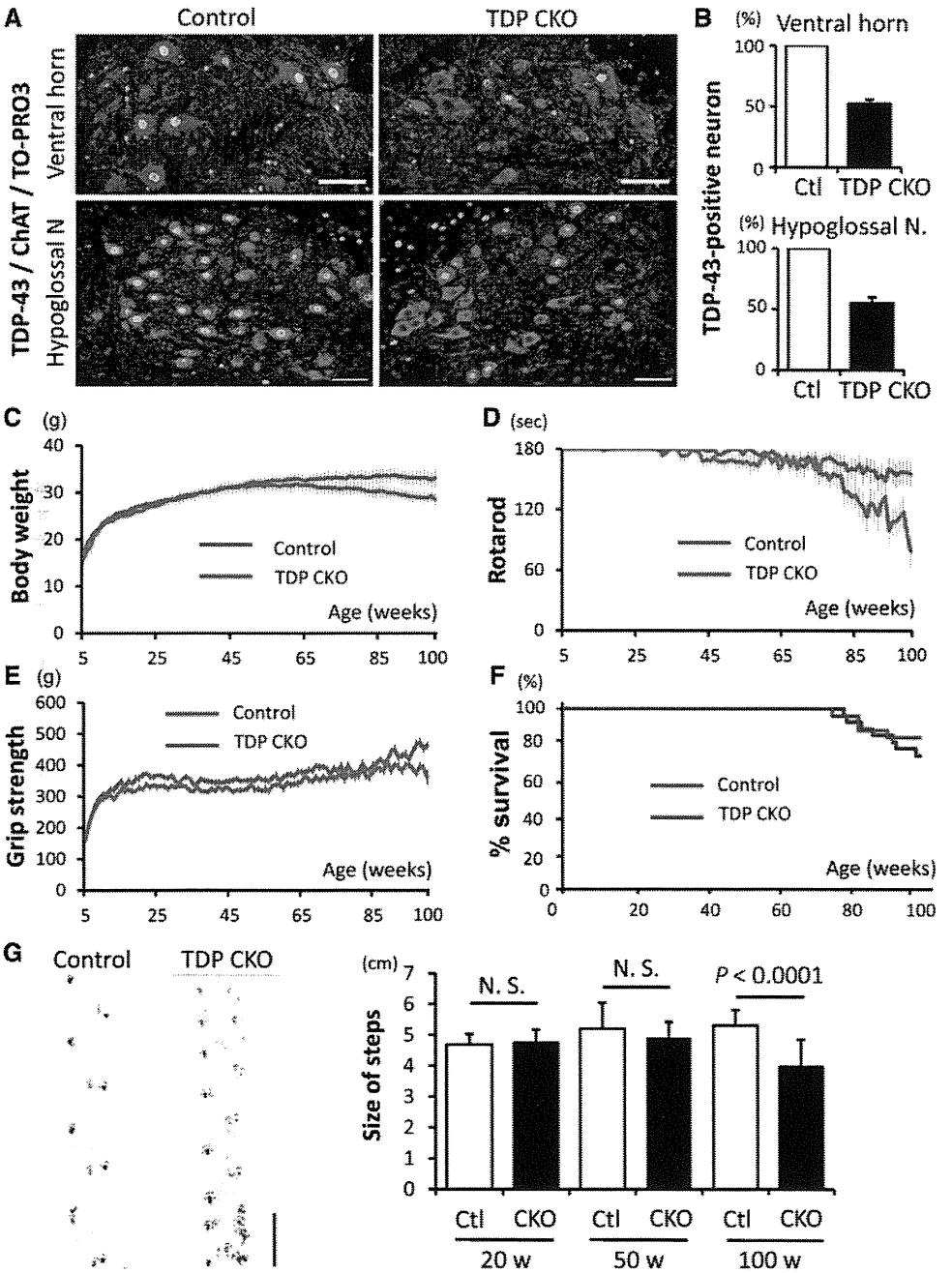

Figure 1 Progressive motor dysfunction in TDP CKO mice. (**A**) Immunofluorescent stainings (TDP-43; green, ChAT; red, TO-PRO3; blue) of lumbar ventral horn and hypoglossal nucleus of 10-week-old control and TDP CKO mice. (**B**) Percentage of TDP-43-positive motor neurons in the lumbar ventral horn and hypoglossal nucleus (N.) of 10-week-old control (Ctl) and TDP CKO mice ($n = 3$ for each group). (**C–E**) Body weight (**C**), Rotarod task (**D**), and grip strength (**E**) phenotypes of control (red line, $n = 21$) and TDP CKO mice (blue line, $n = 20$). Error bars indicate SEM. (**F**) Survival rates of control ($n = 27$) and TDP CKO mice ($n = 26$). (**G**) The average length of hindpaw steps in 20-week-old ($n = 6$ for each), 50-week-old ($n = 6$ for each), and 100-week-old ($n = 15$ for each) control and TDP CKO mice. Error bars indicate SD. Scale bars: **A** = 50 μm; **G** = 5 cm. N.S. = not significant.

lumbar dorsal horn of 10-week-old control, TDP CKO and TDP hCKO mouse showed that all the assessed neurons were positive for TDP-43 (Supplementary Fig. 2). In addition, the analyses of 100-week-old control, TDP CKO and TDP hCKO mice demonstrated that TDP-43 was not excised in the neurons of the primary motor cortex, putamen, deep cerebellar nucleus and cerebellar cortex of TDP CKO or TDP hCKO mouse (Supplementary Figs 3 and 4).

TDP-43 CKO mice develop progressive motor dysfunction

The earliest symptom of motor deficit in TDP CKO mice was tremor, which appeared as early as 50 weeks. TDP CKO mice exhibited progressive weight loss beginning ~60 weeks (Fig. 1C), when muscle atrophy of the trunk and hind limb was detectable. The grip strength and motor performances in the Rotarod task of TDP CKO mice were lower than their control littermates (Fig. 1D and E) beginning at 85 and 75 weeks, respectively. At 100 weeks, TDP CKO mice were significantly different from the control littermates in body weight ($P = 0.04$), rotarod ($P = 0.001$) and grip strength ($P = 0.002$). The average length of hindpaw steps of TDP CKO mice was significantly shorter than that of control littermates in 100 weeks of age ($P = 0.000001$; Fig. 1G). The survival rate of TDP CKO mice, however, was not altered compared with that of control littermates (Fig. 1F). Analyses of TDP-43$^{flox/+}$ and TDP-43$^{flox/+}$/VAChT-Cre (TDP hCKO) mice, which resulted in heterozygous loss of TDP-43 in motor neurons, showed that body weight, Rotarod task, grip strength and length of hindpaw steps were not significantly different between the two transgenic groups (Supplementary Fig. 5A–D).

TDP-43 depletion leads to atrophy of spinal motor neurons

Because TDP CKO mice exhibited progressive motor impairment, we focused on the morphology of spinal motor neurons. The immunofluorescent analysis of the lumbar ventral horn in 100-week-old TDP CKO mice revealed that motor neurons without TDP-43 were significantly smaller than those with TDP-43 and those in control littermates (Fig. 2A–C). Although TDP-43 was knocked-out in 49% of motor neurons in TDP CKO mice, the number of motor neurons in TDP CKO mice did not differ from that in control littermates (Fig. 2D and E). A time course analysis of TDP-43-lacking motor neurons in TDP CKO mice showed that neuronal atrophy was evident at 100 weeks (Fig. 2F). In addition, we measured TDP-43 knockout efficiency in the small ($>250\,\mu m^2$) and large ($<250\,\mu m^2$) lumbar motor neurons. The results showed that there was no difference in the knockout efficiency between the small and large motor neurons (Supplementary Fig. 6A), suggesting that the TDP-43-knockout efficiency in the gamma-motor neurons of TDP CKO mice is similar to that of alpha-motor neurons. The measurement of the average motor neuron number showed that the number of TDP-43-lacking large motor neurons decreased at 100 weeks of age compared with TDP-43-positive motor neurons, whereas the number of TDP-lacking small motor neurons increased, indicating that postnatal deletion of TDP-43 leads to atrophy of the alpha-motor neurons in the aged TDP CKO mice (Supplementary Fig. 6B). This view is supported

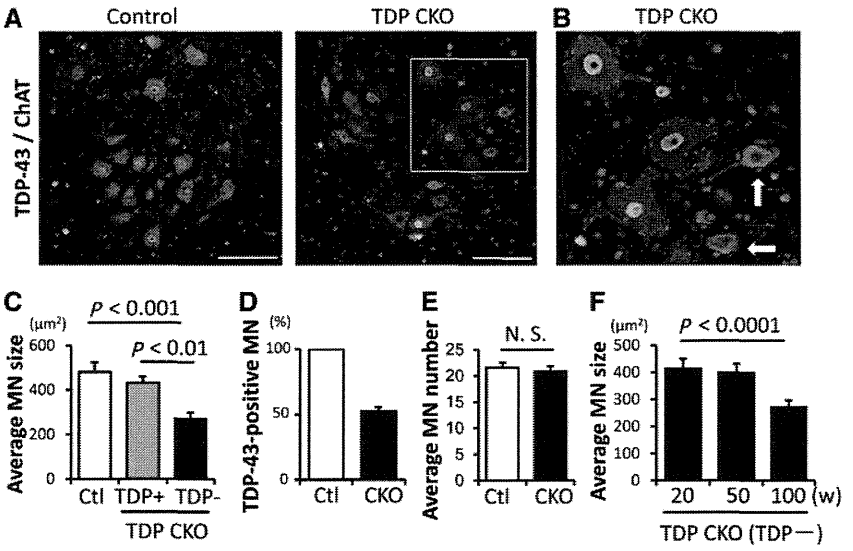

Figure 2 Morphological analysis of spinal motor neurons. (**A** and **B**) Immunofluorescencent stainings (TDP-43, green; ChAT, red) of lumbar ventral horn from 100-week-old control (Ctl) and TDP CKO mice. (**B**) Enlarged image of the area marked in **A** (*left*). TDP-43-lacking motor neurons (arrows) were significantly smaller than TDP-43-positive motor neurons. (**C**) Percentage of TDP-43-positive motor neurons in the lumbar ventral horn of 100-week-old mice ($n = 5$ for each group). (**D**) Average size of spinal motor neurons (MN) in 100-week-old mice ($n = 5$ for each group). Error bars indicate SD. (**E**) Average number of spinal motor neurons in 100-week-old mice ($n = 5$ for each group). Error bars indicate SD. (**F**) Time course of atrophy of TDP-43-lacking motor neurons ($n = 5$ for each age). TDP+ = TDP-43-positive neurons; TDP− = TDP-43-negative neurons. Scale bars = 100 μm.

by the immunofluorescent analysis of the lumbar ventral horn from 100-week-old TDP CKO mice showing that the TDP-43-lacking alpha-motor neuron, which was positive for NeuN and ChAT, was smaller than the TDP-43-positive alpha-motor neuron (Supplementary Fig. 6C). On the other hand, there was no morphological difference in the motor neurons between TDP hCKO and TDP-43$^{flox/+}$ mice (Supplementary Fig. 5E).

TDP-43 depletion affects motor axon, neuromuscular junction and skeletal muscle

The toluidine blue staining of transverse sections of L5 ventral root exhibited axonal degeneration in a subset of large myelinated fibres of TDP CKO mice from 50 weeks (Fig. 3A). Quantitative analyses of the ventral roots demonstrated the decrease of large myelinated fibres and increase of small myelinated fibres in 100 week-old TDP CKO mice (Fig. 3A). The immunofluorescent analysis using anti-ChAT antibody also exhibited the loss of large motor axons in the ventral root of TDP CKO mice (Fig. 3B). Axial sections of the gastrocnemius muscle in 100-week-old TDP CKO mice exhibited grouped atrophy, a neurogenic muscular change (Fig. 3C). Whereas all assessed neuromuscular junctions in the control littermates were innervated, in the TDP CKO mice, the percentage of denervated neuromuscular junctions increased progressively after the age of 50 weeks, concomitant with motor impairment and motor neuron atrophy (Fig. 3D). In analyses of retrograde FluoroGold labelling of the motor neurons in TDP CKO mice, the degree of labelling was significantly less in the TDP-43-lacking motor neurons than in the TDP-43-positive motor neurons (Fig. 3E).

Assessment in motor nuclei of cranial nerves

The histopathology of patients with ALS is characterized by the selective loss of motor neurons with scarcely detectable damage in the extraocular motor nuclei. To examine the region-specific neuropathology in TDP CKO mice, we quantitatively analysed the motor nuclei of cranial nerves. In the trigeminal motor, facial, hypoglossal and abductor nuclei of 100-week-old TDP CKO mice, ~50% of motor neurons were negative for TDP-43, but in the oculomotor nucleus, the efficiency of TDP-43 depletion was only ~25% (Supplementary Fig. 7). Morphological analysis of the trigeminal motor, facial and hypoglossal nuclei in 100-week-old TDP CKO mice revealed that TDP-43-lacking motor neurons were significantly smaller than those with TDP-43 or those of the control littermates (Fig. 4A–C), whereas those in the oculomotor and the abductor nuclei were preserved (Fig. 4D and E), suggesting that this mouse model recapitulates the selective vulnerability of motor neuron in ALS. The time course analysis of the hypoglossal motor nucleus showed that the atrophy of the motor neuron was evident from 50 weeks. The number of motor neurons in these nuclei of TDP CKO mice was not altered compared with the control littermates, as was shown in the spinal cord (Fig. 4A–E).

Astrogliosis in ventral horn and accumulation of phosphorylated neurofilament in motor neurons of TDP CKO mice

Immunohistochemistry of the ventral horn showed that the number of astrocytes progressively increased in TDP CKO mice (Fig. 5A). Phosphorylated neurofilament accumulated in the cytoplasm of TDP-43-lacking motor neurons of TDP CKO mice, but not in motor neurons with TDP-43 in TDP CKO mice or those of control littermates (Fig. 5B).

Formation of autophagosomes in motor neurons of TDP CKO mice

Recent studies indicate that autophagosomes accumulate in motor neurons of patients with sporadic ALS and animal models of motor neuron diseases (Li *et al.*, 2008; Sasaki, 2011; Tian *et al.*, 2011). Therefore, we investigated autophagy-related pathology in 100-week-old control and TDP CKO mice. The immunofluorescent analysis showed LC3-positive puncta in 37% of TDP-43-lacking motor neurons, but not in TDP-43-positive motor neurons in TDP CKO mice or those of the control littermates (Fig. 6A). TDP-43-lacking motor neurons with the puncta were significantly smaller than those without the puncta (Fig. 6B). The ultrastructure of motor neurons from 100-week-old TDP CKO mice demonstrated that autophagy-related structures such as autolysosomes and autophagosomes were accumulated in the cell bodies of motor neurons (Fig. 6C–E), proximal motor axon (Fig. 6F), and sciatic nerve of TDP CKO mice (Fig. 6G–H). These structures were not seen in the control mice as far as we observed.

Discussion

Although TDP-43 is an established pathological hallmark of ALS, it remains unclear how TDP-43 contributes to the pathogenesis. In the present study, we showed that TDP CKO mice, in which TDP-43 was knocked-out specifically in postnatal motor neurons, developed an age-dependent progressive motor impairment such as gait disturbance and muscle atrophy, suggesting that the loss-of-function of TDP-43 in postnatal motor neurons plays a causative role in the neurodegenerative process of ALS. There has been a great deal of debate about whether loss or gain of TDP-43 function causes the neurodegeneration (Lee *et al.*, 2011). Several mouse, rat and primate models overexpressing wild-type or disease mutant TDP-43 recapitulate the phenotype of ALS or FTLD (Wegorzewska *et al.*, 2009; Shan *et al.*, 2010; Stallings *et al.*, 2010; Tsai *et al.*, 2010; Wils *et al.*, 2010; Xu *et al.*, 2010; Zhou *et al.*, 2010; Igaz *et al.*, 2011; Swarup *et al.*, 2011; Uchida *et al.*, 2012); however, redistributions and cytoplasmic inclusions of TDP-43 are generally rare and several models exhibit cytoplasmic mitochondrial aggregation, which is not common in ALS. The expression of endogenous TDP-43 is suppressed in neurons expressing human TDP-43-delta nuclear localization signal as well as those expressing human wild-type TDP-43, suggesting that

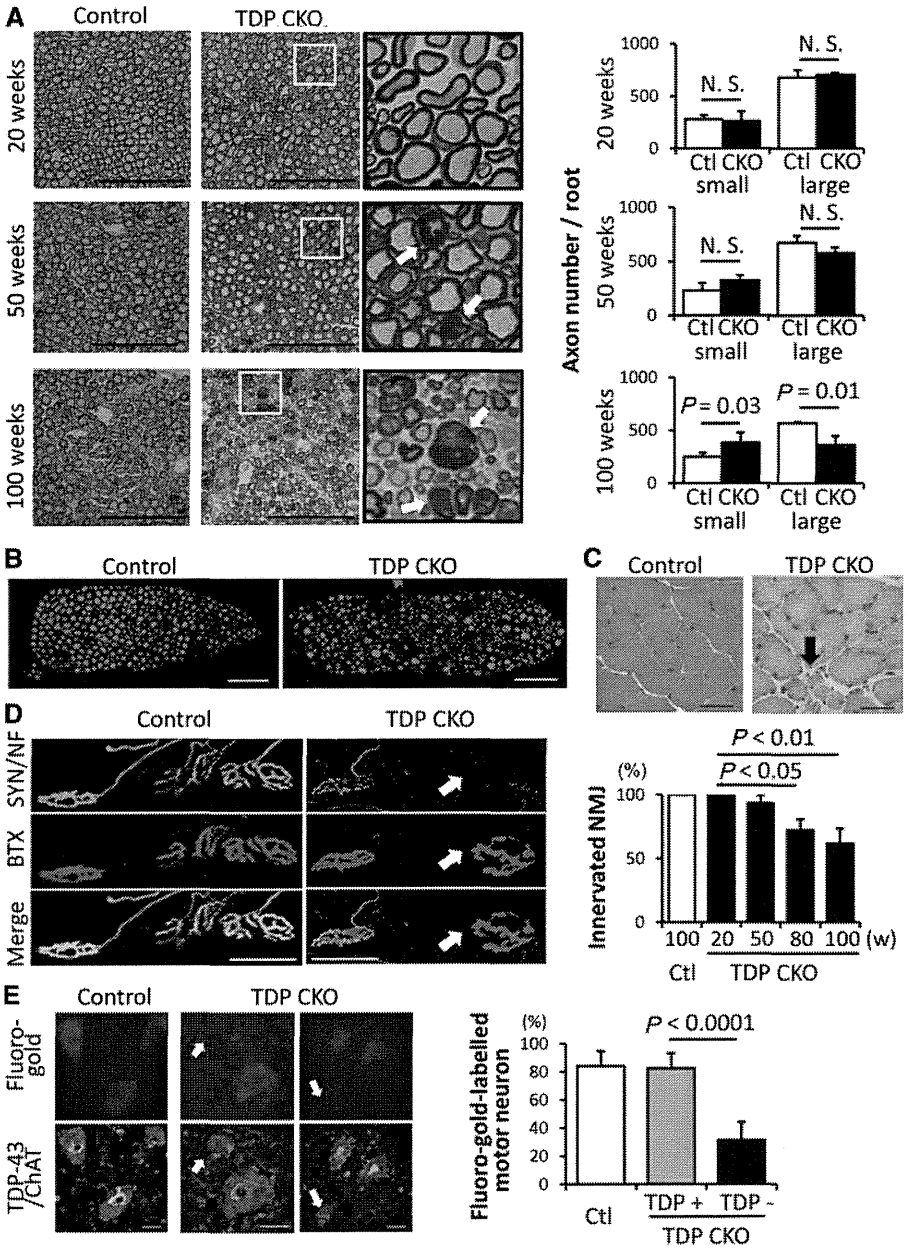

Figure 3 Analysis of motor axons, neuromuscular junctions, and skeletal muscles. (**A**) Toluidine blue staining images and the number of small myelinated fibres (<5 μm) and large myelinated fibres (>5 μm) in the L5 ventral root from 20, 50 and 100-week-old control and TDP CKO mice (n = 6 axons of each). The enlarged image of the yellow-framed area is also shown. Arrows indicate axonal degenerations. Scale bars = 100 μm. Error bars indicate SD. (**B**) Immunofluorescent staining of the L4 ventral root in 100-week-old mice with an anti-ChAT antibody. (**C**) Haematoxylin and eosin staining of gastrocnemius muscles of 100-week-old mice. Axial sections from TDP CKO mice exhibited grouped atrophy (arrow), whereas the control littermates showed no such phenomenon. (**D**) Immunofluorescent staining [synaptophysin (SYN) and phospho-neurofilament (NF), green; bungarotoxin (BTX), red] of neuromuscular junctions (NMJ) in 100-week-old mice and a time course analysis of neuromuscular junctions in TDP CKO mice. Denervated neuromuscular junctions (arrow) are indicated by the lack of synaptophysin and phospho-neurofilament staining. Scale bars = 50 μm. Error bars indicate SD (n = 3). (**E**) FluoroGold labelling (blue) and immunofluorescence staining (TDP-43, green; ChAT, red) of lumbar motor neurons. Retrograde FluoroGold labelling was significantly attenuated in TDP-43-lacking motor neurons but not in TDP-43-positive neurons in 100-week-old TDP CKO mice (arrows). Scale bars = 20 μm. Error bars indicate SD (n = 10).

mutant TDP-43 may cause neurodegeneration through inhibition of normal TDP-43 function (Igaz *et al.*, 2011). On the other hand, TDP-43 knockout mice result in embryonic lethal phenotypes (Kraemer *et al.*, 2010; Sephton *et al.*, 2010; Wu *et al.*, 2010), and systemic postnatal deletion of this molecule led to rapid death (Chiang *et al.*, 2010). Although TDP-43-depleted models of *Drosophila* and zebrafish exhibit neurodevelopmental deficits in motor axons (Feiguin *et al.*, 2009; Kabashi *et al.*, 2011), the

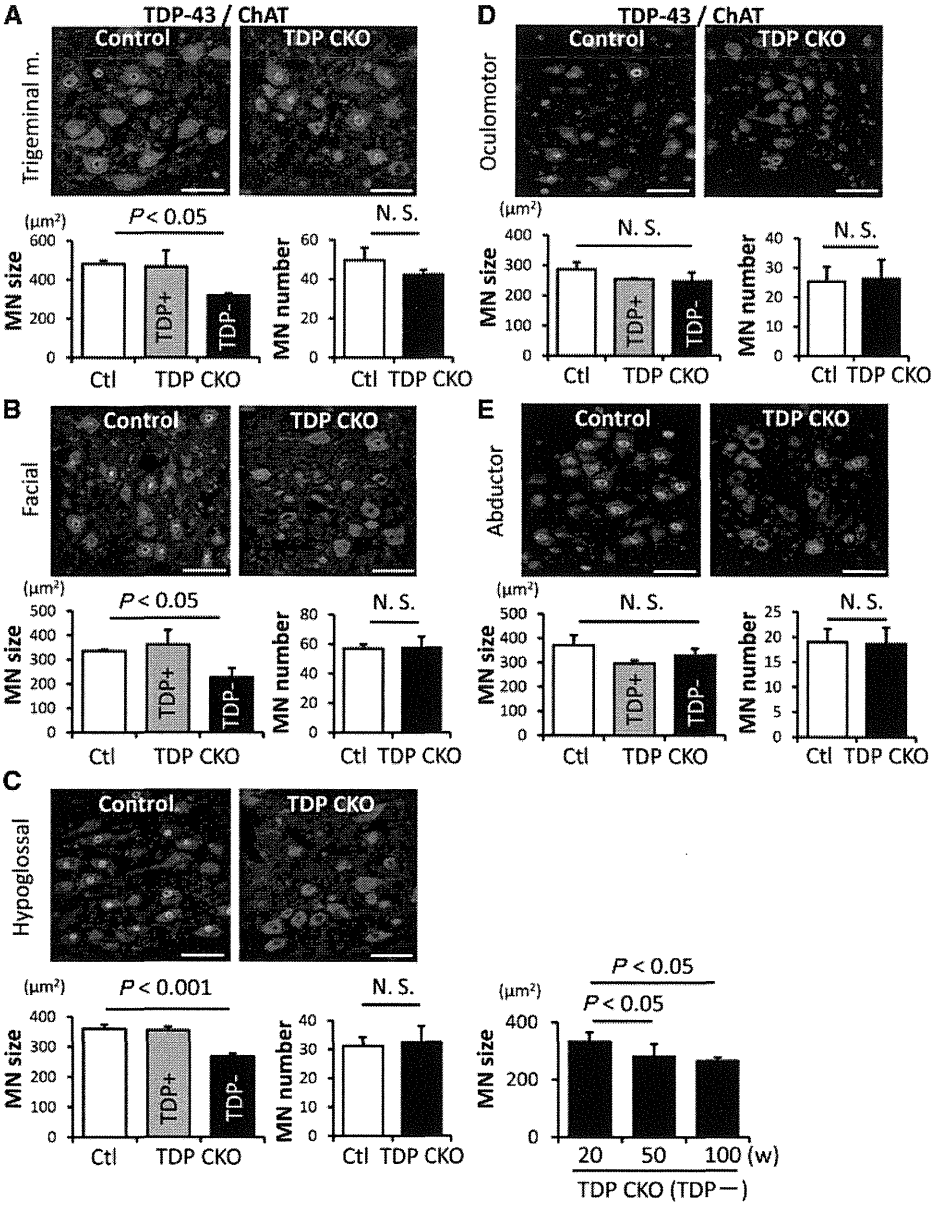

Figure 4 Morphological analysis of cranial motor nuclei. (**A–E**) Immunofluorescent analysis (TDP-43, green; ChAT, red) of motor neurons in the trigeminal motor (m.) (**A**), facial (**B**), hypoglossal (**C**), oculomotor (**D**), and abductor (**E**) nuclei from 100-week-old control ($n = 3$) and TDP CKO mice ($n = 3$). Graphs show the average size and number of motor neurons in each area. TDP+ = TDP-43-positive neuron; TDP− = TDP-43-negative neuron. Error bars indicate SD. Scale bars = 50 μm. MN = motor neuron; N.S. = not significant.

role of TDP-43 in postnatal mammalian neurons has not been fully elucidated. In the present study, we clarified that TDP CKO mice, in which TDP-43 was specifically knocked-out by Cre recombinase in postnatal motor neurons, develops a progressive motor neuronal degeneration as seen in ALS, suggesting that TDP-43 is essential for the long term maintenance of postnatal motor neurons in mice. Although TDP CKO mice developed ALS-like motor impairment, the mortality of the mice was not different from that of control littermates. This might be due to the knockout efficiency of TDP-43, which occurred in ~50% of motor neurons, or due to the life span of mice, which is considerably shorter than the disease duration of patients with ALS. Moreover, there were no significant alterations in body weight, motor function or morphology of motor neurons in our TDP heterozygous CKO (TDP hCKO) mice. Because previous studies demonstrated that the protein expression of TDP-43 was not reduced in various tissues of heterozygous TDP-43 knockout mice (Kraemer *et al.*, 2010; Sephton *et al.*, 2010; Wu *et al.*, 2010), TDP-43 depletion is likely insufficient to affect the motor neurons in our TDP hCKO mice. At the same time, these data suggest that expression of Cre itself did not affect the vulnerability of the mouse motor neurons over 2 years.

An earlier study demonstrates that the motor neuron-specific TDP-43 knockout mouse carrying HB9-Cre exhibits early-onset

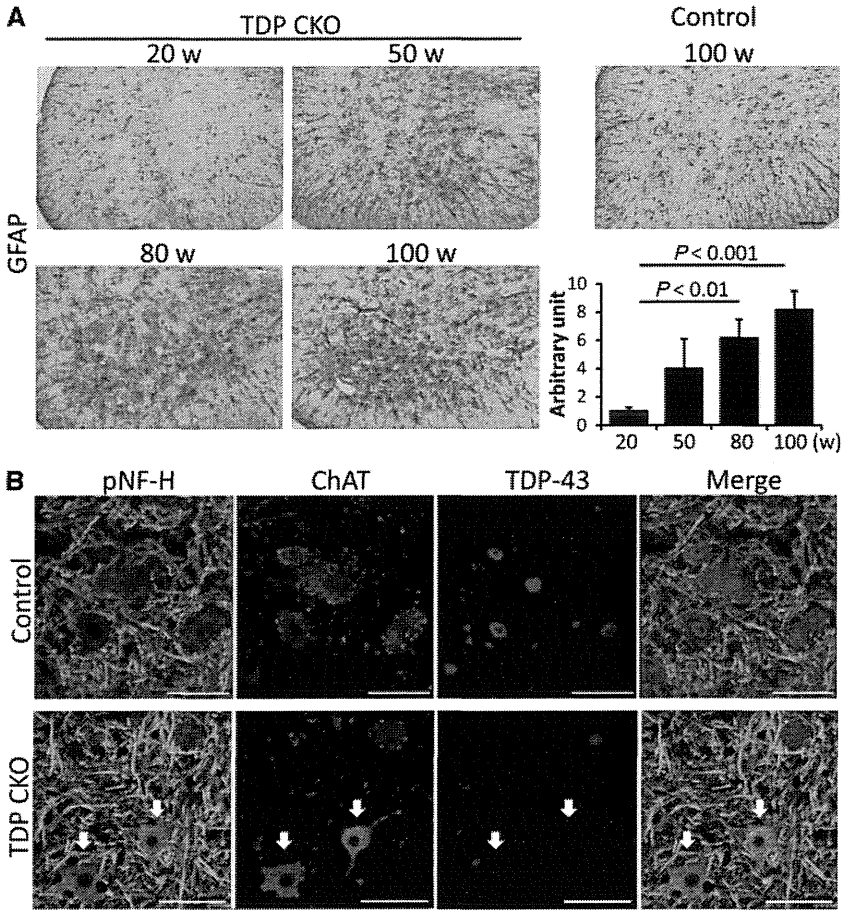

Figure 5 Astrogliosis and neuronal accumulation of phosphorylated neurofilament. (**A**) Immunohistochemistry against GFAP in the ventral horn and a time course analysis of astrogliosis. Error bars indicate SD ($n = 3$ for each age). (**B**) Immunofluorescent staining against pNF-H (green), ChAT (red) and TDP-43 (blue). pNF-H was accumulated in the cell bodies of TDP-43-lacking motor neurons of TDP CKO mice (arrows). Scale bars: **A** = 100 μm; **B** = 50 μm.

motor dysfunction and develops motor neuronal loss earlier than 10 weeks of age (Wu *et al.*, 2012). However, given that the Cre-mediated recombination using the *HB9* promoter began at the developmental stage E9.5 (Arber *et al.*, 1999), this model possibly reflects the loss-of-function of TDP-43 in the motor neuron development. By contrast, because the Cre expression in VAChT-Cre.Fast mice is mediated by the VAChT promoter, the number of Cre-expressing motor neurons in VAChT-Cre.Fast mice is scarcely detected at prenatal stages, but becomes maximum in number at 5 weeks (Misawa *et al.*, 2003). We also confirmed that TDP-43 was not excised in spinal motor neurons of TDP CKO mice at post-natal Day 2, but knocked-out in ~50 % of motor neurons of the 10-week-old mice. This temporal pattern of Cre expression appears to contribute to the late-onset progressive motor dysfunction in our TDP CKO mice and enable the assessment of loss of TDP-43 functions in mouse motor neurons at the postnatal stage. As far as we investigated, TDP-43 was knocked-out in spinal motor neurons beginning at 10 weeks, but the function and morphology of motor neurons were unexpectedly preserved for 1 year in TDP CKO mice, suggesting that the loss of TDP-43 was compensated in motor neurons of young mice, but triggered neuronal vulnerability with the ageing process. Given that ALS is an age-related neurodegenerative disease and that the disease develops after middle age even in inherited cases with TDP-43 mutations (Gitcho *et al.*, 2008; Kabashi *et al.*, 2008; Sreedharan *et al.*, 2008; Yokoseki *et al.*, 2008), our TDP CKO mice appear to be a model that recapitulates the age-dependent phenotypes of ALS. However, as TDP CKO mice lack some aspects of human ALS pathology, such as cytoplasmic inclusions of TDP-43 and the involvement of upper motor neurons, the use of this model for therapeutic research needs further validation.

In the histopathological analyses, TDP CKO mice exhibited the atrophy of motor neurons, degeneration of large motor axons, denervation of neuromuscular junctions and grouped atrophy of skeletal muscles, all of which are common to the pathology of human motor neuron disease. The disruption of retrograde labelling in TDP-43-lacking motor neurons suggests that TDP-43 depletion directly induces neuronal dysfunction. Interestingly, the axonal degenerations were evident in the ventral root of TDP CKO mice at 50 weeks of age, when the morphology of lumbar motor neurons was not altered. These findings are compatible with the fact that ALS pathology initially manifests at the axon

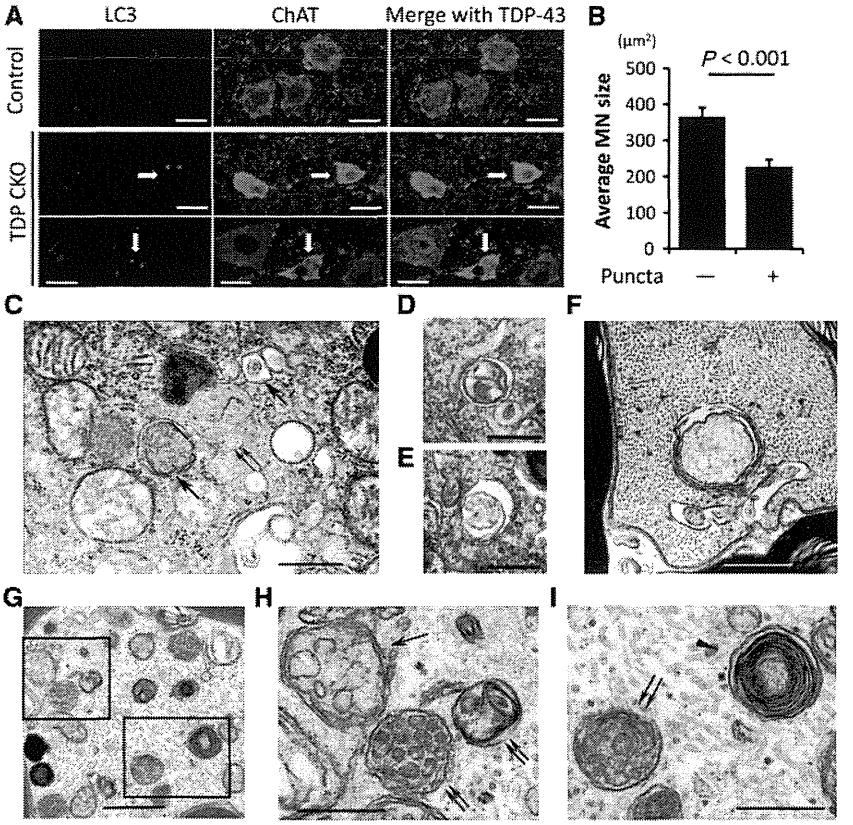

Figure 6 Formation of autophagosomes in motor neurons of TDP CKO mice. (**A**) Immunofluorescent analysis (LC3, green; ChAT, red; TDP-43, blue) revealed LC3-positive cytoplasmic puncta in TDP-43-lacking motor neurons of 100-week-old TDP CKO mice. (**B**) The average size of TDP-43 lacking motor neurons (MN) with ($n = 25$) and without ($n = 48$) LC3-positive puncta. Error bars indicate SEM. (**C–I**) Ultrastructural analysis of 100-week-old TDP CKO mice. Autophagosomes (arrows) and an autolysosome (double arrows) (**C**), autolysosomes surrounded by a single membrane containing mitochondria (**D**) and autophagosomes containing ribosome-like structures (**E**) were observed in the cell bodies of the motor neurons. An autophagic structure in the proximal motor axon (**F**). Accumulation of organelles containing mitochondria, autophagosomes (arrows), autolysosomes (double arrows), and autophagic structure with a multi-lamelleted structure (arrowhead) in the sciatic nerve (**G**) and its enlarged images (**D**, **E**, **H** and **I**). Scale bars: **A** = 20 μm; **C** and **G** = 1 μm; **F**, **H** and **I** = 500 nm.

(Fischer *et al.*, 2004). The increase of small myelinated fibres accompanied by the decrease of large myelinated fibres in the ventral root of 100-week-old TDP CKO mice corresponds to the morphological change in the cell body of the motor neurons, and similar observations were also reported in the patients and mouse models of ALS (Bradley *et al.*, 1983; Zhang *et al.*, 1997). TDP CKO mice also exhibited several features that are shared with patients with sporadic ALS: the involvement in the cranial motor nuclei such as the hypoglossal nucleus, preserved morphology in the extraocular motor neurons, accumulations of phosphorylated neurofilament in motor neurons and astrogliosis in the spinal ventral horn. Dysphagia due to the involvement of the hypoglossal nucleus might enhance the weight loss in aged TDP CKO mice through decreased oral intake. In ALS, extraocular motor neurons are resistant to degeneration compared with other somatomotor neurons, and differences in calcium buffering capacities have been proposed as a possible reason for this selective vulnerability (Alexianu *et al.*, 1994; Reiner *et al.*, 1995; Laslo *et al.*, 2000). Because RNA-seq analysis demonstrates that depletion of TDP-43 affects the calcium signalling pathway in mouse striatum (Polymenidou *et al.*, 2011), it is possible that dysregulation of calcium buffering underlies the pathogenesis of TDP CKO mice.

Our immunofluorescent analysis also demonstrated LC3-positive cytoplasmic puncta in TDP-43-depleted motor neurons, and the presence of these puncta was associated with shrinkage of motor neurons. This finding was confirmed by electron microscopy that revealed the presence of autolysosomes and autophagosomes in the motor neuronal cell bodies and axons of TDP CKO mice, suggesting that TDP-43 depletion resulted in dysregulation of the autophagic pathway. In addition, the accumulation of autophagic structures in the sciatic nerve and the disruption of retrograde labelling in TDP-43-lacking motor neurons suggest that the disruption of retrograde axonal transport may underlie the motor neuronal dysfunction in TDP CKO mice. Although the disruption of constitutive autophagy is shown to instigate the degeneration of certain types of neurons (Komatsu *et al.*, 2006), the causative role of the autophagic dysregulation in the pathogenesis of motor neuron diseases remains controversial. A recent work

demonstrates that motor neuron-specific knockout of the proteasome subunit Rpt3, but not autophagy mediator Atg7, leads to motor neuron degeneration in mice (Tashiro *et al.*, 2012), suggesting that the disruption of autophagic pathway in motor neurons may not be the primary cause of the neurodegeneration. However, accumulation of autophagosomes and autolysosomes was observed in the motor neurons of mice with mutant SOD1 (Li *et al.*, 2008; Tian *et al.*, 2011) and patients with sporadic ALS (Nakano *et al.*, 1993; Sasaki, 2011). In addition, mice carrying mutations of dynein or dynactin exhibit motor dysfunction with accumulation of autophagosomes in the motor neurons (Ravikumar *et al.*, 2005; Laird *et al.*, 2008). These lines of evidence may suggest a possible link between the increased autophagosomes and the process of motor neuron degeneration (Pasquali *et al.*, 2009; Chen *et al.*, 2012), although it remains unclear whether the accumulation of autophagosomes in neurodegenerative diseases results from activation of autophagy, disruption of retrograde transport or decreased lysosome fusion (Shintani and Klionsky, 2004; Baehrecke, 2005; Perlson *et al.*, 2010). Further investigation with regard to the linkage among loss of TDP-43, retrograde axonal transport and dysregulation of autophagy might contribute to our understanding the pathogenesis of ALS.

In conclusion, TDP CKO mice exhibited age-dependent motor impairment and morphological alterations in the motor neuron system that recapitulate several features of sporadic ALS neuropathology, including the accumulation of autophagosomes. These findings suggest that TDP-43 plays an essential role in the long-term maintenance of motor neurons, and that loss of TDP-43 function contributes to the pathogenesis of ALS.

Funding

This work was supported by a Centre of Excellence (COE) grant, a Grant in Aid for Scientific Research on Innovated Areas 'Foundation of Synapse and Neurocircuit Pathology', and Grants in-Aid from Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; grants from the Ministry of Health, Labour and Welfare of Japan; and Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST); Strategic Research Program for Brain Sciences of the MEXT of Japan.

Supplementary material

Supplementary material is available at *Brain* online.

References

Alexianu ME, Ho BK, Mohamed AH, La Bella V, Smith RG, Appel SH. The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis. Ann Neurol 1994; 36: 846–58.

Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 2006; 351: 602–11.

Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 1999; 23: 659–74.

Ayala YM, Misteli T, Baralle FE. TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression. Proc Natl Acad Sci USA 2008; 105: 3785–9.

Ayala YM, Pantano S, D'Ambrogio A, Buratti E, Brindisi A, Marchetti C, et al. Human, *Drosophila*, and *C.elegans* TDP43: nucleic acid binding properties and splicing regulatory function. J Mol Biol 2005; 348: 575–88.

Baehrecke EH. Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol 2005; 6: 505–10.

Bradley WG, Good P, Rasool CG, Adelman LS. Morphometric and biochemical studies of peripheral nerves in amyotrophic lateral sclerosis. Ann Neurol 1983; 14: 267–77.

Buratti E, Brindisi A, Giombi M, Tisminetzky S, Ayala YM, Baralle FE. TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. J Biol Chem 2005; 280: 37572–84.

Buratti E, De Conti L, Stuani C, Romano M, Baralle M, Baralle F. Nuclear factor TDP-43 can affect selected microRNA levels. FEBS J 2010; 277: 2268–81.

Chen S, Zhang X, Song L, Le W. Autophagy dysregulation in amyotrophic lateral sclerosis. Brain Pathol 2012; 22: 110–6.

Chiang PM, Ling J, Jeong YH, Price DL, Aja SM, Wong PC. Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. Proc Natl Acad Sci USA 2010; 107: 16320–4.

Feiguin F, Godena VK, Romano G, D'Ambrogio A, Klima R, Baralle FE. Depletion of TDP-43 affects *Drosophila* motoneurons terminal synapsis and locomotive behavior. FEBS Lett 2009; 583: 1586–92.

Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, et al. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. Exp Neurol 2004; 185: 232–40.

Gitcho MA, Baloh RH, Chakraverty S, Mayo K, Norton JB, Levitch D, et al. TDP-43 A315T mutation in familial motor neuron disease. Ann Neurol 2008; 63: 535–8.

Igaz LM, Kwong LK, Lee EB, Chen-Plotkin A, Swanson E, Unger T, et al. Dysregulation of the ALS-associated gene TDP-43 leads to neuronal death and degeneration in mice. J Clin Invest 2011; 121: 726–38.

Iguchi Y, Katsuno M, Niwa J, Yamada S, Sone J, Waza M, et al. TDP-43 depletion induces neuronal cell damage through dysregulation of Rho family GTPases. J Biol Chem 2009; 284: 22059–66.

Kabashi E, Lin L, Tradewell ML, Dion PA, Bercier V, Bourgouin P, et al. Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits *in vivo*. Hum Mol Genet 2011; 19: 671–83.

Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Vande Velde C, et al. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet 2008; 40: 572–4.

Katsuno M, Adachi H, Kume A, Li M, Nakagomi Y, Niwa H, et al. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Neuron 2002; 35: 843–54.

Katsuno M, Adachi H, Minamiyama M, Waza M, Tokui K, Banno H, et al. Reversible disruption of dynactin 1-mediated retrograde axonal transport in polyglutamine-induced motor neuron degeneration. J Neurosci 2006; 26: 12106–17.

Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 2006; 441: 880–4.

Kraemer BC, Schuck T, Wheeler JM, Robinson LC, Trojanowski JQ, Lee VM, et al. Loss of murine TDP-43 disrupts motor function and plays an essential role in embryogenesis. Acta Neuropathol 2010; 119: 409–19.

Laird FM, Farah MH, Ackerley S, Hoke A, Maragakis N, Rothstein JD, et al. Motor neuron disease occurring in a mutant dynactin mouse