Elevated expression of TDP-43 in the forebrain of mice is sufficient to cause neurological and pathological phenotypes mimicking FTLD-U

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TDP-43 is a multifunctional DNA/RNA-binding factor that has been implicated in the regulation of neuronal plasticity. TDP-43 has also been identified as the major constituent of the neuronal cytoplasmic inclusions (NCIs) that are characteristic of a range of neurodegenerative diseases, including the frontotemporal lobar degeneration with ubiquitin+ inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). We have generated a FTLD-U mouse model (CaMKII-TDP-43 Tg) in which TDP-43 is transgenically overexpressed in the forebrain resulting in phenotypic characteristics mimicking those of FTLD-U. In particular, the transgenic (Tg) mice exhibit impaired learning/memory, progressive motor dysfunction, and hippocampal atrophy. The cognitive and motor impairments are accompanied by reduced levels of the neuronal regulators phospho-extracellular signal-regulated kinase and phosphorylated cAMP response element-binding protein and increased levels of gliosis in the brains of the Tg mice. Moreover, cells with TDP-43*, ubiquitin* NCIs and TDP-43–deleted nuclei appear in the Tg mouse brains in an age-dependent manner. Our data provide direct evidence that increased levels of TDP-43 protein in the forebrain is sufficient to lead to the formation of TDP-43*, ubiquitin* NCIs and neurodegeneration. This FTLD-U mouse model should be valuable for the mechanistic analysis of the role of TDP-43 in the pathogenesis of FTLD-U and for the design of effective therapeutic approaches of the disease.
Despite the rapidly accumulating data on the molecular and cellular properties of TDP-43 in relation to the formation TDP-43\(^+\) NCIs or UBIs (Johnson et al., 2008; Winton et al., 2008; Zhang et al., 2009), a causative role of TDP-43 in the pathogenesis of FTLD-U remains undefined. Biochemical analyses have revealed that TDP-43 is promiscuously modified/processed in the affected regions of the brains and spinal cords of the FTLD-U and ALS patients, respectively. In particular, the following TDP-43–derived polypeptides have been detected on Western blots of the urea-soluble extracts from the pathological samples with TDP-43\(^+\) UBIs: (a) multiple species of high molecular weight, polyubiquitinated TDP-43; (b) phosphorylated TDP-43 migrating at ~45 kD; and (c) ~25- and 35-kD C-terminal fragments of TDP-43 (Arai et al., 2006; Cairns et al., 2007; Kwong et al., 2007; Neumann, 2009; Giordana et al., 2010). In addition, immunohistochemistry analysis of the pathological samples from FTLD-U and ALS patients has revealed the presence of disease cells with NCIs adjacent to TDP-43–depleted nuclei (Arai et al., 2006; Neumann, 2009). The depletion of the nuclear TDP-43 and the formation of the UBIs have been suggested to cause loss-of-function of TDP-43 and cellular toxicity, thus leading to the pathogenesis of FTLD-U and ALS with the TDP-43\(^+\) UBIs (Kwong et al., 2007; Mackenzie and Rademakers, 2008; Wang et al., 2008a).

Using a Thy-1 promoter, Wils et al. (2010) generated transgenic (Tg) mice with overexpression of human TDP-43 in the neurons of the central nervous system, in addition to other cell types (Arai et al., 2006; Neumann et al., 2006), which include the neuronal cytoplasmic inclusions (NCIs) and neuronal intranuclear inclusions (NIIs; for review see Neumann, 2009; Neumann et al., 2007; Wang et al., 2008a). A causative role of TDP-43 in the pathogenesis of ALS with NCIs has been suggested by the association of >30 different point mutations of the TDP genes with this class of ALS patients (for review see Lagier-Tourenne and Cleveland, 2009).

FTLD-U patients of different subtypes are mainly characterized by ubiquitin\(^+\), \(\tau\)- and \(\alpha\)-synuclein\(^-\) NCIs (Mackenzie et al., 2006), and, to a lesser extent, NIIs, both of which are also TDP-43\(^+\) (Neumann, 2007, 2009; Wang et al., 2008a). FTLD-U is the most common neuropathological subtype of FTLD, which refers to a clinically, genetically, and pathologically heterogeneous group of neurodegenerative disorders (Hodges et al., 2004). FTLD is the second most common form of dementia in people under the age of 65, after Alzheimer’s disease, with the prevalence estimated between 3.3 and 25.4 cases per 100,000 people (Ratnavalli et al., 2002). Mutations in the progranulin (PGRN) and VCP genes, respectively, have been identified in a portion of the familial cases of FTLD-U (for review see Cairns et al., 2007; Mackenzie et al., 2006). Clinically, FTLD is characterized by behavioral and/or language dysfunction (Neary et al., 1998). In addition, patients may display movement abnormalities such as Parkinsonism or motor neuron disease (MND; Kertesz et al., 2000; Lomen-Hoerth et al., 2002; Forman et al., 2006). Prominent frontal and temporal lobe atrophy associated with neuronal loss and gliosis are also found in FTLD (Snowden et al., 2007). Currently, there is no drug available for the treatment of FTLD or FTLD-U (Vossel and Miller, 2008).

Figure 1. Generation and characterization of CaMKII-TDP-43 Tg mice. (A) Physical map of the CaMKII-TDP-43 fragment for pronuclei injection. The orientation of transcription is indicated by the arrow. The positions of the short hybrid intron derived from an adenovirus splice donor, an immunoglobulin G splice acceptor, and the SV40 poly(A) addition sequence (pA) are indicated. The approximate locations of the Southern blotting and PCR probes are also indicated. Tg mice were identified by the presence of the 4.4-kb KpnI fragment on the Southern blot and the 523-bp PCR band on gel. The restriction sites on the map are as follows: K, KpnI; E, EcoRV; N, NotI; S, SfiI. (B) Genotyping of the Tg mice. The data from PCR (top) and Southern blotting (bottom) analysis of the tail DNAs are exemplified. NT represents the nontransgenic samples. (C) Western blotting of the protein extracts from the hippocampus, cortex, cerebellum, and spinal cord of the WT, NT, and Tg mice, respectively. (D) In situ hybridization patterns of TDP-43 transcripts in the brains of WT and TDP-43 Tg mice (Tg). Bars, 500 µm. (E) Immunostaining patterns of TDP-43 protein in the brains of WT and Tg mice. CA1, CA1 layer; CA3, CA3 layer; DG, dentate gyrus. Bars, 200 µm. Results in B–E are representative of three independent experiments.
in which the Thy-1 promoter is active, including the thymocytes, myoblasts, epidermal cells, and keratinocytes. Degeneration of the cortical/spinal motor neurons associated with a spastic quadriplegia reminiscent of ALS and degeneration of the nonmotor cortical and subcortical neurons characteristic of FTLD were observed in their Tg mice (Wils et al. 2010). Furthermore, cellular aggregates (NCIs and NIs) containing ubiquitinated and phosphorylated TDP-43, as well as the 25-kD TDP-43 fragment, were detected in association with the disease development and progression of these human TDP-43-overexpressing Tg mice.

In this study, we report the generation of a FTLD-U mouse model with Tg overexpression of TDP-43 in the hippocampus, cortex, and striatum with use of the Ca2+/calmodulin-dependent kinase II (CaMKII) promoter. These Tg mice have developed learning and memory deficits, as well as impairment of their motor functions. The brains of the Tg mice are also characterized with a reduce volume of the hippocampus, giosis, and TDP-43+ ubiquitin+ NCIs. In interesting connection with the finding that TDP-43 expression is up-regulated in some FTLD-U patients (Chen-Plotkin et al., 2008; Mishra et al., 2007; see Discussion), our data suggest that changes of the homocentric concentration of TDP-43, in particular the increase of its protein level, in specific types of cells could be a primary cause leading to the development of FTLD-U, and likely other neurodegenerative diseases with TDP-43+ UBIs as well.

RESULTS

Generation of Tg mice

To test whether elevated expression of TDP-43 in the forebrain could be a cause for the generation of the various disease phenotypes as observed in FTLD-U patients, we have constructed Tg mouse lines carrying full-length mouse TDP-43 cDNA under the transcription control of a 8.5 kb promoter region of the CaMKII gene (Mayford et al., 1996; Fig. 1 A). Genotyping by PCR and Southern blotting was used to identify the transgene-positive mice of the founders and their progenies, as exemplified in Fig. 1 B. DNA samples from both the heterozygotes +/- and homozygotes +/- of the Tg mice gave a 523 bp band in the PCR analysis and a 4.4 kb fragment in the Southern blotting analysis. Neither band was present in the non-Tg (NT) samples (Fig. 1 B). The identities of the homozygotes of the Tg mice were also confirmed by breeding them with the NT mice (unpublished data).

Three independent Tg lines were generated, and they exhibited similarly elevated levels of TDP-43 expression (approximately twofold higher than the NT mice) as directed by the CaMKII promoter in the cortex and hippocampus, which was detected by Western blotting analysis (Fig. 1 C, top left and right). The TDP-43 protein levels in the cerebellum and spinal cord of the Tg mice were similar to those of the WT mice (Fig. 1 C, bottom left and right). Note the higher levels of TDP-43 in the hippocampus and cortex samples from the Tg mice. The similar levels of the TDP-43 protein in the hippocampus or cortices of the Tg +/- and Tg +/+ mice might be caused by a feedback regulatory mechanism on the protein level because the TDP-43 mRNA level of the Tg +/+ mice was approximately twofold of that of the Tg +/- mice (Fig. S1). The details of this observation require further examination. As confirmed by in situ hybridization experiments, the WT mice exhibited basal signals, whereas the TDP-43 Tg mice showed higher signals in both the hippocampus and cortex (Fig. 1 D). As expected, both the endogenous (Fig. 1 E, left) and the overexpressed, exogenous TDP-43 proteins (Fig. 1 E, right) were present in the hippocampus mainly in the neuronal layers, as shown by immunohistochemical staining.

Impaired performances of the TDP-43 Tg mice in the Morris water maze and fear-conditioning tasks

We first used the water maze task to evaluate whether overexpression of TDP-43 in the hippocampus and cortex affected the learning/memory of the mice. As shown, the 2-mo-old Tg +/+ mice exhibited significantly impaired performance in the Morris water maze task (Fig. 2 A). At 2 mo of age, the Tg +/+ mice exhibited significantly impaired performance in the Morris water maze task compared with the WT mice. The time until drop from the rotating rod (20 r.p.m.) are shown for mice at 2, 4, and 6 mo of age. (F) Performance of mice in 1-h novel object recognition tests. The results represent the mean ± SEM of three independent experiments (n = 10 mice/group). *P < 0.05.
test (compare the latencies of the Tg mice to those of the WT mice in Fig. 2 A, and to the NT littermates as shown in Fig. S2). Because the heterozygous+/− and homozygous+/+ Tg mice exhibited similar extents of impairment in their learning/memory capabilities (Fig. S2), we used the homozygous Tg+/+ mice for all subsequent behavior tests and other experimental analyses. As shown in Fig. 2 B, the impairment of the learning/memory capabilities of the Tg+/+ mice were also revealed by the fear-conditioning task. Thus, the data from these two cognition tests together indicated that overexpression of TDP-43 in the hippocampus and cortex of the mice significantly impaired their learning/memory capabilities.

Abnormal limb-clasping and impaired performance of the TDP-43 Tg mice in the rotarod test

We have also analyzed the motor functions of the TDP-43 Tg mice. The Tg mice were born normally and their spontaneous locomotor activities were normal at the age of 2 mo (Fig. 2 C). However, at the age of 6 mo, TDP-43 Tg mice showed limb-clasping reflexes when being suspended by their tails, whereas the control mice extended their limbs (Fig. 2 D). This abnormal reflex was often observed in mouse models of other neurodegenerative diseases, e.g., the Huntington disease (HD; Mangiarini et al., 1996). The mice were further subjected to the rotarod test, and the results indicated that they were severely impaired in motor coordination, balance, and grip strength at the age of 6 mo (WT, 1.80 ± 0.08; Tg, 1.81 ± 0.04; P = 0.88; Fig. 3 B). These data indicated that the receptor gating properties contributing to the EPSC amplitude and that decay kinetics were altered in the TDP-43 Tg mice.

Electrophysiology analysis of TDP-43 Tg mice

In view of the impairment of the learning/memory of the TDP-43 Tg mice, we have performed electrophysiology analysis of their long-term potentiation (LTP) in comparison to the WT mice. LTP between the Schaffer collaterals and principal CA1 pyramidal neurons in the hippocampal slices prepared from the mice were measured. In correlation with the learning/memory test experiments (Fig. 2), tetanic stimulation of the Schaffer collaterals resulted in robust LTP in slices from the WT mice. However, the slices from the TDP-43 Tg mice had impaired LTP maintenance for 60 min after the LTP induction (Fig. 3 A).

We also recorded the miniature excitatory postsynaptic current (mEPSC) from DIV 12–15 cultured hippocampal neurons (Fig. 3 B). No significant difference in the mEPSC frequencies could be detected (WT, 3.16 ± 0.66; Tg, 4.59 ± 0.83; P = 0.20; Fig. 3 B). However, significant difference existed between the mEPSC amplitudes of the WT and Tg mice (WT, 47.30 ± 2.56; Tg, 39.52 ± 2.96; *, P = 0.03; Fig. 3 B). Significant difference in the decay time constant (WT, 3.18 ± 0.30; Tg, 4.99 ± 0.25; ***, P = 0.0006; Fig. 3 B) was also detected but not the rise time constant (WT, 1.80 ± 0.08; Tg, 1.81 ± 0.04; P = 0.88; Fig. 3 B). These data indicated that the receptor gating properties contributing to the EPSC amplitude and that decay kinetics were altered in the TDP-43 Tg mice.
the central nervous system and it is elevated in microglia during gliosis (Moisse et al., 2009a,b). Interestingly, in recent models of nerve injury (Moisse et al., 2009a,b), it was found that axotomy caused both increase of TDP-43 and decrease of PGRN in the neurons, whereas the level of PGRN in the surrounding activated microglia was increased. Thus, likely there could also be a decrease of PGRN in the TDP-43–overexpressing neurons of the Tg mouse forebrains, which would compensate for the increase of the PGRN amount in the activated microglia. Future experimentation should clarify this point.

Interestingly, the protein levels of the glutamic acid de-carboxylase 67 (GAD67) and glial fibrillary acidic protein (GFAP) were also increased, by approximately twofold, in the cortex and hippocampus of the TDP-43 Tg mice. Of the two, GAD67 was known as the principal enzyme for synthesis of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the brain. Its elevated expression in TDP-43 Tg mice was further confirmed by double immunostaining of GAD67 and TDP-43 in the primary neuron cultures from both the Tg mice and the controls (Fig. 4 B). As seen, higher immunostaining signals of GAD67 were present in both the soma and the processes of the neuronal cells of the Tg mice than those of the WT controls (Fig. 4 B, compare the images in the right column to those in the left column), whereas the levels of the neuron-specific nuclear protein (NeuN) were similar between the Tg and control mice (unpublished data).

In view of the impaired performances of the TDP-43 Tg mice in the learning/memory tests and their lowered LTP (Figs. 2 and 3), we have checked the levels in the hippocampus and cortex of several major candidate proteins known to be involved in different signal transduction pathways regulating learning/memory (Kandel, 2001). Although the protein amounts of CaMKII, protein kinase A, growth-associated protein 43 (GAP43), synaptophysin (SYP), and postsynaptic density 95 (PSD95), respectively, were similar between TDP-43 Tg mice and the WT controls, the levels of both pERK and its downstream target pCREB (Carlezon et al., 2005) in the TDP-43 Tg mice were decreased, as shown by the Western blots and histogram in Fig. 4 A.

It should be noted that the level of PGRN in the forebrains of the Tg mice was similar to that of the WT mice (Fig. 4 A). It is not clear at the moment why the level of PGRN was not increased in the FTLD-U mouse brains in view of the elevated level of brain GFAP/gliosis in these mice. PGRN is expressed in neurons and microglia within
forebrains of the TDP-43 Tg mice (Fig. 4 B, bottom right). The results of Fig. 4 suggested that overexpression of TDP-43 impaired the learning/memory of the TDP-43 Tg mice, in part by disrupting the phosphorylation of ERK, but also by up-regulating the inhibitory neurotransmitter GABA.

Neuropathology of the TDP-43 Tg mouse brains
In addition to the behavioral, electrophysiological, and gene expression abnormalities described in Figs. 2–4, the TDP-43 Tg mice also exhibited several neuropathological characteristics similar to those of the FTLD-U patient brains. First, reactive gliosis manifested by increased expression of the astrocytic marker GFAP has been known as a prominent pathological feature of FTLD (Martin et al., 2001; Tan et al., 2005). As shown by Western blotting, the level of the GFAP protein was increased in the TDP-43 Tg mice (Fig. 4 A, bottom). Consistent with this, pronounced increases of the signals of anti-GFAP immunostaining were observed in both the hippocampus (HP) and the cortex (CX) of the Tg+/+ mice as compared with the WT controls (Fig. 5 A).

Second, Western blotting analysis revealed the presence of the high molecular weight TDP-43 species, presumably the polyubiquitinated TDP-43, as well as the enrichment of the 25- and 35-kD fragments in the urea-soluble fraction of brain extracts from the 6-mo-old Tg mice (Fig. 5 B). This pattern on the Western blot was much less prominent in samples prepared from the 2-mo-old Tg mice, and it was not observed in those prepared from the WT mice (Fig. 5 B). Note that the high molecular weight TDP-43 species (***), and the 25-kD (*) and 35-kD (**) fragments of TDP-43 are prominent in the 6-mo-old Tg+/+ mice, but not in the 2-mo-old mice. The 65-kD band (open triangle) has also been observed by others on immunoblots of extracts from cell lines and from patient lymphocyte lysates (Winton et al., 2008; Corrado et al., 2009), but its significance is not yet clear.

We also performed immunofluorescence staining to examine the subcellular distribution of TDP-43 in the Tg+/+ mouse brains in comparison to the WT mice. As shown in Fig. 6 A, TDP-43 was mainly detected in the nuclei, which were positively stained with both anti-NeuN and DAPI, of unaffected neurons of the TDP-43 Tg mouse brains. Notably, however, TDP-43 was absent from the nuclei of neurons with TDP-43+ NCIs (Fig. 6 A, arrows in two leftmost bottom images). The TDP-43+ NCIs were not observed in the brains of the WT mice, as exemplified in Fig. 6 A (right, WT images). Overall, ∼15–20% of the neurons in the cortexes of the Tg+/+ mice contained the TDP-43+ NCIs. Finally, antiantibiquitin immunostaining showed that the TDP-43+ NCIs in the 6-mo-old Tg mouse brains were also positive for the presence of ubiquitin (Fig. 6 B). Overall, the patterns from the proteinopathy analysis of the TDP-43 Tg mice, as displayed in Figs. 5 B and 6, were strikingly similar to those reported for the pathological FTLD-U brain samples with TDP*-UBIs (for review see Forman et al., 2007).

Brain atrophy of the Tg mice
To examine whether brain atrophy developed in the Tg mice as in the FTLD-U patients, we first used MRI to measure the volume of the mouse hippocampus. The result showed that the volumes of the hippocampi of 6-mo-old Tg mice were 17% less than those of the control mice (n = 5 for each group; P < 0.05). This range of the shrinking was similar to that of the frontal and temporal atrophy observed in some of the FTLD patients (Barnes et al., 2006; Snowden et al., 2007). In parallel with the MRI study, we also examined the brain weights and the cortex neuronal numbers of the mice. The results showed that there was neuronal loss in the 6-mo-old Tg mice. On average, the brain weight of the Tg mice was 12% (n = 5; P < 0.05) less than the WT mice (Fig. 7 A), and the number of the cortical neurons was reduced by 24% (Fig. 7 B). The neuronal loss might result in part from apoptosis of the neurons in the Tg mouse brains. Indeed, apoptotic nuclei were detected, by TUNEL staining, in the brains of the 6-mo-old Tg+/+ mice (Fig. 7 C), and this was accompanied with increases of the amounts of both the total and active caspase-3 (Fig. 7 D).
impairment of learning/memory capabilities, the progressive loss of the motor neuron function, abnormal LTP from the electrophysiological measurement, the increase of gliosis, the alternation of the expression levels of proteins known to be involved in learning/memory, the reduction of the hippocampus volume, and, notably, the TDP-43+-UBI–associated proteinopathological features (Table I).

These changes in the CaMKII-TDP-43 Tg mice are strikingly similar to the neurological and pathological features of the FTLD-U patients (Kwong et al., 2007; Vossel and Miller, 2008).

The Tg mice have displayed a pattern of age-dependent loss of the motor function. In respect to this, the FTLD and the MND appear to overlap at several levels, and the FTLD patients share clinical features of MND. It should be noted here that only a portion of the FTLD patients would develop MND (Elman et al., 2008). Development of the motor behavioral deficits in the TDP-43 Tg mice at 6 mo of age (Fig. 2, D and E) parallels the progressive loss of the motor function in the FTLD patients. As deduced from studies of different MNDs, including the ALS (Kwong et al., 2007; Elman et al., 2008), MND could be clinically manifested by signs and symptoms caused by degeneration of the upper motor neurons in the motor cortex, lower motor neurons in the brainstem and spinal cord, or both. However, the size and number of the motor neurons in the spinal cords of the Tg mice are not significantly different from those of the WT (Fig. S4). This is not surprising in view of the forebrain neuron specificity of the CaMKII promoter used by us. Thus, the motor dysfunction of the CaMKII-TDP-43 Tg mice most likely have resulted from damage in the neuronal circuit in the forebrain, which include the not so well-defined rodent motor cortex (O’Leary et al., 2007), and the corticospinal tract, caused by the CaMKII promoter-directed TDP-43 overexpression. It is also possible, but relatively unlikely because of

Life span of the Tg mice

Finally, we measured the survival rates of the mice. The data, as shown in Fig. S3, indicated that the Tg+/− mice had shorter lifespan, with a mean survival of 495 d, than the WT ones, which had an average survival of 632 d.

DISCUSSION

In this study, we have generated Tg mice with overexpression of mouse TDP-43 in the central nervous systems, including the hippocampus and cortex, under the control of the CaMKII promoter. This promoter has been used before to overexpress other proteins in mice and thus establish different mouse models. However, each of these Tg mouse models exhibits unique phenotypes. For example, overexpression of either CREB (Kida et al., 2002) or NR2b (Tang et al., 1999) enhances the learning/memory capabilities of the mice, which is consistent with the known roles of these factors in learning/memory. On the other hand, overexpression of the methyl-CpG–binding protein 2 (MeCP2) leads to a motor dysfunction phenotype, but it has no effect on the cognitive function of the mice (Alvarez-Saavedra et al., 2007). Note-worthily, CaMKII promoter-directed overexpression does not necessarily affect the mouse behaviors, as exemplified by the Tg mice study of the sulfonylurea receptor, or SUR (Hernandez-Sánchez et al., 2001). In the case of TDP-43, its overexpression has resulted in several molecular, cellular, and phenotypic changes in the mice. These changes include the...
clarify and differentiate among the aforementioned possibilities. Future detailed analysis of the CaMKII-TDP-43 Tg mice could originated from some defects in the muscle of the Tg mice. the CaMKII promoter specificity, that the motor dysfunction = 5 mice/group). Bars, 20 µm.

Figure 7. Neuronal loss and apoptosis in the brains of the Tg mice. (A) Reduction of the brain mass of the 6-mo-old Tg mice. Whole brains were dissected and weighed. n = 5; P < 0.05. (B) Loss of neurons in the cortices of the Tg mice. Coronal brain sections from 6-mo-old Tg and WT mice were immunostained with anti-NeuN. The average number of the neurons in the cortices of the Tg mice was compared with that of the WT mice (right; n = 5 for each group and P < 0.05). Bars, 100 µm. (C) TUNEL assay of the brains of 6-mo-old Tg mice. Apoptotic nuclei are shown in green (arrows) and DAPI staining in blue. (D) Western blots of total brain extracts (cortexes + hippocampi) of 6-mo-old WT and Tg mice showing levels of total and active caspase-3 (n = 5 mice/group). Bars, 20 µm.

the CaMKII promoter specificity, that the motor dysfunction originated from some defects in the muscle of the Tg mice. Future detailed analysis of the CaMKII-TDP-43 Tg mice could clarify and differentiate among the aforementioned possibilities.

The CaMKII-TDP-43 Tg mice also exhibit cognitive impairments as reflected by the Morris water maze test (Fig. 2 A), fear conditioning task (Fig. 2 B), novel object recognition test (Fig. 2 F), and LTP electrophysiological recording (Fig. 3). Related to this, the learning/memory deficiency in the FTLD patients is the major factor leading to the dysfunctions of their social behavior and language (Lillo and Hodges, 2009). As a preliminary investigation of the molecular and cellular basis of the effect of the overexpressed TDP-43 on the cognitive functions, we have examined the expression levels of several molecular markers of neuronal plasticity. The results (Fig. 4) indicate that overexpression of TDP-43 has led to the decreases of both p-ERK and p-CREB (Fig. 4), and likely downstream targets such as BDNF (Kandel, 2001). Furthermore, the enhanced gliosis, as observed in the Tg mice (Fig. 5 A), is also known to impair learning/memory (Ridet et al., 1997). Because overexpression of TDP-43 affects a range of biological activities in cell cultures, e.g., transcription (Ou et al., 1995; Wang et al., 2002), alternative splicing (Buratti and Baralle, 2001; Bose et al., 2008), cell cycle progression (Ayala et al., 2008), etc., it is expected that the phenotypes of the Tg mice result from the alternation/ modifications of multiple biological processes as mis-regulated by the overexpressed TDP-43 in the cortex and hippocampus. It should be noted here that one of our previous studies has shown that TDP-43 is distributed in the dendrites of cultured hippocampal neurons as granules/RNA granules (Wang et al., 2008b). However, we have detected no apparent difference in either the distribution pattern or the number of the TDP-43 granules between cultured hippocampal neurons prepared from Tg and WT mice (unpublished data).

In addition to the behavioral phenotypes, the TDP-43 Tg mice also exhibited patterns of neuropathology similar to those of FTLD-U (Arai et al., 2006; Kwong et al., 2007; Neumann, 2009) except for the apparent absence of the phosphorylated 45KD TDP-43 (Fig. 5). Specifically, neurons with TDP-43 depleted nuclei and cytoplasmic TDP-43+, ubiquitin+ inclusions (UBIs) are present in brain sections of the 6-mo-old Tg mice, although they could not be detected in the 2-mo-old Tg mice (Fig. 6 A and B, and Table I). This age-dependent pattern of immunohistochemistry analysis is in good correlation with the immunoblotting data showing the enrichment of the high molecular weight TDP-43 species, presumably the polyubiquitinated TDP-43, and the 25- and 35-kD TDP-43 fragments in the urea-soluble fractions of the brain extracts from 6-mo-old Tg mice (Fig. 5 B and Table I). Thus, it seems that the appearance of the insoluble TDP-43+ UBIs is a priori for the loss of the motor functions, but not the cognitive functions, in the mouse model. On the other hand, the appearance of the NCIs could just be a secondary, age-dependent phenomenon and that some other still unidentified process is responsible for the motor dysfunction of the 6-mo-old Tg mice. The basis for this differential correlation and whether it exists during the development of the human FTLD-U cases require further investigation.

Overall, we have demonstrated that Tg overexpression of TDP-43 in the forebrains of mice leads to development of molecular, cellular, behavioral, and proteinopathological characteristics similar to those identified in FTLD-U. Significantly, there are FTLD-MND patients whose brain TDP-43 mRNA levels are higher than the normal controls (Mishra et al., 2007). A global gene expression study has also found higher level of TDP-43 mRNA in several cases of PGRN mutation–positive FTLD-U (Chen-Plotkin et al., 2008). Notably, immunoblotting analysis of lysates from some ALS pathological samples and myopathic muscles with TDP-43+ inclusions has also revealed higher TDP-43 protein levels than the normal controls (Kabashi et al., 2008; Weihl et al., 2008). Thus, our data on the CaMKII-TDP-43 Tg mice suggest that the elevation of the level of TDP-43 protein could be one of the primary causes leading to the pathogenesis of neurodegenerative diseases with TDP-43+ inclusions. After this scenario, different animal models could be generated by
CaMKII promoter in our study has allowed the detection and follow up of pathogenesis development of the cognitive behaviors and motor function of the mice from their youth to over 2 yr of age. In addition, several hallmarks of FTLD-U, including the cognitive dysfunction, the hippocampal atrophy, and the progressive appearances of the 35- and 25-kD fragments and the high molecular weight species of TDP-43 in the urea-soluble fraction of the disease forebrains (Table I) could be observed in these mice. Thus, although the mice generated by Wils et al. (2010) are more suitable for studies of neurodegeneration reminiscent of ALS, our CaMKII-TDP-43 Tg mice are ideal for future detailed pathological/clinical analysis and drug/therapeutic development for FTLD-U.

MATERIALS AND METHODS

Construction and generation of CaMKII-TDP-43 Tg mice. To generate the Tg mice of FVB/N background, a 1,245-bp full-length mouse TDP-43 cDNA (available from GenBank/EMBL/DDBJ under accession no. NM_145556) was cloned into the EcoRV site of pNN265, a modified form of pcDNAI/Amp provided by E. Kandel (Columbia University, New York, NY; Mayford et al., 1996). A 2.7-kb NotI fragment was isolated from pNN265 and cloned into the NotI site of the vector pMM403 containing 8.5 kb of the mouse CaMKII promoter region (also provided by E. Kandel; Mayford et al., 1996), resulting in pCaMKII-TDP-43.
was then purified from pCaMKII-TDP-43 and injected into the one-cell embryos of FVB/N mice. The injected embryos were then transferred into the oviducts of surrogate mothers. 78 offspring from the surrogate mothers carrying the CamKII-TDP-43 transgene-injected embryos were genotyped, and 10 of them were identified as the founders. These FVB/N founders were bred with WT FVB/N mice, and three of them were germline transmitted. Each of the three germ lines transmitted, CamKII-TDP-43 Tg lines were set to littermate intercrosses within the line so to obtain three homozygous TDP-43 Tg mouse lines. The homozygosity was determined by Southern blotting, and the overexpression of TDP-43 was confirmed by quantitative RT-PCR and Western blotting analyses of the forebrain tissues from 2-mo-old mice. All the mice were bred at the Animal Facility of the Institute of Molecular Biology (IMB), Academia Sinica, Taiwan. They were housed in a room maintained on a 12 h/12 h light/dark cycle (light on at 7:00 a.m.) with continuous supply of food and water. Experimental procedures for handling the mice were approved by the Institutional Animal Care and Utilization Committee, Academia Sinica.

For genotyping of the founders, both Southern blot analysis and PCR were performed according to the standard procedures. For Southern blot analysis, the genomic tail DNAs were digested with KpnI and hybridized with a 543 bp NotI fragment from pGEMT-TDP-43 (Promega). The genomic DNAs of the Tg CaMKII-TDP-43 mice would give rise to a 4.4-kb fragment on the blot. For PCR, the following primers were used: forward primer 5′-GGCTTGGAGATCTGGCCATTACACT-3′ and reverse primer 5′-TAAATCTTCTGGACCTGAACATA-3′. A 523-bp band on gel was expected for the Tg mouse, but not the WT or NT mouse. The breeding test was used to confirm the homozygosity of the Tg+/- mice.

In situ hybridization. In situ hybridization was performed as described previously (Tsai et al., 2002), with minor modifications. 20-µm coronal sections were taken from the mouse brains serially, covering both the hippocampus and cortex of the cerebrum. The antisense probe complementary to the sequence of the TDP-43 mRNA (5′-GGCTTGGAGATCTGGCCATTACACT-3′) and the corresponding sense probe were 3′-end-labeled with α-[35S]dATP and hybridized, respectively, at 42°C for 24 h with Poly-Prep slides (Sigma-Aldrich) containing the brain sections. After extensive washing, the slides were dehydrated with ethanol and exposed to BioMax films (Kodak) for 10 d. The signals from the in situ hybridization were quantified by measuring the optical densities of the relevant fields with the use of the ImageJ program (National Institutes of Health).

Morris water maze task. For spatial learning test, the Morris water maze task was performed as described previously (Tsai et al., 2007). The animals were subjected to four trials per session and two sessions a day, with one session per day. Each of the three germlines transmitted, CamKII-TDP-43 Tg mice of the age 2 mo by homogenization of the tissues in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Igepal CA-630, 2 mM EDTA, pH 8.0, 1 mM Na3VO4, 20 µg/ml pepstatin A, 20 µg/ml leupeptin, 20 µg/ml aprotonin, 1 mM PMSF, 50 mM NaF). The extracts were then analyzed by 8–12% SDS-PAGE, followed by blot hybridization with one or more of the following antibodies: a homomde anti-TDP-43 (Wang et al., 2008a), antitubulin (Millipore), anti-CamKII (Millipore), anti-ERK (Millipore), anti-pERK (Millipore), anti-pCREB (Millipore), anti-GAD67 (Millipore), anti-GAP43 (Millipore), anti-GFAP (Millipore), anti-protein kinase A (Millipore), and anti-PRGN (R&D Systems), respectively. The relative intensities of the bands were normalized against that of the tubulin and expressed as means ± SEM.

For the sequential biochemical fractionation analysis, the forebrain tissue was dissected, weighed, and sequentially extracted with buffers of increasing strength as previously described (Neumann et al., 2006). In brief, the forebrains were extracted sequentially at 5 ml/g (vol/wt) with low-salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 1% sucrose, and a cocktail of protease inhibitors), high-salt Triton X-100 (TX) buffer (LS + 1% Triton X-100 + 0.5 M NaCl), myelin flotation buffer (TX buffer containing 30% sucrose) and, Sarkosyl (SARK) buffer (LS + 1% N-Lauroylsarcosine + 0.5 M NaCl). The SARK-insoluble materials were further extracted in 0.25 ml/g urea buffer (7 M urea, 2 M thiourea, 4% 3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate, 30 mM Tris, pH 8.5). The proteins in the urea-soluble samples were resolved by Tris-glycine/12% SDS-PAGE, transferred to nitrocellulose, and then probed with an anti-TDP antibody (Protein Tech Group) that was used in similar experiments (Winton et al., 2008).

Immunostaining. Immunostaining was used to examine the expression patterns of TDP-43 and GADD67 in primary hippocampal neuron cultures of the WT and Tg mice. Cells were dissected from embryonic day 16.5 embryos for culturing in Neurobasal medium, and the cultured cells (DIV 14) were fixed with 4% paraformaldehyde (PFA). For staining, the cell was incubated overnight with the individual primary antibodies against GADD67 (1:500) and TDP-43 (1:100), respectively, in 1% donkey serum (D9663, Sigma) in PBS (phosphate buffered saline). For immunofluorescence staining of the mouse brains, adult mice were anesthetized and perfused transcardially in PBS with 4% PFA. The brain was removed and then immersed in 4% PFA solution with 20% sucrose overnight. 12-µm-thick sections were incubated with one or more of the following antibodies: anti-TDP-43 antibody (one was previously generated in our laboratory [Wang et al., 2008b]) and another was obtained from the Protein Tech Group), mouse monoclonal anti-GFAP (Millipore), mouse monoclonal anti-ubiquitin (Millipore), mouse monoclonal anti-NeuN (Millipore), and the Alexa Fluor 488-conjugated goat anti-mouse antibodies (Invitrogen). The sections were then incubated with DAPI and coverslipped with the mounting medium (fluorescent mounting medium; Dako). All sections were examined in a laser scanning confocal microscope (LSCM 510; Carl Zeiss, Inc.).
Electrophysiological recordings. The brains of the WT and TDP-43 Tg mice of the age 2 mo were quickly removed and placed in cold cutting buffer. The hippocampus was sliced into 400-µm sections, submerged in artificial CSF (aCSF) buffer, and maintained for 1.5 h before recording. A bipolar tungsten-stimulating electrode was placed in the middle of the stratum radiatum layer of CA1 area, and the extracellular field potentials were recorded by a glass microelectrode (3 MΩ; filled with aCSF). The pulse duration was 100 µs, and the test responses were elicited at 0.05 Hz (GS-3200; Gould). LTP was induced by two trains of 100 Hz stimulation each lasting for 1 s with a 20-s interval between them. The stimulation strength was set to provide field EPSPs (fEPSPs) with an amplitude that was 40–60% of the maximum. When the paired-pulse facilitation (PPF) was examined in the CA1 area, the stimulation was delivered at 0.01 Hz at interstimulus intervals of 20, 50, 80, 100, 200, 300, 400, and 500 ms, with the stainless-steel bipolar electrodes placed in the outer and inner molecular layers, respectively, of the piriform cortex. fEPSPs from the corresponding layers were recorded via the glass pipettes, and they were amplified and filtered at 1 kHz. The PPF ratio was calculated by dividing the amplitude of the second fEPSP by that of the first fEPSP.

Whole-cell voltage clamp recordings were performed on the hippocampal neurons cultured for 12–15 d using an Axopatch 200B amplifier (Molecular Devices). For the miniature post-synaptic current (mEPSC) experiments, 1 µM tetrodotoxin was added to the bath to suppress the action potentials. Only cells that had a resting membrane potentials less than ~50 mV, stable capacitance, and resistance throughout the experiment were considered. The data recorded were digitized with Digidata 1322A (Molecular Devices) and analyzed with Clampfit 9.2 (Molecular Devices).

GABA. To measure the brain GABA levels, the mouse brains were quickly dissected on a chilled dissection board, homogenized on ice (50 mg of tissue with 1 ml of 400 mM HClO4 and 50 µM EDTA), and neutralized with 100 mM borate buffer (1:10). The homogenates were then centrifuged (14,000 rpm, 15 min, 4°C) and filtered with Ultrafree-MC centrifugal filter units (Millipore, 14,000 rpm, 1 mm, 4°C). The concentrations of GABA were then determined by HPLC.

MRI measurement. MRI was acquired in a 7.0 Tesla MRI system (Bruker). High resolution T2-weighted images were acquired for the whole brain region of each mouse using a 3D-RARE (Rapid Acquisition with Relaxation Enhancement) sequence with a field of view of 200 × 150 × 100 mm³ and a matrix size of 200 × 150 × 65 mm³, yielding a voxel size of 100 × 100 × 154 µm³. The repetition time (TR) and the echo time (TE) were 2,500 and 32 ms, respectively. The region of the hippocampus was selected manually from slice to slice, and the volume was then calculated by a homemade code using the MATLAB.

Neuronal counts. To quantitate and compare the numbers of the cortical neurons among the WT and Tg mice, comparable cortical brain sections derived from the septo-striatal, septo-diencephalic, or the caudal diencephalic regions of the cerebral cortices were immunostained with antibody against the neuronal marker NeuN. The numbers of neurons in a total of six comparable areas (2–3 adjacent fields for each area) were counted. The neuronal counts for the Tg mice were then normalized to the WT mice (100%).

TUNEL assay. For TUNEL assay, trypsinized brain sections were reacted on 0.01% polylysine-coated slides, fixed with 4% formaldehyde solution, and fluorescence stained following the protocols of the DeadEnd fluorometric TUNEL system (Promega). The stained samples were analyzed under a fluorescence microscope, and the signals counted in randomly selected views.

Survival analysis. The WT and the TDP-43 Tg+/+ mice born between March 2006 and December 2008 were used to compare the lifespan/survival rates. Five of the animals per cage were maintained in a pathogen-free environment at the Animal Facility of the Institute of Molecular Biology, Academia Sinica, Taiwan. The dates of birth and death of each mouse were recorded. The survival curves were drawn by the Kaplan and Meier method, and compared by the Log-rank test.

Statistical analysis. All data are reported as the mean ± SEM. Independent experiments were compared by the Student’s t test. Differences, indicated by the asterisks, were considered statistically significant at P < 0.05.

Online supplemental material. Fig. S1 shows the levels of TDP-43 in the TDP-43 Tg+/+ and Tg−/− mice in comparison to the WT. Fig. S2 shows the water maze tests of CaMKII-TDP-43 Tg+/+ mice in comparison to the WT, NT, and TDP-43 Tg−/− mice. Fig. S3 shows the survival curves of the TDP-43 Tg−/− and WT mice. Fig. S4 shows the histological analysis of the motor neurons in TDP-43 Tg+/+ mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092164/DC1.

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REFERENCES


SUPPLEMENTAL MATERIAL

Tsai et al., http://www.jem.org/cgi/content/full/jem.20092164/DC1

Figure S1. The levels of TDP–43 in the TDP–43 Tg^{+/+} and Tg^{+/−} mice in comparison to the WT. (top) Representative gel pattern of RT-PCR analysis of the brain RNAs. (bottom) Levels of the brain TDP–43 mRNAs of Tg^{+/+} and Tg^{+/−} mice relative to the WT mice. The results are the mean ± SEM of three independent experiments, and n = 5 for each of the three groups.

Figure S2. Water maze tests of CaMKII–TDP–43 Tg^{+/−} mice in comparison to the WT, NT, and TDP–43 Tg^{+/+} mice. Water maze tests of 2-month-old mice were carried out. The learning/memory capabilities are expressed as the latencies exhibited in six consecutive sessions. There was no significant difference of the littermates between Tg^{+/−} and Tg^{+/+} mice. Results represent the mean ± SEM of three independent experiments, and n = 20 for each of the four groups.
Figure S3. Survival curves of the TDP-43 Tg^{144} and WT mice. The survivals of the mice were followed up as described in the Materials and methods. The data were treated by the log-rank analysis ($X^2 = 9.8; P < 0.01$). Note the significant drop in the average survival of the Tg^{144} mice (495 d) when compared to the WT mice (632 d). $n = 60$ for each of the two groups.

Figure S4. Histological analysis of the motor neurons in TDP–43 Tg^{144} mice. (A) H&E staining was used to analyze the coronal sections of the lumbar spinal cords of 6-mo-old WT and TDP–43 Tg^{144} mice. Bars, 100 μm. The top images show the representative spinal cords at single anatomical level, each one for the WT and Tg^{144} mice. The bottom panels are magnified images of the insets in the top two images. Note the similarity of the staining patterns between the WT and Tg^{144} mice. Results are representative of three independent experiments. (B) Bar graphs of the numbers of motor neurons per lumbar spinal cord section of the WT and TDP–43 Tg^{144} mice. The criteria for selecting the scored motor neurons included their having a round/open/pale nucleus with the diameter of 30–45 μm. For each animal, >25 coronal sections from L1 to L5 of the lumbar spinal cord were counted. The numbers of the spinal motor neurons (MN) are similar between the WT and TDP–43 Tg^{144} mice ($P = 0.05$). Results represent the mean ± SEM of three independent experiments, and $n = 5$ for each group.