Amyotrophic lateral sclerosis-related VAPB P56S mutation differentially affects the function and survival of corticospinal and spinal motor neurons

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The substitution of Proline with Serine at residue 56 (P56S) of vesicle-associated membrane protein-associated protein B (VAPB) has been linked to an atypical autosomal dominant form of familial amyotrophic lateral sclerosis 8 (ALS8). To investigate the pathogenic mechanism of P56S VAPB in ALS, we generated transgenic (Tg) mice that heterologously express human wild-type (WT) and P56S VAPB under the control of a pan-neuronal promoter Thy1.2. While WT VAPB Tg mice did not exhibit any overt motor behavioral phenotypes, P56S VAPB Tg mice developed progressive hyperactivities and other motor abnormalities. VAPB protein was accumulated as large punctate in the soma and proximal dendrites of both corticospinal motor neurons (CSMNs) and spinal motor neurons (SMNs) in P56S VAPB Tg mice. Concomitantly, a significant increase of endoplasmic reticulum stress and unfolded protein response and the resulting up-regulation of pro-apoptotic factor CCAAT/enhancer-binding protein homologous protein expression were observed in the CSMNs and SMNs of P56S VAPB Tg mice. However, only a progressive loss of CSMNs but not SMNs was found in P56S VAPB Tg mice. In SMNs, P56S VAPB promoted a rather selective translocation of VAPB protein onto the postsynaptic site of C-boutons that altered the morphology of C-boutons and impaired the spontaneous rhythmic discharges of SMNs. Therefore, these findings provide new pathophysiological mechanisms of P56S VAPB that differentially affect the function and survival of CSMNs and SMNs in ALS8.

INTRODUCTION

Amyotrophic lateral sclerosis 8 (ALS8) is a slowly progressive and late-onset dominant form of ALS initially identified in a large Brazilian family (1). The affected individuals suffered three distinct conditions: a late-onset slowly progressing form of spinal muscular atrophy, an atypical slowly progressing form of ALS and a typical severe and rapidly progressing form of ALS (1–3). Recently, two missense mutations (Proline with Serine at residue 56, P56S and T46I) at the highly conserved major sperm protein (MSP) domain of vesicle-associated membrane protein B (VAPB) have been identified to associate with ALS8 (1,2), which may provide the molecular clue to understand the pathogenic mechanism of this disease.

VAPB belongs to a family of highly conserved vesicle-associated membrane protein (VAMP)-associated proteins (VAPs) with a highly conserved N-terminal MSP domain located at the cytoplasmic face of the endoplasmic reticulum (ER) and the Golgi apparatus and is presumably involved in the ER-Golgi-mediated vesicle transport (4). The MSP domain of VAPB may also be cleaved, secreted and functions as a ligand for Eph receptors (5). The localization of VAPB in ER suggests a more prominent role in early secretory compartments (6). An in vitro study has shown that the P56S mutation

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is predicted to introduce a kink between two short stretches of β strands, and causes insolubility and accumulation of large ER aggregates in cells (7). This mutation may also induce malfunction of VAPB-mediated unfolded protein response (UPR) or translocation of VAPB protein from conventional ER to organized smooth ER (5,7–10). Additionally, the latest in vitro studies indicate that VAPB is involved in calcium homeostasis, nuclear envelope formation and mitochondrial localization (11–13). However, studies of the more physiologically relevant mouse models are limited, of which VAPB P56S transgenic (Tg) mice failed to develop any obvious motor behavioral phenotypes and motor neuron degeneration (14,15). How P56S VAPB affects the function and survival of motor neurons remains poorly understood.

To investigate the pathogenic mechanism of P56S VAPB in vivo, we developed and characterized new lines of Tg mice carrying wild-type (WT) or P56S mutant human VAPB gene under the control of mouse Thy1.2 promoter (16). No apparent motor behavioral and neuropathological phenotypes were observed in WT VAPB Tg mice, whereas P56S VAPB Tg mice developed various motor behavioral abnormalities, including progressive hyperactivity. The accumulation of P56S VAPB induced ER stress and triggered the UPR in both corticospinal motor neurons (CSMNs) and spinal motor neurons (SMNs), which led to the increase of pro-apoptotic factor C/enhancer-binding protein homologous protein (CHOP) expression in these neurons. However, only a significant loss of CSNMs was detected in aged P56S VAPB Tg mice. In contrast, no obvious degeneration of SMNs was found in P56S VAPB Tg mice. Interestingly, the P56S mutation induced a specific translocation of VAPB protein to the post-synaptic site of C-boutons in SMNs, affecting both the structure and function of C-boutons. Together, we reveal novel pathogenic functions of P56S VAPB that differentially affect the survival and function of CSNMs and SMNs based on the exact context of host cells, which may contribute to the pleomorphic clinical phenotypes associated with ALS8 and related motor neuron diseases.

RESULTS

Generation of human WT and P56S VAPB Tg mice

To investigate the pathogenic mechanism of P56S VAPB in the development of ALS8 and related motor neuron diseases, we generated several independent lines of Tg mice overexpressing either human WT or P56S VAPB complementary DNA (cDNA) under the control of Thy1.2 promoter (Fig. 1A). Thy1.2 promoter has been used previously to drive the expression of transgenes in neurons, including CSNMs and SMNs (16). To facilitate the identification of neurons overexpressing human VAPB, an IRES-EGFP reporter construct was inserted immediately after the stop codon of VAPB cDNA for the bicistronic expression of VAPB and EGFP under the same transcriptional regulation (Fig. 1A).

We have obtained three independent lines of WT and P56S VAPB Tg mice, respectively. The expression of Tg VAPB mRNA was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) with primers that recognize both mouse and human VAPB. More than 20-fold over-expression of VAPB mRNA was observed in the brain of B3 of WT (25.6 ± 1.9 fold) and line D3 of P56S VAPB Tg mice (23.7 ± 0.7-fold) compared with littermate non-Tg (nTg) controls (Fig. 1B). We later refer these two lines as WT and P56S VAPB Tg mice for the following pathological and motor behavioral analyses.

To quantify the expression level of exogenous VAPB protein in Tg mice, we generated a polyclonal antibody (TS5924) that recognized both WT and P56S VAPB (Fig. 1C). The expression of VAPB protein was significantly increased in the cerebral cortex, spinal cord, striatum and hippocampus of WT and P56S VAPB Tg mice compared with nTg mice (Fig. 1C). Interestingly, the steady level of P56S VAPB protein (~7-fold versus endogenous) was significantly less compared with WT VAPB protein (~20-fold versus endogenous) in the Triton X-100-soluble whole brain lysate (Fig. 1D), although the expression levels of VAPB mRNA were comparable between these two lines of mice (Fig. 1B). These data indicate that P56S mutation might affect the stability and/or solubility of VAPB protein in cells.

P56S mutation affects the stability and solubility of VAPB protein and alters the staining pattern of VAPB protein in CSNMs and SMNs

To determine the solubility of VAPB protein in detergent, we extracted proteins from the brain or spinal cord with Triton X-100 and harvested Triton X-100-soluble and insoluble fractions. We found that WT VAPB protein was mainly distributed in the Triton X-100 soluble fraction, whereas P56S VAPB protein was predominantly resided in the Triton X-100 insoluble fraction (Fig. 1D). These data are consistent with previous reports that P56S VAPB is less insoluble in Triton X-100 and tends to form aggregates when over-expressed in cell lines (7,9). Together, these results suggest that the P56S mutation may impair the proper folding of VAPB protein, resulting in degradation and aggregation of misfolded mutant proteins.

At subcellular level, an overall increase of VAPB staining was found in the soma and proximal neurites of CSNMs and SMNs in 3-month-old WT VAPB Tg mice compared with control nTg mice (Fig. 1E). In contrast, a widespread punctate VAPB staining was observed in the soma of CSNMs and SMNs inagematched P56S VAPB Tg mice (Fig. 1E). Occasionally, a few CSNMs and SMNs contained some very large VAPB-positive punctate staining in P56S VAPB Tg mice (arrows, Fig. 1E). Immunostaining also showed accumulation of ubiquitin- and autophagy marker p62-positive aggregates in the soma of CSNMs from P56S VAPB Tg mice (arrows, Fig. 1F and G). The CSNMs were visualized by COUP TF1-interacting protein 2 (CTIP2) staining that specifically recognizes the CSNMs in the anterior cerebral cortex (17). Consistently, the expression of p62 was significantly increased in the brain homogenate of P56S VAPB Tg mice (Fig. 1H). Meanwhile, extensive co-localization of P62 and VAPB-positive inclusions was found in CSNMs of P56S VAPB Tg mice (Fig. 1I). These data are consistent with the notion that misfolded mutant VAPB protein may be degraded through proteasome and autophagy-dependent pathways (18), while the aggregated VAPB protein may potentially affect the function and survival of motor neurons.

P56S VAPB Tg mice develop abnormal motor behavioral phenotypes

A cohort of male nTg (n = 15), WT (n = 12) and P56S (n = 12) VAPB Tg mice were closely monitored for motor and other
Figure 1. Generation of P56S VAPB Tg mice. (A) A schematic outline of the construct used to generate Tg mice. Human WT or P56S mutant VAPB cDNA followed by an internal ribosome entry site (IRES) and green fluorescent protein (GFP) expression cassette is cloned into the mouse Thy1.2 expression vector at the XhoI site. (B) qRT-PCR analysis for VAPB Tg expression in different mouse lines. Three and more mice were analyzed per genotype per line. WT B3 and P56S D3 VAPB lines have the highest and comparable expression level of Tg VAPB. (C) Western blots show the distribution of VAPB protein in the cerebral cortex (CX), spinal cord (SC),
behavioral phenotypes from 2 to 18 months of age. P56S and WT VAPB Tg mice are viable, develop normally and live normal life span. However, we found that the body weight of P56S VAPB mice was significantly less compared with littermate nTg and WT VAPB mice starting at 15 months of age (Fig. 2A). P56S VAPB mice also started to exhibit significant hyperactivity in both horizontal and vertical movements in the Open-field test at 12 months of age (Fig. 2B and C). Meanwhile, Rotarod testing revealed a significant deficit of motor coordination and balance in P56S VAPB mice beginning at 12 months of age (Fig. 2D). By contrast, no significant difference was detected in grip strength testing among these three groups of mice.

Since gait abnormalities are the earlier sign of motor dysfunction in ALS mouse models (19,20), we quantified the gait parameters of P56S VAPB Tg mice using the Treadscan Gait Analysis system. Interestingly, both the stride length and stride time of P56S VAPB mice were significantly shorter than those of the littermate nTg controls and WT VAPB Tg mice (Fig. 2E and F). The shorter stride displayed by P56S VAPB Tg mice was first detected at 2 months of age, and this gait abnormality was persistent without significant deterioration through their life span (Fig. 2E and F). In contrast, no significant difference in gait was found between WT VAPB and control nTg mice (Fig. 2E and F).

P56S VAPB Tg mice develop progressive degeneration of CSMNs but not SMNs

Using an unbiased stereological approach, we counted the number of CSMNs and SMNs in the motor cortex and lumen spinal cord of WT and P56S VAPB Tg mice. No significant CSMN loss was found in the 3-month-old P56S VAPB Tg mice; however, the number of CSMNs as revealed by Nissl staining was significantly decreased in the 10- and 18-month-old P56S VAPB Tg mice compared with the littermate control nTg and age-matched WT VAPB Tg mice (Fig. 3A). In contrast, no apparent degeneration of SMNs was observed in the 3- and 18-month-old P56S VAPB Tg mice (Fig. 3B). In addition, the CSMNs were further visualized by CTIP2 staining that specifically recognizes the CSMNs in the motor cortex (17). More than 60% of CTIP2-positive CSMNs were lost in the 18-month-old P56S VAPB Tg mice (Fig. 3C and D). The loss of CSMNs in the cerebral cortex of aged P56S VAPB Tg mice appeared very selective, since no degeneration of either CTIP2-positive layer V pyramidal neurons in the posterior cerebral cortex or CTIP2-positive striatal medium spiny neurons was found in these mice (Fig. 3E, Supplementary Material, Fig. S1A and B). In correlation with the loss of CSMNs in the motor cortex, a severe reduction of corticospinal tract (CST) was also found in the thoracic spinal cord of 12-month-old P56S VAPB Tg mice (arrows, Fig. 3F). Taken together, here we document a rather selective degeneration of CSMNs in aged P56S VAPB Tg mice.

UPR is activated in CSMNs of P56S VAPB Tg mice

An accumulation of unfolded or misfolded proteins in the lumen of ER triggers the UPR that restores the normal function of cells through attenuation of protein synthesis and induction of molecular chaperones involved in protein folding, whereas a prolonged UPR may lead to cell death (21). Up-regulation of molecular chaperone BiP/Grp78 expression serves as an indicator of ER stress and the UPR in cells (22). Since P56S VAPB causes alterations of the UPR and cell death in vivo (7–9), we co-stained BiP and VAPB in cortical sections of 3-month-old nTg as well as WT and P56S VAPB Tg mice. We found that BiP levels were significantly increased in the CSMNs of P56S VAPB Tg mice that also contained a large amount of VAPB inclusions in the cell body (arrowhead, Fig. 4A). In contrast, no significant increase of BiP expression was found in the CSMNs of nTg and WT VAPB Tg mice (Fig. 4A). When the confocal exposure was adjusted to prevent fluorophore saturation in the neurons that heavily accumulated P56S VAPB, we found that VAPB staining delineated an altered ER morphology (bottom panel, Fig. 4A). Rather than form the small, individual punctate seen in some neurons (asterisks, Fig. 4A), P56S VAPB delineated large convoluted membranous stacks reminiscent of the ER morphology alterations seen with in vitro transfection (10). It may be that these changes in ER morphology affect ER function and contribute to the induction of ER stress and activation of the UPR. We also co-stained 3-month-old cortical sections of P56S VAPB mice with PDI, another marker of ER stress. Again, we found that PDI expression was significantly increased only in large pyramidal CSMNs that heavily accumulated P56S VAPB inclusions (arrowheads, Fig. 4B). These data suggest that an excessive accumulation of P56S VAPB in large pyramidal CSMNs causes ER stress and activates the UPR in these cells.

UPR-induced cell death is mediated by the activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK), which suppresses the protein synthesis, while allowing increased translation of activating transcription factor 4 (ATF4) (21,23,24). ATF4 then turns on the expression of the pro-apoptotic gene CHOP that initiates the cell death pathway (21,23). In line with these earlier findings, we found that CHOP staining was dramatically increased in the CSMNs of 3-month-old P56S VAPB Tg mice that heavily accumulated VAPB in the cell body (arrowhead, arrows, Fig. 5A and B). Western blot analyses also revealed a significant increase of ATF4 and CHOP expression in the nuclear
fraction of cortical homogenates from P56S VAPB Tg mice compared with nTg and WT VAPB Tg mice (Fig. 5C and D). On the other hand, the expression of anti-apoptotic gene Bcl-2 in the cortex was significantly decreased in P56S VAPB Tg mice (Fig. 5E). Additionally, the expression of CTIP2, which is critical to the survival of CSMNs (25), was also significantly decreased in the CSMNs of P56S VAPB Tg mice (Fig. 5B and F). Together, these observations suggest that overexpression of P56S VAPB in CSMNs may cause ER stress and UPR, resulting in PERK activation and cell death. Interestingly, CHOP expression was also increased in the spinal cord of P56S VAPB Tg mice (Supplementary Material, Fig. S2). However, no significant loss of SMNs was found in P56S VAPB Tg mice (Fig. 3B), suggesting that CSMNs and SMNs have a differential response to CHOP-mediated cell death pathway.

P56S mutation induces abnormal translocation of VAPB protein onto the postsynaptic site of C-boutons in SMNs

To further investigate how P56S VAPB affects the function of SMNs, we found that a small fraction of VAPB was juxtaposed with synaptophysin (synap), a maker for presynaptic terminals (26), at the soma and proximal dendrites of SMNs in P56S VAPB Tg mice (Fig. 6A). We further confirmed the postsynaptic location of VAPB protein in the SMNs of P56S VAPB mice by immuno-EM with the TS5924 VAPB antibody (Fig. 6B). In contrast, no postsynaptic location of VAPB was observed in the SMNs of WT VAPB and control nTg mice (Supplementary Material, Fig. S3A).

These VAPB-associated synapses were restricted to the soma and proximal dendrites of SMNs and with a diameter of more than 3 μm (Fig. 6A), which closely resemble the C-bouton of SMNs (27). C-boutons receive cholinergic innervation from a group of cholinergic interneurons near the central canal of the spinal cord (28,29). The type 2 muscarinic (M2) receptors that are evenly distributed along the plasma membrane of large SMNs mediate the postsynaptic response of C-boutons and modulate the excitability of SMNs (28). Accordingly, we found that VAPB staining was juxtaposed with choline acetyltransferase (CHAT) staining in the SMNs of P56S VAPB mice (inset, Fig. 6C). Furthermore, VAPB protein showed specific co-localization with M2 receptors in SMNs, but not with vesicular glutamate transporter (VGLUT2) or glutamic acid decarboxylase 65 (GAD65) (Supplementary Material, Fig. S3B–D), indicating that P56S VAPB may affect the normal structure and function of the C-boutons in SMNs.

P56S VAPB affects the morphology of C-boutons

To examine whether P56S VAPB affects the structure of C-boutons, we compared the volume and length of C-boutons visualized by CHAT staining in the SMNs of WT and P56S VAPB Tg mice as well as control nTg mice. Three-dimensional deconvolution was applied to highlight C-boutons for image analysis (Fig. 6D). The volume of C-boutons appeared comparable.
among these three groups of mice (Fig. 6E). However, the length of the longest axis of C-boutons was significantly increased in P56S VAPB Tg mice compared with the nTg and WT VAPB Tg mice (Fig. 6F). Therefore, these studies demonstrate that the abnormal targeting of mutant VAPB onto C-boutons affects the morphology of these unique synaptic structures in SMNs.

P56S VAPB alters SMN activities

C-bouton-mediated muscarinic transmission has been shown to regulate SMN excitability in the neonatal mouse spinal cord (28). To activate C-boutons, isolated spinal cords from postnatal days 10–11 (P10–P11) P56S VAPB Tg and nTg littermates were stimulated with a suction electrode applied to the medial aspect of the hemi-sected cords near the central canal. We found that the intensity of L5 ventral root (VR) discharges at all stimulus levels (10–500 µA, Fig. 7A and B) was significantly decreased in P56S VAPB Tg spinal cords compared with nTg controls (n = 7, for 10–20 mA, *P < 0.05 and for 40–500 mA, **P < 0.01). At low stimulus currents (100 µA), the intensity of the VR discharge was 36.4 ± 7.2% and at supramaximal stimulus currents (300 µA), it was 75.7 ± 5.0% of the control values recorded from nTg littermates (n = 7, **P < 0.01). To ensure that long-lasting discharges evoked by repetitive stimulation were mediated by the muscarinic transmission of C-boutons, we used atropine, a muscarinic receptor antagonist. We found that atropine completely blocked the discharges in both control and P56S VAPB Tg spinal cords evoked by supramaximal stimulation (300 µA) with a similar dose-dependence (Fig. 7C, n = 4).
DISCUSSION

In this study, we describe some interesting pathophysiological features of ALS-related P56S VAPB mutation that differentially affect the function and survival of CSMNs and SMNs in Tg mice. P56S VAPB Tg mice developed progressive hyperactivity and motor coordination/balance impairments as well as age-independent gait abnormalities. Neuropathological analyses of aged P56S VAPB Tg mice revealed substantial loss of CSMNs, whereas no significant degeneration of SMNs was found. Hyperactivities have been associated with the lesion of CSMNs in other mouse models (30,31). We suspect that the progressive loss of CSMNs in aged P56S VAPB Tg mice may contribute to the increased motor activities developed in these animals. It should be noticed that CSMNs do not directly innervate SMNs in the spinal cord as mice do in humans (32). Therefore, the loss of CSMNs in humans may exhibit different motor phenotypes compared with mice.

Tudor et al. (14) used mouse prion promoter to drive the expression of P56S VAPB in Tg mice. Tg P56S VAPB appeared expressed by both cortical neurons and SMNs (14). However, no quantification of Tg P56S VAPB expression was performed in this study (14). Qiu et al. (15) in their study used chicken β-actin (CAG) promoter to drive the expression of Tg P56S VAPB in a variety of tissues and cell types. They found a 2- to 3-fold increase of P56S VAPB protein expression in the Tg mice compared with the endogenous VAPB (15). In our study, we used Thy1.2 promoter to selectively express Tg P56S VAPB in neurons, particularly in both CSMNs and SMNs. We observed a 7-fold increase of P56S VAPB protein expression compared with the endogenous protein. The higher level of P56S VAPB over-expression in our mutant mice may contribute to the more robust motor behavioral and CSMN degeneration phenotypes observed in these mice. More P56S VAPB mutant protein may exert more ER stress responses in the CSMNs. Alternatively, since VAPB protein functions as dimers (4), the presence of more P56S VAPB mutant protein may sequester more WT VAPB protein, which may further compromise the normal function of VAPB. Therefore, it requires a sufficient amount of P56S VAPB expression to produce any obvious behavioral and neuropathological abnormalities in Tg mice through either a gain of cytotoxicity or a dominant-negative mechanism. Similarly, although the exact levels of Tg P56S VAPB over-expression in the CSMNs and SMNs are unclear, a potentially higher level of Tg VAPB P56S protein expression in the CSMNs may contribute to the selective loss of CSMNs in our P56S VAPB Tg mice.

The onset of gait abnormalities was prior to the degeneration of CSMNs. P56S VAPB was wrongly targeted to the postsynaptic site of C-boutons and compromised the morphology and function of C-boutons in P10 mice, suggesting that the alteration of C-boutons may contribute to the shortening gait phenotype in mutant mice. However, P56S VAPB may impair the activity of CSMNs in young animals long before causing CSMN degeneration in aged mice. Therefore, it cannot preclude the involvement of CSMNs and other neurons in regulating gait properties. Selective expression of P56S VAPB transgene in either CSMNs or SMNs may help to further clarify the involvement of CSMNs and SMNs in regulating locomotion and gait properties.

Compared with SMNs, the pathogenic mechanism of CSMN degeneration is poorly understood in ALS and related motor neuron diseases. In line with the notion that VAPB is involved in ER stress and UPR in cell cultures (7,9), we found that P56S VAPB induced the ER stress markers BiP and PDI in CSMNs,
and activated PERK-mediated UPR and the following ATF4 and CHOP-mediated cell death pathway, indicating that the activation of the UPR may contribute to the loss of CSMNs in P56S VAPB Tg mice. Interestingly, the PERK pathway was also activated in the SMNs of P56S VAPB Tg mice. However, no SMN degeneration was observed in these mice. We suspect that CSMNs may be more vulnerable to PERK-mediated UPR than SMNs in P56S VAPB Tg mice. For example, in addition to the decreased expression of anti-apoptotic factor Bcl-2, the expression of CTIP2, which is essential to the development of survival of CSMNs (17), was concomitantly suppressed in CSMNs. It remains to determine whether CTIP2 expression is directly regulated by ATF4 or CHOP. Nonetheless, since CTIP2 is critical to the development and maintenance of CSMNs (17), we speculate that CTIP2 may be a key downstream molecular target for VAPB P56S-mediated loss of CSMNs in a cell-autonomous fashion. P56S VAPB may also activate other unspecified pathways that cause CSMN degeneration.
Therefore, this new line of P56S VAPB Tg mice provides a useful model system to dissect the molecular pathogenic pathways critical to the loss of CSMNs during ageing.

The C-bouton represents a distinct synaptic structure specialized in regulating the discharge of SMNs (27). However, current knowledge about the formation and composition of the C-bouton in SMNs is very limited. The unexpected translocation of P56S VAPB onto the postsynaptic face of C-boutons may provide a unique angle to study the origin and formation of C-boutons. Since WT VAPB is not presented at C-boutons, we suspect that P56S VAPB, which alters the conformation of cytoplasmic domain of VAPB (10), may gain association with new molecular partners critical to the postsynaptic targeting of C-boutons. It will be interesting to identify proteins that selectively interact with P56S VAPB, which may provide molecular-level insights into the formation of C-boutons.

In conclusion, here we demonstrate that P56S VAPB exerts profound impact on the function and survival of motor neurons through some distinct molecular pathways. This new line of P56S VAPB Tg mice may provide a valuable resource to study

Figure 6. Abnormal translocation of P56S VAPB onto the postsynaptic site of C-boutons alters the morphology of C-boutons in SMNs. (A) Representative images of VAPB (red) and synaptophysin (blue) staining in the SMNs of 3-month-old P56S VAPB Tg mice. Five and more mice per genotype and more than 10 sections per mouse were examined. Scale bar: 10 μm. (B) Representative image shows VAPB located at the postsynaptic membrane of C-boutons in SMNs of 3-month-old P56S VAPB Tg mice. Three mice more than 10 sections per mouse were examined. Scale bar: 100 nm. (C) Representative images show CHAT (green) and VAPB (red) staining in the SMNs of 3-month-old nTg, WT and P56S VAPB Tg mice. Three and more mice per genotype and more than 10 sections per mouse were examined. Arrow points to a C-bouton with CHAT and VAPB staining juxtaposing each other. Scale bar: 10 μm. (D) Representative image shows CHAT staining after 3D deconvolution treatment. Three and more mice per genotype and more than 10 sections per mouse were examined. Scale bar: 10 μm. (E and F) Bar graphs show the average volume (E) and length (F) of C-boutons in the SMNs of 3-month-old P56S VAPB Tg mice. Three mice were used for each genotype. 86, 83 and 92 C-boutons were analyzed for 3-month-old nTg, WT and P56S VAPB Tg mice, respectively. Data are presented as mean ± SEM; ***P < 0.001.
the degeneration of CSMNs and the formation and function of C-boutons. The knowledge gained from these studies may have important implications in both motor neuron development and degeneration.

MATERIALS AND METHODS

Generation of VAPB Tg mice

The P56S missense mutation was introduced into human VAPB full-length cDNA (OriGene Technologies, Inc., Rockville, MD, USA) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the instructions of the manufacturer. The DNA fragment containing IRES2-EGFP coding sequence was isolated from pIRES2-EGFP vector (Clonetech, Mountain View, CA, USA) and inserted immediately after the stop codon of VAPB. The resulting VAPB-IRES2-EGFP DNA fragment was then subcloned into the XhoI site of mouse Thy1.2 expression cassette to generate the VAPB Tg vector (Fig. 1A). The purified insert was injected into oocytes of C57BL/6 strain background. Positive founders were identified by PCR analysis in which three lines of P56S and three lines of WT VAPB Tg mice were established. Mouse genotypes were determined by PCR amplification of the tail biopsies using the forward primers CAC GTA GGT ACT GTG TGA AGT for human VAPB and CAC GTA GGT ACT GTG TGA AGC for endogenous mouse VAPB, and the reverse primer TGT TTC TGT CTT TGA TGC AG for both human and mouse VAPB. The founders were crossbred with WT C57BL6 mice to generate F1 P56S and WT VAPB Tg mice. The mice were housed in a 12-h light/dark cycle and fed regular diet ad libitum. All mouse work follows the guidelines approved by the Institutional Animal Care and Use Committee of National Institute of Child Health and Human Development.

Quantitative reverse-transcripase PCR and RT-PCR

Total RNA from the spinal cord, whole brain or different brain regions from nTg, WT and P56S VAPB Tg mice was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR and RT-PCR analyses. For each sample, 100 ng of total RNA was used for the synthesis of double-stranded cDNA using SuperScript III reverse transcriptase kit (Invitrogen). qRT-PCR was performed in a 10 μl reaction volume in 384-well plates with Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) using the Sequence Detection System 7900 (Applied Biosystems). Each sample was run in technical triplicates and the average values for each sample were normalized to the geometric mean of mRNA levels of reference genes β-actin, GAPDH and HPRT running in separate
reactions from the same cDNA preparations. Genotyping was performed using a pair of primers that recognize both WT and P56S VAPB (forward: CCA CAA CTG CAT CAA AGA CAG; reverse: TCA CCT TGC AGC CTC TTA CAT). An additional pair of primers (forward: CGT TGA CAT CCG TAA AGA CC; reverse: GCT AGG AGC CAG AGT AA) was used as an internal control. In all experiments, the nTg animals were strict littermates.

**Generation of VAPB antiserum**

An anti-VAPB serum (TS5924) was produced via immunization of rabbits with a keyhole limpet hemocyanin-coupled polypeptide (Covance). The polypeptide sequence corresponds to residues 184–204 (REENKQFEEDGLRMKTVQS) of human VAPB.

**Tissue homogenization and western blot**

Brain tissues were homogenized in RIPA buffer (Sigma-Aldrich, St Louis, MO, USA) supplemented with protease inhibitors (Roche Applied) and phosphatase inhibitors (Thermo Fisher Scientific, San Jose, CA, USA). Following 15 min incubation on ice, protein extracts were clarified by centrifugation at 15,000 × g for 30 min at 4°C. The supernatants were quantified for protein content using an assay kit based on bichoninic acid (Thermo Fisher Scientific) and separated by 4–12% NuPage BisTris-polyacrylamide gel electrophoresis (Invitrogen) using MES running buffer (Invitrogen). After transfer to nitrocellulose membranes, the membranes were immunoblotted with the appropriate dilutions of the primary antibody: VAPB, P62 (Sigma), GFP (Roche), β-tubulin (Santa Cruz), ATF4 (Santa Cruz), CHOP (Santa Cruz), Bcl-2 (Streegen), TBP (Santa Cruz), CTIP2 (Abcam, Cambridge, MA, USA), NUP62 (Santa Cruz) and β-actin (Sigma) at 4°C. Signals were visualized by enhanced chemiluminescence development (Thermo Fisher Scientific) and quantified on a Scion Image System (Frederick, MD, USA).

**Histology and immunohistochemical analyses**

The procedures for preparation of mouse brain and spinal cord frozen sections for histological and immunohistological analyses were described previously (33). Antibodies specific to ubiquitin (DAKO, Denmark), CTIP2 (Abcam), p62 (Sigma), BiP/GRP78, PDI, CHOP/GADD153 (Santa Cruz), synaptophysin, GAD65, CHAT, VGLUT2 (Millipore) and M2R (Alomone) were used as suggested by the manufacturer. Fluorescence images were captured using a laser scanning confocal microscope (LSM 510; Zeiss, Thornwood, NJ, USA). Bright field images were captured by Zeiss Axio microscope (Imager A1).

**Behavioral assessment for VAPB Tg mice**

A cohort of 39 mice (WT, P56S and nTg littermate controls; each group comprised of all male animals) was established for behavior testing. At the start of each testing period, mice were weighed and assessed for hind-limb clasping behavior when suspended by the tail. All equipment was wiped clean with ethanol between mice. We performed the Rotarod test, grip strength measurement, Open-field test and gait analysis following the protocols established previously (20,34).

**Differential detergent extraction, centrifugation and western blot analyses**

Brains or spinal cords were homogenized in 1 × TEN (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 100 mM NaCl). Tissue homogenates were mixed at 1 : 1 with buffer A (1 × TEN; 1% Triton X-100; proteinase inhibitor cocktail (Roche)). The mix was sonicated [50% output for 30 s with a probe sonicator (70W; TEKMAR, OH, USA)] and then centrifuged at 17,500 × g for 5 min to separate supernatant S1 and pellet P1. The P1 pellet was washed with buffer B (1 × TEN; 1% Triton X-100) by sonication (50% for 30 s), and centrifuged at 17,500 × g for 5 min to obtain pellet P2, which was resuspended in buffer C (1 × TEN; 1% Triton X-100; 0.5% deoxycholic acid; 0.25% SDS). The P2 fraction was further extracted by sonication (50% for 30 s) and centrifugation (17,500 × g for 5 min) to sediment P3, which was resuspended by buffer D (1 × TEN; 1% Triton X-100; 0.5% deoxycholic acid; 2% SDS). Additional antibodies used for western blot included AFT4 (Santa Cruz), CHOP (Cell Signaling), TBP (Abcam) and Bcl-2 (Stressgen).

**Immunoelectron microscopy**

Spinal cords and cerebellum of WT, P56S and nTg mice (two per group) were processed for immuno-EM as described (35). Sections were examined in a JEOL 1200EX electron microscope. Gold particles within 10 fields for each genotype were counted.

**Stereology**

To examine the number of CSMNs and striatal neurons, series of sagittal brain sections (nine sections, 40 μm in thickness, counting every ninth section) were processed for CTIP2 or Nissl staining. CTIP2 staining was visualized using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The motor cortex was outlined based on the stereotaxic coordinates in the Adult Mouse Brain Atlas (3rd edition, Keith B.J. Franklin and George Paxinos). All brains were processed and sectioned in the same manner so as to produce comparable sagittal sections. The motor cortex was then designated as layer V neurons in the anteromedial cortex. Easily visible and reproducible landmarks (such as the anterior border of hippocampus, anterior and posterior border of the striatum) were used as appropriate to demarcate the layer V boundaries of the motor cortex as outlined in the Adult Mouse Brain Atlas, for each depth of sagittal section. As a control, CTIP2 positive layer V neurons were counted in the posterior cortex. CTIP2 positive layer V neurons from mid-hippocampus to the posterior end of layer V were counted on each section, using the same sections used for counting CSMNs. Spinal cord sections of the L3–L5 lumbar cord were Nissl stained (thirteen sections, 50 μm in thickness, counting every eighth section) to identify the large (>35 μm in diameter) ventral horn SMNs. The number of CTIP positive neurons, large pyramidal (>15 μm) Nissl-stained cortical layer V neurons and Nissl-stained SMNs were assessed using the Optical Fractionator function of Stereo Investigator 8, an unbiased stereological procedure (MicroBrightField, Inc.,

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Williston, VT, USA). Counters were blinded to the genotypes of the samples. The sampling scheme was designed to have coefficient of error <10% in order to get reliable results.

**Measurements for the volume and length of C-boutons**

Fluorescence images and z-series stacks were captured with an LSM 510 META laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) and the built-in Zeiss LSM 510 software. A Zeiss 63× apochromat (1.4NA) oil immersion lens was found to provide the best combination of working distance, resolution and magnification. The setting for exposure time, light source and camera controls were fixed during the acquisition of all the images. The camera gain and offset were both at their minimal settings. The step size was 400 nm in depth. We employed the Velocity software (Improvision, Inc., Lexington, MA, USA) to measure the volume of C-boutons. To determine the volume of C-boutons, a threshold-based segmentation was applied using the object separation option. The procedure works by finding local maxima and separating touching objects in 3D. Touching objects and non-specific stained particles were removed manually to control the accuracy of further analysis and particles with volumes bigger than 0.5 μm³ were collected for analysis. The length (maximal diameter) of C-boutons was measured by the ImageJ software (NIH).

**Electrophysiology**

All surgical procedures were performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the National Institute of Neurological Disorders and Stroke Animal Care and use Committee. The dissection technique was similar to that recently published (36) and will only be briefly described here. Spinal cords from postnatal day 10–11 (P10–P11) mice were isolated and placed in an experimental chamber with continuously recirculating solution containing (in mM): 111 NaCl, 3 KCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgCl₂, 2.6 CaCl₂, 11 d-glucose, pH 7.4, when bubbled with 95% O₂/5% CO₂ at RT (21–23°C). To activate C-boutons, a stimulating suction electrode was placed near the central canal of the spinal cord which was hemisectioned from L1 to L6. Control (nTg) and Tg (P56S VAPB Tg) spinal cords were stimulated identically (5 s train of stimuli: 10–500 μA, 0.5 ms, 10 Hz) and activity from the L5 VR was recorded in the same chamber. Both preparations were from the same litter. Atropine, a muscarinic receptor antagonist, was added to the re-circulating solution to block long-lasting discharges in nTg control and P56S VAPB Tg spinal cords. To activate spontaneous rhythmic bursting (36), we bath-applied bicuculline (BIC, a GABA-A receptor antagonist) together with strychnine (STR, a glycine receptor antagonist). The electrical activity (DC-3 kHz) was recorded with DAM 50 amplifiers (WPI, Sarasota, FL, USA), digitized with Digidata 1322A and saved with the pClamp 9 software (Molecular Devices) and further analyzed with Sigma Plot 12 (Systat Software, San Jose, CA, USA). Chemicals BIC ((−)-bicuculline methiodide), STR (strychnine hydrochloride) and atropine (atropine sulfate salt hydrate) were purchased from Sigma-Aldrich.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) and Sigma Plot 12 (Systat Software). Data are presented as mean ± SEM. Statistical significance was determined by comparing means of different groups using the t test or ANOVA followed by the post hoc test (∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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