Investigating the contribution of VAPB/ALS8 loss of function in amyotrophic lateral sclerosis

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The mutations P56S and T46I in the gene encoding vesicle-associated membrane protein-associated protein B/C (VAPB) cause ALS8, a familial form of amyotrophic lateral sclerosis (ALS). Overexpression of mutant forms of VAPB leads to cytosolic aggregates, suggesting a gain of function of the mutant protein. However, recent work suggested that the loss of VAPB function could be the major mechanism leading to ALS8. Here, we used multiple genetic and experimental approaches to study whether VAPB loss of function might be sufficient to trigger motor neuron degeneration. In order to identify additional ALS-associated VAPB mutations, we screened the entire VAPB gene in a cohort of ALS patients and detected two mutations (A145V and S160D). To directly address the contribution of VAPB loss of function in ALS, we generated zebrafish and mouse models with either a decreased or a complete loss of Vapb expression. Vapb knockdown in zebrafish led to swimming deficits. Mice knocked-out for Vapb showed mild motor deficits after 18 months of age yet had innervated neuromuscular junctions (NMJs). Importantly, overexpression of VAPB mutations were unable to rescue the motor deficit caused by Vapb knockdown in zebrafish and failed to cause a toxic gain-of-function defect on their own. Thus, Vapb loss of function weakens the motor system of vertebrate animal models but is on its own unable to lead to a complete ALS phenotype. Our findings are consistent with the notion that VAPB mutations constitute a risk factor for motor neuron disease through a loss of VAPB function.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive muscular atrophy and the simultaneous degeneration of lower (spinal and bulbar) and upper (corticospinal) motor neurons (1,2). This combined degeneration leads to muscle weakness, fasciculation, speech and swallowing disabilities, and progressive paralysis.

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Approximately 10% of ALS cases, clinically indistinguishable from sporadic cases, are genetically inherited and are termed as familial ALS (fALS) (3) with more than 10 loci currently identified. ALS8 was mapped in a large white Brazilian family and corresponds to a C → T substitution in exon 2 of the gene encoding vesicle-associated membrane protein-associated protein B/C (VAPB) leading to a missense mutation in the encoded protein (4). Since then, one other missense mutation (T46I) has been discovered in an independent fALS family (5).

VAPB is a member of the VAP family, which also includes VAPA, an established regulator of vesicle trafficking (6), and is a protein resident of the endoplasmic reticulum of poorly characterized function, possibly involved in an unfolded protein response (UPR) (7–9). Overexpression of mutant VAPB in cultured cells led to aggregation of the protein in the cytosol and trapping of the wild-type protein in these aggregates, suggesting that VAPB mutations might act as a dominant negative of VAPB function (4,7–10). However, transgenic mice that overexpress mutant VAPB specifically in the nervous system were created and despite displaying obvious VAPB aggregates did not show an apparent behavioural phenotype (11,12), suggesting that the overexpression of mutant VAPB is not sufficient to lead to an ALS phenotype in mammals by toxic gain of function. Interestingly, induced pluripotent stem (iPS) cells prepared from fibroblasts of ALS8 patients displayed decreased levels of VAPB protein, and did not show the cytoplasmic aggregates observed upon overexpression (13). These results suggested that the phenotype associated with VAPB mutations could be due to a loss of VAPB protein function rather than to a toxic gain of function of the mutant VAPB. Supporting this notion are reports of decreased VAPB levels in motor neurons of animal models of ALS1 and in sporadic ALS patients (10,14). Hence, it is currently thought that the partial loss of VAPB protein function through VAPB mutations causes ALS and that a similar loss might be involved in other forms of the disease. Here, we tested the contribution of VAPB loss of function to ALS pathogenesis through multiple genetic and experimental approaches. Our results show that the partial or complete loss of VAPB function is sufficient to lead to motor deficit but is unable to trigger a full-blown ALS phenotype, either in the mouse or in the zebrafish models.

RESULTS
Detection of new VAPB mutations in ALS patients

Two heterozygous mutations were identified in the VAPB gene in the 169 ALS cases screened (Fig. 1A). The Fam113 patient has an in-frame deletion in exon 5 (570_572delCTT) resulting in the loss of a serine amino acid (p.S160Δ), the last of three consecutive serine residues in the protein (Fig. 1B and C). Unfortunately, other affected members have not been collected to confirm the segregation of the mutation with disease in Fam113. While the serine 160 residue is not particularly conserved in other VAP proteins, no other species have an amino acid loss at this position (Fig. 1F). This mutation has been previously described in a cohort of ALS patients and at a very low frequency in the corresponding control cohort (15). In addition, an sALS patient had a nucleotide substitution (c.705C>T) causing an alanine to valine missense mutation (p.A145V), also in exon 5 (Fig. 1D). Alanine 145 is conserved in VAPB protein homologues of all species except zebrafish, which has a lysine at this position (Fig. 1F). The two patients were negative for mutations in SOD1, FUS, TARDBP and/or C9ORF72. Neither of these mutations were detected in the chromosomes of 380 ethnically matched control individuals. Conversely, the variation in the 5’ untranslated region of VAPB (c.239C>G), 33 bp before the ATG start codon, was detected in one sALS patient and also in one of the 380 control individuals. The fALS Fam113 consists of 20 members, including three affected individuals diagnosed with definite ALS of bulbar onset. Symptom onset occurred in the collected patient at age 59 with a 3-year progression. The sALS patient is a male from Quebec, Canada with spinal-onset definite ALS, initially presenting disease at the age of 58. Again no DNA was available from the other members of this family.

Vapb knockdown leads to motor deficits in zebrafish

To investigate the pathogenicity and mechanism of action of VAPB mutations, we first employed the zebrafish model. Zebrafish have a single VAPB orthologue that is closely related to the human gene (75% nucleotide identity). In order to determine loss-of-function properties of these mutations, we injected an antisense morpholino oligonucleotide (AMO) that knocked down the expression of the zebrafish homologue of VAPB, Vapb. Approximately 20% of embryos injected showed developmental and morphological abnormalities and were excluded from motor analysis. The remaining embryos were morphologically normal with no observable abnormalities in major organ development. We tested for motor deficits upon knock down (KD) of zebrafish Vapb using a well-characterized larval behavioural landmark: the touch-evoked escape response (TEER) (Supplementary Material, Fig. S1). Embryos injected with Vapb AMO were separated into three groups: (a) zebrafish lacking a proper TEER and representing motor deficits (motor) (see the image sequence in Supplementary Material, Fig. S1B), (b) zebrafish able to respond to the TEER but with mild motor defects and (c) zebrafish with a normal TEER. Furthermore, we performed a quantitative analysis of the TEER as previously described (16). The duration (Supplementary Material, Fig. S2C), the distance (Supplementary Material, Fig. S2D) and the maximal velocity of TEER swimming episodes (Supplementary Material, Fig. S2E) were significantly reduced solely upon Vapb KD (Supplementary Material, Fig. S2A versus Supplementary Material, Fig. S2B).

Mutant VAPB is unable to rescue the motor phenotype due to Vapb knock down in zebrafish

Upon co-injection of mRNA of human wild-type VAPB with Vapb AMO, we were able to significantly rescue both the mild and severe motor phenotypes (Fig. 2B; as seen in the image sequence in Supplementary Material, Fig. S1C), indicating that these phenotypes are most likely due to KD of the zebrafish Vapb. On the other hand, the percentage of zebrafish with developmental anomalies was not affected upon co-injection of WT human VAPB mRNA (Fig. 2B). These results indicate that the developmental abnormalities obtained are most likely due to non-specific toxicity associated with AMO injection.
We next tested whether VAPB mutations identified in ALS patients were able to rescue the motor phenotypes caused by Vapb KD. Co-injection of the VABP RNA containing a well-characterized P56S mutation was unable to rescue the motor phenotype (Fig. 3B and C). These RNA co-injections were also unable to rescue the percentage of developmentally abnormal zebrafish (Fig. 3A). The two mutations identified in this study, A145V and S160Δ, were similarly unable to rescue the motor phenotype, suggesting similar pathogenic mechanisms for these mutations (Fig. 3B and C). Interestingly, each of the ALS-related VAPB mutations described here, P56S, A145V and S160, were unable to induce a motor
Figure 3. Mutant human VAPB RNA fails to rescue loss-of-function phenotype in the zebrafish model. Zebrafish were separated in the three conditions described in Figure 2: (A) The percentage of zebrafish displaying developmental abnormalities remained unchanged upon co-injection of the Vapb AMO with human wild-type VAPB RNA (indicated hVAPB), or with three human VAPB mRNAs bearing ALS-related mutations P56S, A145V and S160. (B and C) Both motor phenotypes observed upon the TEER assessment at 48 h post-fertilization were not significantly altered upon co-injections of P56S, A145V and S160 mutant RNAs with the Vapb AMO. Only co-injections of the human wild-type VAPB mRNA were able to rescue the TEER deficits.
phenotype when injected on their own (Supplementary Material, Fig. S3), suggesting that loss-of-function rather than toxic gain-of-function mechanisms of toxicity are at play for these mutations.

**Vapb knockdown leads to minor axonal defects in zebrafish**

To assess whether TEER deficits played a functional role in the formation and maintenance of the neuromuscular junction (NMJ), we performed immunohistochemical analysis of axonal projections from the motor neurons. We observed a similar length and arborization of axonal projections of motor neurons in larvae injected with Vapb AMO when compared with control embryos (non-injected and mismatch AMO) (Fig. 4A). As mentioned previously, the embryos injected with Vapb AMO did not display any morphological abnormalities (Fig. 4B).

**Targeted ablation of exon 3 in murine Vapb generates Vapb \(-/-\) mice**

We next sought to determine whether the loss of VAPB function could also lead to motor deficits in a mammalian model. To this aim, we generated mice with insertion of loxP sites flanking both sides of exon 3 because the presence of an ATG codon in exon 3 could lead to the generation of an almost complete VAPB protein (Fig. 5A). On the contrary, excision of exon 3 yielded an out-of-frame mRNA putatively leading to the generation of an aberrant 84 amino-acid long VAPB-derived peptide. Vapb-loxP animals were crossed with CMV-CRE mice to obtain germline transmission and obtain Vapb\(^{\Delta\text{exon}3}\) mice in their progeny. Such Vapb\(^{\Delta\text{exon}3}\) mice were obtained, viable and transmitted the \(\Delta\text{exon}3\) allele to their progeny. We next generated and studied mice with one or two \(\Delta\text{exon}3\) alleles.

RT–PCR analysis from spinal cord tissue using primers spanning the exon 3 sequence detected the presence of mRNA lacking exon 3 in both heterozygous and homozygous Vapb\(^{\triangle\text{exon}3}\) mice (Fig. 5B). RT-qPCR analysis using primers outside exon 3 revealed that the deletion led to a strong decrease in total Vapb mRNA in heterozygous mice, and to barely detectable Vapb mRNA levels in homozygous mice in both cerebral cortex and tibialis anterior (TA) muscle (Fig. 5C), which is likely due to non-sense mRNA decay. To further check that Vapb\(^{\Delta\text{exon}3}\) mice were indeed Vapb \(-/-\) mice, we raised a polyclonal rabbit antibody against a peptide located in exon 1/2 of Vapb since all commercially available antibodies yielded non-specific bands in our hands. Western blot analysis of Vapb\(^{\Delta\text{exon}3}\) mice showed a complete loss of the VAPB signal in homozygous mice and a strong reduction in heterozygous mice (Fig. 5D). We were unable to detect the potential 84-amino acid long Vapb\(^{\Delta\text{exon}3}\)-derived peptide (predicted MW 9 kDa). Vapa mRNA levels were unchanged by Vapb exon 3 ablation (Fig. 5E), suggesting that Vapb loss was not compensated by Vapa transcriptional upregulation. Thus, Vapb\(^{\Delta\text{exon}3}\) mice are complete knockout of Vapb, and are termed Vapb \(-/-\) mice in the rest of this report.

**Vapb ablation leads to mild, late-onset motor deficits in mice**

Vapb \(-/-\) mice were fully viable, fertile and appeared healthy. We then sought to determine whether they displayed behavioural abnormalities. To this aim, we performed a SHIRPA protocol, consisting of a battery of simple tests to rapidly evaluate the existence of a phenotype (phenome.jax.org). The results of the SHIRPA screening in two cohorts of 4–6 months \((n = 8)\) and 18–22 months \((n = 8)\) of +/+,...
of the VAPB signal in using an antibody targeting exons 1 to 2. Note the complete disappearance cortex of three mRNA levels relative to RNA polymerase II in mouse cerebral cortex or been produced from the levels relative to RNA polymerase II in mouse cerebral cortex or TA muscle.

Figure 5. Generation of Vapb −/− mice. (A) Genomic DNA organization of the Vapb-loxP locus (left) and resulting mRNA species before (upper) and after (lower) CRE-mediated excision with loxP sites shown as arrows. Removal of exon 3 generates a transcript with an open reading frame leading to the potential generation of an aberrant 84 amino-acid long peptide. However, older Table S2. Body position appeared unaffected at both ages, as impairing. However, older Vapb −/− mice displayed lower hind limb and ventral tone, meaning that their spontaneous resistance against a small hand pressure on their hind limb and abdomen was decreased. Furthermore, we observed that multiple Vapb +/+ and −/− mice displayed mild tremors in the open field. Thus, Vapb −/− mice seemed roughly normal but might develop age-related central nervous system defects. To further quantify this observation, we performed an accelerating Rotarod protocol to test for motor function, balance and coordination. This test showed that Vapb −/− mice were unable to remain on the Rotarod for as long as Vapb +/+ at 18 months of age, but not at 4 months of age (P < 0.05) (Fig. 6A). However, grip test analysis (Fig. 6B) did not demonstrate significant differences in muscle strength between the three groups at any age tested. Thus, the knockout of Vapb leads to mild, late onset defects in motor performance without affecting muscle strength.

Vapb ablation does not lead to morphological defects at the neuromuscular junction or to muscle denervation in mice

To determine whether this mild behavioural phenotype could be due to an underlying motor neuron disease, we performed electromyographical analysis on Vapb −/− mice. We did not observe stereotypical denervation-related electrical activities in gastrocnemius (GA) or TA muscle of Vapb +/+ and −/− mice (Supplementary Material, Fig. S3A), but irregular abnormal electrical activities were sparsely observed in some TA, but not GA, muscles of Vapb −/− mice (not shown). Diseased SOD1G86R mice analysed in parallel showed typical fibrillations and fasciculations in both muscles. Consistent with the lack of gross denervation in muscle, mRNA levels of denervation-related markers, including the alpha and gamma subunits of the cholinergic receptor, myogenin and Musk were unchanged in Vapb +/+ and −/− tibialis muscle when compared with +/+, while the upregulation of these markers was obvious in muscles of paralysed SOD1G86R mice (Supplementary Material, Fig. S3B). To determine whether individual NMJs were morphologically normal, we studied the morphology of the NMJ in the TA of 18 months and of 12 months mice. The morphology of the post-synaptic apparatus appeared to be normal up to 18 months (Supplementary Material, Fig. S4A). We performed morphometric analysis of the post-synaptic apparatus in these mice and found that the area of the NMJ was stable with age and was not modified by the genotype (Supplementary Material, Fig. S4B). NMJs of 18 months old mice were more fragmented and tended to be less complex, but there was no obvious effect of Vapb genotype (Supplementary Material, Fig. S4C and D). Consistently, NMJs of Vapb −/− and +/+ mice appear to be normally innervated in the GA (Fig. 7A) or the soleus muscles (Fig. 7B). Thus, ablation of Vapb does not trigger denervation of muscles or morphological defects in NMJs up to 18 months of age.

Vapb ablation does not lead to abnormal UPR

VAPB has been involved in UPR and endoplasmic reticulum homeostasis (5,7–9,17). An abnormal UPR is involved in ALS (18,19) and it is therefore possible that loss of VAPB function interferes with UPR signalling. The basal expression levels of BiP (GRP78), CHOP, ATF4 and ATF6 alpha, four master genes in UPR, were, however, similar to controls in 12 months Vapb −/− and +/+ mice in spinal cord (Supplementary Material, Fig. S6A) or TA muscle (Supplementary
Material, Fig. S6B). UPR activation can be elicited with 1 mg/kg of tunicamycin injection and is sustained during several days in mice (20). We found similar levels of UPR gene activation in Vapb+/+/-, +/+/- and +/+/- mice 48 h after tunicamycin injection (Supplementary Material, Fig. S7C). These results suggest that Vapb is dispensable for both basal and tunicamycin-induced UPR.

DISCUSSION

We provide evidence in this study that the loss of function of Vapb weakens the motor system of vertebrate animals without recapitulating the full ALS phenotype. These data suggest that VAPB mutations likely constitute a risk factor for developing ALS.

A first important result of our study is that VAPB mutations, although rare, are found in ALS patients. We found a p.S160D mutation in a fALS patient (family 113) that was previously identified by Landers et al. in another fALS case (15). These authors also observed this deletion variant in about 1% of the control cases studied. We did not detect it in 380 controls, suggesting that this variant is rare in the general population. We further identified a novel p.A145V mutation in an apparently sporadic case, also not detected in our control individuals. Recently a family with a p.V234I mutation (21) and several others with P56S mutations were described (21–25). Altogether, this study and others indicate that VAPB mutations exist in rare cases of ALS and are not restricted to the initial P56S and T46I mutations. Pathogenicity of these VAPB mutations has, however, been conclusively shown only for the P56S mutation. To date, co-segregation was not shown for any other VAPB mutation.

Determining whether VAPB mutations are gain or loss of function is an important step in understanding ALS8. Two lines of argument suggested a potential gain of function: first, the two known mutations were situated very close one from the other, in the MSP domain that, in worms, is cleaved and secreted by neurons to regulate nerve/muscle signalling (26). Second, both P56S and T46I mutations generated large cytosolic aggregates upon overexpression in vitro (8,10,17) and in vivo (11). The p.A145V and p.S160D mutations we identified here lie outside the MSP domain, as does the p.V234I previously identified (21), indicating that ALS mutations spread through the whole coding sequence of VAPB and do not cluster within the MSP domain. This discovery argues against VAPB mutations directly interfering with the signalling elicited by a cleaved MSP domain. These newly identified mutations could, however, modulate, either positively or negatively, VAPB protein levels and/or MSP domain regulation. A second hypothesis recently proposed a loss of function or haploinsufficiency of the protein resulting from ALS-related mutations. This mechanism is indirectly supported by the lack of detectable phenotype in mice overexpressing wild-type or mutant VAPB (11,12). In addition, Caenorhabditis elegans and Drosophila melanogaster showed strong phenotypes upon decreased expression of VAP proteins (26–29). Furthermore, iPS cells-derived neurons...
from P56S patients do not show VAPB cytotoxic protein aggregates as observed upon overexpression, but present decreased VAPB protein levels (13). Our results in both mouse and fish models support the idea that a loss of function of VAPB contributes to ALS pathogenesis. Knockdown of fish vapb led to motor impairments, while the complete knockout of Vapb in mice led to a mild age-dependent decrease in motor performance. Haploinsufficiency experiments, through the loss of one copy of Vapb gene in Vapb+/- mice, did not lead to any detectable phenotype. Furthermore, overexpression of wild-type human VAPB RNA was able to rescue the phenotype of knockdown zebrafish VAPB, while overexpression of a VAPB mutant RNA did not. Despite a motor phenotype being observed in both animal models, neither Vapb knocked down fish nor Vapb +/- mice progressed to a full-blown ALS phenotype. In zebrafish, the phenotype observed upon vapb KD was milder than that observed upon Tardbp KD or human TARDBP mutant overexpression (30). In mice, the NMJ was spared even at very old ages, contrasting with observations from mutant SOD1 mice that develop a stereotypical ALS phenotype (31). The mild phenotypes presented here contrast with the very strong phenotypes observed in the previous invertebrate models. However, invertebrates, and in particular C. elegans and D. melanogaster, possess only one orthologue accounting for both VAPB and VAPA genes. Loss-of-function experiments in these species result in a complete loss of VAP proteins, and are thus more drastic than in single VAPB knock-out vertebrates that retain VAPA normal expression. We did not observe transcriptional upregulation of Vapa upon Vapb ablation. This, however, does not exclude that VAPA and VAPB protein functions are sufficiently redundant for VAPA to compensate for VAPB loss. Thus, we suggest that compensation for the loss of VAPB function through VAPA might underlie the difference in phenotype severity between vertebrates and invertebrates.

The mechanisms underlying the motor phenotype observed in vapb KD fish and Vapb +/− mice warrant further investigation. Since VAPB has been involved in UPR and endoplasmic reticulum homeostasis (5,7–9,17) and an abnormal UPR is involved in ALS (18,19), we hypothesized that a loss of VAPB could interfere with UPR signalling. However, we did not find any transcriptional evidence of UPR activation or deficit in Vapb +/− mice, nor observed massive UPR deficit upon tunicamycin injection. Consistently, recent work showed that VAPB overexpression, either wild-type or mutant, did not modify the UPR (11). Alternatively, the VAPB protein is involved in the regulation of Eph/Ephrin signalling, a major pathway regulating sensory-motor axonal development and regeneration (32). That Vapb silencing might affect the formation and maintenance of the sensory-motor system through altered Eph/Ephrin would be consistent with the recent observation that EphA4 is a risk factor for ALS (33). VAPB is also a key regulator of axonal vesicle transport (6) and its loss could also alter axonal transport leading to axonal deficits, perhaps causative of the observed phenotypes in Vapb KD zebrafish. Future studies using other endoplasmic reticulum homeostasis stressors or axonal transport in loss-of-function animal models could shed light on the function of VAPB in disease-relevant stresses.

In summary, we show here that VAPB mutations are present at low rates in ALS patients, and act, at least partially, through loss of function to impair the vertebrate motor system. Incomplete penetrance of ALS genes might be due to complex inheritance of multiple risk variants (oligogenic theory) (34). In such a scenario, VAPB mutations could be found in patients in combination with other ALS-relevant mutations. Indeed, a fALS patient was recently found to display a double VAPB and C9orf72 mutation (21). We thus postulate that VAPB mutations should be considered as strong risk factors that may lead to ALS, when placed in combination with other genetic risk factors or with environmental agents (35) or nutritional alterations (36) predisposing to ALS.

MATERIALS AND METHODS

Human genetic studies

Patient and control sample collection

Neurologists specialized in ALS diagnosed all cases as definite ALS according to El Escorial criteria and collected all samples. Informed written consent was obtained from each individual and blood samples were collected for DNA extraction. All fALS patients were initially screened for mutations in the SOD1 gene prior to inclusion in this study. In total, DNA from 69 fALS and 100 sALS patients was collected. In the fALS panel, 31 individuals were French, 29 were Canadian and 9 were American. The sALS panel was composed of 44 French patients, 52 Canadian patients and 4 American patients. Ethically matched and unrelated individuals were used as controls in this study.

Gene screening and variation analysis

The coding region of all six exons of VAPB (accession number NM_004738.3) was sequenced in each patient including at least 50 bp of the intronic region at each intron–exon junction. Primers were designed using the PrimerSelect programme (Lasergene) and synthesized by Invitrogen Canada Inc. PCR products were sequenced at the McGill University and Genome Quebec Centre for Innovation. In each case, the forward primer was used for sequencing and variations were confirmed by reverse sequencing. The presence of observed variants was analysed in control individuals. Variants in exon 5 were screened by direct sequencing, while allele-specific oligomerization was employed for variants in exons 1 and 4 as previously described (37,38). Oligomers used for exon 1 were 5’-AGGGGTCTCCCCGCCAA-3’ to detect the common allele and 5’-AGGGGTGCCTCCCCGCCAA-3’ for the rare allele and oligomers used for exon 4 were 5’-GAGAATGATAACCCAG-3’ and 5’-GAGAATGAGAACCAG-3’. The MegAlign programme of the DNASTAR package (Lasergene) was used to align proteins homologous to VAPB from various species by the ClustalW method.

Zebrafish studies

Zebrafish were raised from a colony maintained according to the established procedures, and all experiments were carried out in compliance with the Canadian Council for Animal Care.
Antisense morpholino oligonucleotide (AMO) injections
The morpholino sequences were designed against the zebrafish Vapb orthologue located on chromosome 6. The oligonucleotide 5′-CCATCTCCCCACTGCAAACGCTCGGA-3′ (Vapb AMO) binds to the ATG of the Vapb gene, which prevents its translation, and a 5 bp mismatch oligonucleotide was used as a control. Both were designed and purchased from Gene Tools (Gene Tools, Oregon, USA). AMO injections were performed in one-to-four cell stage zebrafish eggs as previously described, at an optimal concentration of 1.0 mM (30). The rescue experiments were performed as mentioned above, with simultaneous injections of the AMO and human VAPB mRNA at concentrations of 1.0 mM and 80 ng/μl respectively.

Behavioural and immunocytochemical analysis
Morphology and behavioural touch responses were assessed with a stereomicroscope (Zeiss, Oberkochen, Germany). To elicit the escape response at 48 hpf, embryos were touched lightly on the tail with a pair of blunt forceps and their swimming episodes were recorded using a Photron (San Diego, CA, USA) Fastcam PCI high-speed video camera at 125 frames/s. For immunohistochemical analysis of axonal projections of motor neurons, monoclonal antibodies against synaptic vesicle 2 (anti-SV2m Developmental Studies Hybridoma) was used to label pre-synaptic axons at the NMJ and anti-acetylated alpha-tubulin (Sigma) was used to label all neuronal tubulins.

Mouse studies
Animal housing
Transgenic mice were housed in the animal facility of the medicine faculty of Strasbourg University, with 12 h/12 h of light/dark and unrestricted access to food and water. In all experiments, littermates were used for comparison. Transgenic SOD1<sup>GSGr</sup> mice were used at onset of symptoms as previously defined (39) to provide a positive control for ALS-like phenotype.

Generation of Vapb-loxp and Vapb<sup>−/−</sup> mice
Floxed exon 3 Vapb mice were generated in the Institut Clinique de la Souris (ICS, Illkirch) using standard procedures. The Neomycin cassette was deleted through a FRT recombinase step. Complete Vapb<sup>−/−</sup> mice were generated through crossing with CMV-Cre animals. The recombination in double transgenic animals was total and germline transmission of the deleted allele was obtained. The genetic background of all mice used in this study is >99% C57Bl6/N Tac.

Behavioural analysis
We used the SHIRPA (SmithKline Beecham, Harwell, Imperial College School of Medicine at St Mary’s; Royal London Hospital, St Bartholomew’s and the Royal London School of Medicine; Phenotype Assessment) screening procedure to grossly characterize the phenotype of mice. These tests consist of using standard methods such as open field, jar view and grid tests to provide a behavioural and functional profile by rapid and simple observational assessment. The SHIRPA procedure is commonly used for phenotyping purposes (40). Mouse motor performance was assessed using Rotarod (model 7650, Ugo Basile, Comerio, Italy). Each session consisted of three tests of 330 s with an acceleration period (4 to 20 rpm during 150 s) followed by 150 s at constant speed. Results are the mean of four consecutive weekly sessions performed after 2 weeks of habituation. To evaluate muscle strength, we used a gripmeter (ALG01; Bioseb, Vitrolles, France). The muscle force (in Newton) was measured three times per mouse. Results are the mean of three consecutive weekly sessions.

Electromyography
Electromyography was performed as previously described (41,42). Mice (n = 9 +/+; n = 9 +/−; n = 9 −/−) were anaesthetized with a solution of ketamine/xylasine (100 mg/kg; 5 mg/kg) and electrical activity was recorded using a monopolar needle electrode (diameter 0.3 mm; 9013R0312; Medtronic, Minneapolis, MN) inserted into the tail of the mouse. Recordings were made with a concentric needle electrode (diameter 0.3 mm; 9013S0011; Medtronic). Electrical activity was monitored in both GA and TA on both legs for at least 2 min. Spontaneous activity was differentiated from voluntary activity by visual and auditory inspection.

Immunostaining
Immediately after sacrifice, muscle tissues were dissected and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (1 h). Tissues were then rinsed three times with PBS and stored at 4°C until use. The prepared muscles bundles were stained using fluorescently labeled α-Bungarotoxin-TRITC (Sigma, Saint-Quentin Fallavier, France) and an anti-synaptophysin antibody (Epitomics). For post-synaptic morphology quantification, bungarotoxin signals from more than 25 NMJs per animal were imaged under a fluorescent microscope. The data were analysed as previously described (42). For double labelling or post- and pre-synaptic parts of the NMJs, images were acquired using a laser scanning microscope (LSM 510; Carl Zeiss, Thornwood,NY) equipped with a Plan-Apochromat 63x oil DIC immersion lens (numerical aperture 1.4). Synaptophysin-Alexa 488 was excited using the 488 nm ray of the argon laser. The emission signal of Alexa 488 was filtered with a BP505-530nm. Bungarotoxin-TRITC was excited using a 543-nm Helium Neon laser. The emission signal of TRITC was filtered with a BP560-615nm. Fluorescent DNA dye-Draq5 was excited using a 633-nm Helium Neon laser. The emission signal of Draq5 was filtered with a LP 650 nm.

Constructs
Each mutation (P56S, A145V, and S160Δ) was introduced into the human VAPB clone by site-directed mutagenesis. The three mutant VAPB cDNAs, as well as the wild-type cDNA, were cloned into a pCS2 vector and were transcribed in vitro using the SP6 mMESSAGE mMachine kit (Ambion). The protein expression from each of these constructs was validated by western blot analysis with an anti-VAPB antibody (kindly provided by Dr Kanekura). A band at the appropriate molecular weight (∼27 kDa for VAPB) was observed (result not shown).

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Vapb
AMO) binds to the ATG of the previously described, at an optimal concentration of 1.0 mM (30).
Gene Tools (Gene Tools, Oregon, USA). AMO injections were used as a control. Both were designed and purchased from Gene Tools (Gene Tools, Oregon, USA). AMO injections were performed in one-to-four cell stage zebrafish eggs as previously described, at an optimal concentration of 1.0 mM (30). The rescue experiments were performed as mentioned above, with simultaneous injections of the AMO and human VAPB mRNA at concentrations of 1.0 mM and 80 ng/μl respectively.

Behavioural and immunocytochemical analysis
Morphology and behavioural touch responses were assessed with a stereomicroscope (Zeiss, Oberkochen, Germany). To elicit the escape response at 48 hpf, embryos were touched lightly on the tail with a pair of blunt forceps and their swimming episodes were recorded using a Photron (San Diego, CA, USA) Fastcam PCI high-speed video camera at 125 frames/s. For immunohistochemical analysis of axonal projections of motor neurons, monoclonal antibodies against synaptic vesicle 2 (anti-SV2m Developmental Studies Hybridoma) was used to label pre-synaptic axons at the NMJ and anti-acetylated alpha-tubulin (Sigma) was used to label all neuronal tubulins.

Mouse studies
Animal housing
Transgenic mice were housed in the animal facility of the medicine faculty of Strasbourg University, with 12 h/12 h of light/dark and unrestricted access to food and water. In all experiments, littermates were used for comparison. Transgenic SOD1<sup>GSGr</sup> mice were used at onset of symptoms as previously defined (39) to provide a positive control for ALS-like phenotype.

Generation of Vapb-loxp and Vapb<sup>−/−</sup> mice
Floxed exon 3 Vapb mice were generated in the Institut Clinique de la Souris (ICS, Illkirch) using standard procedures. The Neomycin cassette was deleted through a FRT recombination step. Complete Vapb<sup>−/−</sup> mice were generated through crossing with CMV-Cre animals. The recombination in double transgenic animals was total and germline transmission of the deleted allele was obtained. The genetic background of all mice used in this study is >99% C57Bl6/N Tac.

Behavioural analysis
We used the SHIRPA (SmithKline Beecham, Harwell, Imperial College School of Medicine at St Mary’s; Royal London Hospital, St Bartholomew’s and the Royal London School of Medicine; Phenotype Assessment) screening procedure to grossly characterize the phenotype of mice. These tests consist of using standard methods such as open field, jar view and grid tests to provide a behavioural and functional profile by rapid and simple observational assessment. The SHIRPA procedure is commonly used for phenotyping purposes (40). Mouse motor performance was assessed using Rotarod (model 7650, Ugo Basile, Comerio, Italy). Each session consisted of three tests of 330 s with an acceleration period (4 to 20 rpm during 150 s) followed by 150 s at constant speed. Results are the mean of four consecutive weekly sessions performed after 2 weeks of habituation. To evaluate muscle strength, we used a gripmeter (ALG01; Bioseb, Vitrolles, France). The muscle force (in Newton) was measured three times per mouse. Results are the mean of three consecutive weekly sessions.

Electromyography
Electromyography was performed as previously described (41,42). Mice (n = 9 +/+; n = 9 +/−; n = 9 −/−) were anaesthetized with a solution of ketamine/xylasine (100 mg/kg; 5 mg/kg) and electrical activity was recorded using a monopolar needle electrode (diameter 0.3 mm; 9013R0312; Medtronic, Minneapolis, MN) inserted into the tail of the mouse. Recordings were made with a concentric needle electrode (diameter 0.3 mm; 9013S0011; Medtronic). Electrical activity was monitored in both GA and TA on both legs for at least 2 min. Spontaneous activity was differentiated from voluntary activity by visual and auditory inspection.

Immunostaining
Immediately after sacrifice, muscle tissues were dissected and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (1 h). Tissues were then rinsed three times with PBS and stored at 4°C until use. The prepared muscles bundles were stained using fluorescently labeled α-Bungarotoxin-TRITC (Sigma, Saint-Quentin Fallavier, France) and an anti-synaptophysin antibody (Epitomics). For post-synaptic morphology quantification, bungarotoxin signals from more than 25 NMJs per animal were imaged under a fluorescent microscope. The data were analysed as previously described (42). For double labelling or post- and pre-synaptic parts of the NMJs, images were acquired using a laser scanning microscope (LSM 510; Carl Zeiss, Thornwood,NY) equipped with a Plan-Apochromat 63x oil DIC immersion lens (numerical aperture 1.4). Synaptophysin-Alexa 488 was excited using the 488 nm ray of the argon laser. The emission signal of Alexa 488 was filtered with a BP505-530nm. Bungarotoxin-TRITC was excited using a 543-nm Helium Neon laser. The emission signal of TRITC was filtered with a BP560-615nm. Fluorescent DNA dye-Draq5 was excited using a 633-nm Helium Neon laser. The emission signal of Draq5 was filtered with a LP 650 nm.
VAPB Antibody and western blotting

A rabbit polyclonal antibody directed against mouse VAPB was prepared using the double X programme of Eurogentec (Belgium) and the following epitope (SLEPHELKFRGPT+C) present in exons 1/2 of murine VAPB. Antisera were immuno-purified against the peptide. Western blotting was performed as previously described (43). Pre-incubation with an excess of the peptide extinguished the signal in western blots (data not shown).

Real-Time PCR

RNA was reverse transcribed using 1 μg of RNA and the iScript cDNA synthesis kit (Bio-Rad, Marne La Coquette, France). We performed a real-time PCR (IQSYBR GREEN Supermix, BioRad, France) on the cDNA obtained from the RNA samples. Three reference genes were used to compute a normalization factor using Genorm software v3.5: 18S (18S Ribosomal RNA), Pol2 (Pol2 polymerase RNA 2 DNA directed polypeptide A) and Tbp (TATA-box binding protein) (44). Primer sequences are provided in Supplementary Material, Table S1.

Statistical analysis

Statistical comparisons were accomplished with the unpaired Student t-test or ANOVA followed by the post-hoc Newman–Keuls multiple comparisons test using PRISM version 5 for MacOS X software (GraphPad, San Diego) unless otherwise indicated.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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