Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality

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F-actin bundling plastin 3 (PLS3) is a fully protective modifier of the neuromuscular disease spinal muscular atrophy (SMA), the most common genetic cause of infant death. The generation of a conditional PLS3-over-expressing mouse and its breeding into an SMA background allowed us to decipher the exact biological mechanism underlying PLS3-mediated SMA protection. We show that PLS3 is a key regulator that restores main processes depending on actin dynamics in SMA motor neurons (MNs). MN soma size significantly increased and a higher number of afferent proprioceptive inputs were counted in SMAPLS3 compared with SMA mice. PLS3 increased presynaptic F-actin amount, rescued synaptic vesicle and active zones content, restored the organization of readily releasable pool of vesicles and increased the quantal content of the neuromuscular junctions (NMJs). Most remarkably, PLS3 over-expression led to a stabilization of axons which, in turn, resulted in a significant delay of axon pruning, counteracting poor axonal connectivity at SMA NMJs. These findings together with the observation of increased endplate and muscle fiber size upon MN-specific PLS3 over-expression suggest that PLS3 significantly improves neuromuscular transmission. Indeed, ubiquitous over-expression moderately improved survival and motor function in SMA mice. As PLS3 seems to act independently of Smn, PLS3 might be a potential therapeutic target not only in SMA but also in other MN diseases.

INTRODUCTION

As motor growth cones arrive at destined muscle sites, the neuromuscular junction (NMJ) maturation process is initiated by presynaptic release of acetylcholine (ACh) and agrin signals, refining local concentration of ACh receptors (AChR) to form the endplate (1,2). Concomitantly, in a process termed axon pruning, exuberant neuronal branches are removed and endplates finally become innervated by a single axon (3). Disturbance of NMJ maturation and integrity is a classic hallmark of the neurodegenerative disease spinal muscular atrophy (SMA). During SMA progression, NMJ degradation timely precedes the selective loss of spinal α-MNs in the ventral horns of the spinal cord (4), finally resulting in muscle weakness, paralysis and eventual death due to respiratory failure (5,6). Several studies using cellular systems, zebrafish as well as different SMA mouse models have shown severely compromised neuronal specification, defects in axon elongation and pathfinding as well as reduced presynaptic coverage of endplates (7–11). Moreover, a reduction and
abnormal distribution of synaptic vesicles (SV) have been observed in SMA motor nerve terminals, resulting in reduced neurotransmission, retarded AChR clustering and impaired muscle excitability (12–14).

SMA is caused by the loss of the survival motor neuron gene 1 (SMN1) (15), whereas the severity of the phenotype primarily depends on the SMN2 copy number (16). SMN is ubiquitously expressed and has housekeeping functions such as small nuclear ribonucleoprotein assembly (17,18), translational regulation (19) and stress response (20) in various cell types. However, the selective vulnerability of MNs combined with the predominantly axonal localization of SMN early suggested an additional axon-specific function of the protein.

The identification of the F-actin bundling protein plastin 3 (PLS3) as the first fully protective SMA modifier in humans (21) opened the possibility toward understanding the mechanism underlying SMA pathology. High PLS3 levels have been shown to protect individuals from developing SMA despite carrying a homozygous deletion of SMN1. PLS3 has been demonstrated to influence F-actin levels and to rescue axon growth defects associated with SMN reduction in cultured primary SMA MNs and in zebrafish (21,22). In contrast, SMA mice—and likely humans—show no axon outgrowth disturbances of MNs in vivo but rather a failure in NMJ maturation and maintenance (reviewed in 23). At NMJ level, F-actin is involved in actin caging of SVs of the reserve pool (RP) and transport of such to active zones (AZs) (24). Moreover, recent studies performed in SMN-deficient cells and SMA mice provided further evidence for actin dynamics involvement in SMA pathogenesis. In this context, a modified phosphorylation status of the Rho-kinase-downstream targets cofilin, myosin-light chain and profilin IIa, which influences the polymerization and depolymerization of actin and induces neuron-specific changes of the F/G actin ratio, has been demonstrated (25). Furthermore, the treatment of an intermediate SMA mouse model with fasudil, a RhoA/Rho kinase inhibitor, highly increased survival and motor abilities (26). These findings strongly highlight the importance of accurate F-actin networking at NMJ level to counteract SMA.

Despite this wealth of knowledge, the exact mechanisms underlying PLS3-mediated protection in unaffected patients of discordant families remain elusive. To address this question, we generated mice conditionally over-expressing human PLS3 and studied the effects in an SMA mouse model. Most remarkably, PLS3 over-expression resulted in a significant increase of motor axon inputs during the process of axon pruning, allowing prolonged polyinnervation of endplates and maturation of the NMJs. It has been shown that endplates in SMA mice appear either poorly innervated or even denervated (27). Over-expression of PLS3 resulted in improved neuromuscular transmission as well as neurotransmission at the NMJ and, likely as a consequence, in a profound increase in endplate and muscle fiber size. In strong support of these observations, electron as well as confocal microscopy studies revealed a restoration of SV organization and synaptic F-actin amount at the NMJ of PLS3 over-expressing mice. Our data highlight PLS3 as a potential therapeutic target in SMA but also other neuromuscular diseases such as myasthenia gravis or amyotrophic lateral sclerosis (ALS) and, beyond this, demonstrate a yet novel role of PLS3 in NMJ maturation, maintenance and function.

RESULTS

Generation of a conditional PLS3V5 transgenic allele in mice

We inserted the human plastin 3 cDNA fused to a V5 tag at the 3′ end (PLS3V5) into a ROSA26 locus-targeting vector (28) (Fig. 1A). The V5 tag allowed differentiation between the PLS3 transgene and murine Pls3, which are 99% identical. The PLS3V5 expression is driven by a cytomegalovirus (CMV) enhancer/chicken β-actin (CAG) promoter (28). The expression of PLS3V5 is suppressed by a loxP-flanked stop cassette inserted between the promoter and PLS3V5. Only upon removal of the stop cassette by Cre recombination, PLS3V5 will be tissue-specifically expressed (Fig. 2A).

In addition, the targeting construct contains 3′ to PLS3V5 a green fluorescent protein (GFP) cloned behind an internal ribosomal entry site allowing indirect detection of the transgene in vivo (Fig. 1C). Transgenesis and Southern blot analyses, to ensure correct integration of the construct, were performed as described in detail in Materials and Methods and as shown in Figure 1A–G to finally produce the conditional PLS3V5flSt allele in mice.

Generation of a ubiquitously PLS3V5-expressing transgenic mouse (PLS3V5tg)

To allow permanent PLS3V5 expression, the PLS3V5flSt line was crossed with a ubiquitously expressing CMV-Cre deleter line (29) to generate the PLS3V5tg line (Fig. 2A). Once the stop cassette has been removed in germ cells, all subsequent offspring will permanently express the CAG-driven PLS3V5 transgene. To exclude background-dependent modifying effects, prior to further analysis, PLS3V5flSt and PLS3V5tg mice were backcrossed with C57BL/6N wild-type (wt) mice for seven generations to produce pure congenic lines.

Total plastin 3 mRNA (PLS3V5 plus murine Pls3) levels were determined in various tissues of heterozygous mice of the PLS3V5tg line (PLS3V5tg) and compared with wt mice expressing only Pls3. Using primers amplifying both transcripts simultaneously, PLS3V5tg mice showed significantly elevated total plastin 3 mRNA levels with 3.6- to 3.4- and 21.5-fold up-regulation above wt level in brain, spinal cord and muscle, respectively (Fig. 2B). Importantly, intrinsic Pls3 mRNA levels remained unchanged in PLS3V5tg mice, excluding a negative-regulatory feedback loop of PLS3V5 on Pls3 transcription (Fig. 2C).

Staining of proteins isolated from spinal cord and muscle, the two mainly affected tissues in SMA from PLS3V5tg and wt mice with V5 and PLS3 antibodies, revealed clear expression of both the transgene and endogenous Pls3 (Fig. 2D). Additionally, a comparative expression of the transgene in multiple tissues is shown in Supplementary Material, Figure S1A.

To find out whether the PLS3 transgene is functional and the protein properly localized, murine embryonic fibroblasts (MEFs) were isolated from PLS3V5tg and wt embryos and immunostained. In PLS3V5tg MEFs, PLS3V5 stained with V5 was present as a diffuse signal throughout the entire cell body showing slight accumulation in stress fibers and in
perinuclear regions. It co-localized with F-actin and endogenous Pls3 (Fig. 2E and Supplementary Material, Fig. S1B and C). In contrast, wt MEFs exhibited no V5 fluorescent signals, demonstrating specificity of the antibody. PLS3V5 was highly enriched in filopodia at sites of F-actin filament consolidation (Fig. 2E) and exactly matched Pls3 wt localization in PLS3V5tg/0-derived MEFs (Supplementary Material, Fig. S1C), demonstrating functionality of the transgene. It has previously been reported that Pls3 over-expression results in the formation of thicker microvilli on the surface of polarized epithelial cells (LLC-PK1) (30). In line with these findings, PLS3V5tg/0 MEFs exhibited an increased filopodia number and extensive lamellipodial growth, likely as a result of local cytoskeletal stabilization (Fig. 2F and Supplementary Material, Fig. S1D).

Ubiquitous PLS3V5 expression ameliorates neither survival nor motor ability but results in endplate and muscle fiber size increase in severe SMA mice on pure C57Bl/6N background

To investigate the potential beneficial effects of PLS3V5 overexpression on the SMA phenotype, we crossed the transgenic mice with the severely affected Taiwanese SMA model. Since the PLS3V5 transgenic mice were on C57Bl/6N and to reduce background influences, we backcrossed the SMA mice from FVB/N (11) onto a pure C57Bl/6N background for seven generations. Interestingly, SMA mice on pure C57Bl/6N survived 50% longer (15.6 ± 0.6 days) than on pure FVB/N background (9.9 ± 0.5 days) (Supplementary Material, Fig. S4A). Using our breeding scheme (Fig. 3), we were able to obtain
SMA, SMA$_{PLS3V5}$, HET and HET$_{PLS3V5}$ controls with a statistical occurrence of 25% each in a single litter. It therefore offers a highly efficient production of SMA-like mice compared with other existing mouse models and breeding strategies (11).

Unexpectedly, SMA and SMA$_{PLS3V5}$ mice showed almost identical weight progression and mean survival times (Fig. 4A and B). In contrast, and as previously reported (11,31), one functional Smn allele is sufficient to restore weight and survival of HET controls to normal level. HET$_{PLS3V5}$ mice were indistinguishable from HET controls, both showing identical weight progression (Fig. 4B) as well as survival times. Since SMA patients suffer from severe motor impairments, we next analyzed the motor ability of PLS3V5-expressing SMA mice by applying the tube test and the righting reflex test (32). As expected, SMA mice showed
markedly reduced motor abilities in both tests when compared with HET; however, no differences were observed between SMA and SMA
PLS3V5 mice (Fig. 4C and D).

As MNs are most severely affected by low SMN levels, defects of the neuromuscular system are the major hallmark of SMA, ultimately resulting in atrophy of proximal muscles (reviewed in 23). Accordingly, it has been described that different SMA mouse models show markedly reduced postsynaptic endplate and muscle fiber size (12,33). To investigate whether high PLS3 levels affect endplate or muscle fiber size, we performed immunohistochemistry on cross-sections of gastrocnemius (GC) muscle from P10 animals for all four genotypes. Strikingly, but in contrast to our observations from the motor tests, muscle fiber size was highly significantly increased in SMA
PLS3V5 compared with SMA mice (SMA
PLS3V5: 105.0 ± 1.3 μm², SMA: 88.0 ± 1.2 μm², P < 0.001), whereas PLS3V5 had no effect on muscle fiber size of HET controls (HET: 165.2 ± 3.9 μm², HET
PLS3V5: 159.2 ± 2.2 μm²) (Fig. 4E). This effect was accompanied by a clear increase in endplate size in SMA
PLS3V5 and HET
PLS3V5 mice compared with SMA and HET controls, respectively (SMA
PLS3V5: 141.7 ± 1.1 μm², SMA: 121.2 ± 3.4 μm², HET
PLS3V5: 190.4 ± 2.9 μm², HET: 162.1 ± 1.2 μm², P-value in all experiments <0.001) (Fig. 4F).

**PLS3V5 over-expression increases axon input number during axon pruning, improves neuronal connectivity and triggers AChR clustering at severe SMA NMJs**

During NMJ maturation, AChR clusters (endplates) are first present as ovoid plaques but later restrict to sites of direct nerve terminal innervation, resulting in the typical pretzel-like appearance of endplates (34). Given that **PLS3** over-expression led to an increase in endplate size in GC muscle and to examine whether postsynaptic receptor reorganization in SMA mice occurs as a result of loss of innervation, we investigated the axon pruning process in proximal *transversus abdominis* (TVA) muscle, one of the most affected muscle in SMA mice. Normally, in the first 2 weeks of postnatal development in mice, each motor endplate is innervated by several axons (polyinnervation). During axon pruning, however, finally all but one axon retract (35). As expected, the mean number of axons innervating one single endplate decreased constantly from P1 until P8 in all four genotypes (Fig. 5A and B, and Supplementary Material, Fig. S2). At P11, mean axon numbers approached values close to 1 in all tested genotypes, including SMA mice, suggesting a completion of the axon pruning process also in severely affected SMA mice. At all time points measured from P1 until P8, the mean axon number per endplate was significantly reduced in SMA mice compared with HET controls, indicating poorer neuromuscular connectivity in the SMA TVA muscle (Fig. 5B). Most strikingly, SMA
PLS3V5 mice displayed an increased axon number per endplate compared with SMA animals from P1 until P8, which at P1 and P4 exceeded even that of HET mice [P1: SMA
PLS3V5: 2.27 ± 0.04 axon inputs/endplate, HET: 1.93 ± 0.02 axon inputs/endplate (P < 0.001); P4: SMA
PLS3V5: 1.83 ± 0.01 axon inputs/endplate, HET: 1.61 ± 0.01 axon inputs/endplate (P < 0.001)]. Since MN development and axon growth are normal during embryonic and perinatal stages in SMA mice (36), our findings support a possible axon-stabilizing effect of **PLS3V5** during embryonic development and during the postnatal axon pruning process. This, as a consequence, results in a prolonged continuance of exuberant axons on individual endplates, counteracting poor axonal connectivity at SMA NMJs in the two weeks of life during NMJ maturation. Moreover, the observation of increased axon number was not restricted to SMA
PLS3V5 mice but could also be seen in HET
PLS3V5 mice, indicating a general regulatory role of PLS3 on axon growth and stabilization. As SMA
PLS3V5 and HET
PLS3V5 mice showed by trend a similar number of axon connections over the time course, these findings indicate that PLS3 might exert its effects on axon integrity independently of Smn.

We hypothesized that delayed axon pruning might have a beneficial transient impact on endplate development as we previously observed increased sizes for GC muscle of P10 SMA
PLS3V5 and HET
PLS3V5 mice (Fig. 4F). To investigate a possible connection between increased axon number and endplate size, we next determined endplate size of the four genotypes at the given time points and compared our findings with axon input numbers. Starting at P4, the endplate size of SMA
PLS3V5 significantly increased compared with SMA mice and correlated with a higher axon input number, suggesting a beneficial effect of delayed axon pruning on AChR clustering (Fig. 5A–C and Supplementary Material, Fig. S2). At P11, mean axon numbers approached values close to 1 in all tested genotypes, including SMA mice, suggesting a completion of the axon pruning process also in severely affected SMA mice. We detected a significant improvement of the occupancy and arborization level in SMA
PLS3V5 compared with SMA mice (Fig. 5D). According to our findings from axon input quantifications, this effect was clearly visible also in HET
PLS3V5 mice compared with HET controls.
PLS3V5 prevents proprioceptive input loss in severe SMA mice and increases MN soma size

It has been shown that SMA mice display severely diminished connectivity between primary proprioceptive afferents and MN cell bodies (38). To test whether PLS3V5 over-expression is capable of counteracting afferents loss, we immunostained their synapses in the lumbar L4–L5 spinal region, using antibodies against vesicular glutamate transporter 1 (VGlut1), a known marker for proprioceptive primary afferents, and choline acetyltransferase (ChAT) to mark MNs. As previously described in the SMNA7 mouse model (38), we observed a clear reduction of VGlut1 signals per MN in SMA mice compared with HET controls at the late symptomatic time point P10 (SMA: 20 ± 1.9 inputs/MN, HET: 36.4 ± 2.7 inputs/MN, P < 0.001) (Fig. 5E). Afferent input number...
was fully restored to HET control level in SMAPLS3V5 mice (SMAPLS3V5: 36.4 ± 2.9 inputs/MN). We next determined the size of MN somata and found that PLS3V5 restored MN size of SMA mice to control level (SMA: 615.4 ± 56.5 μm²; SMAPLS3V5: 805.1 ± 66.4 μm²; HET: 739.6 ± 47.7 μm²; SMA versus SMAPLS3V5: *P < 0.05; SMAPLS3V5 versus HET: **P < 0.01).

Figure 5. PLS3V5 effects on NMJs and MN in SMA mice on pure C57Bl/6N. (A–C) Motor axon input number per endplate and endplate size in the TVA muscle of SMA, SMAPLS3V5, HET and HETPLS3V5 mice at P1–P11 on pure C57Bl/6N background (input numbers and endplate sizes were determined for 100 NMJs per animal). Three animals were analyzed per genotype and time point; staining: neurofilament and SV2 (NF/SV2, green) and bungarotoxin (BTX, red) (see also Supplementary Material, Fig. S2). (D) The graph shows the occupancy levels of 100 NMJs of P4 mice (*n* = 3 per genotype) classified as weakly occupied (type I), partially occupied (type II) and fully occupied (type III) NMJs, as illustrated in the representative left panels. (E) Proprioceptive input number/MN estimation (VGlut, green) and MN size (ChAT, red) measurements of lateral MNs in L4–L5 spinal sections (100 μm thickness) at P10. The proprioceptive nerves which were in direct contact (inputs) with MN somata were counted. The total distance in the z-axis for all measurements was 1.4 μm [seven optical sections at 0.2 μm intervals (three lower and three upper sections relative to the broadest middle section)]. Inputs were separately counted for each of the seven layers (number in the diagram indicates total number of MNs analyzed, *n*_{SMA} = 3, *n*_{SMA PLS3V5} = 3, *n*_{HET} = 5, *n*_{HET PLS3V5} = 3, scale: 50 μm). Error bars indicate SEM. *P < 0.05, **P < 0.01, ***P < 0.001, n.s. = not significant.
versus HET: $P > 0.05$) (Fig. 5E). An additional impact of PLS3V5 on MN size was observed in HET_{PLS3V5} compared with HET mice (HET_{PLS3V5}: $114.6.8 \pm 94.2 \mu m^2; P < 0.001$). These findings indicate that PLS3V5 exerts similar stabilizing effects on proprioceptive afferents as we previously observed for motor axon terminals at NMJ level, possibly explaining increased MN soma size.

MN-specific expression of PLS3V5 is sufficient to increase endplate and muscle fiber size

We showed that ubiquitous PLS3V5 over-expression results in an increase in endplate size in both SMA and HET animals in the GC and TVA muscles (Fig. 4F and 5C). Additionally, we observed a higher degree of polyinnervation at various postnatal time points in PLS3V5-expressing SMA and HET mice (Fig. 5B). This led us to hypothesize that prolonged abundance of exuberant nerve terminals might positively influence AchR clustering in PLS3V5 transgenic mice, e.g. through improved neurotransmission. To assess the impact of MN-specific PLS3V5 over-expression on endplate size, we crossed the conditional PLS3V5^{fl_st}/0 line to the MN-specific Homeobox9 (Hb9-Cre) line (39). In Hb9-Cre mice, Cre is integrated into the Hb9 gene locus and driven by the intrinsic Hb9 promoter, thereby disrupting Hb9 gene function. Whereas homozygous Hb9-Cre mice (Hb9-Cre^{tg/tg}) display perinatal lethality as well as severe defects in AchR clustering due to motor axon loss, mice heterozygous for the Cre transgene (Hb9-Cre^{tg/0}) are fully viable and fertile (40). After demonstrating MN-specific PLS3V5 expression by immunohistochemistry of spinal cord sections and lack of expression in muscle by western blot analysis (Supplementary Material, Fig. S3A and B), we next assessed endplate size in the TVA muscle of PLS3V5^{fl_st}/0, Hb9-Cre^{tg/0} and PLS3V5^{fl_st}/0;Hb9-Cre^{tg/0} (PLS3V5-Hb9) mice at P21. According to Hb9 function in MN differentiation and axon growth, we found significantly reduced endplate size in Hb9-Cre^{tg/0} mice compared with PLS3V5^{fl_st}/0 controls (Hb9-Cre^{tg/0}: $360.6 \pm 5.4 \mu m^2$; PLS3V5^{fl_st}/0: $394.8 \pm 6.3 \mu m^2$, $P < 0.001$) (Fig. 6A). Interestingly, MN-specific activation of the transgene in PLS3V5-Hb9 mice led to a full restoration of endplate size to normal levels similar to that of PLS3V5^{fl_st}/0 mice (PLS3V5-Hb9: $388.5 \pm 5.5 \mu m^2$, $P > 0.05$). Most strikingly, however, the increase in AchR clustering was paralleled by a significant increase in TVA muscle fiber size of PLS3V5-Hb9 mice compared with Hb9-Cre^{tg/0} mice but also PLS3V5^{fl_st}/0 mice (PLS3V5^{fl_st}/0-Hb9: $246.6 \pm 6.8 \mu m^2$, Hb9-Cre^{tg/0}: $165.6 \pm 3.4 \mu m^2$, PLS3V5^{fl_st}/0: $227.7 \pm 4.6 \mu m^2$, $P < 0.001$ and $P < 0.05$, respectively) (Fig. 6B and C). Furthermore, we observed similar effects of PLS3V5 on endplate and muscle fiber size also in GC and vastus lateralis (VL) muscle (Supplementary Material, Fig. S3B–D). Taken together, these results suggest that PLS3V5 over-expression in the presynapse is sufficient to increase AchR clustering and muscle fiber size.

Smn levels do not affect Pls3 levels and vice versa in mice

We have previously shown in discordant SMA families that asymptomatic SMN1-deleted siblings show high PLS3 expression but similar SMN levels compared with their affected siblings, suggesting an SMN-independent modifying role of PLS3 (21). In contrast, recent studies using zebrafish showed that when Smn is decreased, Pls3 is also decreased and it has been suggested that Smn likely controls Pls3 translation (22). Furthermore, a rather weak association between Smn levels and endogenous Pls3 expression was found in a mild SMA model (SMN^{2B/-} model) (41). To investigate whether Smn regulates Pls3 levels in the Taiwanese SMA mouse model, we determined Pls3 levels in brain and spinal cord of SMA and HET mice. In both tissues, Pls3 was not decreased in SMA mice compared with HET controls (Fig. 7A and B), indicating different regulatory modes in fish but perhaps even between different SMA mouse models. Furthermore, to investigate a possible effect of PLS3V5 on the Smn amount, we isolated brain and spinal cord tissue from PLS3V5^{tg/0} and wt mice and measured Smn protein levels. In line with studies in zebrafish (22), the Smn amount was unaffected by PLS3V5 over-expression (Fig. 7C and D). Taken together, our results are in line with our previous findings in discordant SMA families (21) and further support the view of an Smn-independent ameliorative action of PLS3V5 on the SMA phenotype in mammals.

PLS3V5 over-expression ameliorates the phenotype of mixed50 SMA mice

According to the threshold theory of SMA, MNs are most severely affected by low SMN levels and thus demarcate the one end of a vulnerability-resistance spectrum (reviewed in 23). As SMN protein levels further decrease, other tissue types such as bone, heart, liver, intestine, lung, vasculature, hippocampal structures of the brain or sensory neurons get involved in SMA pathology, highlighting the systemic character of the disease (38,42–51). Here, we show that PLS3V5 over-expression leads to significant improvements of neuromuscular connectivity and muscle fiber size in the Taiwanese SMA model on pure C57Bl/6N background, however, without ameliorating motor impairment or the survival phenotype. Therefore, we speculated that the severe multi-organ defects associated with low SMN levels, as recently shown by us in the Taiwanese SMA model (48), might mask the observed positive effects of high PLS3V5 levels on the neuromuscular system. To test our hypothesis, we next over-expressed PLS3V5 in a milder SMA mouse model. As we observed a clear amelioration of SMA symptoms in SMA mice of mixed genetic background that is independent of the Smn amount (Supplementary Material, Fig. S4A–F), we developed a breeding scheme allowing PLS3V5 over-expression in statistically 50%FVB/N/50%C57BL/6N background in F1 mice, herein after referred to as mixed50 mice (Fig. 3). Although the use of mice with randomly mixed background has been shown to result in large phenotypic differences (31,33), our breeding scheme guarantees an identical ‘mixed’ background in the F1 generation, rendering a reduced phenotypic variability. In support of our hypothesis, we found a moderate but significant increase in mean survival of SMAPLS3V5 compared with SMA mice on this background [SMA: 16.9 ± 1.1 days (100 ± 6.1%), SMAPLS3V5: 19.4 ± 1.2 days (114.7 ± 6.4%), $P < 0.05$] (Fig. 8A). Additionally, SMAPLS3V5 mice showed an improved weight gain compared with SMA mice [e.g. P14: SMA: 5.0 ± 0.2 g (100 ± 5.0%), SMAPLS3V5: 6.1 ±
0.3 g (12.2 ± 5.0%), P < 0.01; P20: SMA: 5.5 ± 0.4 g (100 ± 7.3%), SMA: 5.5 ± 0.4 g (100 ± 7.3%), P < 0.001, n.s. = not significant.

**Figure 6.** HB9-specific PLS3V5 expression and its effect on endplate size and muscle fiber size on wt mice. (A and B) Endplate and muscle fiber size measurements in PLS3V5-expressing HB9-Cre and PLS3V5-Hb9 mice at P21 (n = 3 per genotype and time point). (C) Representative picture of the TVA muscle from PLS3V5-expressing HB9-Cre and PLS3V5-Hb9 mice (H & E staining, scale: 50 μm) (see also Supplementary Material, Fig. S3 for specific V5 expression in MN and effects at P10). Error bars indicate SEM. *P < 0.05, ***P < 0.001, n.s. = not significant.

**Figure 7.** PLS3V5 does not influence the expression of SMN. (A and B) Quantitative western blot analysis of murine P33 and brain spinal cord tissue of P10 SMA and HET mice (n = 3 per genotype). (C and D) Quantitative western blot analysis of Smn/SMN amounts in brain and spinal cord tissue of P10 PLS3V5tg/0 and wt mice (n = 3 per genotype). Protein sizes in kilodalton. Error bars indicate SEM. n.s. = not significant.

PLS3V5 over-expression results in restoration of SV content in mixed SMA mice

Previous reports have shown that the amount of SVs at motor nerve terminals of SMN7 mice are reduced compared with wt terminals (12–14). To confirm this finding in the mixed SMA mouse model and to test whether PLS3V5 over-expression affects SV content and distribution, we performed immunostaining on the TVA muscle, using a primary antibody against vesicular ACh transporter (VACHT) at P4 and P14 (Fig. 9A and B). As previously reported (14), at P4, SVs appeared clustered, whereas at P14, vesicles were scattered throughout the terminal. The area occupied by SVs in SMA terminals was significantly smaller than in HET at P4 and P14 (33%, P < 0.05 and 34%, P < 0.01, respectively) (Fig. 9C). On the other hand, PLS3V5 restored SV area in mutants to HET levels at both ages (P < 0.05), although it had no influence in HET mice (P > 0.05) (Fig. 9C). To test whether the reduction in the area occupied by SVs in SMA mice could be due to reduced NMJ size, the SV/endplate area ratio was calculated. No significant differences were found among genotypes at P4; however, at P14, a significant increase in the SVs/endplate area ratio was detected in SMAPLS3V5 compared with SMA and HET terminals (both P < 0.05) (Fig. 9D). The distribution of the absolute values of SV area versus postsynaptic area for each genotype at P14 showed a clear correlation between SV content and endplate size (Supplementary Material, Fig. S6). Together, these data show that the over-expression of PLS3V5 restored SV content in SMA terminals to normal levels.

PLS3V5 over-expression restores AZ number in mixed SMA mice

Among the proteins that form the cytomatrix of AZs, Piccolo has been related with F-actin dynamics (52). A decrease in Piccolo reduces the amount of actin polymerization. Given that F-actin dynamics seem to be altered in SMA (25,33,41), we next investigated the abundance of Piccolo in all four genotypes on mixed SMA genetic background at P14. In all mice, Piccolo appeared as bright spots distributed homogeneously throughout the entire motor nerve terminal (Fig. 9E). To
estimate the relative amount of Piccolo, we next established an intensity threshold 3-fold the standard deviation of the fluorescence background of the terminal. This allowed us to determine Piccolo intensity and area at each terminal. Mean Piccolo intensity and area were significantly lower in SMA compared with HET terminals \((P, 0.001)\) (Fig. 9F and G), but were restored to normal levels in SMAPLS3V5 mice. Interestingly, PLS3V5 over-expression did not affect Piccolo signals in HET terminals. When the area of Piccolo was normalized to the postsynaptic size (Fig. 9H), no differences were evident among genotypes, suggesting that the low mean intensity of Piccolo in SMA (Fig. 9F) was due to the small size of SMA terminals.

**PLS3V5 over-expression restores F-actin amounts in presynapses of mixed\textsubscript{50} SMA mice**

To investigate F-actin distribution and abundance in presynaptic terminals, we labeled F-actin, using Phalloidin-Alexa647 in all four genotypes of mixed\textsubscript{50} genetic background mice at P14. The criteria for considering the Phalloidin signal belonging to the presynaptic terminal was to be within the NMJ boundary and in association with SVs (Fig. 9I). In all four genotypes, actin was found both in the sub-plasmalemmal region and deep in the cytoplasm forming a network (Fig. 9J). The fluorescence signal was strikingly less intense in SMA compared with HET terminals, and much higher in SMAPLS3V5 than in SMA mutants \(\sim 26\%\) reduction in SMA versus HET terminal \((P < 0.05)\), and \(\sim 34\%\) increase in SMAPLS3V5 versus SMA \((P < 0.001)\) (Fig. 9K). Indeed, PLS3V5 over-expression restored F-actin intensity of SMA presynapses to control levels \((P > 0.05)\).

**PLS3V5 over-expression restores organization of the readily releasable pool of vesicles in mixed\textsubscript{50} SMA mice**

It has been proposed that synaptic F-actin organizes vesicles into the readily releasable pool (RRP) as well as into the RP. SVs are tethered to F-actin in close proximity to the synaptic membrane via the phosphorylation-dependent linker protein synapsin (53). Previously, SMA mice have been shown to exhibit significantly reduced RRP vesicle numbers (14). This, in combination with the observation of severely impaired quantal content (QC) in SMA mice, suggested that reduced density of SVs in the RRP results in a reduced probability of vesicles to fuse with the membrane at SMA NMJs (12–14). To test whether PLS3V5 acts on vesicle organization in the RRP, we performed ultrastructure analysis using transmission electron microscopy (TEM) and determined the number of membrane-docked or -fused vesicles in SMA and SMAPLS3V5 mice of mixed\textsubscript{50} genetic background. Consistent with previously published data (12), SMA mice showed a strong reduction in the number of docked or fused vesicles compared with HET controls (67\%, \(P < 0.001\)) (Fig. 10). Most strikingly, however, we observed a complete rescue of the vesicle fusion phenotype in SMAPLS3V5 to normal levels (SMAPLS3V5: 6.3 ± 1.5 docked or fused vesicles/\(\mu\)m, HET:...
Figure 9. PLS3V5 effects on presynaptic vesicle content, AZ number and F-actin caging of mixed12 SMA mice. (A and B) Representative confocal maximal projection images of nerve terminal SVs, labeled with anti-VAChT antibody from TVA muscles of SMA, SMAPLS3V5, HET and HETPLS3V5 mice at P4 (A) and P14 (B) (scale: 5 μm). (C) Mean SV area (normalized to HET) of SMA at P4 (n = 19) and P14 (n = 29), SMAPLS3V5 at P4 (n = 23) and P14 (n = 28), HET at P4 (n = 21) and P14 (n = 28) and HETPLS3V5 at P4 (n = 19) and P14 (n = 26). (D) SV and postsynaptic area ratio. (E) Representative confocal maximal projection images showing Piccolo-stained nerve terminals from SMA, SMAPLS3V5, HET and HETPLS3V5 TVA muscles at P14. Insets illustrate Piccolo spots at higher magnification (scales: 5 and 1 μm, respectively). (F) Mean Piccolo intensity of SMA (n = 25 terminals), SMAPLS3V5 (n = 27 terminals), HET (n = 22 terminals) and HETPLS3V5 (n = 21 terminals) normalized to the HET value. (G) The mean Piccolo area normalized to the HET area. (H) The mean Piccolo and postsynaptic area ratio. (I) Single image showing actin–SV spatial relationship in a representative terminal. Note an actin loop (gray) surrounding a cluster of vesicles (green) (scale: 5 μm). (J) Representative confocal maximal projection images showing Phalloidin-Alexa647-stained nerve terminals from SMA, SMAPLS3V5, HET and HETPLS3V5 TVA muscles at P14. Red discontinuous lines mark the presynaptic perimeter. Signal outside the red boundary was not considered for presynaptic actin quantification (scale: 5 μm). (K) Phalloidin mean intensity for P4 SMA (n = 26 terminals), SMAPLS3V5 (n = 20 terminals), HET (n = 32 terminals) and HETPLS3V5 (n = 45 terminals) mice normalized to the HET value. Error bars indicate SEM. *P < 0.05, ***P < 0.001, n.s. = not significant.
5.9 \pm 1.9 \text{ docked or fused vesicles/}\mu\text{m, } P = \text{n.s.}) \text{ (Fig. 10A and B). Interestingly, PLS3V5 did not further increase the number of docked or fused vesicles in HET mice (HET}_{\text{PLS3V5}}: 7.4 \pm 2.8 \text{ docked or fused vesicles/}\mu\text{m, } P = \text{n.s.}). Together with the observations of restored vesicle and AZ number as well as increased F-actin levels in motor terminals of SMA}_{\text{PLS3V5}} \text{ mice, these findings indicate an essential role for PLS3V5 in proper presynaptic cytoskeletal regulation and, as a consequence, vesicle organization and release.}

**PLS3V5 over-expression ameliorates electrophysiological defects of mixed_{50} SMA mice**

By intracellular evoked endplate potential (EPP) recordings of muscle fibers, it has previously been shown that SMA motor terminals exhibit significantly reduced QC as well as RRP vesicle numbers, resulting in reduced neurotransmission and in part explaining insufficient NMJ maturation as well as motor defects in SMA mice \((12-14,26). Here, we observed a restoration of presynaptic F-actin amount, SV and AZ numbers as well as a rescued organization of RRP vesicles in SMA}_{\text{PLS3V5}} \text{ mice (Figs 9 and 10). These observations together with improved motor abilities of such mice (Fig. 8C and D) led us to investigate whether electrophysiological...
measurements would confirm our morphological findings in mixed \textsubscript{50} genetic background mice. As it has previously been reported for the SMN\textsubscript{Δ7} mouse model (13), our SMA mice showed an increased rise and decay time compared with HET controls (Fig. 11A). PLS3V5 over-expression in SMA mice, however, led to rise and decay times comparable with those of HET and HET\textsubscript{PLS3V5} mice (Fig. 11A), indicating improved kinetics of neuromuscular transmission.

It is well established that cortical F-actin of presynaptic terminals regulates SV accessibility to release sites and, therefore, impacts on vesicle release probability (54, 55). Indeed, we detected a significant increase in the QC and RRP size in SMA\textsubscript{PLS3V5} compared with SMA mice (\(P < 0.001\) and \(P < 0.05\), respectively), although below HET levels (Fig. 11B and C). Taken together, our electrophysiological data suggest that PLS3V5 might support vesicle release by positively influencing the presynaptic F-actin network.

**DISCUSSION**

PLS3 has been identified as a fully protective SMA modifier in humans (21). According to its function in F-actin bundling, our previous results from cell culture as well as zebrafish experiments suggested that high PLS3 levels increase the F-actin amount in MNs, finally resulting in improved axon outgrowth.

This observation has recently been confirmed in an SMA zebrafish model over-expressing pls3 (22). While reduced smn levels in zebrafish result in axon growth defects including truncations and early branching of motor axons (7, 21), SMA mice exhibit severe defects at NMJ level affecting NMJ integrity and function (12–14, 27). As NMJ defects timely precede spinal MN loss, a ‘dying back’ mechanism has been proposed to account for MN death at advanced developmental stages (36, 56, 57). More recent data showed that also afferent proprioceptive inputs on MN somata are massively reduced in SMA mice (28). Since over-expression of PLS3 protects against SMA and as PLS3 is an F-actin bundling protein, we assumed that misregulated actin dynamics should be the main cause underlying SMA pathology. The generation of a conditional PLS3 over-expressing mouse and its breeding into an SMA background allowed us to finally propose a model for the exact biological mechanism underlying PLS3-mediated SMA protection.

Here, we show that over-expression of human PLS3 caused a significant delay in axon pruning until P8 which counteracted the poor synaptic connectivity seen in SMA mice. We found increased F-actin amount in presynapses, significant improvement of neuromuscular connectivity, restoration of SV and AZ content, restored organization of the RRP, increased endplate and muscle fiber size and improved neurotransmission as shown by our electrophysiological data. In addition, MN cell body size significantly increased and a higher number of afferent proprioceptive inputs were counted in SMA\textsubscript{PLS3} compared with SMA mice. All these findings provide strong evidence for a protective effect of PLS3 on MN and NMJ development and functionality (Fig. 12).

Recent preclinical trial data using either antisense oligos restoring correct SMN2 splicing or adeno-associated viruses (AAV9) expressing SMN protein strongly support the view that only early administration (P0–P3) of these molecules/recombinant viruses is able to rescue the SMA phenotype (58–62). Postsymptomatic administration (after P6) has only weak or no effects in the severe SMA mouse model (61–63). These data suggest that severe SMA is a developmental disease in which rescuing mechanisms need to act right after birth in order to have the desired impact. In this respect, PLS3-mediated increase in axon input number seems to positively influence NMJ maturation and development, pointing at a new therapeutic target pathway to counteract SMA pathology.

As MN development and axon growth are normal during embryonic and perinatal stages in SMA mice (36), we showed that PLS3 over-expression results in a stabilization of existing incoming motor axons during the axon pruning process and thus delays axon pruning. Alternatively, as previously shown, PLS3 has an important role on axon growth in PC12 cells, zebrafish and cultured MNs (21, 22), and therefore the observed increase in axon input number in PLS3-expressing mice at perinatal stages might be caused by an enhanced initial axon outgrowth during early embryonic stages.

Unfortunately, we neither detected any improvements in motor ability nor survival in severely affected SMA\textsubscript{PLS3} mice on pure C57Bl/6N background. Only when PLS3 was over-expressed in milder affected SMA mice (50%/C57Bl/6N / 50%/FVB/N), highly significantly improved motor ability, improved NMJ functionality as shown by electrophysiological measurements and moderately increased survival were observed compared with SMA mice. There is increasing evidence from patients and SMA mouse models that, next to MN dysfunction, defects in various other tissue types strongly contribute to the lethal phenotype (reviewed in 23). Indeed, our detailed analysis of the Taiwanese SMA mouse model showed massive abnormalities and functional impairment of the intestine, heart and lung (48). As we did not observe an improved morphology in sections of heart, lung and intestine tissue in PLS3-over-expressing SMA mice, we assume that the observed positive effects of PLS3V5 expression on NMJ structure and function are not sufficient to overcome the detrimental global defects in the severe SMA mouse model. This hypothesis is in line with our previous observation in humans, where PLS3 provides full protection against SMA only in SMN1-deleted individuals carrying three to four SMN2 copies, but not in those with two SMN2 copies. This suggests that a certain amount of SMN is required in individuals lacking SMN1 in order to fully benefit from the over-expression of PLS3 (21). We detected similar Smn levels between Taiwanese SMA mice on pure C57Bl/6N background and the moderately affected mixed\textsubscript{50} SMA mice, excluding Smn expression as the cause for prolonged survival. In addition, PLS3 over-expression had no effect on SMN expression. Together, these findings suggest an SMN-independent mechanism of PLS3 protection. In contrast, in the zebrafish SMA model, reduced smn levels correlate with decreased pls3 levels, whereas restoration of smn increased pls3 levels (22), suggesting a potential interaction. However, in mice (this study), humans (21) and zebrafish (22), increased plastin3 expression has no influence on SMN levels. Taken together, we
therefore concluded that, at least in mammals, PLS3 seems to exert its modifying effects rather independently of Smn.

We further showed that ubiquitous PLS3 expression in SMA mice results in significantly increased endplate and muscle fiber size. In order to test whether presynaptic PLS3 over-expression is sufficient to cause these effects, we generated MN-specific PLS3-over-expressing mice, using Hb9-Cre. Indeed, MN-specific PLS3 over-expression resulted in increased endplate and muscle fiber size in TVA, GC and VL muscle. These results, together with the observation of restored SV and AZ number, RRP vesicle organization as well as an amelioration of electrophysiological deficits in SMA<sub>PLS3V5</sub> mice, strongly implicate that improved release of neurotransmitter or other secreted molecules might cause enhanced AChR clustering. As significantly more axons are present at the NMJs of SMA<sub>PLS3V5</sub> compared with SMA mice during the axon pruning process, the longer reminiscence of axons might additionally allow longer exposure to secreted factors supporting NMJ maturation and development. As a consequence, the increase in endplate size might then stimulate muscle fiber growth. It has been demonstrated that Smn is essential for proper β-actin mRNA transport and translation in motor axons (9). It is also known that F-actin by a caging mechanism regulates the presynaptic organization of vesicles into the RP and RRP and that this process is disrupted in SMA (12,14,64). In addition, vesicles are tethered to F-actin filaments proximal to the membrane region and transported along such filaments to fusion sites prior to release. Therefore, it is plausible that diminished neurotransmission as a key feature of SMA might be attributed to a disturbance of cytoskeletal organization in the presynapse. In view of these results and supported by the finding of restored F-actin amounts in SMA<sub>PLS3V5</sub> synapses, it is therefore possible that PLS3 compensates for the consequences of cytoskeletal

![Figure 12. Model of PLS3 protection in SMA. High PLS3 levels increase F-actin amount in motor axons and presynaptic terminals, resulting in an increased axon input number as well as terminal arborization. As shown by confocal as well as TEM imaging and supported by our electrophysiological findings, high PLS3 levels lead to increased AZ and overall vesicle numbers as well as a restoration of RRP organization and vesicle content, likely as a consequence of increased amounts of cortical F-actin. Possibly, PLS3 might also act on the RP of vesicles as caging of such vesicles occurs in a strictly F-actin-dependent manner. Together, these changes support vesicle fusion and thus neurotransmission as was demonstrated by improved release kinetics and increased QC in SMA<sub>PLS3V5</sub> compared with SMA mice. At postsynaptic sites, increased neurotransmitter release, e.g. of agrin, supports AChR clustering, which in turn might cause the observed muscle fiber size increase. As F-actin formation in the cortex of muscle cells is a prerequisite for correct AChR clustering, a muscular involvement of PLS3, as based on our experiments, cannot be excluded. RP, reserve pool; RRP, readily releasable pool.](https://academic.oup.com/hmg/article-abstract/22/7/1328/592895)
defects on neurotransmission through directly acting on the stability and organization of F-actin filaments in the presynapse. Importantly, however, we detected high PLS3 levels also in muscle tissue, raising the possibility that PLS3 acts directly in muscle to increase endplate size and, as a consequence, muscle fiber size. In this context, it has been shown that membrane-associated F-actin assembly timely precedes agrin-induced AChR clustering and that Latrunculin, a toxin that sequesters globular (G)-actin and prevents F-actin formation, is able to inhibit proper AChR clustering (65). Furthermore, it is well established that upon agrin/Lrp4/MusK signaling, postsynaptic rapsyn tethers and cross-links freely diffusing AChR to the cortical actin cytoskeleton of muscle cells and that several F-actin-stabilizing factors are enriched at the postsynaptic membrane (66,67). Therefore, based on these findings and in combination with our observation of high PLS3V5 levels in muscle, an additional muscle-specific effect of PLS3 on AChR clustering in SMA

PLS3V5 mice cannot be excluded.

In summary, our detailed analyses provide the in vivo proof-of-concept of PLS3-mediated protection against SMA in the mouse system, underscoring the importance of PLS3 as a potential drug target in SMA therapy. Moreover, our experiments identify important roles of PLS3 in NMJ development, maturation, integrity and function. As our analyses indicate a rather Smn-independent mechanism of PLS3 on presynaptic cytoskeletal regulation (Fig. 12), high PLS3 levels might exert neuroprotective effects also in other neurodegenerative diseases such as myasthenia gravis or ALS.

MATERIALS AND METHODS

Generation of a conditional PLS3V5 transgenic allele and of PLS3V5-expressing mice

Human and mouse plasin 3 proteins are 99% identical (68). Human T-plastin (PLS3, also termed T-fimbrin; MIM 300131, Xq23) coding sequence (NM_005032) missing the stop codon was amplified from fibroblast cDNA, using the primers 5′GAGGTGCAGAAGTTGTCTGA3′ and 5′CACTCTCTTCATCCCTGC3′, subcloned into pcDNA3.1-V5/HIS-Topo (Invitrogen) to add the 5′-tag, PCR-amplified using the ASCl-restriction overhang primers 5′GGCCGGCCACCATGGATGAGATGGTCACTACACTG3′ and 5′GGCGCCCTCAATGGATGAGATGGTCACTACACTG3′, the correct sequence confirmed via Sanger sequencing and the product cloned into SERCA ASCI-linearized targeting vector (28). Embryonic stem (ES) cell culture, transfection and selection of transfected clones were performed as previously described (69). In brief, 10⁷ V6.5 hybrid F1 ES cells (C57Bl/6J × SV129) were transfected with 50 μg of Asis 1 linearized construct, and correctly targeted ES cell clones were identified by Southern blot analysis. To proof the inducibility of the transgene, the positive ES cell clones A6 and A11 were in vitro-treated with recombinant His-TAT-NLS-Cre (HTNC) as has been described elsewhere (71) and subsequently analyzed for GFP fluorescence using microscopic and fluorescence-activated cell sorting (FACS) analysis and the presence of PLS3V5 via western blot analysis (Fig. 1C and D). Injection of the parental A6 and A11 ES cell clones into blastocysts and transplantation into pseudo-pregnant FVB females were carried out at the mouse engineering facility, Institute for Genetics, University of Cologne, Germany. Finally, by F1 screening, one PLS3 transgenic founder (PLS3V5-fl line) could be identified via genotyping PCR (see what follows). Prior to further analysis, transgenic mice were backcrossed to pure C57Bl/6N wt mice for seven generations to produce a congenic strain. To obtain ubiquitously PLS3V5-expressing mice (PLS3V5lf line), the original PLS3V5lf line containing a stop cassette between promoter and transgene was crossbred with the CMV-Cre deleter line (29) (Fig. 2A). To specifically activate PLS3V5 in MNs, PLS3V5lf mice were bred to Hb9-Cre (Hb9-Cre+/−) transgenic mice (39).

Animal care and all surgical procedures were performed according to the institutional animal care committee guidelines and German animal protection laws and were approved under the reference number 9.93.2.10.31.07.186 of the ‘Landesamt für Natur, Umwelt und Verbraucherschutz NRW’.

SMA mice

To achieve over-expression of PLS3V5 in the SMA background, we used the Taiwanese SMA mouse model originally purchased from the Jackson Laboratory on pure FVB background (FVB.Cg-Tg(SMN2)2Hung Smnfltm1Hung/J, stock number: 005058). Additionally, these mice were congenically backcrossed for seven generations to C57Bl/6N background mice. The breeding colony was maintained by interbreeding Smn−/−;SMN2fl/fl mice and Smn−/− mice with wt mice. All offspring were genotyped using PCR assays on tail-tip DNA with primer combinations described in what follows. The breeding scheme for the generation of SMAPLS3V5 mice and respective controls is given in Figure 3.

Genotype analysis of mice and cells

Genomic DNA was isolated from mouse tail-tip biopsies, embryos or ES cells as previously described (71). Genotyping PCR was conducted using the following primers and conditions: PLS3V5 (Triplex-PCR, Forw1: 5′AAAGTCGCTCTAGTGTATATC3′, Rev1: 5′GATATGAAGTACTGGGCTC3′, annealing 56°C, elongation 1 min); murine Smn knockout (Triplex-PCR, Forw1: 5′ATAAACACACCACCTTATC3′, Rev1: 5′AGCTTAAGGAGATCGAGC3′, Rev2: 5′TAGCCGTTGAGCCATTCG3′, annealing 59°C, elongation 1 min); murine Smn knockout (Triplex-PCR, Forw1: 5′ATAAACACACCACCTTATC3′, Rev1: 5′AGCTTAAGGAGATCGAGC3′, Rev2: 5′TAGCCGTTGAGCCATTCG3′, annealing 59°C, elongation 1 min); human SMN2 (Forw1: 5′CGAATCACTTGAGGGCAGG3′, Rev1: 5′AATGCTGAGCGTGTTCCAG3′, Rev2: 5′GTCGAATCTGGATCTGAG3′, annealing 59°C, elongation 1 min); human Smn1 (Forw1: 5′GTCGAATCTGGATCTGAG3′, Rev1: 5′GTCGAATCTGGATCTGAG3′, Rev2: 5′GTCGAATCTGGATCTGAG3′, annealing 59°C, elongation 1 min); Hb9-Cre (Forw1: 5′GGCCGGCCACCATGGATGAGATGGTCACTACACTG3′, Rev1: 5′GTCGAATCTGGATCTGAG3′, Rev2: 5′GTCGAATCTGGATCTGAG3′, annealing 59°C, elongation 1 min). PLS3V5-specific PCR to confirm the presence of PLS3V5 in first PLS3V5lf founder: Forw1: 5′GCCAAAGCTAGTGTTCCAG3′, Rev1: 5′GCCAAAGCTAGTGTTCCAG3′.

Southern blot analysis

Correct insertion of the targeting vector in ES cells and germline transmission in mice were investigated applying Southern
Western blot analysis
Mice were humanely sacrificed and respective tissues harvested. To quantify protein amounts, lysates were homogenized in RIPA buffer (Sigma), using an Ultra Turrax homogenizer (IKA), and western blot analysis was performed as previously described (73). Following antibodies were used: mouse α-actin (1:10 000, Sigma), mouse α-tubulin (1:80 000, Sigma), mouse α-SMN (1:2000, BD Transduction Laboratories), rabbit α-PLS3V5 [1:10 000 (21)], mouse α-V5 (1:5000, Invitrogen).

Quantitative real-time PCR
Total RNA was isolated from mouse organ biopsies by using RNasy kit (Qiagen) and QIAshredder (Qiagen), according to the manufacturer’s protocols. For the isolation of RNA from fatty organs like the brain or spinal cord, preparative steps with Qiazol (Qiagen), according to the manufacturer’s protocol, were included. DNasel digest was carried out using RNase-Free DNase kit (Qiagen), according to the manufacturer’s instructions. For subsequent analysis of Pls3 and PLS3V5/Pls3 transcript levels with the LightCycler 1.5 (Roche), following primer combinations were used: Pls3: Forw1: 5′AGCTGATCTGTACGACAC3′, Rev1: 5′ATA CAAAAGGACCAGAATG3′ annealing 63°C, elongation 35 s. PLS3V5/Pls3: Forw1: 5′ATGAGCTTATGTAATGAACTC AAA3′, Rev1: 5′TGGCATATTAGCTTCCTTGA3′, annealing 66°C, elongation 35 s). The quantification program was followed by a melting step to detect the melting points for every PCR product. Analysis of the PCR curves was performed with the second-derivative maximum method of the LightCycler software. All sample measurements were repeated at least three times.

Fluorescence-activated cell sorting
For FACS analysis, HTNC-deleted cells were trypsinized and then sedimented at ~200 g for 5 min. The pellet was reconstituted in 500 μl of PBS and pipetted up and down several times to completely re-suspend cells. Cells were kept on ice until FACS analysis using the BD FACS Vantage SE and CellQuest analysis software.

Motor ability tests of SMA_PLS3V5 mice
To evaluate SMA progression, SMA_PLS3V5 mice and respective controls were weighted daily in the morning. Motor performance was estimated applying the tube test as well as a modified version of the righting reflex test as described elsewhere (32). Although the tube score was given according to the original method, righting times were related to a score in the following manner: t < 1 s = 0, 1–2 s = 1, 3–4 s = 2, 5–6 s = 3, 7–8 s = 4, 9–10 s = 5, t > 10 s = 6. Survival times were recorded in a time frame of maximum 30 days. SMA or SMA_PLS3V5 mice surviving beyond 30 days were humanely sacrificed due to usually obvious necrosis of the nose, ear, tail and legs.

Immunohistochemistry of MNs and NMJs
Proprioceptive input staining on MN cell bodies
The spinal cord was dissected from euthanized mice and fixed in 4% paraformaldehyde (PFA)/PBS for 2 days. The spinal cord was quickly rinsed in PBS, embedded in 4% low-melting agarose and lumbar L4–L5 region sliced into 100 μm thick sections, using a vibratome (Leica). Samples were permeabilized in PBS containing 2% Triton X under constant shaking for 30 min. Samples were blocked in PBS containing 4% BSA/1% Triton/PBS for 2 h. Finally, goat anti-ChAT (1:100, Millipore) and rabbit anti-VGlut1 (1:100, Millipore) antibodies in blocking solution were added overnight (o.n.). Samples were rinsed six times in PBS for 10 min under constant shaking. Media was exchanged with secondary antibodies [donkey anti-rabbit Alexa fluor 488 (1:500, Invitrogen), donkey anti-goat Alexa fluor 568 (1:500, Invitrogen)] in PBS and incubated for 2 h at room temperature (RT) on a rocking platform. Following five times of 10 min washing steps in PBS, samples were mounted in Mowiol (Kuraray) for further analysis. Quantification details are given in Figure 5.

SV and neurofilament staining of NMJs
To stain SVs and neurofilament in NMJs, TVA, GC and VL muscles were fixed in 4% PFA o.n. Although TVA was used as whole, GC and VL muscles were embedded in 4% low-melting agarose (Qiagen) and sliced into 100 μm thick sections prior to the actual staining protocol using the VT 1200 S vibratome (Leica). Tissues were rinsed 3 times in PBS for 10 min to remove excess PFA. From this point on, the staining protocol followed the procedure previously described for MN staining. Primary antibody dilutions were mouse anti-SV2 (1:100, Hybridoma bank) together with mouse anti-neurofilament (1:100, Hybridoma bank). The secondary antibody dilution was donkey anti-mouse Alexa fluor 488 (1:500, Invitrogen). To outline endplates in muscle tissue, 10 μl of BTX labeled with Rhodamine stock solution (1 μl) was diluted in 7 μl of PBS and samples incubated for 10 min under shaking at RT prior to the final washing steps. Quantification details are given in Figure 5.

Piccolo, SVs and F-actin staining of NMJs
To stain the AZ marker Piccolo, SVs, and F-actin in NMJs of the TVA muscle, fixed tissue sections were bathed in 0.1 mm glycine in PBS for 20 min, then permeabilized with 1% (w/v) Triton X-100 in PBS for 30 min and incubated in 5% (w/v) BSA, 1% Triton X-100 in PBS for 2 h. Samples were incubated overnight at 4°C with primary antibodies (see what follows). The following day, TVA was rinsed for 1 h in PBS containing 1% Triton X-100, incubated for 1 h both with the corresponding secondary antibodies and 0.5 μg/ml rhodamine-BTX (Sigma) and rinsed again with 0.05% Triton X-100 in PBS for 90 min. Presynaptic terminals were labeled with antibodies against the vesicular acetylcholine (ACh) transporter (1:750, Synaptic System) and Piccolo.
Isolation and immunohistochemistry of MEFs

Primary MEFs were isolated from E13.5 embryos derived from intercrosses between female PLS3V5tg/0 and male wt mice, resulting in 50% PLS3V5tg/0 and 50% wt embryos. Embryos were dissected, head, heart and liver were removed and the remaining tissue was homogenized through a 70 mm cell strainer (BD Falcon) which was subsequently flushed with 10 ml of DMEM medium (Invitrogen) supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin (PAA), 100 mg/ml streptomycin (PAA), 100 μg/ml non-essential amino acids (PAA), 2 μM glutamine (PAA) and 1 μM sodium pyruvate (PAA). Cells were sedimented and resuspended in 5 ml of medium before plated on gelatinized 6 cm tissue culture dishes (Sarsted). Cells were cultured at 37°C, 5% CO2 and 90% humidity. MEFs of the desired genotypes PLS3V5tg/0 and wt were identified by genotyping the corresponding embryos.

For the staining of MEFs, cells were trypsinized and replated in 12-well plates provided with sterile cover slips (Thermanox). After settling o.n., MEFs were rinsed once in PBS and fixed in 4% PFA for 15 min at RT. Following fixation, cells were rinsed once in PBS for 5 min. Antigen retrieval was conducted by addition of 80°C 0.01 M citrate buffer (pH 6.0) and incubation at RT for 20 min. After another washing step with PBS, cells were permeabilized in 0.2% Triton X in PBS for 5 min. For blocking, cells were incubated in 5% of the secondary antibody’s host serum plus 5% BSA in PBS for 2 h at RT. Next, primary antibodies rabbit α-PLS3 [1:40 (21)], mouse anti-V5 (1:100, Invitrogen), mouse anti-Vinculin (1:150, Sigma), were diluted in 5% BSA in PBS and given to the cells o.n. Following three washing steps in PBS for 15 min each, secondary antibodies donkey anti-rabbit Alexa fluor 350 (1:500, Invitrogen), donkey anti-mouse Alexa fluor 488 (1:500, Invitrogen) were co-diluted with Phalloidin Alexa 647 (1:40, Invitrogen) in PBS containing 5% BSA and incubated for 4 h at RT. Finally, samples were mounted in mounting medium containing DAPI (Vectorshied).

H & E staining

TVa, GC and VL muscle samples were dehydrated using an infiltration machine (Leica), embedded in paraffin and finally cut into 7 μm sections, using a microtome (Leica). In order to stain with H & E, muscle sections were de-paraffinized by incubation in Xylol for 30 min. Following incubation in decreasing EtOH concentrations (100, 96, 70, 50%, 3 min each), sections were washed once in PBS and then incubated in H2O for 1 min. Next, sections were incubated in hematoxylin (Sigma) for 5 min and afterwards washed in H2O for 15 min. The sections were rinsed quickly in H2O once to remove excess dye and then placed into eosin (Sigma) solution for 1 min. To clarify the staining, sections were rinsed in H2O six to seven times and then de-hydrated in increasing EtOH concentrations (50, 70, 96, 100%, 3 min each). Finally, sections were air-dried and embedded in Entellan (Merck).

Image acquisition and analysis

For SV, AZ and F-actin measurements, TVA muscle fiber images from different genotype preparations were taken with similar conditions (laser intensities and photomultiplier voltages) with an upright Olympus FV1000 confocal laser scanning microscope equipped with three excitation laser lines (argon–krypton laser with 488, 561 and 633 nm excitation lines). During image acquisition, an alternating sequence of laser pulses was used for the activation of the different fluorescent probes. Images were taken using a 63× oil-immersion objective with a numerical aperture of 1.4. Morphometric analysis of the fluorescently labeled structures was performed offline with ImageJ (W. Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Postsynaptic terminal and SV area were determined semi-automatically by finding outline masks based on brightness threshold, from maximal projected confocal images. For Piccolo area analysis, an intensity threshold over 3-fold the standard deviation of the background intensity was established. Phalloidin single images had been masked and cleared outside the postsynaptic area before doing the Z-projection. Brightness/contrast was enhanced in the figures to enable easier visualization of the signals. For the determination of endplate size and axon input number, the Axioplan 2 fluorescent microscope (Zeiss) together with the Axiovision software (Zeiss) was used. Representative pictures were taken with the META 510 confocal microscope (Zeiss). Proproceptive input numbers on MNs and MN sizes were quantified as described in the main text, using the Velocity 3D image analysis software (PerkinElmer). All these experiments were double-blinded.

Transmission electron microscopy

TVa muscle was fixed in 4% PFA for 30 min and postfixed in 0.6% glutaraldehyde for another day. Samples were then prepared as previously described (74). Thickness of semi-thin and ultra-thin sections was 0.5 and 0.1 μm, respectively. Image acquisition was performed as described in Supplementary Material. Image acquisition was performed using the TEM CM10 (Phillips) microscope with the Orius SC200W 1 gatan camera and the Digital Micrograph software.

Electrophysiological recordings

Electrical stimulation and intracellular recordings were performed as described in Ruiz et al. (13). The TVa muscle was dissected with its nerve branches intact and pinned to the bottom of a 2 ml chamber, over a bed of cured silicone rubber. Preparations were continuously perfused with saline, according to the following composition (in mM): 125 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 25 NaHCO3 and 15 glucose. The solution was continuously gassed with carbogen (95% O2 and 5% CO2), which maintained the pH at 7.35. Recordings were performed at RT (22–23°C).
The nerve was stimulated with a bipolar hook electrode (75). The nerve was isolated with silicone oil (Baysilone-Paste hochviskos; GE Bayer Silicones, Leverkusen, Germany). The stimulation consisted of square-wave pulses of 0.3 ms duration and 0.01–5 mA amplitude at frequencies of 0.5 or 20 Hz.

Intracellular recordings were conducted using glass microelectrodes (GB100TF-8P; Science Products, Hofheim, Germany), filled with a solution of 3 M potassium acetate with 100 mM KCl (electrode resistance: ~20 MΩ). Signals were amplified using an SEC-10 L amplifier (NPI, Tamm, Germany). Evoked EPPs and miniature EPPs (mEPPs) were recorded from different NMJs within the muscle as described previously (13). Muscular contraction was prevented by including 3–4 μM µ-conotoxin GIIIB (Alomone Labs Ltd, Jerusalem, Israel) in the bath. µ-Conotoxin GIIIB specifically blocks muscular voltage-gated sodium channels.

All muscle fiber recordings with rise times (10–90% of the EPP) of <1.2 ms for TVA muscles from SMA and SMA-PLSVS; mice and 1 ms for TVA muscles from HET and HET-PLSVS mice were considered close enough to the NMJ and therefore suitable for analysis. For TVA muscles from SMA and SMA-PLSVS mice, longer rise times were allowed, as Ruiz et al. (13) showed that TVA muscle fibers from SMA mice in general had EPPs with significant slower rise times. The mean amplitudes of the EPP and mEPPs recorded at each NMJ were linearly normalized to ∼70 mV resting membrane potential. EPP amplitudes were corrected for non-linear summation (76) as follows: EPPc = Average Peak EPP/(1 - Average Peak EPP/(V_m - E_r)), where V_m is the resting membrane potential and E_r the reverse potential (assumed to be -5 mV for all of our experiments). QC was estimated by the direct method, which consists of recording mEPPs and EPPs (nerve stimulation 0.5 Hz) simultaneously and then calculating the ratio: QC = Average Peak EPP/Average Peak mEPP. The RRP was estimated as described in Torres-Benito et al. (14). QC during a 5 s 20 Hz stimulation train was plotted against total released quanta. The initial decay was fitted linearly and the x-axis intercept determined the RRP.

For analysis, data were pooled within one group. Therefore, all electrophysiological data are given as group mean ± SEM, unless otherwise stated, with N being the number of muscle fibers per group and n the number of mice per group. All experiments reported include the results of at least three animals per genotype. Statistical comparisons between mutant and wt measures were made using t-test.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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