Amelioration of Muscle and Nerve Pathology in LAMA2 Muscular Dystrophy by AAV9-Mini-Agrin

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LAMA2-related muscular dystrophy (LAMA2 MD) is the most common and fatal form of early-onset congenital muscular dystrophies. Due to the large size of the laminin α2 cDNA and heterotrimeric structure of the protein, it is challenging to develop a gene-replacement therapy. Our group has developed a novel adeno-associated viral (AAV) vector carrying the mini-agrin, which is a non-homologous functional substitute for the mutated laminin α2. A significant therapeutic effect in skeletal muscle was observed in our previous study using AAV serotype 1 (AAV1). In this investigation, we examined AAV9 vector, which has more widespread transduction than AAV1, to determine if the therapeutic effects could be further improved. As expected, AAV9-mini-agrin treatment offered enhanced therapeutic effects over the previously used AAV1-mini-agrin in extending mouse lifespan and improvement of muscle pathology. Additionally, overexpression of mini-agrin in peripheral nerves of dy/dy mice partially ameliorated nerve pathology as evidenced by improved motor function and sensorimotor processing, partial restoration of myelination, partial restoration of basement membrane via EM examination, as well as decreased regeneration of Schwann cells. In conclusion, our studies indicate that overexpression of mini-agrin into dy/dy mice offers profound therapeutic effects in both skeletal muscle and nervous system.

INTRODUCTION
Congenital muscular dystrophy (CMD) is a group of rare genetic muscle disorders that appear early in life from birth to 2 years of age. Characteristic signs include congenital hypotonia, delayed motor development, progressive muscle weakness, and dystrophic features visualized on muscle biopsy.1,2 The most common form is muscular dystrophy type 1A (MDC1A, also named LAMA2-related muscular dystrophy, LAMA2 MD), which is responsible for 40% of all CMD cases. This subset is characterized by a primary deficiency in the laminin α2 chain of merosin (laminin-2, also termed Laminin211) caused by mutations within the LAMA2 gene.3,4 In general, complete laminin α2 deficiencies are functionally null mutations, leading to severe and non-ambulatory phenotypes; however, partial laminin α2 deficiencies tend to present with variable but comparatively milder phenotypes.5 The advent of next-generation sequencing technology has substantially improved CMD diagnosis, allowing for rapid and cost-effective CMD molecular testing in the clinic.6 Consequently, the spectrum of LAMA2 MD has expanded in recent years partially due to the definitive diagnosis of molecular genetics.7,8 Additionally, the International Standard of Care Committee for CMD recently published two consensus statements on standard of care and diagnostic approach for CMD to improve quality of life in CMD patients.9,10 Evidence-based guidelines for CMD diagnosis and care were also released by the Review Panel of the American Association of Neuro muscular & Electrodagnostic Medicine.11,12

The molecular pathogenesis of LAMA2 MD has not been fully elucidated, but several mechanisms have been proposed. Ervasti and Campbell13 suggested that the laminin α2 chain confers a structural link from the extracellular matrix (ECM) to the cytoskeleton, stabilizing the muscle-cell membrane and imparting protection against contraction-induced damage. This hypothesis was challenged by Hall et al.14 By using time-lapse photomicroscopy in a LAMA2 MD zebrafish model, they demonstrated that pathology of LAMA2 MD was through mechanically induced fiber detachment rather than sarcolemma rupture or fiber denervation.15 Nevertheless, cell membranes are ruptured to some extent in mouse models with complete laminin α2 deficiency.15

Despite these recent advances in the diagnosis and understanding of LAMA2 MD, there is no approved treatment available for this debilitating disease.16,17 The current clinical approach is centered on supportive care.10,11 Over the past decade, various studies have been carried out on LAMA2 MD mouse models to test potential treatment options. Because the basement membrane is affected in LAMA2 MD, many of these approaches have targeted the expression of ECM...
Figure 1. Lifespan, Growth Rate, and Pathology Improvement in the LAMA2 MD Mouse Model

(A) Lifespan of the treated mice was significantly extended. The vectors (2 × 10^11 vg/pup) were delivered into homozygous dy/dy neonatal pups via intraperitoneal injection. n = 22 for untreated dy/dy group; n = 6 for AAV1-CMV-mini-agrin group; n = 9 for AAV9-CMV-mini-agrin group; n = 6 for AAV9-CB-mini-agrin group; n = 5 for wild-type (WT) control mice. **p < 0.01; ***p < 0.001. (B) Body weight of the treated mice was significantly increased. n = 7 for the untreated dy/dy and AAV9-CMV-mini-agrin mice; n = 6 for AAV9-CB-mini-agrin mice. *p < 0.05; ***p < 0.001. (C) Histology examination of triceps muscle. Mice were sacrificed at around 3 months of age. Agrin was stained (legend continued on next page)
proteins. Transgenic expressions of the laminin α1, α2 chains, mini-agrin (a basement membrane-associated heparin sulfate proteoglycan), and other linker molecules in dyw/dyw mice, a model of LAMA2 MD, have been found to compensate for laminin-α2-chain deficiency. Several approaches aimed to alleviate the secondary defects rather than to target the primary deficiency have been undertaken in LAMA2 MD. These approaches include inhibition of apoptosis, interference with proteasomal and autophagy-mediated protein degradation, as well as targeting fibrosis or inflammation in dystrophic muscle of LAMA2 MD mice. It is worth mentioning that omigapil, an anti-apoptosis drug, is in phase I clinical trial with assessment of safety and tolerability for CMD (ClinicalTrials.gov Identifier: NCT01805024).

Ideally, gene replacement therapy would offer curative treatment for LAMA2 MD patients. However, the size (>9 kb) of both laminin α1 and laminin α2 cDNA precludes their packaging into commonly used gene therapy vectors, such as adeno-associated viral (AAV) vectors. It is also unfeasible to generate a miniaturized laminin α chain, as it must incorporate into the laminin heterotrimer to be functional. A potential solution to the packaging size limitation is the utilization of mini-agrin, a functional substitute gene. Mini-agrin shares with wild-type agrin the ability to bind to α-dystroglycan, a protein that is involved in the linkage of basement membranes to the muscle sarcolemma. Additionally, the immunological rejection of the protein will be minimal for clinical application because LAMA2 MD patients express agrin endogenously. Our group was the first to attempt somatic gene transfer of mini-agrin in mice. Systemic delivery of mini-agrin into multiple vital muscles of LAMA2 MD mice using AAV vector significantly improved whole-body growth and motility and quadrupled the lifespan of the treated mice. One question from our previous study that remains unanswered is whether mini-agrin offers any beneficial effects to the nervous system and if so, to what extent. In this study, we set out to investigate whether the therapeutic effects offered by AAV-mini-agrin could be further augmented by utilizing a more robust AAV serotype.

RESULTS

Improvement of Growth Rate and Lifespan after AAV9-Mini-Agrin Treatment

Previously, we utilized the AAV1 vector to deliver the mini-agrin gene because at that time AAV1 demonstrated superior transduction ability in skeletal muscle. More recently, new AAV serotypes have been discovered, and AAV9 has demonstrated robust transduction of both skeletal muscle and nervous systems with greater efficiency than AAV1. In this study, we performed a side-by-side comparison of these two serotypes to deliver mini-agrin gene. Additionally, we utilized a different ubiquitous promoter, CB (CMV enhancer and chicken β-actin promoter) versus the previously used CMV (cytomegalovirus) promoter to drive mini-agrin gene (Figure S6). The vectors (2 × 1011 vector genomes [vgl/mouse]) were delivered into homozygous dyw/dyw neonatal pups (2 to 3 days of age) via intraperitoneal injection. The average lifespan of the untreated homozygous dyw/dyw mice was 10.18 ± 7.12 weeks (n = 22) (Figure 1A). Both AAV9-CMV-mini-agrin and AAV9-CB-mini-agrin treatments dramatically improved mouse lifespan (42.1 ± 10.6 weeks for CMV group, n = 9, p < 0.001 as compared to untreated control; 45.8 ± 3.4 weeks for CB group, n = 6, p < 0.001), and there was no significant difference in lifespan extension between AAV9-CMV-mini-agrin and AAV9-CB-mini-agrin groups (Figure 1A).

The average lifespan of the AAV1-CMV-mini-agrin-treated mice was 24 ± 5.6 weeks (n = 6), which was significantly shorter than that of the AAV9-CMV-mini-agrin group (p < 0.001) (Figure 1A). In addition, AAV9-CMV-mini-agrin and AAV9-CB-mini-agrin treatment significantly increased mouse body weight (Figure 1B). At 13 weeks, the average body weight of the untreated dyw/dyw mice was 11.31 ± 1.41 g (n = 7). The mean body weights of the AAV9-CMV-mini-agrin and AAV9-CB-mini-agrin-treated dyw/dyw mice were 15.65 ± 1.3 g (n = 7, p < 0.01) and 15.11 ± 1.03 g (n = 6, p < 0.01), respectively (Figure 1B). Taken together, our results indicate that AAV9-mini-agrin (driven by either CB or CMV promoter) improved therapeutic effects compared to AAV1-cmv-mini-agrin.

As expected, systemic delivery of both AAV9-mini-agrin and AAV1-cmv-mini-agrin in dyw/dyw mice resulted in overexpression of mini-agrin protein in body-wide muscle groups. Triceps (Figure 1) and diaphragm muscles (Figure S1) are presented as examples here. The wild-type agrin is expressed in the neuromuscular junction (appearing as punctuated staining) and blood vessel wall in the wild-type and untreated dyw/dyw mice. The treatment led to overexpression of mini-agrin in the ECM of the skeletal muscles as revealed via immunofluorescent staining, with more uniform distribution in the AAV9 group than the AAV1 group (Figure 1C). We additionally examined muscular dystrophic pathology amendment by the treatment through H&E staining for general morphology, Masson’s trichrome stain, and Sirius red stain for collagen deposition. As compared to the wild-type muscle, the dystrophic muscle displayed varied muscle fiber sizes and mononuclear cell infiltration on H&E staining. It also showed increased collagen accumulation in ECM of the skeletal muscles on both Masson’s trichrome stain and Sirius red/fast green stain (Figures 1C and S1). Overexpression of mini-agrin in both groups led to more uniform muscle fiber sizes, reduced mononuclear cell infiltration, and decreased collagen deposition (Figures 1C, S1, S5, and S7). Collagen quantification data further confirmed decrease of the collagen accumulation in the treated muscle. The collagen content of wild-type triceps muscle was 1.04% ± 0.12% (p < 0.001 as compared to untreated homo), and the collagen content of the untreated dyw/dyw triceps muscle was 1.04% ± 0.12% (p < 0.001 as compared to untreated homo). Scale bar, 50 μm. Error bar indicates SEM.
muscle was 2.01% ± 0.27% (Figure 1D). The collagen content of the two treated groups, AAV1-mini-agrin and AAV9-mini-agrin, was 1.5% ± 0.21% (p < 0.05) and 1.29% ± 0.205 (p < 0.001), respectively (Figure 1D). This clearly indicates muscle pathology improvement by overexpression of mini-agrin.

Improvement of Motor Function and Sensorimotor Processing in the LAMA2 MD Mouse Model

To evaluate whether AAV9-mini-agrin treatment results in any behavioral improvement in dy/wdy” mice, the marble-burying and open-field tests were used. For this study, the AAV9-CB-mini-agrin vector was delivered into neonatal homozygous dy/wdy” pups via temporal vein injection (2 × 10^11 vg/pup). The marble-burying test takes advantage of the proclivity of mice to dig in natural settings, while the open-field test assesses both horizontal and vertical (i.e., rearing) activity levels.35 As shown in Figure 2A, untreated dy/wdy” mice could barely bury any marbles (0.83 ± 1.6) even at a young age (6 to 8 weeks of age). The AAV9-mini-agrin-treated mice could bury as many as 16 marbles (10.16 ± 4.40) at 13 weeks of age (two-way ANOVA, p < 0.0001). Exploratory motor activity in a novel environment of the mice was assessed by an open-field test. The untreated dy/wdy” mice were hypoactive, as revealed by progressive decline in fine movements (an index of horizontal movements; Figure 2B) and rearing movements (Figure 2C). In contrast, the treated dy/wdy” mice maintained normal levels of fine movements until the end of the experiments (n = 3–7, two-way ANOVA, p < 0.0001). Vertical rearing movements by the treated mice were significantly improved at earlier ages (6 to 8 weeks of age); however, the activity decreased with age (Figure 2C). We also examined mini-agrin expression in brain and spine, including the nerve roots. There was no difference of agrin expression in the brain and spinal cord between treated and control mice; however, there was remarkable and even transgene expression in the AAV9-mini-agrin-treated nerve roots (Figure 1E). Patchy mini-agrin expression was observed in the AAV1-mini-agrin treated nerve roots as compared with the untreated group. This indicated that the treatment delayed the hind leg paralysis of the mice but could not cure it. General behavioral improvement of the treated mice is also demonstrated in video recordings (Figures S2 and S3).

Additionally, we measured the acoustic startle responses, which can provide general information regarding sensorimotor processing. Measurement of the reflex under conditions that engage the influence of higher brain centers can predict forebrain function. In the case of acoustic startle responses, presentation of lower intensity acoustic stimuli immediately prior to the acoustic startle stimulus decreases the response to the startle stimulus. This phenomenon, called prepulse inhibition (PPI) of the acoustic startle response, can be used to assess forebrain function. In the case of acoustic startle responses, presentation of lower intensity acoustic stimuli immediately prior to the acoustic startle stimulus decreases the response to the startle stimulus. This phenomenon, called prepulse inhibition (PPI), is regulated by forebrain neural circuits and is considered an operational measure of sensorimotor gating, a filtering mechanism to modulate information flow in the brain, allowing selective allocation of attentional resources to salient stimuli.35 Deficits in PPI are indicative of deficiencies in the cognitive processes underlying this sensorimotor gating.
In the present study, the untreated dy/dy mice had overt decreases in PPI, in comparison to the wild-type mice, across all prepulse sound levels (Figure 2D). However, the AAV9-mini-agrin-treated mice showed significantly enhanced PPI at the higher prepulse levels: 86 dB (15.6 ± 9.5 in untreated versus 40.4 ± 12.7 in treated versus 68.1 ± 16.9 in the wild-type mice) and 90 dB (9.6 ± 15.7 in untreated versus 39.2 ± 10.4 in treated versus 70.5 ± 11.7 in the wild-type mice) (Figure 2D). This clearly demonstrates the AAV-mini-agrin treatment led to partial rescue of impaired sensorimotor processing in the LAMA2 MD model.

Partial Restoration of Schwann Cell Basement Membrane and Improvement of Regeneration of Schwann Cells

Since we had observed much delayed hind-leg paralysis in the treated mice, we next sought to investigate whether the congenital hypomyelination had been amended. At 4 months of age, the control (n = 2) and treated mice (n = 3) were sacrificed, and their sciatic nerves were examined by transmission electron microscopy (TEM). Laminin α2 is a major component in both skeletal muscle and Schwann cells. In the present study, laminin α2 deficiency in dy/dy mice resulted in patchy or discontinuous basement membrane of Schwann cells in sciatic nerves (Figure 3A, black arrowhead) under TEM examination. The wild-type basement membrane displayed dense and continuous staining (Figure 3A, gray arrow). As expected, the phenotype of Schwann cells basement membranes was vastly improved by treatment; we observed a continuous membrane in most parts with occasional patchy spots (Figure 3A).

To further examine the amelioration of nerve pathology, we also employed light microscopy to investigate the myelinated nerve fibers of small area(s) of unmyelinated nerve fibers (white star). This clearly indicated improvement of myelination of the treated nerve fibers.

Laminin α2 appears to have both a structural role in formation of the Schwann cell basal lamina and a signaling role through dystroglycan and integrin receptors. Here, we intended to study how overexpression of mini-agrin affected Schwann cell development in the sciatic nerve of the laminin α2-deficient mice. Immunofluorescent staining against a myelinating Schwann cell proliferation marker (Oct6, a critical transcriptional factor for Schwann cell proliferation) on sciatic nerve cryo-sections was performed. As displayed in Figure 3B, the sciatic nerves of untreated dy/dy mice showed segregation of a large area of unmyelinated nerve fibers (black star), while the nerves from wild-type mice exhibited evenly distributed unmyelinated and myelinated axons. The nerve fibers from the treated dy/dy mice displayed relatively even distribution between myelinated and unmyelinated nerve fibers with occasional segregation with the gold standard method of toluidine blue staining.

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DISCUSSION

Among different subtypes of CMDs, LAMA2 MD is the most common subtype and accounts for approximately 40% of all cases. Currently, there are no effective treatment options for any of the CMDs. Because of the large size of the laminin α2 cDNA and the heterotrimERIC feature of the laminin α2 protein, it is particularly challenging to develop gene replacement therapy for LAMA2 MD. In our previous study, systemic delivery of an alternative functional gene, mini-agrin, utilizing AAV1 vector had a profound therapeutic
effects in skeletal muscle. In this manuscript, we extended our previous investigation by using the more robust AAV9 serotype to examine whether therapeutic effects could be further improved. Additionally, we investigated whether nerve pathology could also be ameliorated by overexpression of mini-agrin.

We now report several key discoveries in our study. First, AAV9 vector offers superior therapeutic effects compared to the AAV1 serotype in dyw/dyw mice. Second, treatment not only ameliorates muscle pathology, but also improves peripheral neuropathy and cognitive processing. Third, we show that phenotyping approaches, such as the marble-burying assay, open-field test, and acoustic-startle procedure, may be used to systematically evaluate motor dysfunction and sensorimotor gating deficits in the dyw/dyw mice. Unlike other dystrophic animal models, which only exhibit mild debilitation, the phenotype of dyw/dyw mice is severe. Untreated dyw/dyw mice rarely survive past 3 months of age. Due to the severity of the dyw/dyw phenotype, traditional motor functional tests such as treadmill running are not suitable. On the other hand, the approaches described in this investigation provide objective methods for muscle and nerve functional evaluation for CMD research that are both feasible and reliable.

Indeed, our data indicated that AAV9-mini-agrin treatment resulted in motor functional improvements in a marble-burying assay and delayed hind leg impairment in an open-field test, as well as improved sensory processing in brain, demonstrated via the acoustic-startle assay. Furthermore, electron microscopic examination revealed partial restoration of basement membrane of Schwann cells in peripheral nerves of the treated mice. Light microscopy revealed less segregation of unmyelinated nerve fibers as compared to the untreated counterpart. Immunofluorescent staining against a pro-myelinating Schwann cell marker (Oct6) indicated improvement of regeneration of Schwann cells in the treated peripheral nerves. All together, this study indicates that systemic delivery of AAV9-mini-agrin vector into dyw/dyw mice offers therapeutic effects in both skeletal muscle and nervous systems.

Interestingly, overexpression of mini-agrin in dyw/dyw mice was able to facilitate their fertility, suggesting widespread therapeutic benefits, since the homozygous dyw/dyw mice are not fertile. We did obtain one homozygous dyw/dyw pregnant female after treatment with AAV9-mini-agrin vector (Figure S4). Genotyping showed that all pups were homozygous dyw/dyw, indicating both treated male and female parental dyw/dyw mice were fertile. Although we only obtained one pregnant female, this data indicates mini-agrin can partially compensate for the function of laminin α2 in reproductive organs as well as overall health. Consistently, it was reported that overexpression of laminin α1 chain in testis by transgenic technology significantly reversed the histopathologic feature of the testis in laminin α2-deficient dyw/dyw mice. However, only pathological studies were described, and no actual fertility experiments were performed.

It is important to note that overexpression of mini-agrin in peripheral nerves of dyw/dyw mice only delayed hindlimb paralysis, and it is not a cure. Most of the AAV9-mini-agrin-treated dyw/dyw mice still developed progressive paralysis 3 months after treatment, although they were free to move around by crawling with forelimbs. In fact, the progressive lameness of hindlimbs also appeared in both laminin α2 and mini-agrin transgenic dyw/dyw mice. In this study, we purposely utilized the ubiquitous CMV or CB promoter to achieve transgene expression in peripheral nerves. However, in the clinic, neuropathy damage in most of the CMD patients may not be reversible.

Figure 4. Reduction of Schwann Cell Regeneration by the Treatment
The treated mice were delivered with AAV9-CB-mini-agrin at neonatal age. (A) Immunofluorescent staining against pro-myelinating Schwann cell marker Oct6. Nuclei were stained blue with DAPI. (B) Quantification of Oct6-positive cells. All positive cells were counted, and at least three mice were utilized for each group. ***p < 0.001. Error bar indicates SEM.
**MATERIALS AND METHODS**

**Plasmid Construction and AAV Vector Production**

The original mouse mini-agrin cDNA was generated by a RT-PCR method from mouse kidney tissue as described previously. The mini-agrin cDNA was then cloned into AAV vector plasmid under the transcriptional control of CB promoter (chicken β-actin promoter and CMV enhancer), and the final construct was named pXX-CB-mini-agrin. AAV serotype 9 was chosen as our delivery vehicle to achieve robust transgene expression in both skeletal muscle and peripheral nervous system, and the final vector was named AAV9-CB-mini-agrin. The AAV vector was produced by a triple transfection (IACUC). The heterozygous breeding pairs of dyw/dyw mice (Stock no. 013786/dyw) were purchased from The Jackson Laboratory (Bar Harbor, ME). The pregnant females and neonatal pups were bred at UNC animal facilities. The AAV1-CMV-mini-agrin and the AAV9-CMV-mini-agrin vectors were delivered into neonatal dyw/dyw mice via intraperitoneal injection (100 μL of 2 to 5 × 10^{12} vg/mL). The AAV9-CB-mini-agrin vector was delivered into the neonates (2 to 3 days age) of dyw/dyw mice by temporal vein injection with 80 μL of the vector (2 to 5 × 10^{12} vg/mL) per mouse.

**Marble-Burying Assay for Digging Ability**

Mice were tested in a Plexiglas cage located in a sound-attenuating chamber with ceiling light and fan. The cage contained 5 cm of corncob bedding, with 20 black glass marbles (14 mm diameter) arranged in an equidistant 5 × 4 grid on top of the bedding. Subjects were given access to the marbles for 30 min. Measures were taken of the number of buried marbles (two-thirds of the marble covered by the bedding).

**Open-Field Test for Activity and Motor Function**

Mice were assessed by 60-min trials in an open-field chamber (41 cm × 41 cm × 30 cm), crossed by a grid of photo beams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photo beams broken during the trial, with separate measures for horizontal activity (repeated breaking of photo beams) and rearing movements.

**Acoustic-Startle Procedure for Sensorimotor Gating**

The acoustic startle measure was based on the reflexive whole-body flinch, or startle response, following exposure to a sudden noise. Animals were tested with a San Diego Instruments SR-Lab system, using published methods. In brief, mice were placed in a small Plexiglas cylinder within a larger, sound-attenuating chamber (San Diego...
The cylinder was seated upon a piezoelectric transducer, which allowed vibrations to be quantified and displayed on a computer. The chamber included a ceiling light, fan, and a loudspeaker for the acoustic stimuli (bursts of white noise). Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments). Each test session consisted of 42 trials, presented following a 5-min habituation period. There were seven different types of trials: the no-stimulus trials, trials with the acoustic startle stimulus (40 ms; 120 dB) alone, and trials in which a prepulse stimulus (20 ms; either 74, 78, 82, 86, or 90 dB) had onset 100 ms before the onset of the startle stimulus. The different trial types were presented in blocks of seven, in randomized order within each block, with an average inter-trial interval of 15 s (range, 10–20 s). Measures were taken of the startle amplitude for each trial, defined as the peak response during a 65-ms sampling window that began with the onset of the startle stimulus. Levels of PPI at each prepulse sound level were calculated as 100 – [(response amplitude for prepulse stimulus and startle stimulus together/response amplitude for startle stimulus alone) × 100].

**TEM Method**

Mice were perfused with a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M sodium phosphate buffer (pH 7.4). After perfusion, sciatic nerves were dissected and stored for several days in the fixative before processing for electron microscopy. Following three rinses with 0.15 M sodium phosphate buffer, the samples were post-fixed for 1 hr in 1% osmium tetroxide in sodium phosphate buffer. The nerves were dehydrated through increasing concentrations of ethanol (30%, 50%, 75%, 100%, 10 min each) and two changes of propylene oxide (15 min each). Samples were infiltrated in a 1:1 mixture of propylene oxide: Spurr and the color was read at OD540 and OD605 by spectrophotometer. The collagen (μg/sec) = (OD540 – [OD605 × 0.291])/37.8 × 1,000. Non-collagen protein (μg/sec) = OD605 values/2.04 × 1,000. The goat anti-Type III Collagen-UNLB (cat. no. AF550, R&D Systems, Minneapolis, MN) was used at 1:100.

**Statistical Analysis**

Values are expressed as means ± SEM. Welch’s t test was applied when comparing two groups. When comparing three groups, one-way ANOVA plus Dunnett post-test was applied using Graph Pad Prism software. p < 0.05 was considered statistically significant. For the experiments involving multiple groups at different time points, two-way ANOVAs plus Bonferroni post-tests were used for statistical analysis. The mouse experiments were not done in a blinded fashion.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes eight figures, one table, and two movies and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.01.005.

**AUTHOR CONTRIBUTIONS**

C.Q., S.S.M., and X.X. were actively involved in conceptualization, methodology development, and writing of this manuscript. Q.J. was responsible for the critical editing of the manuscript. C.Q., Y.D., B.X., and V.D.N. directly performed animal experiments. Jianbin Li and Juan Li were involved in AAV vector production and titration for this study.

**Collagen Staining and Quantification and Immunofluorescent Staining**

Three methods, Masson’s Trichrome staining, Sirius red/fast green staining, and immunofluorescent staining against collagen III were used for collagen display. The Masson trichrome stain kit was commercially available (IMEB, cat. #K7228), and the instructor’s protocol was strictly followed. For Sirius red/fast green staining, the 8-μm-thin cryo-section tissues were fixed in prewarmed Bouin’s solution for 20 min at 37°C followed by three times washing with tap water. Then, the slides were put in 0.2% (w/v) aqueous phosphomolybdic acid for 2 min, followed by washing prior to staining. The slides were stained in 0.1% fast green for 15–20 min, washed with tap water. Prior to Sirius red staining, the slides were dipped in 1% acetic acid for 2 min followed by washing with tap water. The slide was stained in 0.1% Sirius red for 15–20 min, washed with water. Next, the slides were dehydrated in 100% ethanol for three times, cleared in xylene, and mounted to the slides by permount. Sirius red was dissolved in saturated picric acid, and fast green was dissolved in distilled water. To quantify the collagen, the stained slides were eluted with dye extraction solution (a 1:1 mixture of 0.1 N NaOH and methanol), and the color was read at OD540 and OD605 by spectrophotometer. The collagen (μg/sec) = (OD540 – [OD605 × 0.291])/37.8 × 1,000. Non-collagen protein (μg/sec) = OD605 values/2.04 × 1,000. The goat anti-Type III Collagen-UNLB (cat. no. AF550, R&D Systems, Minneapolis, MN) was used at 1:100.

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**Toluidine Blue Staining of Sciatic Nerves**

For light microscopy, 1 μm cross-thin-sections of the nerves were cut, mounted on 200 mesh copper grids, and post-stained with 4% aqueous uranyl acetate for 15 min, followed by Reynolds’ lead citrate for 7 min. Sections were observed using a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss SMT, Peabody, MA) and photographed using a Gatan Orius SC1000 Digital Camera and Digital Micrograph 3.11.0 (Gatan, Pleasanton, CA).

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Supplemental Information includes eight figures, one table, and two movies and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.01.005.

**AUTHOR CONTRIBUTIONS**

C.Q., S.S.M., and X.X. were actively involved in conceptualization, methodology development, and writing of this manuscript. Q.J. was responsible for the critical editing of the manuscript. C.Q., Y.D., B.X., and V.D.N. directly performed animal experiments. Jianbin Li and Juan Li were involved in AAV vector production and titration for this study.
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