Defining the optimal dose and therapeutic window in SMA with respiratory distress type I model mice, FVB/NJ-Ighmbp2\textsuperscript{nmd-2J}

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Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is an autosomal recessive disorder that develops in infancy and arises from mutation of the immunoglobulin helicase \(\mu\)-binding protein 2 (IGHMBP2) gene. Whereas IGHMBP2 is ubiquitously expressed, loss or reduction of function leads to alpha motor neuron loss and skeletal muscle atrophy. We previously developed a gene therapy strategy for SMARD1 using a single-stranded AAV9-IGHMBP2 vector and compared two different delivery methods in a validated SMARD1 mouse model. An important question in the field relates to the temporal requirements for this or any potential treatment. To examine the therapeutic window, we utilized our recently developed SMARD1 model, FVB/NJ-Ighmbp2\textsuperscript{nmd-2J}, to deliver AAV9-IGHMBP2 at four different time points starting at post-natal day 2 (P2) through P8. At each time point, significant improvements were observed in survival, weight gain, and motor function. Similarly, treatment improved important hallmarks of disease, including motor unit pathology. Whereas improvements were more pronounced in the early-treatment groups, even the later-treatment groups displayed significant phenotypic improvements. This work suggests that an effective gene therapy strategy could provide benefits to pre-symptomatic and early-symptomatic individuals, thereby expanding the potential therapeutic window for SMARD1.

INTRODUCTION

Spinal muscular atrophy with respiratory distress type 1 (SMARD1) is an infantile autosomal recessive neurodegenerative disease that is characterized by loss of alpha motor neurons and muscle atrophy.\textsuperscript{1,2} In contrast to 5q-linked spinal muscular atrophy (SMA), the first clinical symptom in SMARD1 is respiratory distress that develops between 6 weeks and 13 months of age.\textsuperscript{1,3,4} Respiratory distress is a result of diaphragm muscle atrophy,\textsuperscript{1,3,5,6} whereas in the related disease, SMA, intercostal muscle atrophy is typically the cause of respiratory failure. The respiratory complications in SMARD1 typically lead to complete paralysis of the diaphragm and artificial ventilation. Additional symptoms for SMARD1 include intratuerine growth delay, autonomic dysfunction, distal-to-proximal spread of muscle atrophy, and decreased diameter of myofibrils in skeletal muscles including the diaphragm.\textsuperscript{2,4,6,7}

SMARD1 is caused by loss-of-function mutations in the housekeeping gene immunoglobulin \(\mu\)-DNA binding protein 2 (IGHMBP2), located on chromosome 11q13.3.\textsuperscript{3,5,9} The ubiquitously expressed IGHMBP2 gene is comprised of 15 exons encoding 993 amino acids corresponding to a \(\sim\)110 kDa product. IGHMBP2 consists of an ATP-binding motif, a helicase-like motif, and two nucleic-acid-binding motifs. Patient mutations are predominately located in these functional motifs; however, the helicase domain is the region most frequently mutated.\textsuperscript{2,4,8,11} Based on in vitro and in vivo studies, the IGHMBP2 protein functions in immunoglobulin-class switching,\textsuperscript{9} pre-mRNA maturation,\textsuperscript{10} transcription regulation by either DNA binding activity\textsuperscript{11,14} or interaction with TATA-binding protein,\textsuperscript{15} and translation by direct interaction with tRNA and other components of translational machinery.\textsuperscript{16,17} However, the precise disease-causing role of IGHMBP2 mutation that leads to selective motor neuron loss remains unclear.

A spontaneous mutation in Ighmbp2 resulted in the initial identification and characterization of the neuromuscular degeneration (nmd\textsuperscript{2J}) mouse model.\textsuperscript{18,19} The original nmd\textsuperscript{2} mouse is on the C57BLKS background and contains a mutation in Ighmbp2 intron 4, giving rise to a cryptic splice site, resulting in an aberrant splicing event in \(~75\%–80\%\) of the transcripts. The altered splicing causes an addition of 23 nucleotides to the transcript that creates a premature...
translational termination codon. At 3 weeks of age, nmd mice become symptomatic and rapidly develop hindlimb muscle weakness to the extent that mice were paralyzed by 5 weeks of age with survival to 14 weeks. By postnatal day 10, nmd mice demonstrated over a 40% loss in motor neurons that progressed until death.

Our lab and another group have developed a single-stranded AAV9 (ssAAV9) vector containing human IGHMBP2 cDNA that demonstrated excellent efficacy in rescuing the nmd mice. We compared the efficacy of two delivery methods (intravenous [i.v.] and intracerebroventricular [i.c.v.]) in the nmd model using a single low dose, which demonstrated that i.v. injections are not as effective as i.c.v. injections in rescuing motor deficits. We recently developed a closely related strain of nmd mice on the FVB background called FVB/NJ-Ighmbp2nmd-2 J or “FVB-nmd” from this point on, resulting in a model that was consistent, severe, and well-suited for therapeutic studies, with a median survival of ~18–20 days. To date, AAV-mediated gene replacement has been shown to be the most effective means to rescue the SMARD1 mouse model. To advance the pre-clinical animal studies, we examined the therapeutic window to determine the relative efficacy of AAV9-IGHMBP2 at various stages of disease development by delivering vector at postnatal day 2 (P2), P4, P6, and P8. Our results suggest that the therapeutic window of IGHMBP2 gene replacement is not limited to early asymptomatic stages such as P1 or P2; rather, significant phenotypic benefit was observed in all treatment groups, including the cohort that received treatment at P8. This was surprising, since the vector is single-stranded, and maximal expression typically occurs more than 10 days after delivery. These results provide the first evidence of the effectiveness of SMARD1 gene therapy at later time points following disease onset.

RESULTS

Delivery of AAV9-IGHMBP2 at all time points significantly increased FVB-nmd lifespan

A single low dose of AAV9-IGHMBP2 at P2 via i.c.v. or i.v. injection at P2 significantly rescues the nmd phenotype. Here, we analyzed the phenotype of the severe FVB-nmd model following i.c.v. delivery of two different doses of AAV9-IGHMBP2 (Figure 1A) at 4 different time points starting at P2–P3 (asymptomatic) through P8–P9 (early symptomatic). A single low dose (1.25e11 vector genomes [vg]) was injected at P2, P4, P6, and P8, whereas the high dose (total of 2.5e11 vg) was applied on two consecutive days for each time point (P2; P4, P6; P8). Interestingly, all treated groups displayed significantly extended lifespans compared to untreated animals (Figures 1B and 1C; Figures S1A–S1D). Within the low-dose cohort, earlier P2 and P4 were similarly effective, while P6 and P8 significantly improved survival compared to the early injections. The same pattern was also observed in the cohorts that received the high-dose treatment, although the high dose reduced the number of early deaths in the P6 and P8 cohorts (Figure 1C). While one death occurred prior to P80 in the P2 and P4 low-dose treatment cohorts, all of the high-dose-treated animals in the P2 and P4 groups lived beyond 100 days (Figures 1B and 1C). Delivery of the vector at P6 and P8 time points still resulted in a significant extension in survival for treated FVB-nmd mice, albeit not as robust as the earlier treatment groups (p = 0.0002 for P6 and P = 0.001 for P8) (Figure 1B). The endpoint for these experiments was 132 days, and all remaining cohorts were euthanized.

Delivery of AAV9-IGHMBP2 at all time points significantly increased FVB-nmd weight

The weight of untreated FVB-nmd mice fluctuates between 3–4 g, whereas the nmd mice can weigh up to 10 g. To investigate the effect of the low- and high-dose AAV9-IGHMBP2 on the weight of FVB-nmd mice treated at different time points, daily weight was recorded starting at P2 until P132, when the survival and weight analyses were terminated. Consistent with the extension of the lifespan, both low and high doses of the viral vector led to a considerable increase in the weight of every treated group compared to untreated (p < 0.001) (Figures 1D and 1E). All treated groups gained weight until 40–50 days of age and then reached a plateau. Weight gain for each group was directly correlated to the time point of therapy rather than the dose of the vector. Treatment at earlier time points led to higher weight gain than the later time points, indicating a significant difference between each treated group regardless of the dose (P2 versus P4, p < 0.001; P4 versus P6, p < 0.001; P6 versus P8, p < 0.05; P2, P4, P5, P6, P7, P8, p < 0.01; P4, P5 versus P6, P7, p < 0.01; P6, P7 versus P8, p < 0.01) (Figures 1D and 1E). Additionally, there was no significance in the weight gain of any individual group treated with either low or high dose (Figures S2A–S2D) except for P4-treated mice, in which the increased dose led to higher weight gain during the age of 85–115 days (p < 0.05).

Early AAV9-IGHMBP2 treatment improved motor function in FVB-nmd more than later time points

Rotarod and grip strength were performed starting at P40 for 7 consecutive days following a training and acclimation period. Untreated FVB-nmd animals were not included in these studies focused upon motor function, since younger animals are not capable of performing these tasks and their lifespan is too short. Therefore, each treatment group was compared to the heterozygous unaffected littermates (HET: FVB-Ighmbp2nmd/+) and to each other in low- and high-dose-treated groups. Rotarod performance, as determined by the time an animal remained on the rotating cylinder, of early-treated animals (P2 and P4 treated) were indistinguishable from unaffected HET counterparts in both low- and high-dose groups (Figures 2A and 2B; Figures S3A and S3B). The rotarod performance of P6- and P8-treated mice, though improved, was significantly less compared to the HET and early-treated littermates in the low- and high-dose groups (p < 0.001) (Figures 2A and 2B). High-dose delivery slightly improved the rotarod performance of the P6- and P8-treated compared to low dose, but it did not differ statistically (Figures S3C and S3D).

In contrast to the rotarod performance, forelimb strength in all treated groups was significantly weaker than unaffected animals...
This reduced level of rescue in forelimb strength is consistent with the original nmd mice, demonstrating that the i.c.v. delivery of the vector is capable of repairing hindlimb activity more efficiently than forelimb, although the grip strength in each treated group improved significantly using the high dose of the viral vector when compared to low-dose group (Figures 2C and 2D). Single comparison of each time point treatment in low- and high-dose groups revealed that improvements in forelimb strength, resulting from high-dose administration, are more obvious in early-treated (P2 versus p2,3 and P4 versus P4,5, p < 0.001) (Figures S4A and S4B) but still significant in the P6-treated (P6,7 versus P6, p < 0.01) (Figure S4C) and P8-treated mice (P8,9 versus P8, p < 0.05) (Figure S4D). Nevertheless, the high dose could not sufficiently increase the grip strength to reach the level of the HET littermates in any treated group (p < 0.001) (Figures 2D; Figures S4A–S4D). Western blot analysis of early- and late-treated mice (P2, P8) showed no statistical difference among the various cohorts (Figure S5).

Muscle pathology improved in all treatment groups

FVB-nmd mice have a severe hindlimb phenotype characterized by contracture in the hindlimbs and an inability to splay their legs by P17. In our previous study, we showed that FVB-nmd mice have significantly decreased gastrocnemius muscle fiber size compared to FVB mice. Additionally, we have previously reported that P2-treated nmd/2J mice have a high level of rescue in their gastrocnemius muscle. To investigate whether the early (P2) and late (P8) time point treatment led to muscle fiber rescue in FVB-nmd, we compared laminin-immunostained cross-sectioned gastrocnemius muscles of high-dose-treated animals with those of the wild-type (WT) FVB at 42 days of age. The muscles of phenotypically unaffected age-matched HET mice were used as a control for untreated
FVB-nmd harvested at 14 days of age, a late symptomatic stage. Consistent with our previous results, muscle fiber area and perimeter of the gastrocnemius muscle are significantly decreased in untreated FVB-nmd mice compared to HET (p < 0.0001) (Figures 3A–3C). A blinded quantitative assessment of muscles revealed that muscle fiber area and perimeter of the gastrocnemius in P2-treated mice were significantly larger than P8-treated mice (p < 0.0001) but still notably smaller than WT (p < 0.0001) (Figures 3A–3C). The grip strength of all the treated animals, in both low- and high-dose groups, was significantly weaker than HET (one-way ANOVA, p < 0.001). Early-treated groups were significantly stronger than late-treated littermates (one-way ANOVA, P2 and P4 versus P6 and P8, p < 0.001; P2 versus P4, p < 0.001 in low dose). High-dose P2- and P4-treated mice did not differ statistically (p > 0.05); P6- and P8-treated mice had similar grip strength in low- and high-dose groups (p > 0.05).

Neuromuscular junction (NMJ) pathology of the gastrocnemius improved in early- and late-treated FVB-nmd mice

NMJs from the gastrocnemius muscle exhibit severe denervation defects in FVB-nmd mice, but these can be partially rescued by delivery of AAV9-IGHMBP2 on P1.23,24 To examine how early and late delivery of AAV9-IGHMBP2 impacts the FVB-nmd NMJ phenotype, we conducted a blinded quantitative assessment of NMJ pathology from the gastrocnemius of P2- and P8-treated FVB-nmd mice and age-matched, untreated WT mice at P42. The untreated FVB-nmd and their age-matched HET counterparts harvested at P14 were compared to each other at a relatively early time point. As expected, NMJs from untreated FVB-nmd mice displayed severe pathology, with less than 40% fully innervated endplates and more than 50% fully denervated endplates compared to age-matched HET mice (p < 0.0001) (Figures 4A and 4B). The P2-treated mice had similar level of innervation and denervation to age-matched WT controls at P42. The untreated FVB-nmd and their age-matched HET counterparts harvested at P14 were compared to each other at a relatively early time point. As expected, NMJs from untreated FVB-nmd mice displayed severe pathology, with less than 40% fully innervated endplates and more than 50% fully denervated endplates compared to age-matched HET mice (p < 0.0001) (Figures 4A and 4B). The P2-treated mice had similar level of innervation and denervation to age-matched WT controls at P42.
WT and P2-treated, p < 0.0001) and approximately 22% fully denervated endplates (P8-treated versus WT, p < 0.01; P8-treated versus P2-treated, p < 0.05) (Figures 4A and 4B). These results are consistent with the other phenotypic parameters and further demonstrate that early delivery is the most efficacious, but even the symptomatic cohort (P8) significantly benefits from this effective treatment.

AAV9-IGHMBP2 increases the motor neuron numbers in the spinal cord (SP) of the early- and late-treated FVB-nmd mice

Based on our previous analysis, the number of motor neurons in the lumbar region of the original nmd2J mice was reduced nearly 65% at 8 weeks of age.21 We investigated whether the delivery of AAV9-IGHMBP2 prevents motor neuron loss and pathology of motor neurons in the FVB-nmd model. Lumbar spinal cord sections representing L3–L5 regions from P14 cohorts, FVB-nmd and HET, and P42 cohorts, WT, P2 treated, and P8 treated, were analyzed through a blinded quantification of immunofluorescent images. As predicted, FVB-nmd contained a significantly reduced number of motor neurons, with an average of ~5 motor neurons per section, compared to ~6.6 or 8 motor neurons per section in the age-matched HET or WT mice (p < 0.0001) (Figures 5A and 5B). However, the area and perimeter of the FVB-nmd motor neurons were only slightly smaller than those in HET (p > 0.05), suggesting that even though motor neurons at P14 are reduced in numbers, the remaining motor neurons do not yet exhibit dramatic cellular pathology (Figures 5A–5D). Interestingly, the average motor neuron numbers in P2-treated, P8-treated, and WT mice were similar to each other, with an average of 8 motor neurons per section (p > 0.05) (Figures 5A and 5B). Although the area and perimeter of P2-treated and P8-treated motor neurons were significantly smaller than WT (p < 0.05), they were significantly larger than the P8-treated in area (p < 0.01) and similar to the P8-treated in perimeter (p > 0.05) (Figures 5A–5D). P8-treated motor neurons were significantly smaller than WT in area and perimeter (p < 0.0001) (Figures 5A–5D). These results confirm the effectiveness of early treatment in rescuing the numbers and, to some extent, the size of motor neurons, whereas late treatment was only able to rescue the number of motor neurons but not the size.

DISCUSSION

While there is no approved treatment for SMARD1, AAV9-IGHMBP2 gene therapy has made significant progress in pre-clinical
mouse models. However, understanding the temporal requirements of this, or any, potential therapeutic is critical as it advances through pre-clinical evaluation. To begin to understand the temporal requirements of IGHMBP2 from a therapeutic perspective, we devised a series of experiments that delivered a previously characterized gene therapy vector, AAV9-IGHMBP2. I.c.v. injections of the vector were administered to FVB-nmd cohorts on P2, P4, P6, and P8. This time frame spanned pre-symptomatic through early symptomatic days. Additionally, two different doses were administered: a low dose and a high dose, corresponding to 1.25e11 and 2.5e11 vector genomes, with mice receiving either a single injection or double injection to accommodate the small volume of the neonatal ventricles, respectively. Previous studies in nmd2J mice have shown a significant extension of lifespan, improvements in hindlimb splay, and significant reduction of cellular pathology following a single treatment with AAV9-IGHMBP2 immediately after birth. While we have previously observed that P2 pre-symptomatic delivery of this vector protects from disease development in the B6.BKS-nmd mouse model, it was surprising to observe a robust response in the later treatment time points of P6 and P8, with symptomatic cohorts significantly responding to treatment. The FVB-nmd mice present with a severe phenotype and a relatively short lifespan, with an average lifespan of ~18–20 days. This dramatic extension in survival was unexpected due to the severity of the disease and because single-stranded AAV vectors such as the AAV-IGHMBP2 vector typically do not achieve maximal levels of expression until 10 days post-delivery. It is important to note that in this animal model, there is a baseline (~20%) of Ighmbp2 protein that is full-length and WT. This baseline level may be very important as it relates to therapies that further increase IGHMBP2 levels, since achieving a therapeutic threshold should theoretically be easier than if the genetic context was purely mutant in nature, such as any number of the SMARD1-causing IGHMBP2 point mutations. The development of additional models that reflect these patient-based contexts would be another important step toward understanding the requirements of a potential AAV9-IGHMBP2 therapeutic.

We examined Ighmbp2/IGHMBP2 protein levels in the spinal cord from P2 and P8 at 10 days after treatment, respectively. In this mouse model, we did not see a significant change in protein between the unaffected, the untreated SMARD1 model mice, and the two treated cohorts. Clearly, there is a difference in the phenotype of these animals; however, the western blots do not appear to be capturing these differences. A potential explanation for this is that the relative amount of
protein is tightly regulated by the cell, but in specific cell types (perhaps motor neuron populations), a critical increase in full-length IGHMBP2 is achieved that significantly improves the phenotype.

In the 5q SMA context, several studies have examined the temporal requirements of SMN-dependent therapeutics in severe SMA mice (SMNΔ7 mice), including a gene therapy strategy. Consistent results were observed in different labs, each demonstrating that SMNΔ7 mice do not respond well to SMN induction past ~P6. While improvements were observed in the cohorts that received treatment at P7–P8, the magnitude of rescue was significantly reduced. The SMA phenotype was largely recalcitrant to SMN increases when delivered post-symptomatically, suggesting that SMNΔ7 mice with lifespans of 14–15 days may have progressed to a point in their disease state that has permanent damage despite rapid expression of SMN1. However, in the instance of the P7–P8-treated FVB-nmd cohorts, AAV9-IGHMBP2 results in ~40% survival past 50 days of age. This suggests that in the FVB-nmd cohort, P7–P8 delivery of full-length IGHMBP2 is sufficient to have a protective effect.

Through our time point study, we were able to demonstrate that the early injection time points, P2 and P4, resulted in a more robust phenotypic rescue compared to P6- and P8-treated mice. Our cellular pathology analyses show that P8 AAV9-IGHMBP2-treated mice do not differ in the number of lumbar motor neurons when compared to WT and P2-treated mice, which indicates that the specific motor neurons targeted by AAV9-IGHMBP2 are present at the time of delivery. Additionally, we show that NMJ innervation is significantly protected in P8 AAV9-IGHMBP2-treated mice, with ~20% increase in innervated endplates compared to FVB-nmd. The gastrocnemius and diaphragm muscle fiber size additionally showed improvement in P2-treated and P8-treated mice; however, the muscle fiber size did not reach WT levels, indicating that AAV9-IGHMBP2 does not result in a full rescue but does have a protective effect.

Like many other related neurodegenerative diseases, the disease-specific role for IGHMBP2 is poorly understood. The best-described function for IGHMBP2 is its role in the translational machinery

Figure 5. I.c.v. delivery of AAV9-IGHMBP2 in high dose improved the motor neuron number and area size in the lumbar region of FVB-nmd mice at early and late time points
(A) Nissl body (NeuroTrace) and anti-choline acetyl transferase (ChAT) were used to immunostain motor neurons from cross-sections of lumbar spinal cord (L3–L5). High-dose P2- and P8-treated mice (n = 4) were compared to age-matched WT littermates (P42) (n = 3), while untreated FVB-nmd mice (n = 4) were evaluated in comparison to the age-matched HET mice (P14) (n = 3). Fluorescent representative images were taken at 40× magnification. (B) The number of motor neurons in FVB-nmd mice was drastically less than HET (one-way ANOVA, p < 0.001), while the motor neuron numbers in P2 and P8 were similar to the WT cohort (p > 0.5). The area and perimeter of the motor neurons in FVB-nmd mice was not statistically different from HET (p > 0.5). P2- and P8-treated motor neurons were smaller than WT in area and perimeter with varying degrees (one-way ANOVA, P2 versus WT, p < 0.05; P8 versus WT, p < 0.001). P2-treated and P8-treated mice differed only in the area of the motor neuron soma (p < 0.05) (C and D). Scale bar, 50 μm. Error bars represent mean ± SEM.
and ribosome biogenesis. IGHMBP2 has been shown to interact with tRNA$^{3'\text{VT}}$ and TFIIC220, which are essential factors for tRNA transcription. Additionally, IGHMBP2 also interacts with Reptin/Pointin and activator of basal transcription 1 (ABT1), which has an important role for ribosome biogenesis. Although it still remains unknown, we speculate that AAV9-IGHMBP2 allows for sufficient restoration of translation-related pathways that are dysregulated in Ighmbp2-deficient tissues, such as sub-populations of motor neurons. It remains unclear why motor neurons are the most susceptible cell in SMARD1, though dysregulation of pre-mRNA, rRNA, and tRNA appears to be a common feature among motor neuron diseases.

We have demonstrated that a higher dose of AAV9-IGHMBP2 in FVB-nmd mice resulted in higher peak weight gain, lowered the number of early deaths following treatment, and improved their overall forelimb grip strength. The next step would be to understand the relationship between dosage and the extent of rescue in cellular pathology defects. Collectively, this pre-clinical study addresses the temporal requirements for AAV9-IGHMBP2 in FVB-nmd mice by illustrating that treatment pre-symptomatically results in the most therapeutic benefit but that a significant phenotypic improvement was also observed in the cohorts that received the vector even after symptoms developed. While it is important to stress that these are experiments performed in mice, it is a step forward for this potential therapeutic. This is particularly important as, unlike SMA, the majority of SMARD1 patients are diagnosed after symptomatic onset due to lack of a newborn genetic test and the infrequency of SMARD1 cases.

MATERIALS AND METHODS

Animal procedures, viral vector, and injections

All experimental procedures were approved by the University of Missouri’s Institutional Animal Care and Use Committee and were performed following the regulations established by the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. The FVB-nmd mouse model was created in the Animal Modeling Core of the University of Missouri, Columbia as previously described in detail. FVB-nmd animals were genotyped at P1. The viral vector (single-stranded AAV9-IGHMBP2) has been previously described in detail. Vector was delivered via an i.v. injection with 1.25 × 10^{11} (low dose) viral genomes at four time points (P2, P4, P6, and P8) and 2.5 × 10^{11} (high dose) on two consecutive days for each time point (P2.3, P4.5, P6.7, P8.9). At 14 days of age, two groups of FVB-nmd mice (unaffected heterozygous HET [n = 3] and homozygous untreated [n = 4]) and at 6 weeks of age, three groups (WT [n = 3], P2.3-treated [n = 4], and P8.9-treated [n = 4]) at 14 days of age were transcardially perfused and gastrocnemius muscles harvested after 24 h. 16-μm cross-sections were prepared from gastrocnemius and diaphragm muscles followed by immunohistochemistry with anti-laminin. Images were collected using a Leica DM5500 B fluorescent microscope (Leica Microsystems). A blinded quantification assessments of muscle fibers were performed using ImageJ as previously described.

Motor neuron count

Two groups of FVB-nmd (HET [n = 3] and homozygous untreated [n = 4]) at 14 days of age and three groups of FVB-nmd (WT [n = 3]),
P2.3-treated \([n = 4]\), and P8.9-treated \([n = 3]\)) at 42 days of age were transcardially perfused with ice-cold 4% PFA followed by subsequent post-fixing at 4% PFA for 24 h at 4°C. Lumbar (L3–L5) regions from spinal cord tissue were dissected and cryoprotected in 30% sucrose solution overnight before being embedded in optimal cutting temperature (OCT) media. Embedded tissues were cryosectioned at 16 μm thickness with every 10th section from the spinal cord tissue being collected for immunohistochemistry. Sections were stained with choline acetyltransferase (ChAT) primary antibody (1:100; catalog R25GM064120 (J.M.).

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AUTHOR CONTRIBUTIONS

M.S., C.E.S., and C.L.L. designed the experiments and wrote and edited the manuscript. M.S., C.E.S., E.V., J.M., and M.O.G.-K. conducted the experiments; Z.A. and S.M.R.H quantified the immuno-stained images.

DECLARATION OF INTERESTS

C.L.L. is co-founder and Chief Scientific Officer of Shift Pharmaceuticals. All other authors declare no competing interests.

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