

## ARTICLE

# AAV9 supports wide-scale transduction of the CNS and TDP-43 disease modeling in adult rats

Kasey L Jackson<sup>1</sup>, Robert D Dayton<sup>1</sup> and Ronald L Klein<sup>1</sup>

AAV9 has emerged as an efficient adeno-associated virus (AAV) serotype for gene transfer to the central nervous system. We have used this technique to study aspects of amyotrophic lateral sclerosis (ALS) by administering AAV encoding the ALS-related gene transactive response DNA binding protein of 43 kDa (TDP-43) to neonatal rats. However, inducing the expression in adult subjects would be preferable to mimic the adult onset of symptoms in ALS. We expressed either green fluorescent protein (GFP) or TDP-43 in adult rats after an intravenous (i.v.) route of administration to attempt wide-scale transduction of the spinal cord for disease modeling. In order to optimize the gene transfer, we made comparisons of efficiency by age, gender, and across several AAV serotypes (AAV1, AAV8, AAV9, and AAV10). The data indicate more efficient neuronal transduction in neonates, with little evidence of glial transduction at either age, no gender-related differences in transduction, and that AAV9 was efficient in adults relative to the other serotypes tested. Based on these data, AAV9 TDP-43 was expressed at three vector doses in adult female rats yielding highly consistent, dose-dependent motor deficits. AAV9 can be delivered i.v. to adult rats to achieve consistent pathophysiological changes and a relevant adult-onset system for disease modeling.

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## INTRODUCTION

Viral vector gene transfer is an important approach in the neurosciences for basic functional studies, gene therapy, and disease modeling. Recombinant adeno-associated virus (AAV) is often chosen for viral vector gene transfer due to its lack of pathogenicity, ability to transduce nondividing cells, and long-term gene expression. AAV gene transfer in the brain and spinal cord has historically employed localized, focal gene vector delivery. With the advent of AAV serotype 9 (AAV9),<sup>1,2</sup> relatively less invasive systemic administration has been used to transduce the central nervous system (CNS) on a widespread basis.<sup>3–5</sup> Of note, peripheral intravenous (i.v.) delivery of AAV9 has been used successfully to correct pathology in the CNS in preclinical mouse models. Preclinical gene therapy is robust in mouse models of spinal muscular atrophy,<sup>6–11</sup> lysosomal storage diseases,<sup>12,13</sup> and stroke.<sup>14</sup> Preclinical work with i.v. AAV9 in spinal muscular atrophy has progressed to a clinical trial (NCT02122952).<sup>15</sup> We have administered AAV9 systemically to express transactive response DNA binding protein of 43 kDa (TDP-43) and model amyotrophic lateral sclerosis (ALS).<sup>16–18</sup> For further research into these areas, it is important to understand some of the governing parameters of gene transfer, for example, whether the subject's age or gender influences gene transfer efficiencies and if different AAV serotypes perform as well as AAV9. Most wide-scale CNS gene transfer has been studied in mice,<sup>3,4,19</sup> but relatively less is known in rats. Rats are more similar to humans than mice with respect to a variety of physiological parameters, e.g., cardiovascular,

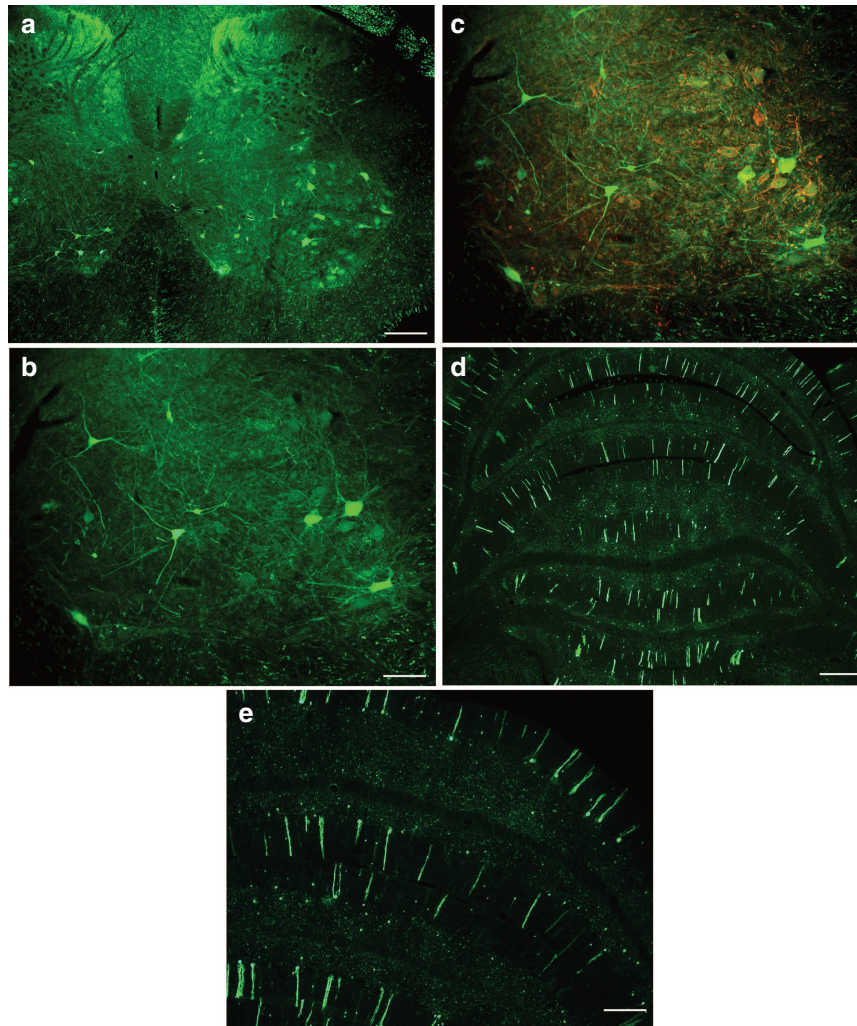
respiratory, and metabolic rates as well as being larger in size. Rats also provide specific, advantageous test systems for experimental approaches in pharmacology, toxicology, and behavioral neuroscience. Understanding gene transfer parameters in rats is important as more and more advantageous rat transgenic lines become available.

AAV9 was initially reported to confer widespread neuronal transduction in neonatal but not adult mice.<sup>4</sup> However, other studies reported widespread neuronal gene transfer in adult mice.<sup>3,19,20</sup> We have used the i.v. AAV9 approach to consistently express green fluorescent protein (GFP) or the ALS-related protein TDP-43 in rats on a wide-scale basis after gene delivery to neonatal rats.<sup>16–18</sup> However, ALS is an adult-onset disease so it is more relevant to express TDP-43 in adults. We therefore tested if AAV9 could support consistent gene expression and TDP-43-induced paralysis in adults as we previously saw after neonatal gene transfer.<sup>16</sup> Adult gene transfer is preferable for several reasons, an important one being that it will better avoid developmental effects which could complicate interpretations. We tested if consistent, wide-scale CNS expression could be achieved in adults as we had previously seen with neonates in order to explore a more relevant adult-onset model of ALS. We also wanted to determine if relatively lower level expression is to be expected in adults versus neonates when compared under equal conditions.

It is relevant to include both genders in biomedical studies of health and disease. There are reports of gender differences in mice with respect to AAV gene transfer efficiency. For example, Maguire

<sup>1</sup>Department of Pharmacology, Toxicology, and Neuroscience, Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA. Correspondence: RL Klein (Klein@lsuhsc.edu)

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**Figure 1** AAV9 administered via tail vein to adult rats transduces neurons in the spinal cord and cerebellum. Green fluorescent protein (GFP) was expressed in spinal lower motor neurons in the ventral horn throughout the spinal cord. (a–c) A section from the lumbar region. GFP immunoreactivity is shown in a and b and merged with immunoreactivity for nonphospho neurofilament, a marker for motor neurons, in c. (d, e) In the cerebellum, the Purkinje neurons showed relatively high levels of GFP expression. Bar in a = 268  $\mu\text{m}$ . Bar in b = 134  $\mu\text{m}$ . Same magnification in c. Bar in d = 536  $\mu\text{m}$ . Bar in e = 268  $\mu\text{m}$ .

*et al.*<sup>20</sup> reported greater efficiency in female mice after systemic AAV gene delivery. It is important to know if there is an effect of gender in interpreting data including both genders as well as for optimizing gene transfer efficiency for modeling.

Another key factor controlling gene transfer efficiency is AAV serotypes or engineered vectors. In adult mice, several AAV serotypes support widespread gene transfer.<sup>19</sup> It is important to know if other serotypes can support widespread expression in adult rats, for example, to determine if similar expression is to be expected upon applying different serotypes. This might be done to avoid an immune response and the potential for muted expression after a second, subsequent vector application to the same animal. In this study, we compared AAV1, AAV8, AAV9, and AAV10 for the efficiency of GFP gene transfer after i.v. administration to rats to determine if AAV9 was uniquely efficient among this small vector set. The overall goal is to understand if different levels of expression are to be expected across ages, genders, and AAV serotypes in order to expand ALS disease modeling to adults. The experiments address if i.v. delivery to adults can be used for consistent expression and disease modeling as well as assess the overall feasibility and utility of this peripheral-to-central gene transfer approach.

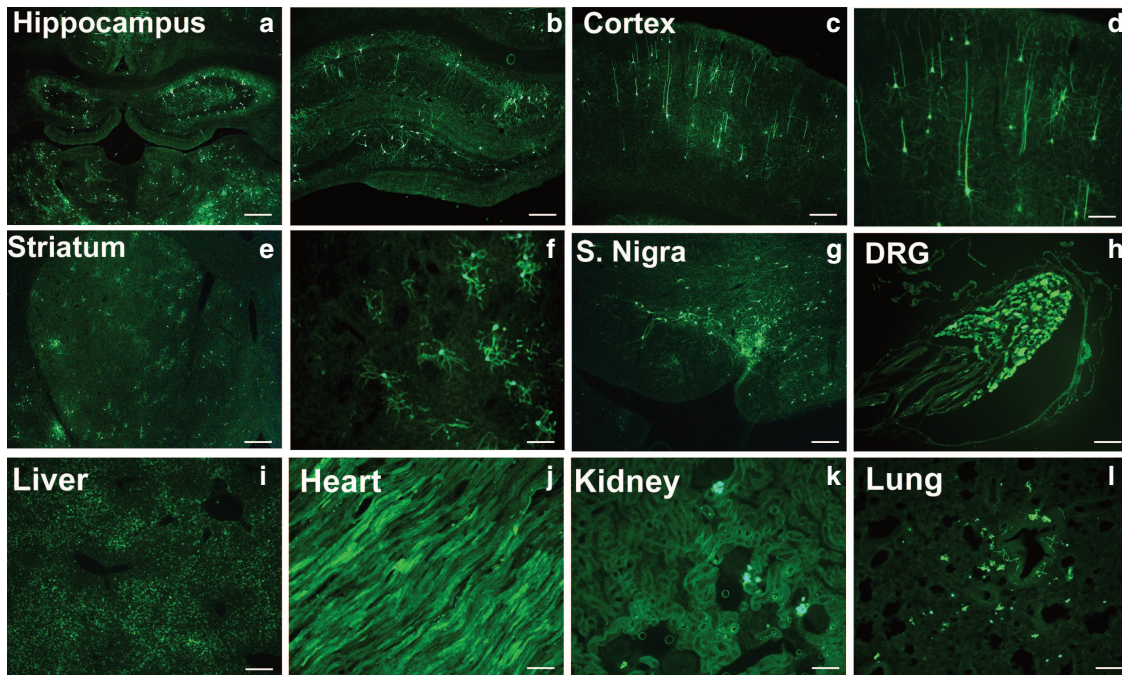
## RESULTS

### AAV GFP gene transfer in neonates and adults

In our initial attempts with i.v. injections to adult rats, we only observed sporadic neuronal transduction in the CNS. In light of studies in which widespread transduction was achieved in adult mice,<sup>3,19</sup> we increased our vector dose and found efficient expression in the CNS.

AAV9 GFP at a dose of  $1 \times 10^{14}$  vector genomes (vg)/kg produced wide-scale neuronal transduction throughout the spinal cord and the cerebellum (Figure 1 and Supplementary Figure S1), two areas that are also highly transduced with the neonatal approach. Transduction of spinal motor neurons and cerebellar Purkinje neurons are shown in Figure 1. We counterstained adjacent sections with an astroglial marker and observed that very few if any of the transduced cells were astrocytes in the adult rats. Thus, the same transduction pattern of AAV9 within the CNS resulted after tail vein injections to adults as occurs after either i.v. administration to neonatal rats or with stereotaxic injections (Figures 1 and 2). Viewing the Purkinje cell layer in the cerebellum of adult rats with robust expression in this area, we did not notice signs of cellular infiltration relative to controls by staining with hematoxylin and eosin





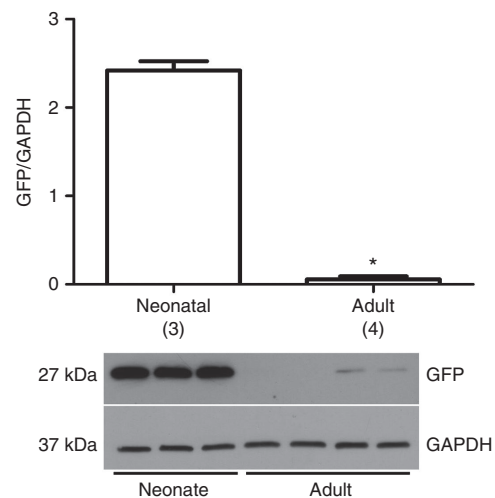
**Figure 2** Wide-scale neuronal transduction in the CNS and other tissues after AAV9 GFP tail vein injections to adult rats. Transduced neurons were seen in all areas of the CNS that were examined. Some of the transduced regions included (a, b) hippocampus and thalamus, (c, d) cortex, (e, f) striatum (note morphology of medium spiny neurons), (g) substantia nigra, and (h) dorsal root ganglia (DRG). Outside of the CNS, there was substantial transduction of (i) the liver, and (j) the heart. Sparse GFP-labeling was seen in the (k) kidney and the (l) lung. Bars in a and e = 536  $\mu$ m. Bars in b, c, g, and i = 268  $\mu$ m. Bars in d, f, h, and j–l = 134  $\mu$ m. CNS, central nervous system; GFP, green fluorescent protein.

(Supplementary Figure S2). Outside of the CNS, with this promoter system and i.v. administration in either neonates or adults, there is significant expression in several peripheral organs, such as the liver and the heart (Figure 2).

The i.v. administration to neonates results in efficient expression, but if similar expression could be achieved in adults, it would be more relevant for adult-onset diseases. We studied if gene delivery in adults would produce physiologically relevant levels of expression, or if gene delivery in neonates is required for strong expression. Thus, we compared gene transfer in adults versus neonates. The same per kilogram dose was administered to neonatal and adult rats. GFP expression levels were quantified on western blots of spinal cord from the lumbar region (Figure 3). The survival interval for this experiment was 4 weeks after gene transfer. By this analysis, there was a clear difference in expression (Figure 3,  $P < 0.001$ , *t*-test), estimated to be about 40-fold higher in neonates at this vector dose and time interval.

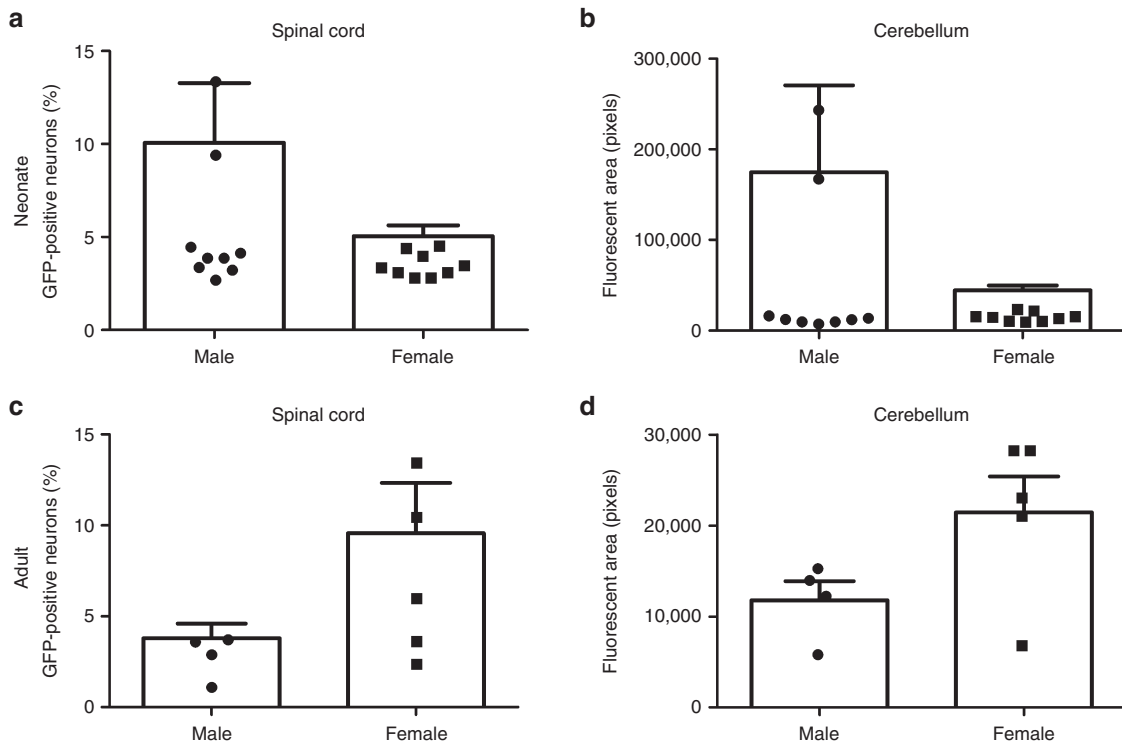
Potential effects of gender on AAV transduction efficiency were evaluated in both neonatal and adult rats using AAV9. There have been reports of sex hormone involvement in AAV transduction efficiency.<sup>20–22</sup> However, in this study, there was no significant difference in transduction efficiency between genders at either the neonatal (Figure 4a,b) or adult age (Figure 4c,d). However, it is worth mentioning a trend of increased expression in adult female rats relative to adult male rats ( $P = 0.09$ ; *t*-test), which would be consistent with the increased expression seen in adult female mice by Maguire *et al.*<sup>20</sup> Each data point is shown to depict the variability of the approach, which was considerable in the neonatal males and the adult females as shown in Figure 4.

Next, we compared several AAV serotypes that have been used extensively in animals before, such as AAV1, AAV8, AAV9, and AAV10. The same GFP expression plasmid DNA was packaged into each type, and the same dose of viral particles were administered.



**Figure 3** GFP expression levels in the spinal cord in neonates and adults after similar dose-matched injections. The GFP levels in the lumbar spinal cord samples of neonates were estimated to be ~40-fold higher than that of the matched adult samples ( $*P < 0.001$ , *t*-test,  $N = 3–4$ /group). Top, quantification of the bands. Bottom, western blots with 3–4 subjects per group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein.

GFP fluorescence was evaluated in the cerebellum and spinal cord 4 weeks post-administration. With neonatal administrations, we found no significant differences in transduction efficiency in the cerebellum or spinal cord (Figures 5 and 6). The AAV administration and GFP expression had no untoward effect on motor performance in these rats as studied by rotarod (Supplementary Figure S3). AAV8, AAV9, and AAV10 were compared in adult rats. AAVs were administered into the tail vein, and fluorescence was evaluated 4 weeks post-administration. AAV9 produced greater expression levels of



**Figure 4** AAV9 GFP gene transfer in females and males after i.v. vector administration to either (a, b) neonates or (c, d) adults. In the spinal cord, the percentage of motor neurons expressing GFP was estimated. In the cerebellum, an imaging program was used to estimate the intensity of GFP immunoreactivity (Supplementary Figure S1). There was no significant effect of gender on transduction efficiency using AAV9 at either age ( $N = 4-9$  group). GFP, green fluorescent protein; i.v., intravenous.

GFP compared to AAV8 and AAV10 (Figures 5 and 6). AAV9 therefore appeared stronger in the adult but not in neonatal administrations. AAV1 was excluded from the analysis in adults due to an insufficiently low titer, although AAV1 GFP was applied to one adult subject. The AAV1 GFP adult subject did not appear to be strongly transduced relative to AAV9 (Figure 5).

#### Adult onset of motor paralysis and AAV9 TDP-43 vector dose dependence

In order to study if expression in adults was physiologically significant, we administered female rats with AAV9 encoding wild-type TDP-43 (TDP-43), a protein found aggregated in the majority of patients with ALS.<sup>23</sup> Rats were administered AAV9 TDP-43 in two separate dose groups:  $1 \times 10^{13}$  vg/kg (low dose TDP-43) and  $3 \times 10^{13}$  vg/kg (medium dose TDP-43). One rat was administered a higher dose of AAV9 TDP-43 ( $6 \times 10^{13}$  vg/kg). The rats were monitored for motor deficits and then for TDP-43 expression postmortem.

There was a clear vector dose dependence of the TDP-43-induced paralysis in adult rats over an 8-week time interval after gene transfer. The low-dose AAV9 TDP-43 group showed no significant differences in weight gain compared to uninjected animals and displayed no signs of motor deficits over 8 weeks (Figure 7). In contrast, the medium dose AAV9 TDP-43 produced a progressive disease state with reduced weight gain, rotarod impairments, and reduced rearing over 3 weeks (Figure 7). The one high dose rat became severely immobilized and died by 3 weeks. Upon evaluating TDP-43 levels in the tissues by western blots, we found that the level of impairment in the low- and medium-dose groups corresponded with TDP-43 expression. Both total and human TDP-43 levels were significantly higher in the medium-dose group than those in the low-dose group

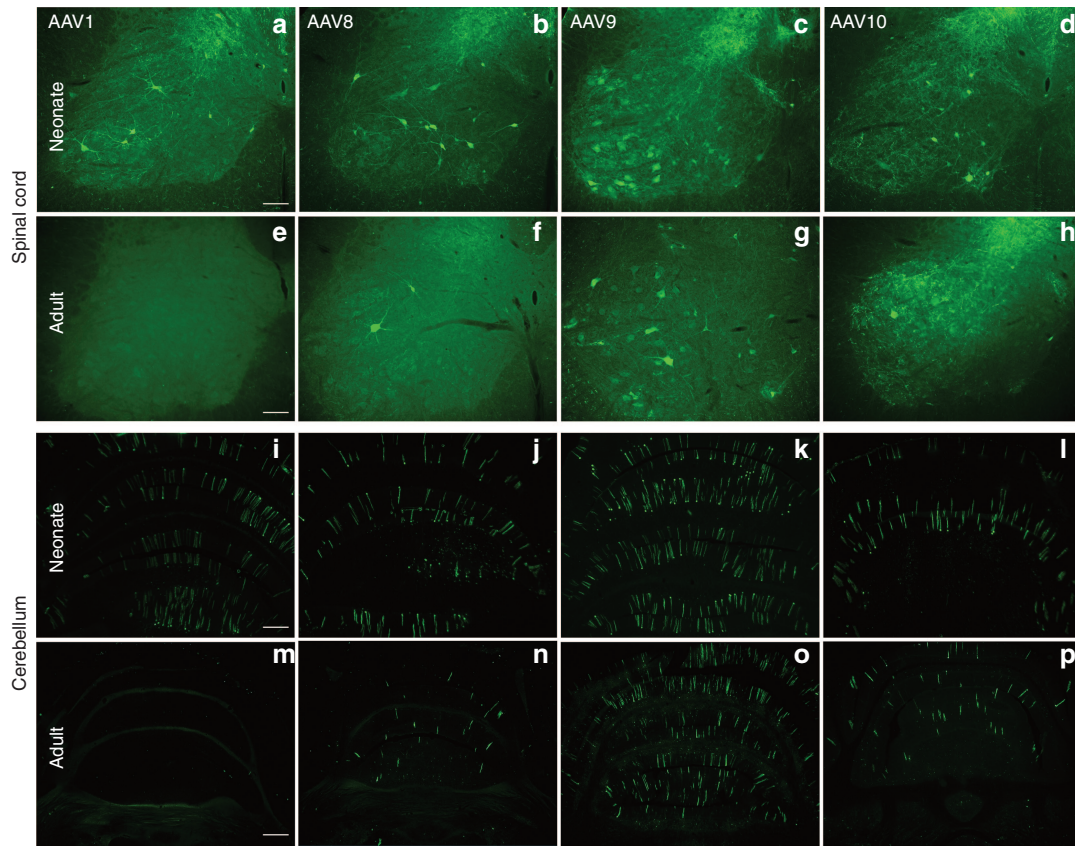
(Figure 8;  $P < 0.05$ ), demonstrating fine dose control of consistent and physiologically relevant TDP-43 levels.

#### DISCUSSION

This study determined that greater neuronal expression levels could be achieved in younger versus older rats after i.v. administration of AAV9. This finding agrees with data from mice by Foust *et al.*<sup>4</sup> although we did not observe a change in the cellular tropism as was found in the aforementioned paper which saw more astroglial transduction in adults. The GFP expressed within the CNS was found mainly in neurons after gene transfer in either neonates or adults. Despite some degree of variability as detailed above, no significant effect of gender was determined at either age. This agrees with our previous work with neonatal rats using both genders in which we have not noticed any trend in favor of a gender difference in gene transfer efficiency.<sup>16,17</sup> In this study, there was data to suggest that AAV9 was more efficient in the CNS of adults relative to AAV8 and AAV10. However, we do not know if this represents a true age-related difference or a technical aspect with respect to the overall lower level expression in adults. For example, the adult data may be lower on the dose-response curve of expression, which may be more sensitive to tease out small differences among serotypes than in neonates that may be closer to the maximal levels. Regarding the greater efficiency that we observed in neonates, it is worth noting that we used two different sites of injections at the two ages, and we cannot rule out that there may be inherently greater transduction from the temporal vein site used in the neonates.

Based on these results, we used AAV9 to express TDP-43 in adult females at several vector doses, which resulted in consistent, dose-dependent effects of the induced disease state. The low dose of AAV9 TDP-43 was well tolerated by the rats, while the medium dose caused





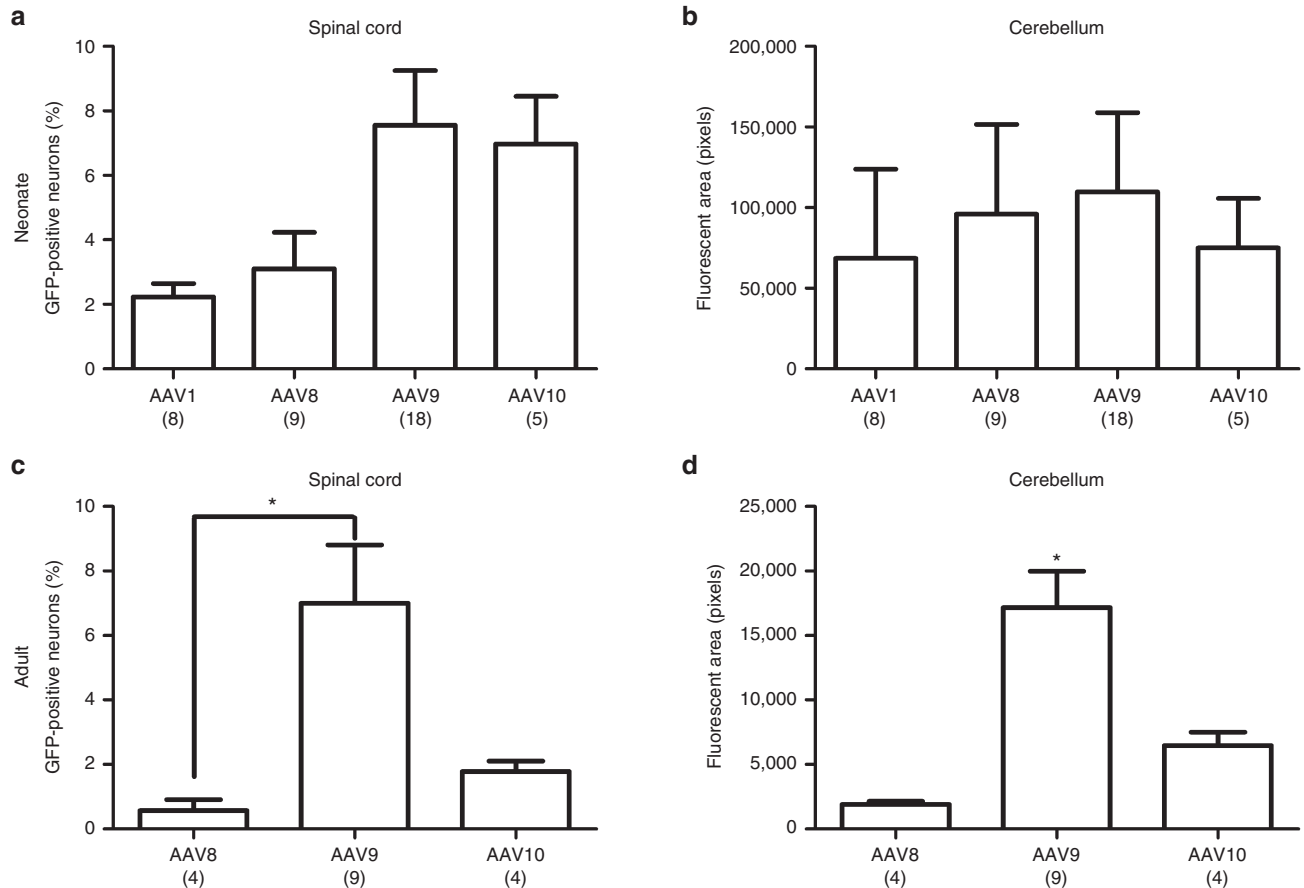
**Figure 5** GFP expressed by AAV1, AAV8, AAV9, or AAV10 after i.v. vector administration to either neonates or adults. The GFP expression in the CNS appeared to be neuronal with any of the serotypes tested at either age. The different serotypes are shown in the different columns, and the spinal cord and cerebellum are shown at each age. The serotypes performed similarly for GFP expression in the neonates, but in adults, AAV9 appeared to result in the strongest GFP expression. Quantifications of the GFP expression are in Figure 6. Bar in **a** = 134  $\mu\text{m}$ . Same magnification in **b–h**. Bar in **i** = 268  $\mu\text{m}$ . Same magnification in **j–l**. Bar in **m** = 536  $\mu\text{m}$ . Same magnification in **n–p**. CNS, central nervous system; GFP, green fluorescent protein; i.v., intravenous.

progressive paralysis, and the disease state was even more severe at the high dose. We were able to detect significantly greater expression levels in the medium-dose group compared to the low-dose group as expected, which underscored the fine control of consistent expression levels by this method. Despite the reduced gene transfer when using older subjects, the adult approach was sufficiently potent to detect pathophysiological effects of TDP-43. Only very small fold overexpression of TDP-43, less than twofold over endogenous rat TDP-43 in the spinal cord, is sufficient to induce motor impairments and paralysis.<sup>18</sup> In fact, we used higher doses of AAV9 to visualize GFP ( $1 \times 10^{14}$  vg/kg) than we did to track TDP-43-induced motor paralysis ( $3 \times 10^{13}$  vg/kg); there is sensitive detection of TDP-43-induced deficits in this system. This method was sufficiently consistent to determine dose-dependent phenotypic differences (unimpaired to lethal) within a very narrow dosing window.

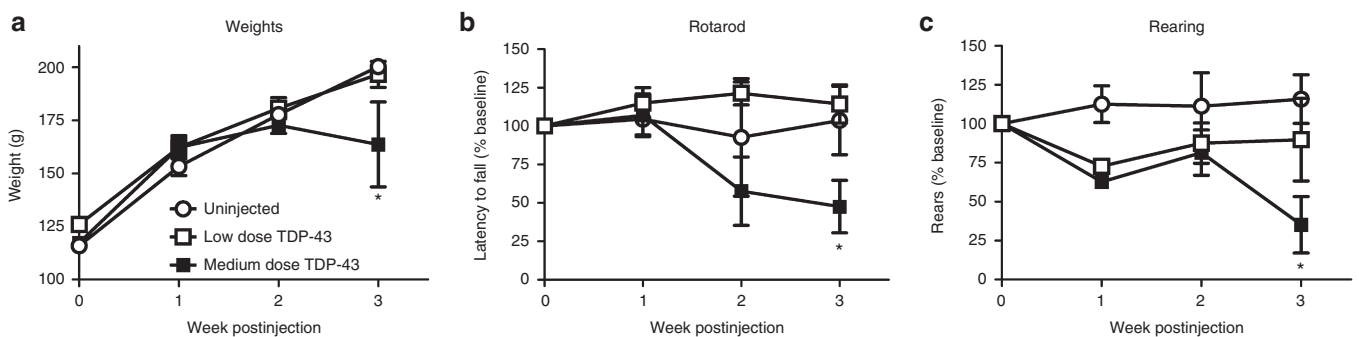
Adult CNS transduction via peripheral administration of AAV is advantageous because it is more relevant to adult-onset diseases like ALS, but there are a number of other advantages. Adult treatments will reduce the potential for neurodevelopmental complications. Tail vein injections are relatively easy and less invasive than i.v. injections to neonates. Relying on a one-time treatment in neonates to achieve efficient neuronal expression is more restrictive for treatment design. For example, adult administration allows for baseline pretreatment behavioral assays to be conducted. A corrective gene therapy intervention after the onset of symptoms will require gene transfer to adults.

Despite the consistent gene transfer and induced phenotype that was achieved in adults, there are two important caveats to this approach. The overall significance of the transduction of non-CNS peripheral organs is not fully understood although we have some data to suggest that spinal cord expression is essential for TDP-43.<sup>17</sup> We are currently attempting more selective targeting strategies to avoid peripheral organ transduction using different tissue-specific promoter systems. Another caveat is that i.v. injections to adults require large amounts of viral vector. The viral titers needed to achieve these doses could be nonfeasible and cost-prohibitive as was the case with the AAV1 in this study. The potent toxicity of TDP-43 supported and facilitated a relevant phenotype of progressive paralysis. However, given the large amounts of vector needed for affecting the CNS after i.v. delivery to adult rats, disease modeling is more challenging using disease-related genes that are not as potently toxic as TDP-43. In terms of gene therapy, AAV9 stood out as efficient for CNS gene transfer in adults. For a preclinical gene therapy in adult rats that affects the CNS after a peripheral, systemic delivery, the approach will be facilitated by potent secreted factors or cross-correcting enzymes that can exert their beneficial effect beyond the transduced cells.

Several studies suggest that administration of AAV to the cerebrospinal fluid will improve targeting and decrease the amount of vector needed.<sup>24–27</sup> However, with further refinement, the i.v. approach can be a rapid and relatively noninvasive manner to affect the function of the CNS in relevant adult subjects in a highly consistent manner, as



**Figure 6** Quantification of GFP expression levels after i.v. administration of AAV1, AAV8, AAV9, or AAV10 to either neonatal or adult rats. There was no difference in serotype transduction efficiency in the (a) neonatal spinal cord or (b) cerebellum, but AAV9 resulted in significantly higher GFP levels in adult rats. AAV9 showed significantly increased motor neuron transduction in (c) the lumbar spinal cord compared to AAV8 (\* $P < 0.05$ , ANOVA/Bonferroni) and had significantly higher transduction efficiency in the (d) cerebellum compared to AAV8 or AAV10 ( $P < 0.01$ – $0.05$ , ANOVA/Bonferroni). *N* values are listed for each group. ANOVA, analysis of variance; GFP, green fluorescent protein; i.v., intravenous.



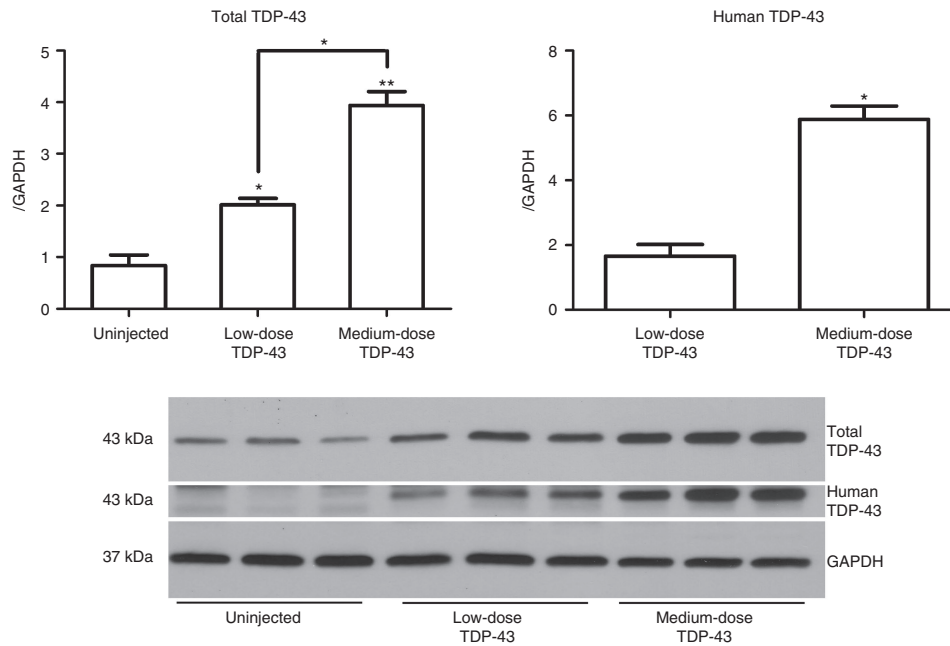
**Figure 7** Progressive and dose-dependent TDP-43-induced disease state after i.v. administration of AAV9 TDP-43 to adult female rats. (a) The medium-dose of AAV9 TDP-43 (closed squares), but not the low-dose (open squares), reduced weight gain compared to a group of uninjected rats (open circles) on week 3 (\* $P < 0.05$ ;  $N = 3$ – $4$ /group, ANOVA/Bonferroni). (b) The medium-dose AAV9 TDP-43 rats were significantly impaired on the rotarod at week 3 compared to baseline (\* $P < 0.05$ , ANOVA/Bonferroni) and (c) also impaired in terms of rearing at week 3 compared to baseline (\* $P < 0.05$ , ANOVA/Bonferroni). ANOVA, analysis of variance.

shown here using TDP-43 to mimic aspects of ALS. The tail vein delivery method to adult rats supported highly consistent results that allowed us to discern vector dose–response. This method will therefore support rapid studies of different genes, gene isoforms, gene variants, and gene combinations that will expedite hypothesis testing relative to comparing or combing genes in germline transgenic animals.

## MATERIALS AND METHODS

### AAV vector production

The transgene cassette was composed of AAV serotype 2 inverted terminal repeats, the hybrid cytomegalovirus/chicken  $\beta$ -actin promoter, a GFP construct or TDP-43 construct, the woodchuck hepatitis virus posttranscriptional regulatory element, and the bovine growth hormone polyadenylation sequence.<sup>28</sup> This cassette was packaged into AAV1, AAV8, AAV9, or AAV10



**Figure 8** Intravenous administration of AAV9 TDP-43 to adults causes a dose-dependent increase in both recombinant human and total TDP-43. The recombinant human TDP-43 can be distinguished with a human TDP-43-specific antibody while a non-species-specific TDP-43 antibody recognizes and detects both human and endogenous rat TDP-43 (total TDP-43). Both the low- and the medium dose AAV9 TDP-43 groups had significantly increased levels of total TDP-43 compared to uninjected animals (low-dose  $*P < 0.01$ ; medium-dose  $**P < 0.001$ ; ANOVA/Bonferroni;  $N = 3$ /group). The medium-dose AAV9 TDP-43 group had increased human TDP-43 ( $*P < 0.01$ , *t*-test) and total TDP-43 ( $*P < 0.01$ , ANOVA/Bonferroni) levels compared to low-dose TDP-43. Top, quantification of the bands. Bottom, western blots with three subjects per group. ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

using helper and capsid plasmids from the University of Pennsylvania.<sup>1,29</sup> Viral stocks were sterilized via Millipore (Billerica, MA) Millex-GV syringe filter, aliquoted, and frozen. Stocks were titered by dot blot assay, and equivalent viral titers were created by diluting in lactated Ringer's solution (Baxter Healthcare, Deerfield, IL). Through the course of these studies, we made and used three batches of AAV1 GFP, one batch of AAV8 GFP, seven batches of AAV9 GFP, two batches of AAV10 GFP, and one batch of AAV9 TDP-43. Consistent results across vector batches were found in the cases where more than one preparation was used.

## Animals

**Neonatal.** For neonatal i.v. administration studies, six litters of Sprague-Dawley rats (Harlan, Indianapolis, IN) totaling 43 animals were used. Animals were injected with 100  $\mu$ l of dilute AAV-encoding GFP on postnatal day 1. The diluent buffer was lactated Ringer's solution. Animals were administered  $\sim 1 \times 10^{12}$  vector genomes (vg) equaling  $1.5 \times 10^{14}$  vg/kg of AAV1-encoding GFP (AAV1 GFP,  $n = 8$ ), AAV8 (AAV8 GFP,  $n = 9$ ), AAV9 (AAV9 GFP,  $n = 18$ ), or AAV10 (AAV10 GFP,  $n = 5$ ). For the comparison to adult animals, one group of rats ( $n = 3$ ) was administered  $1 \times 10^{14}$  vg/kg of AAV9 GFP, the vector dose used in adults. One hundred microliters of diluted virus was loaded into a 1 ml syringe attached to a 30-gauge needle. The AAV vector was injected into the temporal vein, and the paws were tattooed for identification (Spaulding Color, Voorheesville, NY). Animals were monitored after injections then returned to the mother's cage. Weight and general health were evaluated until the endpoint of the study, 4 weeks after gene transfer. Motor performance was evaluated by a rotarod assay at 4 weeks.

**Adult.** For i.v. administration studies to adults, 18 adult Sprague-Dawley (Harlan) rats were used, with an age range of 6–8 weeks. Virus was diluted to a final volume of 200  $\mu$ l. Animals were administered a dose of  $1 \times 10^{14}$  vg/kg via tail vein injection. For the tail vein injections, the animals were anesthetized under isoflurane. The AAV vector was loaded in a 1 ml syringe attached to a 30-gauge needle. The rat was placed on a heating pad, and the tail was swabbed with isopropanol to help visualize the tail veins. The vector was injected to one of the lateral tail veins over a period of about 5 seconds per injection. Weight and general health were evaluated until the endpoint of 4 weeks after gene transfer.

Female Sprague-Dawley rats at 6 weeks of age remained uninjected ( $n = 4$ ) or were administered AAV9 TDP-43 at doses of  $1 \times 10^{13}$  vg/kg ( $n = 3$ ),  $3 \times 10^{13}$  vg/kg ( $n = 3$ ), or  $6 \times 10^{13}$  vg/kg ( $n = 1$ ) via tail vein. Rats were evaluated weekly for weight gain, rotarod performance, and locomotor performance. Additionally, escape reflex, rearing posture, and gait were monitored.

All animal procedures followed protocols approved by the institutional Animal Care and Use Committee and the NIH Guide for Care and Use of Laboratory Animals.

## Rotarod

For the neonatal rats administered AAV GFP, rotarod testing was conducted at 4 weeks of age. For adult rats administered AAV9 TDP-43, rats were tested the day before injection (baseline) and weekly after injection. Rotarod (Rota-rod/RS; Leticia Scientific Instruments, Barcelona, Spain) testing was conducted by measuring the amount of time the rat could remain walking on the rod before falling (latency to fall). The rod accelerated from 4–40 rpm over 2 minutes. The average of three trials for each rat was taken at each time point.

## Locomotor behavior

For adult rats administered AAV9 TDP-43, rats were evaluated for locomotor performance at baseline and weekly after injection. Locomotor testing was conducted in an open field chamber with a photobeam activity-monitoring system (Truscan 2.0; Coulbourn Instruments, Whitehall, PA). Rats were allowed to acclimate to a dark room for 30 minutes before testing began. Testing was conducted in a dark room over a 30-minute time period where the rat was allowed to freely move about the chamber.

## Immunohistochemistry

At 4 weeks post-injection, animals were anesthetized with ketamine (100 mg/ml; Fort Dodge Animal Health, Fort Dodge, IA), xylazine (20 mg/ml; Butler, Columbus, OH), and acepromazine (10 mg/ml; Boehringer Ingelheim, St. Joseph, MO). Animals were transcardially perfused with phosphate-buffered saline followed by cold 4% paraformaldehyde in phosphate-buffered saline. Tissues were removed and immersed overnight in 4% paraformaldehyde at 4 °C. All tissues were cryopreserved in 30% sucrose. Fifty micrometer sections were cut on a sliding microtome with a freezing stage (Leica Biosystems, Buffalo Grove, IL). Primary antibodies used were anti-GFP (Life Technologies,



Eugene, OR) and SMI 311 (Convance, Princeton, NJ) for nonphosphorylated neurofilament at dilutions of 1:500 and 1:2,000, respectively. The astroglial marker antiligal acidic fibrillary protein was also used as a primary antibody (Chemicon EMD, Billerica, MA; 1:2,000). Secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 594 (Life Technologies) at dilutions of 1:300.

### Measurements of GFP expression in the cerebellum

Cerebellar sections were stained for GFP and analyzed via Scion Image (Scion Corporation, Frederick, MD). Six evenly spaced sections per animal were analyzed. The GFP fluorescent immunoreactivity (Supplementary Figure S1A) in the cerebellar vermis was captured at  $\times 5$  or  $\times 2.5$  magnification and converted to grayscale (Supplementary Figure S1B). The Density Slice option in Scion Image was used to highlight the fluorescent pixels (Supplementary Figure S1C). Only highlighted pixels were counted. The GFP signal that was quantified was highly specific and only found in AAV GFP transduced samples. Nontransduced tissues produced negligible background readings in this analysis. The average for six sections per animal was taken.

### Transduction of spinal motor neurons

Six evenly spaced spinal cord sections from the lumbar region were stained for GFP and co-stained for nonphosphorylated neurofilament (SMI 311) to mark the motor neurons. The numbers of SMI 311 immunoreactive cells in the ventral horn were manually counted, as were the number of cells immunoreactive for both GFP and SMI 311. The number of GFP/SMI 311 immunoreactive motor neurons was divided by the total number of SMI 311 immunoreactive cells to determine the percent transduction or transduction rate of spinal motor neurons (Supplementary Figure S1D–F).

### Western blot

Tissues were dissected from the animals and frozen on dry ice. The samples were Dounce-homogenized in RIPA buffer (1% nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, phosphate-buffered saline) with protease inhibitors (Halt protease inhibitor cocktail kit from Pierce, Rockford, IL) and then centrifuged. Protein content of the supernatant was determined by Bio-Rad Protein Assay Dye. Samples were normalized for protein content and electrophoresed in 12% polyacrylamide containing SDS (Bio-Rad). Primary antibodies used to probe blots were antihuman TDP-43 (Abnova, Taipei City, Taiwan), anti-TDP-43 (ProteinTech, Chicago, IL), anti-GFP (Chemicon EMD, Billerica, MA), and anti-GAPDH (Ambion Life Technologies, Grand Island, NY). Secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX), and chemiluminescence reagents were from Amersham (Buckinghamshire, UK). Primary antibody dilutions were 1:1,500, and secondary antibody dilutions were 1:10,000.

### Statistical analyses

Statistical analyses were conducted on GraphPad Prism 5.0. Statistical tests include one-way ANOVA, repeated-measures ANOVA, and *t*-test. Data are expressed as mean  $\pm$  SEM.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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