Chapter 7

Lentiviral and Adeno-Associated Vector-Based Therapy for Motor Neuron Disease Through RNAi

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Abstract

RNAi holds promise for neurodegenerative disorders caused by gain-of-function mutations. We and others have demonstrated proof-of-principle for viral-mediated RNAi in a mouse model of motor neuron disease. Lentivirus and adeno-associated virus have been used to knockdown levels of mutated superoxide dismutase 1 (SOD1) in the G93A SOD1 mouse model of familial amyotrophic lateral sclerosis (fALS) to result in beneficial therapeutic outcomes. This chapter describes the design, production, and titration of lentivirus and adeno-associated virus capable of mediating SOD1 knockdown in vivo. The delivery of the virus to the spinal cord directly, through intraspinal injection, or indirectly, through intramuscular injection, is also described, as well as the methods pertaining to the analysis of spinal cord transduction, SOD1 silencing, and determination of motor neuron protection.

Key words: Amyotrophic lateral sclerosis, superoxide dismutase (SOD1), lentivirus, adeno-associated virus (AAV), RNA interference (RNAi), gene therapy, G93A SOD1.

1. Introduction

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder that results from the progressive and irreversible degeneration of motor neurons in the spinal cord, brain stem, and cerebral cortex. This loss of motor neurons leads to gradual muscle weakness and atrophy, with death often occurring 2–5 years after diagnosis as a result of respiratory failure. Ten percent of ALS cases are familial with one-fifth of those being caused by mutations in the gene encoding for superoxide dismutase 1 (SOD1). Current evidence supports that disease-linked

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mutations confer SOD1 a toxic gain-of-function, independent of its enzymatic activity, which triggers motor neuron death through a still unclear mechanism *(1)*. In light of this direct toxic effect, reduction of mutated SOD1 protein levels via RNA interference (RNAi) is an attractive therapeutic approach as it targets the cause of the disease regardless of the specific pathogenic mechanism responsible for motor neuron death.

1.2. Viral-Mediated RNAi Against SOD1

RNAi is an innate gene silencing mechanism that utilizes small single- or double-stranded RNA (dsRNA) to promote the degradation of specific mRNA targets, effectively reducing the levels of the corresponding protein *(2)*. Saito et al. *(3)* have demonstrated the potential for RNAi as a therapeutic approach for familial amyotrophic lateral sclerosis (fALS). By crossing transgenic mice expressing dsRNA against SOD1 with the G93A SOD1 mouse model, the development of the ALS-like phenotype was completely prevented with the double transgenic mice having normal spinal cord histology and behaving as wild-type controls.

The current challenge facing RNAi-based therapy for fALS is the delivery of the dsRNA silencing instructions in vivo. Two strategies exist towards this goal. The first approach is to synthesize dsRNA in vitro prior to their in vivo delivery. An example of this is to deliver the dsRNA or antisense oligonucleotides directly to the cerebrospinal fluid (CSF) through ventricular infusion. Indeed, osmotic pumps have been used to deliver antisense oligonucleotides against SOD1 into the ventricles of G93A SOD1 rats leading to silencing of SOD1 within the spinal cord and subsequent therapeutic gains *(4)*. The limiting factor of this intervention, however, is the continual synthesis and delivery of oligonucleotides that would be required to maintain this therapeutic silencing throughout the lifetime of the patient. The second approach, that overcomes this hurdle, is to provide cells with the DNA templates that they may use to indefinitely transcribe short hairpin RNAs (shRNAs) *(5)*. shRNA consist of two complementary RNA strands linked by a hairpin loop that are processed into dsRNA capable of mediating RNAi. Viral vectors can be used to deliver these transgenic cassettes directly to the genome of cells to result in stable and long-lasting expression of shRNA following a single intervention. Three landmark studies have used recombinant viral vectors to deliver shRNAs to result in SOD1 knockdown and achieve therapeutic gain in the G93A mouse model of ALS.

1.2.1. Intramuscular Delivery of Lentivirus Based on EIAV into Newborn SOD1 Mice

Ralph et al. *(6)* delivered lentivirus based on the equine infectious anemia virus (EIAV) to multiple muscle groups of newborn G93A SOD1 mice. As EIAV is capable of efficient retrograde transport

from the muscle to the motor neuron soma, this technique facilitated transduction and SOD1 silencing within motor neurons at multiple levels of the spinal cord and brain stem. This resulted in delaying disease onset by 115% (and subsequently increased survival age by 77%) although did not slow disease progression from time of onset.

1.2.2. Intraspinal Delivery of VSV-G-Pseudotyped Lentivirus into Presymptomatic SOD1 Mice

Lentivirus pseudotyped with vesicular stomatitis virus G (VSV-G) were injected directly to the L3 and L4 lumbar spinal cord of presymptomatic 42-day-old G93A SOD1 mice *(7)*. This silenced SOD1 in the motor neurons and glial cells of the ventral horn resulting in a delay of both disease onset and progression, as measured by various electromyographical and behavioral outcomes, in the hindlimb muscles innervated by this region of spinal cord. The observation that this RNAi strategy was capable of retarding disease progression concurs with current evidences that SOD1 expression within glial cells is an important determinant of disease progression *(8)*.

1.2.3. Intramuscular Delivery of AAV Serotype 2 into Presymptomatic SOD1 Mice

Miller et al. *(9)* also capitalized on viral retrograde transport to transduce motor neurons following intramuscular injection, albeit using adeno-associated virus (AAV) serotype 2. Presymptomatic 45-day-old G93A SOD1 mice were injected directly into the lower hindlimb muscles with the vector to result in transduction and silencing of SOD1 in lumbar-level motor neurons. This conferred behavioral improvement in the injected limbs as determined by preservation of grip strength.

The present chapter will detail the experimental procedures required to recapitulate the studies mentioned above. We will describe the design, production, and titration of both VSV-G-pseudotyped lentivirus used in the study by Raoul et al. *(7)* and AAV serotype 6 (AAV6), a vector that we have found to be capable of particularly efficient muscle-to-motor neuron retrograde transport. Next, the intraspinal and intramuscular delivery of these vectors to adult mice will be described, as well as the intramuscular injection to neonates. Finally, the methods required to analyze in vivo transduction, SOD1 silencing, and conferred neuroprotection will be provided.

2. Materials

2.1. Virus Production

1. pSIN-W-PGK: Lentivirus genomic plasmid.
2. pCMVΔR8.92: Packaging plasmid encoding viral genes in *trans*.
3. pMD.G: Plasmid encoding the VSV-G envelope.
4. pRSV-Rev: Plasmid encoding the rev protein of HIV-1.
5. pAAV-CMV-MCS: AAV genomic plasmid.
6. pDF6: Packaging plasmid encoding for the AAV6 capsid.

7. AAV-293 cells (#240073, Stratagene, La Jolla, CA) and 293T cells.
8. Six-well Multiwell™ Tissue Culture Plate ((#166508, Becton Dickinson, Frankin, NJ).
9. 500 cm^2 Nunclon™Δ polystyrene dish (#166508, Nalg Nunc International, Rochester, NY).
10. Cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Odgen, UT). Episerf (Gibco BRL).
11. Trypsinization medium: Hanks' balanced salt solution (HBSS; red phenol 0.4 g/L KCl, 0.06 g/L KH_2PO_4, 8 g/L NaCl, 0.35 g/L $NaHCO_3$, 0.048 g/L Na_2HPO_4, 1 g/L D-glucose) containing 0.05% trypsin and 0.53 mM EDTA.
12. Transfection reagents: 0.5 M $CaCl_2$ (73.5 g $CaCl_2 \cdot 2H_2O$; complete to 500 mL with H_2O; store in aliquots at –20°C) and 2x HBS (28 mL of 5 M NaCl, 11.9 g of HEPES, 750 μL of 1 M Na_2HPO_4 in 400 mL H_2O; adjust the pH to 7.1 with NaOH and complete to 500 mL; store in aliquots at –20°C).
13. Ultracentrifugation of lentivirus: ultracentrifuge, SW28 rotor, SW60 rotor, and polyallomer tubes (SW28 rotor, 25 × 89 mm, SW60 rotor, 11 × 60 mm, Beckman Coulter, Fullerton, CA).
14. Centricon® Plus-20 centrifuge filters (#UFC2BHK08, Millipore, Billerica, MA).
15. PBS/1% BSA: Add 5 g of bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO) to 500 mL pH 7.4 phosphate-buffered saline (PBS) and pass through a 0.22-μm filter.
16. 1.6 mL Maxymum Recovery, Clear Microtubes (Axygen Scientific, Union City, CA).
17. 4% paraformaldehyde (PFA): Add 40 g of paraformaldehyde to 900 mL of PBS and heat at 60°C (*see* **Note 1**). Add a few drops of 5 M NaOH to dissolve the paraformaldehyde and allow to equilibrate to room temperature. Filter the solution, adjust pH to 7.4, and complete to 1 L.
18. 5 mL Polystyrene Round-Bottom Tube with Cell-Strainer Cap (#352235, Becton Dickinson).

2.2. Animal Experiments

1. Anesthesia: Anesthesia system, O_2 cylinder, induction chamber, surgical table with anesthesia mask, isoflurane.
2. Intraspinal injections: Small Animal Stereotaxic Instrument (Model 963 Ultra Precise, David Kopf Instruments, Tujunga, CA), Mouse Spinal Adaptor (#51690, Stoelting, Wood Dale, IL), Mouse Transverse Clamps (#51695, Stoelting), Scalpel Handle No. 4 (#02-036-040, allgaier instrumente GmbH, Tuttlingen, Germany), Scalpel Blades

(#02-040-021, allgaier instrumente GmbH), Adson Tissue Forceps (#08-265-120, allgaier instrumente GmbH), Derf Needle Holder (#20-116-125, allgaier instrumente GmbH), Bulldog clamps (#18050-28, Fine Science Tools GmbH, Heidelberg, Germany), Friedman-Pearson Rongeur (#16021-14, Fine Science Tools GmbH), self-regulating heating pad (#21061-00, Fine Science Tools GmbH), 27G needle (Terumo®, Leuven, Belgium), 70 mm × 34G (0.23×0.127) needle (Phymed, Paris, France), 50 mm × 26G (0.457×0.254) needle (Phymed), Viscotears® Ophthalmic Gel (Novartis, Basel, Switzerland), Microinjection pump (CMA/100, CMA Microdialysis, Solna, Sweden), Polyethylene Tubing (0.38× 1.1 mm) (A-M Systems, Carlsborg, WA), 6-0 Vicryl™ Suture (Ethicon, Norderstedt, Germany), Betadine® standard solution (Mundipharma GmbH, Basel, Switzerland), electric shaver and 500 mg Paracetamol effervescent tablets.

3. Intramuscular injections of adults: Hamilton 25 μL syringe (#702, Hamilton, Reno, NV), Scalpel Handle No. 4, Scalpel Blades, 6-0 Vicryl™ Suture, Derf Needle Holder, Adson Tissue Forceps, Betadine® standard solution, and electric shaver.
4. Intramuscular injections of neonates: 30G × 1 mm Insulin Needle (Becton Dickinson), Polyethylene Tubing (0.28×0.61 mm) (A-M Systems), and a Hamilton 10 μL syringe (#701, Hamilton).

2.3. Histology

1. Heparin (Liquemin, Roche, Basel, Switzerland).
2. Spinal cord dissection: McPherson-Vannas spring scissors (#52130-01, Stoelting), Friedman-Pearson Rongeur, Tissue Forceps, and blunt-dissection probe.
3. 25% sucrose: Dissolve 25 g of sucrose in 100 mL of PBS and pass through a 0.22-μm filter.
4. Cryosections: Peel-A-Way® Disposable Embedding Molds (#18646A, Polysciences Inc, Warrington, PA), Shandon Cryomatrix™ (#6769006, Thermo Electron Corporation, Pittsburgh, PA), goal polyclonal anti-GFP Ab (ABCAM, Cambridge, UK), rabbit polyclonal anti-GFP Ab (ABCAM), monoclonal anti-SOD1 Ab (clone SD-G6, Sigma-Aldrich), monoclonal anti-SOD1 Ab (clone G215-1, BD Pharmingen, Franklin, NJ), polyclonal goat anti-SOD1 Ab (sc-8636, Santa Cruz, Santa Cruz, CA), rabbit polyclonal anti-VAChT Ab (Sigma-Aldrich).
5. Tris-buffered saline (TBS): Dissolve 12.11 g of Tris-base (MW= 121.14) and 8.76 g NaCl in 1 L of H_2O adjusting the pH to 7.6.
6. Paraffin sections: Cresyl Violet (Sigma-Aldrich), Toluidine Blue (Sigma-Aldrich), osmium tetroxide (Sigma-Aldrich), Leica Histomold (2×15 mm, Leica Microsystems), Leica Historesin Embedding Kit (#702218500, Leica Microsys-

tems, Heidelburg, Germany) containing 500 mL basic resin liquid, 5 g activator powder, and 40 mL hardener.
7. Differentiation buffer for Cresyl Violet staining: Mix one volume 2.72% sodium acetate (2.72 g/100 mL) with four volumes 1.2% acetic acid (1.2 g/100 mL).
8. 10 mM sodium acetate buffer pH 6.5: 13.3 g sodium acetate trihydrate (MW=136.1) in 900 mL H_2O and pH to 6.5 with glacial acetic acid. Complete to 1 L.
9. Mounting media: Merkoglass® (Merk, Darmstadt, Germany) for paraffin-prepared sections and Mowiol (Calbiochem, San Diego, CA) for cryosections.

3. Methods

3.1. Design and Construction of Viral Vectors Encoding Small Hairpin RNA

Bipartite cassettes containing green fluorescent protein (GFP) and an shRNA sequence under control of the H1 promoter are cloned into the pSIN-W-PGK and pAAV-CMV-MCS genomic plasmids using standard molecular biology techniques to generate pSIN-PGK:GFP-H1:shRNA and pAAV-CMV:GFP-H1:shRNA, respectively (**Fig. 7.1**). The shRNA sequence consists of the 19–23 bp stem sequence targeting SOD1, the loop sequence (*TTCAAGAGA*), the reverse complementary stem sequence, and the pol III transcription termination sequence (TTTTT). Examples of complementary oligos used to clone shRNA sequence (from Raoul et al. *(7)*) are given below. Observe the 5′ overhangs that facilitate ligation into restriction-digested DNA.

```
5′ GATCCCC AAGGATGAAGAGAGGCATG TTCAAGAGA CATGCCTCTCTTC
    ATCCTT TTTTTGGAAA 3′
3′ GGG TTCCTACTTCTCTCCGTAC AAGTTCTCA GTACGGAGAGAAGTAG
    GAA AAAAACCTTTGATC 5′
```

The design of shRNA to target SOD1 (or any gene for that matter) is not trivial. Several algorithms have been published that can predict the silencing efficacy of siRNA sequences *(10–15)* and these have been used to create multiple online programs capable of identifying effective siRNA targets within a selected gene. Unfortunately, these algorithms for siRNA design have little or no efficacy at predicting shRNA knockdown outcome *(16)*. However, such software still offers an easy method to identify oligonucleotide sequences that may then be screened for silencing efficacy in vitro.

1. Use siRNA design software (*see* **Note 2**) to identify multiple target sequences ($n > 10$) that are located across different regions of the selected gene.

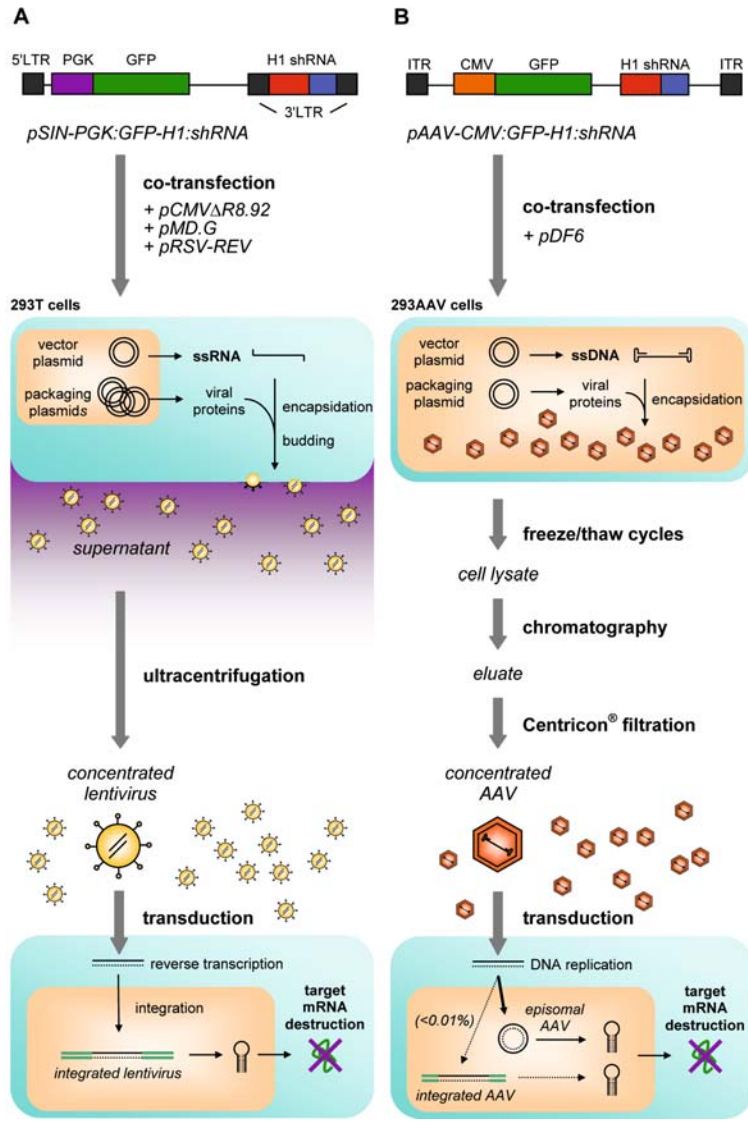

Fig. 7.1. Comparison of lentivirus (**A**) and adeno-associated virus serotype 6 (**B**) production and transduction.

2. Perform BLAST searches to eliminate those sequences that have less than three mismatches with off-target genes within the genome of the species being examined.
3. Order the oligo pairs required to generate the shRNA sequences ($n > 8$) (use template described above), anneal, and clone into an H1 expression vector.
4. Transfect the vectors into a cell line expressing the target protein and determine knockdown efficiency by Western blot, immunocytochemistry, or reverse transcription quantitative PCR (RT-QPCR).

5. Select the best two shRNA sequences for subsequent in vivo studies and design corresponding scrambled nucleotide shRNA sequences as negative controls.

3.2. Production and Titration of Recombinant Virus

Recombinant lentivirus and AAV are produced through calcium-phosphate transfection of HEK293-derived cells. HEK293 cells are human embryonic kidney cells that have been transformed by sheared adenovirus type 5 DNA *(17)*. We use AAV-293 cells that are derived from HEK293 cells for AAV production although they produce higher viral titers (technical data sheet, #240073, Stratagene). Our lentiviral production utilizes 293T cells, which express the SV40 large T antigen allowing for episomal replication of plasmids containing an SV40 origin of replication. Indeed, the packaging and genomic plasmids used for lentivirus production contain the SV40 *ori*, leading to greater copies of plasmid and therefore increased yields of virus.

Following transient transfection, AAV particles are produced and remain within the AAV-293 cell nucleus. The cells are then lysed and the virus is purified from the lysate by high-pressure liquid chromatography on heparin columns. This is made possible by the high heparin-binding affinity of AAV6. Not all AAV serotypes, however, have an affinity for heparin and may alternatively be purified by cesium-chloride ultracentrifugation or ion-exchange chromatography *(18)*. VSV-G-pseudotyped lentiviral vectors are packaged at the membrane following 293T transfection and delivered directly to the cell culture media. The media is collected and the virus concentrated by ultracentrifugation. **Figure 7.1** summarizes the production protocol for the two viruses.

3.2.1. Cell Culture and Transfection

1. Maintain 293 (AAV- or -T) cells with DMEM/FCS in a 37°C humidified incubator with 5% CO_2.
2. Passage cells 1:3 twice per week towards maintaining stable high-quality cell line (*see* **Note 3**).
3. Trypsinize cells from confluent tissue culture flasks the afternoon prior to transfection and centrifuge at 800 *g* for 5 min at 4°C to remove trypsin. Resuspend in DMEM/FCS and plate 40×10^6 cells per 500 cm^2 dish. The cells should be 70–80% confluent on the day of transfection.
4. Prepare the vector plasmid and packaging plasmid/s in 2.5 mL 0.5 M $CaCl_2$ per 500 cm^2 plate (lentivirus, 130 μg SIN-PGK:GFP-H1:shRNA, 130 μg pCMVΔR8.92, 37.5 μg pMD.G, and 30 μg pRSV-Rev; AAV, 100 μg pAAV-CMV:GFP-H1:shRNA, and 200 μg pDF6). Bring the volume to 5 mL with H_2O. Add 5 mL of 2x HBSS dropwise (one drop every other second) to the DNA/$CaCl_2$ solution while gently vortexing.

5. Incubate the solution for 10 min (*see* **Note 4**). The solution should turn opaque but no solids should be visible.
6. Add the precipitate solution gently to the cells by distributing the drops uniformly across the plate. Mix the solution in a cross 'T' manner. Return the cells to the incubator.

3.2.2. Lentivirus Isolation and Concentration

1. Remove the 293T cells from the incubator 8–15 h following transfection and aspirate the media. Replace with 70 mL of serum-free Episerf and return the dish to the incubator.
2. Collect the media (containing the virus) from the dish 48–72 h post-transfection and filter through a 0.45-μm bottle-top membrane.
3. Ultracentrifuge the filtrate at 50,000 *g* at 4°C for 90 min. Aspirate the supernatant and resuspend the pellet in 50 μL to 2 mL of PBS/1% BSA (depending on desired concentration). Leave the suspensions on ice for 1 h.
4. Aliquot the lentivirus and store at –80°C.

3.2.3. AAV Isolation

1. Remove the 293T cells from the incubator 6–8 h following transfection and replace media with 100 mL of serum-free Episerf. Return to incubator.
2. Collect the media from the plates 48 h following transfection and store. Trypsinize the cells and inactivate with Episerf.
3. Combine the harvested cells and collected media and centrifuge for 5 min at 800 *g*. Discard the supernatant and resuspend the pellet in 3 mL per 500 cm^2 plate.
4. Freeze–thaw the cells three times by cycling between a dry ice/ethanol and a 37°C water-bath.
5. Add 50 U/mL Benzonase and incubate for 37°C for 30 min.
6. Centrifuge the sample at 1,500 *g* for 15 min. Collect the supernatant and add 0.5% deoxycholic acid.
7. Incubate for 30 min and then centrifuge at 3,000 *g* for 15 min. Collect the supernatant and pass through a 0.8-μm filter.

3.2.4. AAV Purification via HPLC

1. Equilibrate the heparin column by washing with PBS for 10 min (*see* **Note 5**).
2. Load viral lysate onto the column and monitor the 280 nm absorbance. A large peak should arrive representing the non-heparin binding flow-through from the cell lysate. At this stage the AAV6 should remain bound to the column.
3. Continue to wash the column with PBS for 10 min until the peak returns to the starting absorbance or levels out in a parabolic shape.
4. Elute the virus with 0.4 M NaCl in PBS. Collect the eluate in 5 mL aliquots using the automated function of the HPLC

system. A peak smaller than the first should appear. Allow to elute until the peak levels out.
5. Strip the column of any remaining virus or sundries by washing with 1 M NaCl in PBS for 10 min.
6. Rinse the column with H_2O for 10 min and sterilize over 10 min with 20% EtOH. Leave the system including the column in 20% EtOH.

3.2.5. AAV Concentration

1. Prepare the Centricon® Plus-20 filter column by washing with 19 mL H_2O and then with 19 mL PBS. Centrifuge at 4,000 *g* for 5 min.
2. Load the HPLC eluate onto the column and centrifuge at 4,000 *g* until approximately 1 mL of volume remains (10–15 min). Do not let the column dry out.
3. Wash the sample by adjusting the volume to 19 mL with PBS and re-centrifuging until 100–200 μL of the volume remains.
4. Aliquot the AAV and store at –80°C.

3.2.6. Virus Titration Using FACS Analysis

Several methods are available to titrate lentivirus and AAV. Lentivirus particle content is typically performed by p24 (capsid antigen) ELISA assay (*see* **Note 6**). Similarly, AAV genomic particle content may be determined by quantitative PCR against the viral genome. These methods do not, however, quantify vector particles capable of infecting cells. When a fluorescent reporter is expressed by the vector, flow cytometry is a simple procedure that can be used to accurately titrate the vector in terms of transducing units (tu).

1. Plate 293T cells at a density of 3×10^5 cells per six-well tissue culture dish.
2. Infect the following morning with serial dilutions of the vectors. The dilutions will ensure that there are wells with a low multiplicity of infection (i.e., <15% transduction rate) that are desirable for accurate titration.
3. *Lentivirus only*: Harvest an uninfected well and determine total number of cells at time of infection (*C*).
4. Harvest the cells after 48 h with trypsin and inactivate with DMEM/FCS. Centrifuge for 5 min at 800 *g* and discard the supernatant.
5. Resuspend the pellet in 1 mL of PBS.
6. *AAV only*: Take an aliquot and quantify the total number of cells at time of analysis (*D*).
7. Centrifuge again for 5 min at 800 *g* and discard the supernatant. Resuspend the pellet in 1 mL of 4% PFA and incubate for 20 min (*see* **Note 7**).
8. Centrifuge for 5 min at 1,000 *g* and discard the supernatant. Resuspend the pellet in 1 mL of PBS and pass

through the cell-strainer cap of a 5 mL polystyrene round-bottom tube.

9. Perform flow cytometry to quantify the number of transduced cells.
10. As a population of transduced cells will include cells that have been transduced more than once, the percentage of transduced cells (A) will follow a Poisson's distribution in relation to transduction events per cell (B) (*formula 1*). The titer of lentivirus (T_L) in tu per microliter is calculated from transduction events in relation to the number of cells at time of infection (C) (*formula 2*, where V is the volume of virus added (μL)). The titer of AAV (T_{AAV}) in tu per microliter is calculated from transduction events in relation to the number of cells at time of analysis (D) (*formula 3*) (*see* **Note 8**).

$$\text{Formula 1} : B = \ln\left(\frac{1}{1-A}\right)$$

$$\text{Formula 2} : T_L = \frac{BD}{V}$$

$$\text{Formula 3} : T_{AAV} = \frac{BC}{V}$$

3.3. Viral-Mediated RNAi Against SOD1 in the G93A SOD1 Mouse Model of fALS

The hindlimb muscles of G93A SOD1 mice are the first muscles to display motor deficits (day 90) and become paralyzed (day 130) and invariably determine the age of death in this experimental paradigm. The hindlimb muscles and the lumbar spinal cord are therefore ideal targets for viral-mediated therapeutic silencing of SOD1. Indeed, the intraspinal and intramuscular injections described in this section specifically target the motor neurons at the level controlling hindlimb function (lumbar spinal cord). These protocols could, however, be modified to target motor neurons at different spinal cord levels to increase the therapeutic effect.

3.3.1. Intraspinal Delivery of VSV-G-Pseudotyped Lentivirus into Adult Mice

1. Place the 34G needle into the 26G needle and seal the overlap with adhesive glue. Connect the needle to the 10 μL 26G Hamilton syringe with polyethylene tubing (0.38×1.10). Replace the dead volume in the system with sterile H_2O.
2. Place the mouse in the induction chamber and anesthetize with 4% isoflurane. Remove the mouse and place on the surgery platform with the nose of the rodent in the anesthesia mask. From this point the anesthesia is maintained with 1.5% isoflurane (*see* **Note 9**). Make sure to apply and

reapply Viscotears® to the eyes to prevent ocular damage to the mouse.

3. Shave the back of the animal with an electric razor. Be careful to remove excess hairs that may contaminate the surgery. Swab the skin with Betadine® solution and allow to dry.
4. Make a 30 mm incision with the scalpel down the middle of the back. Use the tissue forceps to separate the skin as much as possible from the underlying muscle.
5. Identify the third and fourth lumbar vertebrae (L3 and L4) and mark the region by making a small scratch in the muscle surface with a scalpel (*see* **Note 10**). Make approximately 4 mm deep and 10 mm long incisions on either side of the vertebral column. Be careful not to axotomize the animal by penetrating the tissue too deep.
6. Transfer the mice to the stereotaxic frame and secure the spinal cord by placing the transverse clamps between the cranial articular process and costal process at L3 and L4 (**Fig. 7.2C,D**). The spinal column should be perpendicular the injection plane and firmly fixed such that it is unable to move upon applying downward pressure with the tissue forceps.
7. Place bandages under the mouse to support the weight of its body and reduce force exerted by the clamps.
8. Expose the vertebral column by removing the overlying muscle. This is achieved by making tiny lateral incisions down the column and carefully scraping the muscle to the side. Do not tear away or transversally cut the muscle as it may increase local inflammation.
9. Use the bone rongeurs to delicately remove the spinous process, caudal articular process, and cranial articular process at L3 and L4 (**Fig. 7.2E**) (*see* **Note 11**). The spinal cord is visible at this point.
10. Lower the needles at the desired lumbar level such that they touch the surface of the spinal cord (*see* **Note 12**). There is a small blood vessel running down the middle of the spinal cord that can help align the bilateral injection.
11. Raise the needles 1 mm from the surface and puncture holes in the dura mater with a 27G beveled needle. Place the needles back on the spinal cord surface and then slowly lower them 0.75 mm into the tissue.
12. Inject the desired amount of virus in a volume between 1 and 2 μL at 0.2 μL/min. The amount of lentivirus normally required to achieve efficient transduction is approximately 1×10^6 tu per site ($\approx$ 100 ng p24).
13. Wait for 5 min after the injection has finished and then slowly remove the needles.

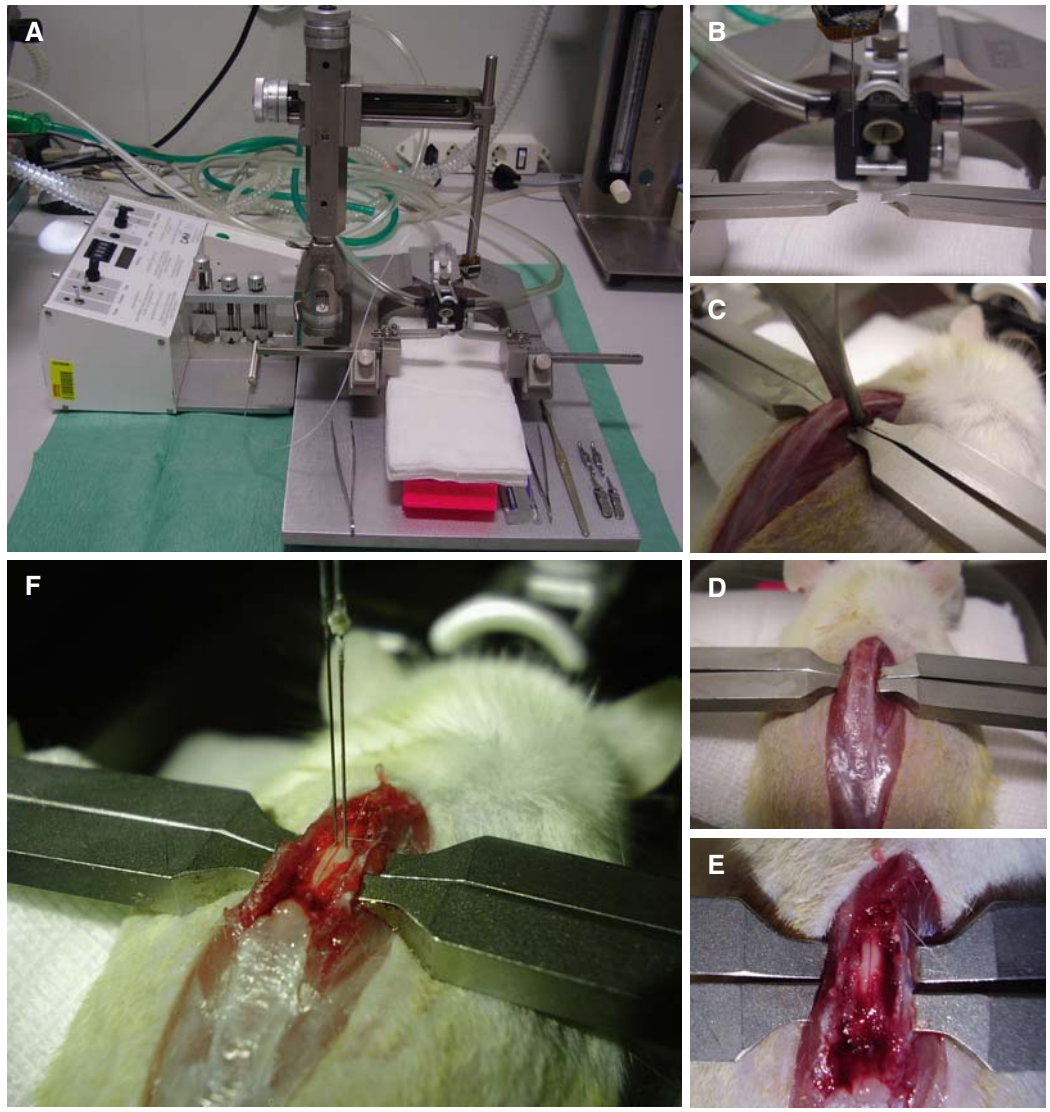

Fig. 7.2. Intraspinal injection of lentivirus into G93A SOD1 mice. (**A**) Experimental setup and required instruments. (**B**) Macrophotograph of 34G blunt needle intersecting with injection plane. (**C, D**) The vertebral column being raised with forceps to allow fixation with the transerve clamps. (**E**) The spinal cord being revealed following removal of the dorsal vertebral bones. (**F**) Macrophotograph of spinal cord being bilaterally injected with lentivirus.

14. Close the skin with 6-0 Vicryl® suture and return the animal to the housing cage (*see* **Note 13**).

3.3.2. Intramuscular Delivery of AAV6 into Adult Mice

1. Anesthetize the mouse in the induction chamber and transfer to the surgery table as described above.
2. Use an electric razor to remove the hair from the back of the lower leg and apply Betadine® solution to the surface of the skin.

3. Make an 8 mm incision with a scalpel to expose the gastrocnemius muscle. Use the tissue forceps to separate the skin from the underlying muscle.
4. Secure the legs of the animal by taping the feet to the surgery table (**Fig. 7.3B**).
5. Insert the 26G beveled needle of the 25 μL Hamilton syringe at a 45° angle into the belly of the gastrocnemius (**Fig. 7.3C**,**D**). Administer the desired amount of virus (diluted in 25 μL of PBS) via bolus injection (*see* **Note 14**). The amount of AAV6 required to achieve efficient transduction of motor neurons via retrograde transport is approximately 1×10^6 tu.
6. Close the skin with 6-0 Vicryl® suture and return the animal to the housing cage.

3.3.3. Intramuscular Delivery of AAV6 into Neonate Mice

1. Break off the needle from a disposable 30G insulin syringe with a needle holder by bending (not twisting) it back and forth. Connect the needle to a 26G beveled 10 μL Hamilton syringe via 20 cm of polyethylene tubing (0.28×0.61) (*see* **Note 15**).
2. Place on a clean pair of gloves and rub them in the earth of the cage of the animals. Transfer the mother of the neonates into an empty cage and remove the pup. Return the mother to the cage immediately (*see* **Note 16**).
3. Place the newborn on a latex-covered bed of crushed ice to induce hypothermic anesthesia.
4. Remove the pup from the ice and inject the desired amount of virus in 2 μL via bolus injection with the help of a second operator (**Fig. 7.3F**,**G**). The amount of AAV6 required to achieve efficient transduction of neonate motor neurons via retrograde transport is between 10^5 and 10^6 tu.
5. Return the pup to the cage and quickly roll it in the soiled earth. Do not return the pup directly in the nest. Instead, place the pup at least 10 cm away such that the mother is forced to return it. This will ensure that the mother does not reject the newborn.

3.4. Analysis of Transduction, SOD1 Silencing, and Neuroprotection Within the Spinal Cord

In order to adequately interpret any behavioral improvement or extended survival observed within the mouse model following viral-mediated RNAi, it is necessary to determine the transduction profile of the vector, confirm SOD1 silencing within the transduced cells, and determine whether the effects were as a result of motor neuron protection. This may be achieved via spinal cord and ventral root histology.

3.4.1. Spinal Cord Dissection

1. Administer an overdose of sodium pentobarbital and transcardially perfuse the mouse with ice-cold PBS containing 5,000 U heparin for 1 min followed by 4% PFA for 5 min.

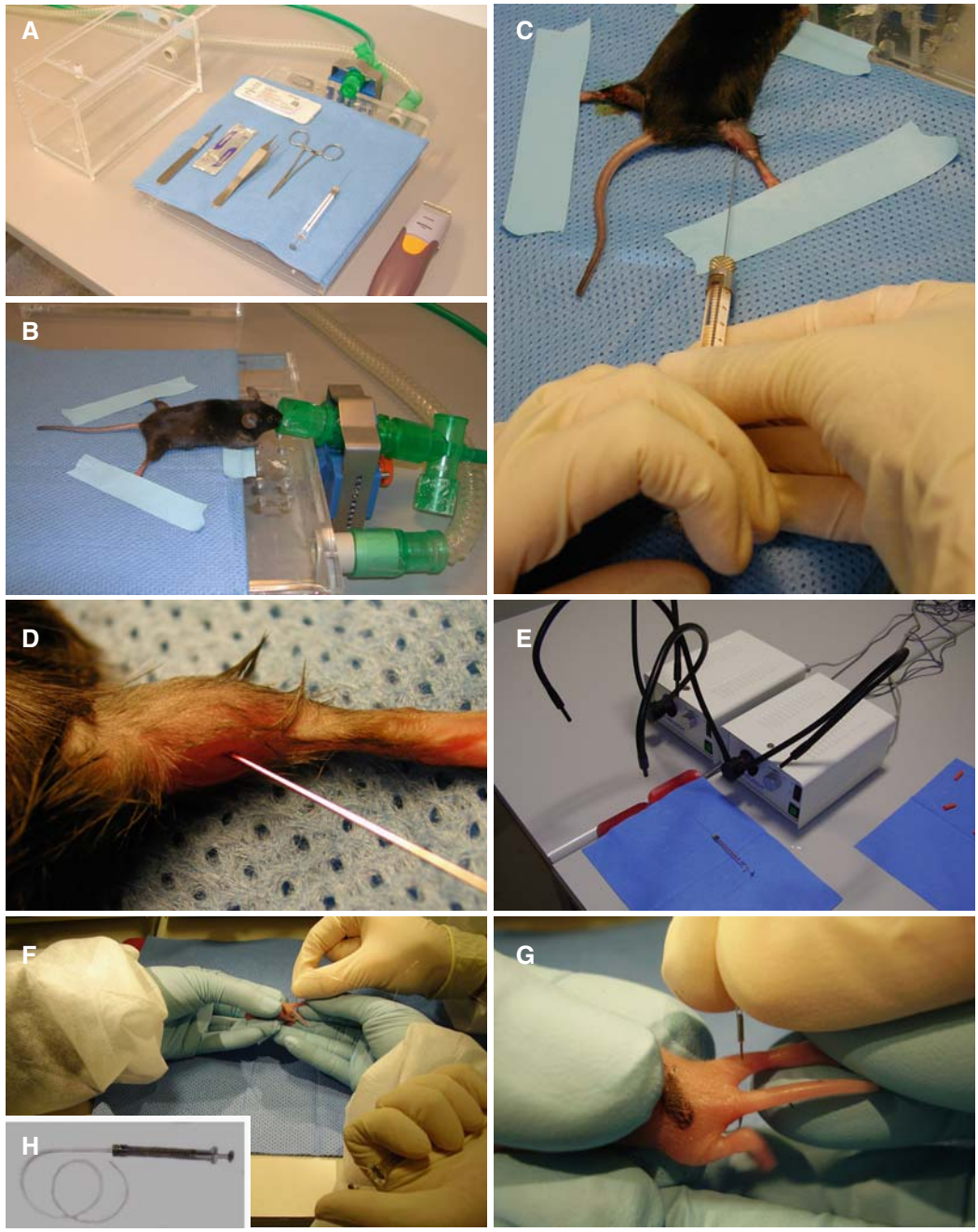

Fig. 7.3. Intramuscular injection of AAV6 into adult and neonate G93A SOD1 mice. (**A**) Experimental setup for intramuscular injection into adult mice. (**B**) Anesthetized mouse in prone position. (**C**) AAV6 being delivered into gastrocnemius muscle with 26G Hamilton syringe. (**D**) Macrophotograph of 45° needle entry into gastrocnemius. (**E**) Experimental setup for neonate intramuscular injections. (**F**) Two person coordination for ease of animal manipulation and vector injection. (**G**) Macrophotograph of needle entry into neonate gastrocnemius muscle. (**H**) 30G insulin needle connected to a 10 μL Hamilton syringe through polyethylene tubing.

2. Remove the muscle overlying the vertebral column with tissue forceps and then use the bone rongeurs to remove the vertebrae and expose the spinal cord (*see* **Note 17**).
3. Cut the spinal cord above and below the region to be dissected and then carefully remove the spinal cord with a blunt dissection probe. Use small spring scissors to cut any nerves as required to free the spinal cord.

3.4.2. Evaluation of Transduction and SOD1 Silencing; Immunohistology on Frozen Sections

1. Place the freshly dissected spinal cord in 4% PFA in PBS and postfix for 6–18 h at 4°C.
2. Transfer the spinal cords to 25% sucrose in PBS and cryoprotect overnight at 4°C. The spinal cords are ready once they have sunk to the bottom of the tube.
3. Place the spinal cord in a Peel-A-Way® disposable embedding mold containing Cryomatrix™ embedding fluid. Freeze the spinal cord by placing the mold in a beaker filled with liquid nitrogen (*see* **Note 18**).
4. Place the embedded spinal cord in a cryostat and allow to equilibrate to –20°C. Cut 20 μm transverse sections and dry for 30 min on glass slides. The sections may be stored at –80°C at this point for several months.
5. Wash slides three times with PBS over 30 min to remove cryoembedding solution and then block for 3–6 h. Incubate the sections with the primary antibodies in blocking solution overnight at 4°C. Wash the slides three times with PBS for over 45 min and then incubate with the appropriate fluorescent secondary antibodies in PBS/1% BSA for 2 h. Wash again three times with PBS for over 45 min and then rinse briefly in H_2O prior to mounting in Mowiol.
6. Examine GFP expression (corresponding to virus transduction and shRNA delivery) using either native GFP expression or enhance the signal using antibodies against GFP (1:800 goat polyclonal anti-GFP Ab with 5% NDS, 0.1% BSA, 0.1% Triton X-100 blocking solution). Analyze the GFP expression with a confocal microscope for increased resolution (**Fig. 7.4A**).
7. Quantify motor neuron transduction by counting the total number of GFP-positive cells that colocalize with VAChT-positive cells and express these as a percentage of total VAChT-positive cells (**Fig. 7.4B**) (1:800 goat polyclonal anti-GFP Ab and 1:2500 rabbit polyclonal anti-VAChT with 5% NDS, 0.1% BSA, 0.1% Triton X-100 blocking solution).
8. Examine SOD1 silencing in motor neurons/glial cells by double labeling sections from silencer and mismatch injected animals for SOD1 and GFP. SOD1 staining is achieved in TBS using either 1:1500 anti-SOD1 clone SD-G6, 1:1500 anti-SOD1 clone G215-1, or 1:100 polyclonal goat anti-SOD1 Ab in combination with 1:800 rabbit polyclonal

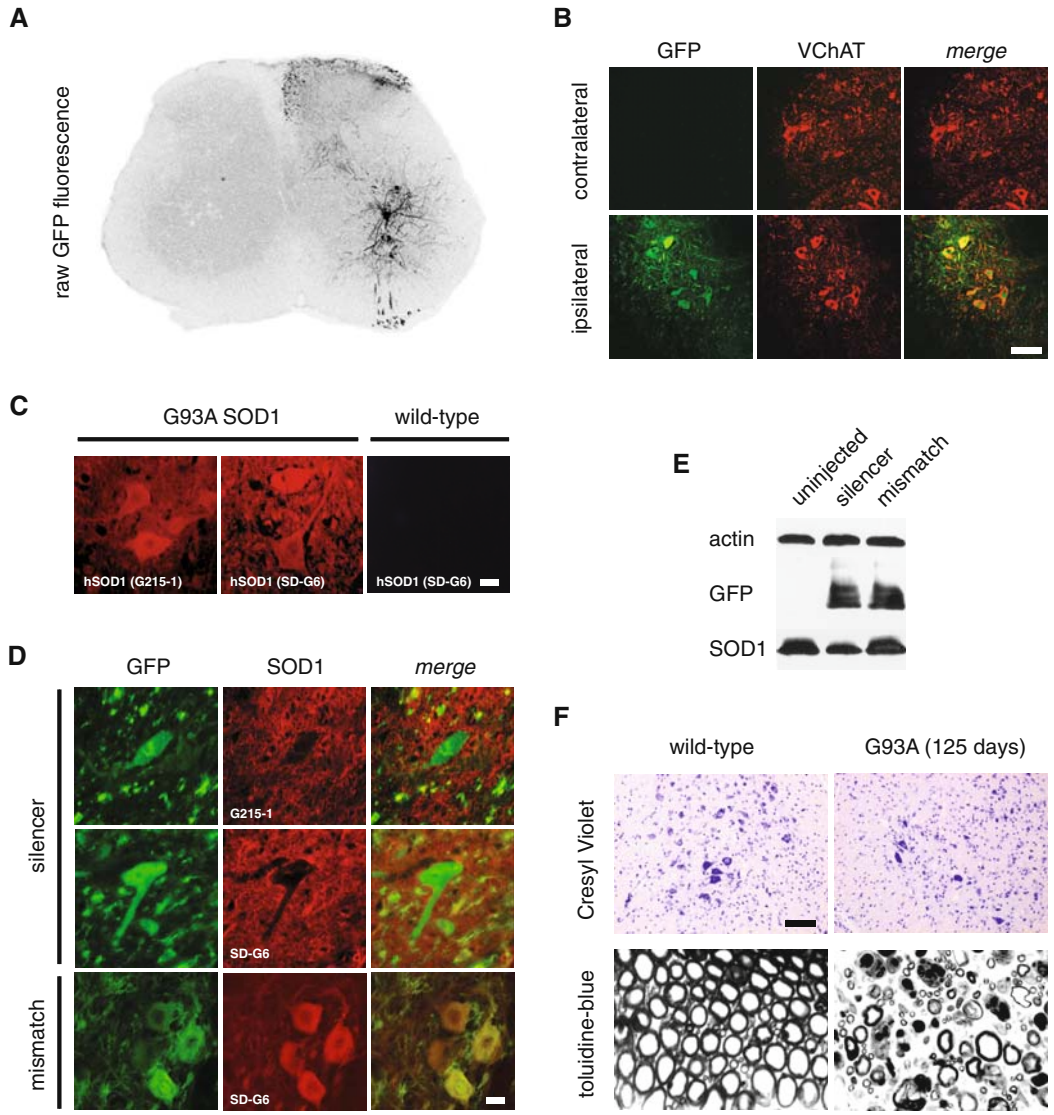

Fig. 7.4. Analysis of transduction, SOD1 silencing, and neuroprotection following viral-mediated-RNAi in the G93A SOD1 mouse. (**A**) GFP fluorescence of lumbar spinal cord following unilateral intramuscular injection of AAV6. (**B**) Double labeling demonstrating colocalization between GFP and the motor neuron marker, VAChT, in the ventral horn. Scale bar, 80 μm. (**C**) Specificity of SOD1 staining with two different antibodies comparing G93A SOD1 and wild-type mice. Scale bar, 20 μm. (**D**) Immunostaining for SOD1 and GFP in the ventral horn of mice injected with lentivirus expressing either the silencer or mismatch shRNA against SOD1. Scale bar, 20 μm. (**E**) Immunoblot evaluation of vector-mediated silencing in the lumbar spinal cord following direct intraspinal delivery. (**F**) Cresyl Violet and toluidine-blue sections of ventral horn spinal cord and ventral roots, respectively, from wild-type and G93A SOD1 mice at 125 days of age. Scale bar, 100 μm. (**C–F** reproduced fromk Ref. *(7)* with permission from Nature Publishing Group).

anti-GFP Ab with 4% NDS, 4% BSA, 0.1% Triton X-100 blocking solution. Merge images from the GFP and SOD1 channels to best illustrate SOD1 knockdown (**Fig. 7.4C**) (*see* **Note 19**).

3.4.3. Evaluation of Neuroprotection; Motor Neuron Counts on Paraffin-Embedded Sections

1. Place the freshly dissected spinal cord in 4% PFA in PBS and postfix for 6–18 h at 4°C.
2. Prepare spinal cord for paraffin inclusion by rinsing two times with PBS for 15 min; two times with 25% EtOH/75% PBS for 10 min; two times with 50% EtOH/50% PBS for 10 min; two times with 70% EtOH for 20 min.
3. Perform the following cycles using an automatic embedding machine: 70% EtOH for 90 min; 80% EtOH for 90 min; 96% EtOH for 90 min; 100% EtOH for 90 min; toluol for 90 min; paraffin for 2 h at 60°C.
4. Embed the spinal cord in the embedding mold and solidify the paraffin at 4°C.
5. Cut 8 μm transverse spinal cord sections and place on a 1% gelatin solution heated at 42°C. Mount the sections onto glass slides and dry for 30 min.
6. Deparaffinize the slides for heating for 1 h at 60°C and place immediately in toluol. Leave for 10 min and transfer to a fresh batch of toluol for a further 5 min.
7. Rehydrate the slides by rinsing: two times with 100% EtOH for 2 min; one time with 96% EtOH for 2 min; one time with 80% EtOH for 2 min; one time with 70% EtOH for 2 min; one time H_2O for 5 min.
8. Place slides for 1 min in 0.5% Cresyl Violet in differentiation buffer and then leave 30 s in differentiation buffer alone. Wash two times for 30 s in 100% EtOH and then two times for 30 s in toluol.
9. Mount the slides in Merkoglass® and allow to dry overnight.
10. Count only large-sized cells (>25 μm) (motor neurons) with a visible nucleolus on every fifth section across the desired region of spinal cord. Examples of stained sections are shown in **Fig. 7.4F** (top panel).

3.4.4. Evaluation of Neuroprotection; Myelinated Fiber Counts on Resin-Embedded Sections

One pathological characteristic of ALS observed in the clinic and in rodent models is the demyelination of large axons (motor neurons) in the spinal cord ventral roots. Quantifying the numbers of myelinated fibers therefore provides another measure of protection from motor neuron degeneration.

1. Dissect the ventral roots from the dissected spinal cord under a stereomicroscope and postfix in 4% PFA/2.5% glutaraldehyde for 6–18 h at 4°C.

2. Incubate ventral roots in the dark for 4 h in 2% osmium tetroxide, 50 mM cacodylate (*see* **Note 1**).
3. Wash the ventral roots rapidly in H_2O and dehydrate with 80% EtOH for 1 h, 96% EtOH for 1 h, 96% EtOH for 1 h, 96% EtOH/infiltration solution (1:1) for 1 h, and then infiltration solution overnight at 4°C.
4. Embed ventral roots in 2 × 15 mm molds by mixing the infiltration solution with the hardener and allow to set.
5. Cut 2 μm transverse sections using an ultramicrotome and place sections on a glass slide (*see* **Note 20**).
6. Place the slides on a heating plate at 120°C for 10 min and let dry.
7. Incubate the slides in 0.5% Toluidine Blue O, 10 mM sodium acetate pH 6.5 for 1 h.
8. Wash slides two times for 30 s in H_2O, two times for 60 s in 100% EtOH, two times for 60 s in toluol, and mount in Merkoglass®.
9. Count the numbers of small (<5 μm) and large (≥5 μm) myelinated motor fibers in the ventral roots. The lipid-rich myelin is stained black by the lipophilic osmium tetroxide. Examples of stained sections are shown in **Fig. 7.4F** (bottom panel).

4. Notes

1. Paraformaldehyde and osmium tetroxide are extremely hazardous. Observe all warning and precautions listed on the MSDS for these compounds.
2. The Whitehead Institute for Biomedical Research offers an excellent siRNA target designer that is free (http://jura.wi.mit.edu/bioc/siRNAext/). Registration by email is required.
3. Viral production yield may decrease as passage number increases in the 293-derived cells. We recommend producing a cell bank with cells at low passage to allow regular thawing of new vials. Cell may be frozen in 80% serum and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.
4. Unless otherwise stated, all incubations, washes, drying steps, and centrifugations are performed at room temperature.
5. New heparin columns should be washed with PBS for 10 min, followed by 0.4 M NaCl in PBS, followed again by PBS for 10 min.
6. There are many commercially available kits to titrate p24 antigen. We have previously used with success the

RETROtek HIV-1 p24 Antigen ELISA kit (#0801200, ZeptoMetrix Corporation, Buffalo, NY).

7. Gently flick the tube one or two times prior to adding the PFA to disperse the pellet. This reduces the number of cell clumps observed following addition of the fixative.
8. The reason for the difference between the calculations results from the fact that lentivirus integrates whereas AAV does not. AAV genomes remain mostly episomal within the nucleus of cells and will be diluted as the cells divide.
9. The percentage of isoflurane required to induce and maintain anesthesia may vary between strain, age, and sex of the rodents as well as the apparatus being used.
10. L3 and L4 are identified by using the index finger and thumb to localize the pelvic bone at the hip level which corresponds to L6. The vertebrae are then counted backwards to localize the desired level.
11. Knowledge of the anatomy of mouse vertebrae will help at this point. We recommend consulting a mouse anatomy book prior to performing this procedure.
12. The use of a stereomicroscope or magnifying glass will aid visualization of the injection.
13. 1 g/L of effervescent paracetamol can be added to the water supply of the animal in order to minimize postoperative pain and hasten recovery.
14. The muscle should expand in volume following virus delivery. This is easily observed visually and indicates a successful injection.
15. We have found that this setup is the most practical for neonate injections. One hand can be used to hold and insert the needle into the muscles while the other hand remains free to inject the virus with the syringe. This increases the dexterity of the manipulation.
16. There is always a chance that experimental neonate manipulations may provoke the mother to kill the newborns. The animal handling method described in this section is the best in our experience to avoid this.
17. Removing the vertebrae is the most difficult procedure in spinal cord dissection. Great care must be taken to avoid damaging the spinal cord that, despite being fixed, remains extremely delicate. After clearing the muscle from either side of the vertebra, use the bone rongeurs to carefully 'crack' the column at each vertebral level (in the same way as one would crack open a walnut). *Be careful not to apply so much force that the vertebrae collapse onto the underlying spinal cord*. After this step, use the bone rongeurs to carefully remove the piece of bone comprising the spinous process and cranial articular process at the level below the region of spinal cord that is to be dissected. This action

will be transversal to the column. Next, remove the piece of bone comprising the cranial articular process with the caudal articular process. This action will be lateral to the column. Repeat these actions (transversal, lateral, transversal, etc.) moving up the spinal column until sufficient spinal cord is revealed.

18. Follow the appropriate safety procedures for handling liquid nitrogen as specified by your institution.
19. SOD1 silencing can also be demonstrated via Western blot (**Fig. 7.4D**) or RT-QPCR (not shown).
20. Placing a drop of water on the glass slide prior to placement of the resin section will help to unwrinkle and flatten the section.

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