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Chapter Title	Design and Cloning of Short HairpinRNAs (shRNAs) into a Lentiviral Silencing Vector to Study the Function of Selected Proteins in Neuronal Apoptosis

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Abstract	Double-stranded RNA-mediated interference (RNAi) is a new simple and fast research tool for shutting down genes and characterizes function of their respective proteins. Many strategies for design and delivery of siRNA to target cells are available. Here, we describe the use of lentiviral short hairpinRNA (shRNA) RNA silencing to identify the involvement of D-serineracemase (SR)- an enzyme that syntheses D-serine to modulate glutamate-*N*-methyl-D-aspartate receptor- in regulating ratcerebellar granule neurons (CGN) apoptosis. Apoptosis is induced by serum and KCl withdrawal and is detected with fluorometric caspase 3assay.

Keywords (separated by “-”)	siRNA - ShRNA - Lentivirus - Cerebellar granule cells - D-serine racemase

Chapter 9 1

Design and Cloning of Short Hairpin RNAs (shRNAs) into a Lentiviral Silencing Vector to Study the Function of Selected Proteins in Neuronal Apoptosis 2 3 4

[AU1] Nadia Canu 5

Abstract 6

Double-stranded RNA-mediated interference (RNAi) is a new simple and fast research tool for shutting down genes and characterizes function of their respective proteins. Many strategies for design and delivery of siRNA to target cells are available. Here, we describe the use of lentiviral short hairpin RNA (shRNA) RNA silencing to identify the involvement of D-serine racemase (SR)- an enzyme that syntheses D-serine to modulate glutamate- *N*-methyl-D-aspartate receptor- in regulating rat cerebellar granule neurons (CGN) apoptosis. Apoptosis is induced by serum and KCl withdrawal and is detected with fluorometric caspase 3 assay. 7 8 9 10 11 12

Key words **siRNA,** ShRNA, Lentivirus, Cerebellar granule cells, D-serine racemase 13

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1 Introduction 14

Gene knockout is used to study the function of specific gene, detect its protein product, and link it to physiological or pathological processes. Knockout can be deliberately made using different molecular techniques some of which, like homologous recombination, are lengthy and expensive. RNA interference (RNAi) has appeared as a novel pathway to knockdown specific mRNAs, thus preventing translation of the respective protein from occurring. 15 16 17 18 19 20 21

RNAi is a natural process—used in many different organisms to regulate endogenous gene expression—in which non-translated, long, double-stranded RNA (dsRNA) led to a strong, long lasting and specific silencing of selected genes [1]. Further studies revealed that small dsRNA of 21–25 bp (small interfering RNA = siRNA) derived from endonuclease Dicer-mediated processing of long dsRNAs interact with a protein complex to form the RNA-induced silencing complex (RISC) [2, 3]. This complex has nuclease activity and digests mRNA containing a base pair sequence identical to that in the siRNA. Thus, the siRNA serves 22 23 24 25 26 27 28 29 30 31

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32 as a target sequence that allows RISC to recognize specific
33 mRNAs and to prevent their translation by cleaving them [4].
34 Another method to produce siRNA is based on the use of short
35 hairpin RNAs (ShRNAs) that trigger RNAi [5, 6]. Short (60–
36 75 bp long) DNA oligodesoxynucleotides that form hairpins are
37 cloned into a plasmid under the control of the U6 or H1 pro-
38 moter for RNA polymerase III. Transfection of such a plasmid
39 promotes the expression of ShRNAs that induce RNAi. Non-
40 replicating recombinant viral vectors (adeno, adeno-associated
41 and lentiviruses) are commonly used for ShRNA expression in
42 primary neuronal cells. Lentiviruses may be particularly suited for
43 long-term ShRNA and expression and gene silencing in vivo since
44 the viral DNA gets incorporated in the host genome.

45 Commonly used lentiviral vector systems belong to the
46 second or third generation, ensuring safe application, as these
47 viruses are unable to self-replicate, since the spontaneous self-
48 assembly is prevented by distributing the least necessary num-
49 ber of virus elements on three and four plasmids, respectively.
50 Here, we describe the methods used in our laboratory to silenc-
51 ing SR in rat CGNs as a tool for identifying the role of this
52 enzyme during apoptosis.

53 2 Materials

54–58 2.1 Design, Production, and Cloning of shRNAs and Preparation of Lentiviral Vectors

54–56 1. Packaging cell line: HEK (human embryonic kidney)-293T (Invitrogen™—Life Technologies™, Gaithersburg, MD). *See* **Note 1**.
57 2. 15 cm plates (Becton Dickinson Labware, Franklin Lakes, NJ).
58–60 3. Dulbecco's modified Eagle's medium (DMEM, Gibco™—Life Technologies™) with 2 and 10 % fetal bovine serum (FBS). *See* **Note 2**.
61 4. Lipofectamine 2000 (Invitrogen™—Life Technologies™).
62 5. Opti-MEM 1× (Gibco™—Life Techonologies™).
63–65 6. Plasmids: pLVTHM; pCMVdR8.74; pMD2G (available from Addgene: http://www.addgene.org/) for second lentivirus generation. For third lentivirus generation, refer to Dull et al. [7].
66–67 7. 10× Tris-buffered EDTA buffer (TBE buffer): 1 M Tris, 0.9 M boric acid, 0.01 M EDTA.
68–69 8. Extraction Kit (DNA 70–10 kb): e.g., QIAquick Gel 8 (Quiagen GmbH, Hamburg, Germany).
70 9. Endotoxin-free plasmid maxipreps columns (Quiagen).
71–72 10. Tris-EDTA buffer (TE buffer 1×): 10 mM Tris, 1 mM EDTA, pH 8.0 with HCl.
73–74 11. Primer, 5′ forward must contain an MLu I site; Primer, 3′ reverse must contain a Cla I site.

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12. Restriction endonucleases: MLu I, Cla I (New England Biolabs, Ipswich, MA). 75 76
13. T4 DNA ligase 400,000 U/mL (New England Biolabs). 77
14. 10× T4 DNA ligase buffer: 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5. 78 79
15. Bacterial growth strain(s): DH5α for pMD2G and pCMVdR8; HB101 for pLVTHM lentiviral vector. *See* **Note 3**. 80 81
16. LB agar ampicillin plates: Use a 2 L flask to prepare 1 L of LB broth with agar (Lennox) (Sigma Chemicals, St. Louis, MO). To 1 L of distilled water add 35 g of LB agar. Swirl to dissolve and autoclave for 15 min at 120 °C to sterilize. Cool medium to 50 °C, and add 50–100 μg/mL ampicillin. Pour into Petri dishes and allow to solidify, store at 4 °C. 82 83 84 85 86 87
17. Hank's Buffered Salt Solution (HBSS): 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.1 g glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$. 88 89 90
18. 20 % [w/v] sucrose in HBSS. 91

2.2 Primary Cerebellar Granule Neuron Culture, Induction, and Detection of Apoptosis

1. Basal medium Eagle (BME; Life Technologies™). 92
2. Bovine serum albumin (BSA, Sigma Chemicals). 93
3. Krebs-Ringer bicarbonate medium (KRB): 120 mM NaCl, 5 mM KCl, 1.22 mM KH_2PO_4, 25.5 mM, 14 mM glucose, 4.2 mM phenol red. 94 95 96
4. Solution A: KRB supplemented with 1.2 mM $MgSO_4$, 3 mg/mL BSA. 97 98
5. DNAse I (Sigma Chemicals). 99
6. Soybean trypsin inhibitor (Sigma Chemicals). 100
7. Trypsin type III (Sigma Chemicals). 101
8. L-Glutamine. 102
9. Gentamicin sulfate. 103
10. Fetal bovine serum (FBS, Gibco™). 104
11. CGN culture medium: BME, 10 % FBS, 25 mM KCl, 2 mM glutamine, 100 mM gentamicin sulfate. 105 106
12. 1β-Arabinofuranosylcytosine (Sigma Chemicals). 107
13. Caspase 3 substrate: Ac-DEVD-AMC [*N*-Acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methyl coumarin)] (Biomol International, Plymouth Meeting, PA). 108 109 110
14. Caspase 3 lysis buffer A: 10 mM HEPES, pH 7.4, 42 mM KCl, 5 mMMgCl$_2$, 1 mM , 1 mM PMSF, 0.5 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 1 μg/mL leupeptin. 111 112 113 114
15. Caspase 3 assay buffer B: 25 mM HEPES, 1 mM EDTA, 0.1 % CHAPS, 10 % sucrose, 3 mM DTT, pH 7.5. 115 116

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2.3 Western Blotting and Immuno-fluorescence for D-Serine Racemase

1. Lysis buffer: 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS.
2. 10 % SDS-PAGE (Sodium Dodecyl Sulfate—PolyAcrylamide Gel Electrophoresis)- Laemmli protocol:
   (a) 10 % lower gel (resolving gel): 4.9 mL distilled H_2O, 2.5 mL 40 % acrylamide/Bis-acrylamide (29:1), 2.5 mL 1.5 M Tris, pH 8.8, 50 μL 20 % SDS, 50 μL 10 % ammonium persulfate, 10 μL TEMED (total volume = 10 mL). Mix well and quickly transfer the gel solution by using 1 mL pipette to the casting chamber between the glass plates. Once the gel has polymerized, prepare stacking gel.
   (b) 3.75 % stacking gel: 2.44 mL distilled H_2O, 0.46 mL 40 % acrylamide/Bis- acrylamide (29:1), 1 mL 0.5 M Tris, pH 6.8, 40 μL 10 % SDS, 15 μL 10 % ammonium persulfate. [AU2] Righ before pouring the gel add 1.5 μL TEMED.
3. Agarose gel: agarose 1 % in TBE buffer 0.5×.
4. Phosphate-buffered saline 1× (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.
5. Normal goat serum (NGS) (Jackson ImmunoResearch, Europe Ltd., Newmarket, UK).
6. 100 % methanol.
7. Antifade mounting medium (ProLong® Gold Antifade—Life Technologies).
8. Mouse D-serine racemase antibody (BD Transduction laboratories™, San Jose, CA).
9. Affinity purified-goat D-serine racemase antibody (Santa Cruz Biotechnology, Dallas, TX).
10. Secondary TRITC-conjugated donkey anti-goat antibody (Jackson ImmunoReseach Europe Ltd.).

2.4 Equipment

1. Centrifuge (e.g., Beckman Coulter Inc, Brea, CA).
2. Tissue culture 15 cm dishes.
3. Tissue culture 6-well dishes.
4. Tissue-culture 24-well dishes for CGNs (Nunc A/s, Roskilde, Denmark).
5. Filters (0.22- or 0.45-μm).
6. Incubators preset to 37 °C (5 % CO_2).
7. Microcentrifuge.
8. PCR thermocycler.
9. SW 28 and SW 55 rotors (Beckman Coulter).
10. Sterile round-bottom polypropylene tubes 5 mL (e.g., BD Falcon, BD Biosciences, San Jose, CA).

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11. 50 mL tubes. 158
12. Centrifuge, polyallomer, 5 mL tubes (Beckman Coulter). 159
13. Centrifuge, polyallomer, 12 mL tubes (Beckman Coulter). 160
14. Microcentrifuge tubes. 161
15. Vortexer. 162
16. 96-Well plate fluorescence reader (EnVision, PerkinElmer, Wellesley, MA). 163 164
17. Spectrofluorometer (e.g., Kontron AG, Zurich, Switzerland). 165
18. Protein electrophoresis/Western blotting apparatus. 166
19. Acrylamide gel electrophoresis apparatus. 167
20. Fluorescence microscope. 168

3 Methods 169

3.1 Design, Production, and Cloning of shRNAs and Preparation of Lentiviral Vectors

3.1.1 Design of shRNAs

ShRNA oligonucleotide design describes the process of identifying target sequences within a gene of interest and designing the corresponding oligonucleotides to generate the ShRNA. 170 171 172

A number of algorithms may been utilized to predict effective siRNA sequences and design ShRNA (e.g., http://www.ambion.com/ or http://sfold.wadsworth.org/; http://eu.idtdna.com/Scitools/Applications/shRNA etc). Here are general guidelines for ShRNA design based on the work of Tuschl et al. [8] and Elabishir et al. [9, 10] (see also: http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.htmL). 173 174 175 176 177 178 179

1. Select a region of 19 nt within the gene to be silenced [in our case, rat D-serine racemase (NCBI accession number NM_198757)] do not opt for region near the start codon (within 50-100 bases), nor untranslated regions [9, 10]. *See* **Note 4**. 180 181 182 183
2. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence appear to have increased knockdown activity. *See* **Note 5**. 184 185 186
3. Ensure the content of GC of the 19 bases oligonucleotide between 40 and 60 %, and a GC content of approximately 45 % is ideal. 187 188 189
4. Examine the 19 bases oligonucleotide for secondary structure and long base runs, both of which can interfere with the process of annealing. 190 191 192
5. Filter out, by appropriate database search, candidate targets that are present in other genes to avoid silencing of these loci. *See* **Note 6**. 193 194 195
6. Add the 7–9 nt hairpin loop sequence between sense and antisense strand [11–14]. One of the most effective loop sequences for H1 promoter is TTCAAGAGA [15]. 196 197 198

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a

446
GCGCAATCTCTTCTTCAAA

597
GCTCTCACCTATGCTGCTA

772
GCATCTTGGTCCATCCCAA

888
GGAGGAATGGTTGCTGGAA

b

```
5'-cgcgtGCGCAATCTCTTCTTCAAATTCAAGAGATTTGAAGAAGAGATTGCGCTTTTTTat-----3'
5'-cgatAAAAAAGCGCAATCTCTTCTTCAAATCTCTTGAATTTGAAGAAGAGATTGCGCa-----3
```

and the annealed oligos are:

```
5'-cgcgtGCGCAATCTCTTCTTCAAATTCAAGAGATTTGAAGAAGAGATTGCGCTTTTTTat-----3'
3'-----aCGCGTTAGAGAAGAAGTTTAAGTTCTCTAAACTTCTTCTCTAACGCGAAAAAAtagc-5'
```

Fig. 1 (**a**) Potential RNAi target sequences identified in the rat SR-coding region. (**b**) For one of the chosen sequences (GCGCAATCTCTTCTTCAAA) the complementary oligonucleotide pair for the hairpin siRNA and the annealed oligonucleotides are reported. The TTCAAGAGA loop sequence is highlighted in *yellow*, the stretch T is highlighted in *light blue* and the restriction sites (MLu I and Cla I) in red lowercase

7. Place a stretch of 5–6 T at the end of ShRNA to guarantee the termination of RNA polymerase III transcription.
8. Add to the end of two complementary oligonucleotides restriction sites (in our case MLu I at 5′ and Cla I at 3′) (*see* Fig. 1).
9. Include a negative control ShRNA. Usually ShRNA design online tools returns a scrambled sequence with the same nucleotide composition as your siRNA/shRNA input sequence.
10. Sense and antisense oligos must be phosphorylated and PAGE purified in order to increase cloning efficiency. When ordering, be sure to require that oligonucleotides are supplied after PAGE purification.

3.1.2 Annealing of shRNA Oligonucleotides

For expedience, annealing can be done in a thermal cycler.

1. Resuspend each PAGE-purified oligonucleotide in TE buffer to a concentration of 100 μM.
2. Mix the oligos for the sense strand and the anti strand at a 1:1 ratio. This will ultimately give 50 μM of ds oligonucleotide (assuming 100 % theoretical annealing).
3. Heat the mixture to 95 °C for 30 s. *See* **Note 7**.
4. Heat at 72 °C for 2 min.
5. Heat at 37 °C for 2 min.
6. Heat at 25 °C for 2 min.
7. Store on ice or at −20 °C until use.

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3.1.3 Cloning ShRNA Oligonucleotides into pLVTHM

1. Dilute the annealed oligonucleotides with TE buffer to obtain a concentration of 0.5 μM.
2. For each ligation, add the following reagents in a microfuge tube:
   - 2 μL digested (MLu I/Cla I) and dephosphorylated pLVTHM vector (100 ng/μL).
   - 4 μL diluted, annealed oligonucleotide (0.5 μM).
   - 2 μL 10× T4 DNA ligase buffer.
   - 0.5 μL BSA (10 mg/mL).
   - 11 μL Nuclease-free H_2O.
   - 0.5 μL T4 DNA ligase (400 U/μL).
   - For a 20 μL total volume.
3. Set up separate ligation using 2 μL of the negative scramble control ShRNA annealed oligonucleotide.
4. Set up separate ligation using 2 μL of digested (MLu I/Cla I) pLVTHM vector (50 ng/μL) without annealed oligonucleotide.
5. Incubate ligation mixture at room temperature for 3 h. *See* **Note 8**.
6. Transform immediately competent bacteria (with high transformation efficiency) and select on ampicillin plates.
7. Digest plasmid DNA from colonies with MLu I/Cla I and run on a 12 % DNA polyacrylamide gel in TBE 1× buffer gel (*See* **Note 9**). Positive clones will contain an approximately 60-bp insert compared to 17 bp for colonies without an insert.
8. Sequence the insert with human H1 primer (TCGNTATGTG TTCTGGGAAA) to check hairpin integrity.
9. Validate, by Western blot analysis or indirect immunofluorescence, the cloned ShRNA cassettes by transfecting pLVHTM-ShRNA as well as scramble vector in cell line that coexpresses the target gene. In our case, the cDNA for D-serine racemase together with ShRNA silencing cassette were transfected in an highly transfectable cell line (e.g., HEK-293). *See* **Note 10**.

3.1.4 Preparation of Lentiviral Vectors

You need to observe Biosafety-level-2since application of this protocol leads to the production of pseudoviral particles capable of infecting mammalian cells.

1. For a 10 cm dish lentiviral preparation: plate lentivirus HEK-293-T cells at a density of approximately 100 cells/mm^2 in 10 mL of DMEM, supplemented with 10 % FBS, 12–24 h before transfection. Addition of antibiotic solution does not interfere with transfection.
2. To increase cell adherence, precoat twelve 15 cm dishes with 10 mL of poly-L-lysine, incubate for 30 min at room temperature

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262 under UV, and aspirate off the liquid. Grow the cells
263 overnight at 37 °C in 5 % CO_2. *See* **Note 11**.

264 3. Aliquot in 200 µL of Opti-MEM the three plasmids into a
265 sterile polypropylene tube. For a 10 cm dish, use:

266 • –10 µg of lentivector pLVTHM.
267 • –3.5 µg of pMD2G (Gag-Pol).
268 • –6.5 µg of pCMVdR8.74.

269 4. In a separate tube, dilute 20 µL lipofectamine 2000 in 200 µL
270 of Opti-MEM 1×.

271 5. Add diluted lipofectamine reagent drop-wise to the DNA
272 solution while gently vortexing the DNA-containing tube and
273 incubate for 30 min at room temperature. *See* **Note 12**.

274 6. Remove medium from cell plate, wash cells twice with Opti-MEM
275 and add 5 mL of Opti-MEM without antibiotics. *See* **Note 13**.

276 7. Add the transfection mixture to each plate. Swirl the plates
277 gently to distribute the complex and incubate overnight at
278 37 °C in a 5 % CO_2 atmosphere.

279 8. Approximately 6–8 h after transfection, remove media. Add
280 15 mL of fresh DMEM plus 2 % heat-inactivated FBS and
281 penicillin-streptomycin to each plate and incubate overnight
282 at 37 °C in a 5 % CO_2 atmosphere. *See* **Note 14**.

283 9. Collect the supernatant from the plates and centrifuge at
284 500×*g* for 10 min to remove cell debris and filter through
285 0.45 µm filters. *See* **Note 15**.

286 10. Add 15 mL of fresh medium to each plate and incubate over-
287 night. Filtered supernatants can be stored for several days at 4 °C.

288 11. Collect media and filter as in **step 9**. *See* **Note 16**.

289 12. Pool collected supernatants from steps 9 and 11. Transfer to
290 Beckman tubes using 25–29 mL per tube.

291 13. Concentrate viral particles by centrifuging in a Beckman SW
292 28 rotor at 65,000×*g* or 2 h at 20 °C.

293 14. Resuspend all pellets in a total of 1 mL of HBSS and wash
294 tubes a second time with 1 mL of HBSS.

295 15. Increase the combined volume from 2 to 3 mL with HBSS
296 and layer the resuspended pellets on 1.5 mL of a 20 % sucrose
297 (in HBSS) cushion in Beckman tubes.

298 16. Centrifuge using a Beckman SW 55 rotor at 53,500×*g* for
299 1.5 h at 20 °C.

300 17. Resuspend the pellet in 100 µL of HBSS containing 1 % BSA and
301 wash the tube with an additional 100 µL of HBSS containing 1 %
302 BSA. Shake the resuspended viral preparation on a low-speed
303 vortexer for 15–30 min. Centrifuge for 10 s to remove debris.

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18. Aliquot the cleared viral solution and store at -80 °C. It can be stored for many months. Avoid repeated freeze-thaw cycles.
19. Titrate the viral preparations by biological titration using GFP, which is the marker contained in the lentivector. The fraction of GFP fluorescent cells can be counted by FACS (fluorescence activated cell sorting). GFP fluorescence may be also visualized under a fluorescence microscope. Usually 10–15 random fields of view are used to estimate the overall fraction of fluorescing cells in each well.

3.2 Primary Cerebellar Granule Neuron Cultures and Lentivirus Transduction

Cultures enriched in CGNs are obtained from dissociated cerebella of 8-day-old Wistar rats according to Levi et al. [16]. The preparation of CGN cultures is carried out at *Day 1*, transduction at *Day 2*, induction and detection of apoptosis at *Days 6–7*.

3.2.1 Primary Cerebellar Granule Neuron Cultures

1. Remove 4–5 cerebella from 8-day-old rats and slice them (0.4 mm thickness) with a mechanical tissue chopper.
2. Suspend in 10 mL solution A, centrifuge for 15 s at 150 × *g*.
3. Resuspend the tissue in 10 mL solution A containing 0.25 μg/mL trypsin III and incubate at 37 °C for 15 min in a shaking water bath at rate of 125 rpm.
4. Add to the suspension 10 mL solution A containing 12.8 μg DNAase I and 83 μg soybean trypsin inhibitor.
5. Centrifuge immediately for 15 s at 150 × *g*.
6. Resuspend the pellet in 2 mL of solution A containing 80 μg DNAase I, 0.52 mg soybean trypsin inhibitor and 2.7 mM $MgSO_4$. Triturate the tissue with a Pasteur pipette (25 strokes).
7. Allow the suspension to stand for 15 min, aspirate carefully the upper 1.5 mL, readjust the volume to about 2 mL ad dissociate as above. After allowing the suspension to stand for 15 min, take off the supernatant, leaving only 0.2 mL containing clumps and debris.
8. Transfer the supernatant into 3 mL Solution A containing 0.1 mM $CaCl_2$. After about 10 min decant the supernatant, allow to stand for another 10 min and resuspend the pellet in CGN culture medium.
9. Count the cell in the suspension.
10. Plate 4×10^5 CGN per well in a NUNC 24-well plate in 800 μL CGN culture medium. Incubate the cells at 37 °C with 5 % CO_2.
11. After 24 h add 10 μM 1β-Arabinofuranosylcytosine to CGN culture medium to prevent proliferation of non-neuronal cells.

3.2.2 Transduction of Primary Cerebellar Granule Neuron Cultures

1. Transduce CGN cells with lentivirus. For each well, prepare 50 μL of virus suspension diluted in CGN culture medium (*See* **Note 17**). To transduce CGN reduce the volume of the

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346 medium to one-third; add the recombinant lentivirus at different
347 dilutions. Allow the virus to adsorb for 1–2 h, thus render back
348 the medium to its original volume. Then cultivate neurons up
349 to 6–7 days in vitro (DIV) when apoptosis will be induced
350 (Fig. 1).

351 3.3 Induction and Detection of Apoptosis

Induction of apoptosis is carried out in serum-free medium at low (5 mM) KCl [17].

3.3.1 Induction of Apoptosis

1. Wash cultures twice and maintain in serum-free low (5 mM) KCl CGN culture medium for 8 h.
2. Wash and maintain control cultures in serum-free CGN culture medium for 8 h.

3.3.2 Detection of Apoptosis

Measure caspase 3 activity as follows:

1. Wash 500,000 CGNs with PBS once.
2. Add 100 μL of caspase 3 lysis buffer A to lyse cells.
3. Combine 25 L of lysate with 75 μL of caspase 3 assay buffer B containing 30 μM Ac-DEVD-AMC.
4. Incubate for 20 min at room temperature.
5. Measure fluorescence at an excitation of 380 nm and an emission of 460 nm using a spectrofluorometer (Fig. 2).

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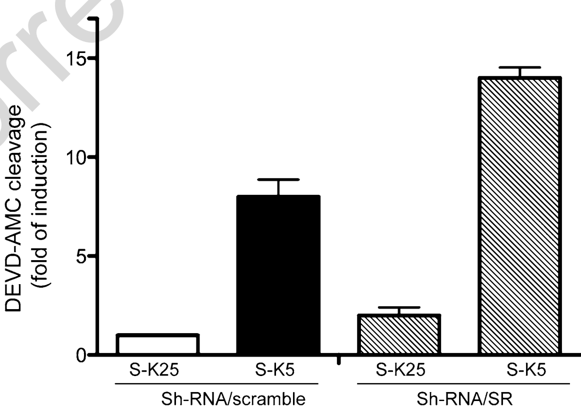

Fig. 2 In vitro CGNs (2 DIV) were transducted either with Sh-RNA/scramble and Sh-RNA/SR lentivirus at MOI 40. At 6DIV they were induced to undergo apoptosis by serum and KCl deprivation (S-K5); control cells were maintained in serum-free medium supplemented with 25 mM KCl (S-K25). Eight hours after apoptosis induction neurons were lysed and assayed for DEVD-MCA cleavage. Fold-induction of caspase-3 activity is the mean (±SEM) of triplicate determinations from three independent experiments. Note that silencing of SR increases caspase-3 activity in CGNs undergoing apoptosis, suggesting that this enzyme has a protective role in survival of CGN (*see* ref. 17)

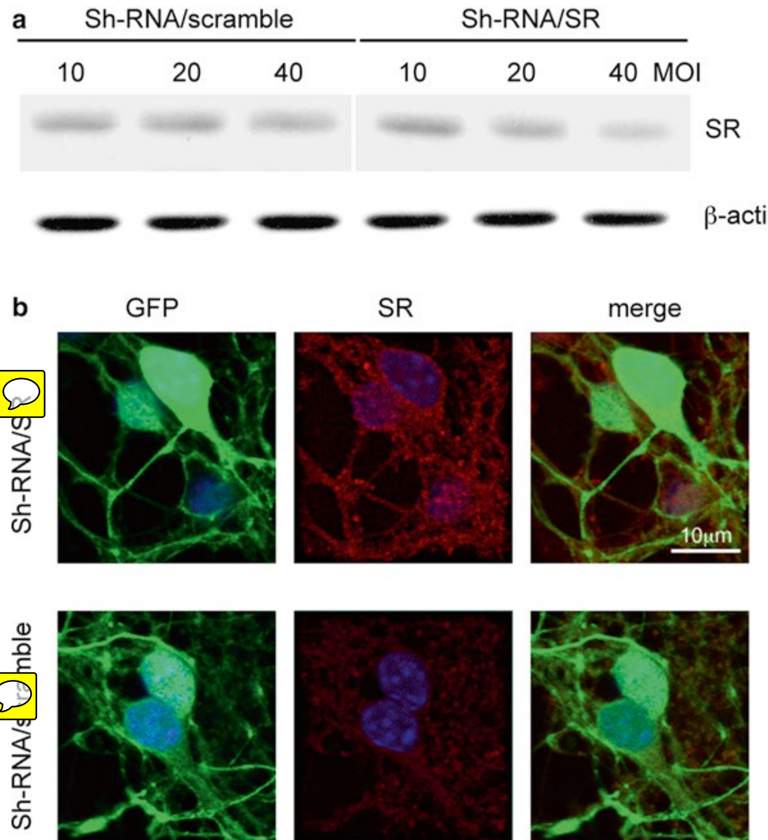

Fig. 3 (**a**) In vitro CGNs (2 DIV = day in vitro) were transduced either with Sh-RNA/scramble and Sh-RNA/SR lentivirus at MOI indicated. At 6DIV lysates were processed for SDS-PAGE and Western blot for immunodetection of SR and β-actin as control of silencing efficiency and specificity. (**b**) Confocal microscope analysis of SR expression at 6 DIV (*red*) in Sh-RNA/scramble and Sh-RNA/SR transducted CGNs at 2DIV at MOI 40. ~~Transdu/recommended guidelines~~

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3.4 Western Blotting and Immunofluorescence for D-Serine Racemase

3.4.1 Western Blotting

1. Extract total proteins by scraping cells in SDS-reducing sample buffer. 365 366
2. Boil for 5 min. 367
3. Perform western blot analysis with mouse anti-D-serine racemase antibody (*see* Fig. 3a). 368 369

3.4.2 Immunofluorescence

1. Fix and permeabilize CGN cultured in the chamber slide with methanol 100 % for 20 min at −20 °C. 370 371
2. Block with 4 % NGS in PBS for 1 h at room temperature. 372
3. Incubate slides with affinity purified-goat anti-D-serine racemase antibody diluted 1:50 in PBS overnight at 4 °C. 373 374
4. Wash three times with PBS. 375

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5. Add secondary TRITC-conjugated donkey anti-goat antibody diluted 1:200 in PBS and incubate at room temperature for 30 min in a humid chamber.
6. Wash three times with PBS.
7. Remove excess moisture from the slide before adding anti-fade mounting medium.
8. Examine slide under fluorescence/confocal microscope (*see* Fig. 3b).

4 Notes

1. Cells should be of low-passage number and should not be used after passage 20 or if growth is slow.
2. Certain brands of FBS do not support efficient transfection and can result in low viral titers. We routinely use FBS, Qualified, Australia Origin from Gibco™.
3. There is an additional Cla I site in pLVTHM vector that is blocked by Dam methylation. The plasmid needs to be grown in a Dam^+ bacterial strain such HB101 in order to use Cla I for cloning.
4. Although it is recommended to avoid to select target sequence in the untranslated regions, since regulatory protein binding to regions in and near the untranslated region might interfere with the RNAi process, in some case targets within the untranslated regions (UTR) have been also reported [18].
5. Avoid selecting target sense or antisense sequences that contain a consecutive run of three or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination of the shRNA transcript.
6. Many online tools to design shRNA gives a link to the NCBI BLAST server to search for similarity of the suggested target against the mRNA database of the organism of interest.
7. Heating to 95 °C is essential to remove all secondary structure, disrupt the internal hairpin of each oligonucleotide and promote intermolecular annealing.
8. If you are unable to perform immediately transformation, store ligation at −20 °C.
9. See Tables 5 and 6 in Sambrook and Russell, Molecular Cloning 3rd Ed VIII, p5.42 for different acrylamide concentrations and the effective range of DNA fragment sizes separated.
10. We have transfected 200 ng of target cDNA plasmid (HA-D-serine racemase) plus 500–1,000 ng of the plasmid containing the silencing cassette per 6-well plate and harvest the cells for immunoblot analysis 48–72 h after transfection.

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11. For best result and to optimize viral titer cells must be at 70–80 % confluence, equably distributed and with flat morphology before transfection.
12. The DNA-lipofectamine complex must be formed in the absence of proteins even though the complex is able to transfect cells in the presence of proteins such as 10 % FBS. Opti-MEM I is recommended for diluting both DNA and lipofectamine reagent. The ratio of 2.0 μL of lipofectamine 2000 per 1 μg of plasmid has been found to be optimal. Increasing the ratio does not further improve transfection efficiency.
13. Though the complex is able to transfect cells in the presence of proteins such as 10 % FBS, we found an improved transfection efficiency in the absence of serum.
14. To increase the lentivirus titer we have added, caffeine to a final concentration of 2–4 mM for 17–40 h post-transfection [19].
15. Do not use nitrocellulose filters, as nitrocellulose is known to bind lentivirus and reduce titers. Use 0.45 μm polyethersulfone (PES) low protein-binding filters.
16. Peak of virus production is normally achieved 24–48 h post-transfection; however collecting medium at multiple times at 36, 48, and 60 h post-transfection increases the viral yield.
17. Use several multiplicity of infection (MOI) virus stock to find the more suitable MOI to obtain silencing of you gene of interest. In addition, include a transduction with the scramble control and other appropriate positive and negative controls. Mix the virus with the medium gently by inverting the tubes several times. Do not vortex.

Uncorrected Proof

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Author Queries

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Queries	Details Required	Author's Response
AU1	Please check whether the affiliation is appropriate as typeset.	
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