Published in final edited form as:

Methods Cell Biol. 2016 ; 131: 177–197. doi:10.1016/bs.mcb.2015.06.004.

Optimization of cerebellar Purkinje neuron cultures and development of a plasmid-based method for Purkinje neuron-specific, miRNA-mediated protein knockdown

C. J. Alexander¹, J. A. Hammer III¹

¹Cell Biology and Physiology Center, National Heart, Lung Blood Institute, National Institutes of Health, Bethesda, Maryland, 20892

Abstract

We present a simple and efficient method to knockdown proteins specifically in Purkinje Neurons (PN) present in mixed mouse primary cerebellar cultures. This method utilizes the introduction via nucleofection of a plasmid encoding a specific miRNA downstream of the *L7/Pcp2* promoter, which drives PN-specific expression. As proof-of-principle, we used this plasmid to knock down the motor protein myosin Va, which is required for the targeting of smooth endoplasmic reticulum (ER) into PN spines. Consistent with effective knockdown, transfected PNs robustly phenocopied PNs from *dilute-lethal* (myosin Va-null) mice with regard to the ER targeting defect. Importantly, our plasmid-based approach is less challenging technically and more specific to PNs than several alternative methods (e.g. biolistic- and lentiviral-based introduction of siRNAs). We also present a number of improvements for generating mixed cerebellar cultures that shorten the procedure and improve the total yield of PNs, and of transfected PNs, considerably. Finally, we present a method to rescue cerebellar cultures that develop large cell aggregates, a common problem that otherwise precludes the further use of the culture.

Keywords

cerebellar cultures; Purkinje Neurons; *L7/Pcp2*; miRNA knockdown; nucleofection

Introduction

The cerebellum, which means ‘little brain’, is an extremely dense, highly structured region of the brain that contains ~50% of the neurons present in the CNS (Wang and Zoghbi, 2001). In addition to its fundamental roles in controlling fine motor coordination, voluntary movement and the maintenance of posture and balance (Llinas, 1985; Kashiwabuchi, Ikeda, Araki, Hirano, Shibuki, Takayama et al., 1995; Miall and Wolpert, 1996; Mauk, Medina, Nores and Ohyama, 2000), the cerebellum has been linked to higher cognitive functions, with implications in autism spectrum disorder (Leiner, Leiner and Dow, 1993; Piven, Saliba, Bailey and Arndt, 1997; Fatemi, Halt, Realnuto, Earle, Kist, Thuras et al., 2002; Fourier, Hass, Naik, Lodha and Cauraugh, 2010). Purkinje neurons (PNs) are the central players in cerebellar function, as they receive the vast majority of inputs into the cerebellar cortex (primarily from granule neurons and climbing fibers), and they represent the cortex’s sole output (Ito, Sakurai and Tomgroach, 1982; Hansel, Linden and D’Angelo, 2001).

A large number of related neurological conditions, which include spinocerebellar ataxias, result directly or indirectly from PN dysfunction (Manto, 2005; Dueñas, Goold and Giunti, 2006; Soong and Paulson, 2007). One such condition is Griscelli's Syndrome Type II (Ménasché, Ho, Sanal, Feldmann, Tezcan, Ersoy et al., 2003), and its mouse equivalent, the *dilute-lethal* mouse, (Mercer, Seperack, Strobel, Copeland and Jenkins, 1991), both of which result from mutations in the motor protein myosin Va. These mutations, which result in severe ataxia and PN degeneration, are likely due to aberrant calcium signaling within the dendritic spines of PNs (Llano, Dreessen, Kano and Konnerth, 1991; Miyata, Finch, Khiroug, Hashimoto, Hayasaka, Oda et al., 2000; Wagner, Brenowitz and Hammer, 2011a).

Given the central role played by PNs in cerebellar function, methods to successfully culture these neurons using dissociated embryonic cerebella as a starting point have been developed (Linden, Dickinson, Sneyne and Connor, 1991; Linden, 1996; Tabata, Sawada, Araki, Bono, Furuya and Kano, 2000; Wagner, McCorskey and Hammer, 2011b). This approach, in conjunction with the introduction of exogenous DNAs into PNs by nucleofection, were pivotal in demonstrating that myosin Va serves as a point-to-point organelle transporter to translocate tubules of smooth endoplasmic reticulum (ER) into the dendritic spines of PNs (Wagner et al., 2011a).

In depth structure: function studies in PNs rest on one's ability to complement mutant PNs with wild type and mutated versions of one's protein of interest. This can be achieved by reintroducing the protein into PNs isolated from the corresponding mouse knockout (see, for example, Wagner et al, 2011b). If a mouse knockout is not available (or results in early lethality), one must resort to knocking down the protein in wild type PNs, and then complementing with an RNAi-immune version to characterize structure: function relationships. Indeed, several laboratories have had considerable success using this approach, where siRNA oligonucleotides were introduced into cultured PNs using a biolistic method (Eto, Bock, Brarutigan and Linden, 2002; Chung, Steinberg, Haganir and Linden, 2003; Leitges, Kovac, Plomann and Linden, 2004; Shima, Kengaku, Hirano, Takeichi and Uemura, 2004; Cho, Park, Wolff, Xu, Hopf, Kim et al., 2008; Tanaka, Yanagawa and Hirashima, 2009). That said, this approach is technically quite challenging, not particularly efficient, and does not result in PN-specific knockdown or re-expression.

Relevant to the issue of PN-specific knockdown and re-expression, the use of the PN-specific promoter *L7/Pcp2* to drive the expression of fluorescent fusion proteins in live PNs has proven very powerful for dissecting molecular mechanisms in these cells (see, for example, De Zeeuw, Hansel, Bian, Koekkoek, van Alphen, Linden et al., 1998; Wagner et al., 2011b). Moreover, a number of labs have developed lentiviral vectors that drive the expression of miRNA using a truncated version of the *L7/Pcp2* promoter (Uesaka, Mikuni, Hashimoto, Hirai, Sakimura and Kano, 2012; Miki, Hirai and Takahashi, 2013). While this approach has yielded effective protein knockdown in PNs, the truncation of the *L7/Pcp2* promoter results in significant expression in cells other than PNs, complicating efforts to interpret phenotypes in mixed cerebellar cultures (unpublished observations). Moreover, defining the exact conditions required to generate a lentivirus that will effectively and specifically infect PNs is very difficult. For example, small batch-to-batch variations in

serum, or slight changes in media pH, can dramatically alter the tropism of the virus (Groenawan and Hirai, 2012; Torashima, Yamada, Itoh, Yamamoto and Hirai, 2012).

To address the various issues with PN-specific protein knockdown, we have developed a custom plasmid-based vector system that drives the PN-specific expression of miRNAs, and we provide a proof-of-principle for their use. To augment the use of these plasmids, we have also optimized conditions for preparing primary mixed mouse cerebellar cultures. This modified method yields at least 10-fold more PNs per culture than our previous approach (Wolfgang et al., 2011a) while being significantly faster to perform.

Material and Methods

2.0 Media components for preparing primary mixed cerebellar cultures and performing immunofluorescence (IF) staining.

Basal DFM Medium is DMEM/Ham's F12 (DFM) (Sigma D2906) supplemented with 25 mM HEPES (Sigma H4034), 1.2 g/l NaHCO₃ (Sigma S5761), 100 μ M putrescine (Sigma P5080), 30 nM, Na₂SeO₃ (Sigma S5261), 1.4 mM L-glutamine (Gibco 25030-081), and 5 μ g/ml Gentamycin (Gibco 15710-064), pH 7.2.

Complete DFM Medium is Basal DFM Medium supplemented with 2 μ M cytosine β -D-arabinofuranoside (ARA-C) (Sigma C6645), 40 nM progesterone (Sigma P6149), 1 ng/ml tri-iodothyronine (T3) (Calbiochem 64245), 100 μ g/ml insulin growth factor I (IGFI) (Shenandoah Biotechnology 100-34), 200 μ g/ml transferrin (Sigma T1147), 100 μ g/ml bovine serum albumin (BSA) (Sigma A3156) and 20 μ g/ml insulin (Sigma I6634). Complete DFM is filter sterilized using a 0.22 μ m filter (Millipore SLVG 025 LS). Note that once this medium has been made, it can be used for a maximum of only 3 days. It is advisable, therefore, to make just 50 ml at a time.

Hank's Balanced Salt Solution (HBSS) (Life Technologies 14025092) is used for harvesting and washing steps. Appropriate numbers of 240 μ l aliquots (one per embryo) should be made prior to starting.

Papain Solution is 10 U/ml of papain from Papaya Latex (Sigma P4074) diluted in HBSS.

Papain Stop Solution is HBSS containing 18.25% (v/v) fetal bovine serum (FBS) (Gibco 15710-064).

DNase Solution is 10 U/ml of DNase (Roche 776 785) diluted in HBSS and supplemented with 24 mM MgCl₂.

PN Recovery Media is Complete DFM Medium containing 10% (v/v) FBS. Twenty 300 μ l aliquots per time pregnant female should be made prior to starting and incubated at 37°C in the presence of 5% CO₂ and 90% relative humidity (RH).

Complete DFM Feeding Medium is Complete DFM Medium supplemented with Antibiotic-Antimycotic (Gibco 15240-096).

IF Fixative is 4% paraformaldehyde (PFA) made by diluting a 16% stock solution (Electron Microscope Sciences 15710) to 4% in 1 x PBS using 10 x PBS (pH 7.4) (Gibco 70011044) and supplemented with 0.2% (w/v) picric acid (Sigma 197378)

IF Blocking Solution is 10% (v/v) normal goat serum (EMD Millipore S26-LITER), 1% (w/v) BSA, 0.5% (v/v) Triton X-100 (Sigma T9824), and 2% (w/v) sucrose (Sigma S7903) diluted to 1 x PBS using 10 x PBS. The solution is filter sterilized using a 0.22 μ m filter.

2.1 Primary mixed cerebellar cultures

2.1.1 Harvesting cerebella from E18 embryos—The successful nucleofection of cultured PNs requires that cultures be prepared from E17-E18 embryos. While older embryos and even P0 to P2 pups can yield mixed cultures containing healthy PNs, E17-E18 embryos should be used if efficient PN nucleofection and subsequent live cell imaging are the goals (Wagner et al., 2011a). Indeed, we tried repeatedly without success to express exogenous proteins via nucleofection in PNs present in mixed cultures made from P0-P2 pups, as this saves the breeding pair (unpublished observations). That said, PNs can be prepared from P0 to P2 pups for immunofluorescence studies or for viral transduction (Yuzaki, Forrest, Curran and Connor, 1996; Ahlemeyer and Baumgart-Vogt, 2005). It is also worth noting that we have prepared useful PN cultures from embryos as early as E13 (unpublished data). That said, we typically prepare cultures from E18 embryos as the larger embryo allows for easier processing of the cerebella.

1. Prepare aliquots of Complete DFM, HBSS, and PN Recovery Media, as well as the Papain, Papain Stop, and DNase solutions. Given that a timed-pregnant female yields on average 8–10 embryos, reagents and aliquots are prepared for 10 embryos per mouse preparation. Because each cerebellum is divided in two for subsequent processing, 20 PN Recovery Medium aliquots should be prepared for each timed pregnant female. Store the Complete DFM and PN Recovery Media aliquots at 37°C in the presence of 5% CO₂ and 90% RH until required later.
2. Coat glass-bottom, 14 mm aperture, 35 mm culture dishes (Invitro Scientific D35–14–1–N) with 0.01% (w/v) poly-l-lysine (Sigma P8920) supplemented with 1 mg/ml fibronectin (Life Technologies 33010–018) and 300 μ g/ml rat tail collagen I (Gibco A1048301). Incubate the dishes at 37°C in the presence of 5% CO₂, and 90% RH for at least 30 minutes. Note that the specific premade 0.01% PLL solution specified is required for primary mixed cerebellar cultures to attach to the coverglass. Diluting 0.1% PLL to 0.01% has not proven successful as an extracellular matrix.
3. Sterilize tools by soaking in 90% (v/v) ethanol for 10 minutes and allow them to dry in a laminar flow hood.
4. For each timed-pregnant female, place two 6-well dishes (Corning 353046) and one 10 cm dish (Corning 351029) on packed ice and fill with ice-cold HBSS.
5. Timed-pregnant females are anesthetized by inhalation of the anesthetic Isoflurane (Baxter 1001936060). In a fume hood, soak a cotton swab in

Isoflurane and place it under a metal cage in a bell jar to ensure that the animal does not come into direct contact with liquid anesthetic. Mice are exposed to Isoflurane in the presence of oxygen until immobile (approximately 1 minute).

6. Euthanize the mouse via cervical dislocation by holding the base of the tail and firmly squeezing at the base of the skull while pulling the head and tail apart (Figure 1A). Mice are anesthetized and euthanized in accordance with NIH Animal Research Advisory Committee guidelines.
7. Turn the mouse onto its back and spray the abdomen with 70% (v/v) ethanol to sterilize.
8. Using a pair of curved forceps (Fine Science Tools 11052–10), lift the skin at the base of the abdomen and gently cut across, and up, the abdomen wall with a pair of Wagner scissors (Fine Science Tools 14068–12) (Figure 1B).
9. Gently remove the uterine horns with forceps by cutting the connective tissue where necessary and place the uterus into the 10 cm dish containing ice-cold HBSS (Figure 1C).
10. Using straight forceps (Fine Science Tools 11050–10) and fresh Wagner scissors, gently remove the uterine tissue and placenta from each embryo.
11. While firmly holding the embryos body with forceps, swiftly decapitate the embryo and remove the body. Place the head into a well in the 6-well dish containing ice-cold HBSS.
12. Repeat steps 9 and 10 for each embryo.
13. You will need a pair of fine-point #5 forceps (Fine Science Tools 11251–20), extra-fine Bonn scissors (Fine Science Tools 14084–08), and a pair of curved serrated #7b forceps (Fine Science Tools 11270–20) to remove the brain from the skull.
14. Use the Bonn scissors to stabilize the head while piercing the forceps into the orbits of the skull, being extra careful not to squeeze the forceps together (Figure 1D).
15. Using the same scissors, carry out a sagittal cut starting at the base of the skull. The skull is soft and therefore little force is required. The cut should be guided by the tension of the blade against the tissue (Figure 1E).
16. Using serrated forceps, pull apart the skull and the meninges to reveal the brain while still stabilizing the head with the forceps.
17. Gently slide the serrated forceps along the curve of the brain until it is underneath the frontal lobe. Carefully use the forceps to lift the whole brain out of the skull and place it in a well of the 6-well dish.
18. Repeat steps 13 through 16 for each embryo.

19. Using the same serrated forceps and scissors, carefully cut the cerebellum from the rest of the brain (Figure 1F) and place it into a microfuge tube containing 240 μ l of ice-cold HBSS.
20. Dice the cerebellum into small pieces of approximately 1 mm square using the Bonn scissors. Wipe the blade between each cerebellum if harvesting cerebella from mice of different genotypes.
21. Add 250 μ l of room temperature (RT) papain solution to each diced cerebella and incubate for 10 minutes at RT. During this incubation period, prepare DNA aliquots (10 μ g of each DNA to be nucleofected, see below)
22. Save the remainder of the brain for genotyping or preparation of an extract for Western blotting, if required.

2.1.2 Preparation of cells for nucleofection—The remainder of the procedure should be carried out under aseptic conditions in a laminar flow hood to avoid contamination.

1. Stop the papain digestion by adding 1 ml of Papain Stop Solution and invert the tube.
2. Centrifuge the samples at RT for 4 minutes at 700 x *g* to pellet the tissue fragments.
3. For each cerebellum, remove the supernatant and gently triturate the tissue fragments ~40–50 times using a P1000 pipette in 250 μ l of DNase Solution until the sample appears as a homogenous cell suspension. Be careful not to introduce air bubbles at this point.
4. Filter the cells through a 0.4 μ m mesh strainer (Small Parts Inc. CMN-0210-A) to remove remaining tissue or cell clumps.
5. Pellet the cells by centrifugation for 4 minutes at 700 x *g*, discard the supernatant, and gently resuspend the pellet in 1 ml of RT HBSS.
6. Repeat step 6 for each embryo as a second wash step.
7. Divide the cell suspension into two 500 μ l aliquots and collect the cells by centrifugation at 700 x *g* for 4 minutes.
8. Remove as much of the supernatant as possible to prepare the cells for nucleofection.
9. Before proceeding to the next step, prepare the glass-bottom, 35 mm culture dishes for plating by aspirating off all of the coating solution and placing the dishes back into the incubator. Note that the dishes should also not be allowed to dry out, as this will reduce cell attachment.

2.1.3 Nucleofection with Purkinje neuron-specific promoter constructs—To ensure highest efficiency, the following should be carried out as quickly as possible.

1. Add 100 μ l of nucleofection solution (Amaxa, Mouse Neuron Nucleofector Kit, VPG-1001), to 10 μ g of each DNA to be nucleofected in a total volume of less than 130 μ l. For efficient nucleofection, DNA of less than 1 μ g/ μ l should not be used.
2. Use this solution to resuspend the cell pellet until a homogenous cell suspension is obtained (usually requiring 10–15 passes through a P1000 pipette tip). Any large clumps of cells that remain should be removed by gently pressing them against the tube wall with the pipette tip and then sliding them out of the tube.
3. Transfer a maximum of 100 μ l of cell suspension into an electroporation cuvette supplied with the Amaxa Mouse Neuron Nucleofector Kit and nucleofect using program O-003 on an Amaxa Nucleofector 2b (Amaxa AAB-1001).
4. Immediately after nucleofection, add 300 μ l of PN Recovery Medium pre-warmed to 37°C to the cuvette with the supplied transfer pipette and transfer the resulting cell suspension to a coated 35 mm glass-bottomed dish. Note that it is very important to remove the cells from the cuvette as soon as possible for optimal cell survival.
5. Incubate the culture for 2 hours at 37°C in the presence of 5% CO₂ and 90% RH to allow the cells to adhere to the glass dish.
6. Feed the culture by adding 2 ml of Complete DFM Feeding Medium and incubating overnight at 37°C, in the presence of 5% CO₂ and 90% RH.
7. 24–48 hours post-plating, replace 1 ml of the medium with 1 ml of fresh Complete DFM Feeding Medium, and repeat this step every 7 days thereafter.

2.1.4 Alternative method for cultures subjected solely to IF staining—While cultures that have been subjected to nucleofection can be processed for IF staining, problems with adherence of the mat of cells to the glass during the multiple incubation and wash steps associated with staining can arise. Therefore, we use the following protocol for cultures where IF staining is all that is required.

1. Harvest cerebella exactly as described above except that the cerebella should be harvested into 240 μ l of RT Papain Solution that has been diluted with HBSS to half the concentration used in the standard protocol. Additionally, cerebella do not need to be diced as described above- they can be incubated whole for 10 minutes at RT.
2. Add 1 ml of Papain Stop Solution to the cerebella and pellet the tissue and cells by centrifugation at RT for 4 minute at 700 x *g*.
3. Triturate the tissue in DNase Solution as described above until there is a mostly homogenous cell suspension.
4. Divide the material in half by filtering 150 μ l of the solution through 0.4 μ m mesh strainer into fresh microfuge tubes and collect the cells by centrifugation at 700 x *g* for 4 minutes at RT.

5. Remove the supernatant and resuspend the cell pellet in 300 μ l of prewarmed PN Recovery Medium.
6. Plate the cells onto pre-coated 35 mm glass-bottom dishes as described above.
7. Incubate the cultures for 2 hours at 37°C in the presence of 5% CO₂ and 90% RH to allow the cells to adhere.
8. Feed the culture by adding 2 ml of Complete DFM Feeding Medium and incubating overnight.
9. 24–48 hours post-plating, replace 1 ml of the medium with 1 ml of fresh Complete DFM Feeding Medium, and repeat this every 7 days thereafter.

2.1.5 Replating cultures which form aggregates—The method outlined below is used to recover primary mixed cerebellar cultures that have started to form aggregates rather than the desired uniform monolayer of cells. Why this happens is unknown and the frequency is unpredictable. There are times when 50 to 75% of cultures will form aggregates and other times when all cultures form a more uniform monolayer.

1. Identify cultures that appear to be forming aggregates using a standard inverted light microscope fitted with a 40 x lens. A good indication that a culture will aggregate is noticeable areas of cell clearing on the dish. If a culture has not been identified as forming aggregates by DIV 5, it cannot be recovered should it form aggregates later on.
2. Carefully remove the medium from the dish leaving just enough to cover the cells. Save this medium at 37°C to re-feed the cells later.
3. Quickly aspirate off the remaining medium and add 50 μ l of TrpLE (Life Technologies 12563–011) to dissociate the cells and aggregates from the dish.
4. Incubate the dish at 37°C in the presence of 5% CO₂ and 90% RH for 30 seconds.
5. Gently pipette 800 μ l of 1 x PBS containing 10% (v/v) FBS pre-warmed to 37°C onto the cells to dislodge them from the culture dish.
6. Collect the cells by centrifugation at 37°C and 700 x *g* for 4 minutes.
7. Very carefully aspirate the supernatant and then gently resuspend the pellet with 300 μ l of the pre-conditioned medium pre-warmed to 37°C until a homogenous cell suspension has been obtained. It is critical that no air bubbles are introduced at this stage.
8. Transfer the ~300 μ l of cell suspension to a freshly-coated 35 mm glass-bottomed dish.
9. Incubate for 2 hours at 37°C in the presence of 5% CO₂ and 90% RH to allow the cells to re-adhere to the culture dish.
10. Gently feed the culture by adding the remaining 2 ml of the conditioned medium.
11. Feed cells with Complete DFM Feeding Medium as normal.

2.2 Generation of Purkinje neuron-specific micro RNA (miRNA) knockdown constructs

The plasmid-based approach we describe here for the PN-specific knockdown of proteins using miRNA is based on the BLOCK-iT miR RNAi system from Life Technologies (K4935–00). In this system, short, complementary, gene-specific oligonucleotides are introduced into a plasmid which then contains the miRNA sequence followed by an mGFP reporter. In order to achieve PN-specific knockdown, we have introduced the required elements needed for miRNA expression immediately following the full length PN-specific promoter *L7/Pcp2*.

1. Design gene-specific complimentary oligonucleotides for miRNA-mediated protein knockdown using Life Technologies BLOCK-iT RNAi Designer tool (<https://rnaidesigner.lifetechnologies.com/rnaiexpress/>). It is important to select the miR RNAi Target Design option to ensure that the correct oligonucleotide sequences are generated to allow for the duplexed oligonucleotides to contain the correct overhangs required for ligation into the supplied linearized vector. Using the accession number as the identifier for ones protein of interest (e.g. X57377 for mouse myosin Va), the program will generate 10 possible miR RNAi sequences rated for predicted knockdown efficiency.
2. It is advisable to select 5 predicted sequences in different regions of the protein to test for knockdown efficiency. Selecting the specified sequences will generate complimentary oligonucleotides required to generate the specific knockdown plasmid. Table 1 shows the selected miRNA target sequence for myosin Va knockdown, the first associated base number and subsequent name of plasmids generated in this study. Each of the target sequences selected had a predicted knockdown efficiency of 5/5, as determined by the BLOCK-iT website.
3. miR RNAi knockdown plasmids should be generated according to the kit manual. Briefly, anneal the two complementary oligonucleotides by heating equal parts of each oligonucleotide at a concentration of 50 μ M diluted in Oligo Annealing Buffer to 95°C for 5 minutes and then allowing the mixture to cool to RT. All the oligonucleotides used in this study were purchased from Eurofins Genomics at 50 nM scale and purified by standard desalting.
4. Once the oligonucleotides have reached RT, place them on ice and carry out all subsequent steps on ice.
5. Dilute the annealed oligonucleotides by serial dilution to a final concentration of 50 nM, as described in the manufacturer's guidelines.
6. Ligate the annealed oligonucleotides into the linearized pcDNA6.2/emGFP plasmid provided in the BLOCK-iT Inducible Pol II miR RNAi Expression Vector Kit (Life Technologies K4939–00) and transform the DNA into the TOP10 competent *E. coli* provided, as described in the kit's manual.
7. Prepare DNA from 10 colonies for each knockdown plasmid using a Qiagen Miniprep Kit (Qiagen 27104).

8. Screen clones for insertion of the duplexed oligonucleotides by PCR using primers oCA29 (miR_pL7_Fwd_NotI, TAATCCCCGGGGCTAAGCACTTCGTGGC) and oCA31 (miR_pL7_Rev_NheI, TAAAGCTAGCGGGCCATTTGTTCCATGTGAG) and using the polymerase Expand High-Fidelity (Roche 11732641001), an annealing temperature of 50°C, and an extension time of 30 seconds was used for each PCR reaction. Note it is important to use a Taq-based polymerase for PCR reactions to allow for subsequent TA cloning.
9. Run the resulting PCR reactions on a 3% 5 mM LiAc agarose gel using a 50 bp DNA ladder (NEB N3236S).
10. Clones positive for oligonucleotide insertion will exhibit a 64 bp band shift, from 106 bp to 170 bp.
11. Clones positive for oligonucleotide insertion are gel purified using a GeneClean III kit (MP Biomedicals 111001600) following the manufacturer's guidelines.
12. Purified fragments are then cloned into the pGEM-T-Easy vector (Promega A1360) following the manufacturer's instructions. Resulting ligations are transformed into One-Shot Stbl3 (Life Technologies C7373-03) chemically competent *E. coli*, as outlined in the manufacturer's instructions. Note that it is important to use a strain of *E. coli* like Stbl3, as the miR RNAi insert contains an unstable, direct repeat.
13. Prepare DNA from three independent clones using a Qiagen Miniprep kit.
14. A minimum of three clones for both the original miR RNAi plasmid and the newly generated miR RNAi pGEM plasmid should be sent for sequencing to confirm that the insert is correct.
15. The pGEM clones generated contain the miR RNAi sequences flanked by NheI and NotI restriction sites, which are used for subsequent subcloning into the PN-specific pL7 vector.
16. Simultaneously digest 200 ng of a sequence-confirmed miR RNAi pGEM clone and 200 ng of pL7.mGFP (or the fluorophore of choice) with 1 U NheI-HF (NEB R3131S) and 1 U NotI-HF (NEB R3189S) in the supplied CutSmart buffer for 1 hour at 37°C (Figure 2A).
17. Run the restriction digests on a 3% 5mM LiAc agarose gel and gel purify the resulting 170 bp band from the pGEM digest, and the 6.7 kb fragment from the pL7.mGFP digest, using a GeneClean III Kit.
18. Ligate the miR RNAi insert at a molar ratio of 4:1 into the pL7.mGFP backbone using 1U T4 DNA Ligase (NEB M0202S) diluted to a final volume of 10 µl and 1 x T4 DNA Ligase buffer. Incubate overnight at 4°C (Figure 2B).
19. Transform 5 µl of the ligation reaction into Stbl3 chemically competent *E. coli* following the manufacturer's guidelines.
20. Prepare DNA from 5 clones using a Qiagen Miniprep kit.

21. Screen the clones for the insert by PCR or restriction digest, as described above.
22. The pL7.mGFP knockdown constructs are now ready to be nucleofected into primary mixed cerebellar cultures for knockdown studies as described in Section 2.2.3. If so desired, the pcDNA6.2 knockdown constructs can be used to quantitate knockdown efficiency in cells other than PNs (see below).

2.3 Improved immunofluorescence staining of PN cultures

PN cultures are typically fixed using 4% PFA to preserve the structure of the cell (Dekker-Ohno, Hayasaka, Takagishi, Oda, Wakasugi, Mikoshiba, et al., 1996; Sakamoto, Mezaki, Shikimi, Ukena and Tsutsui, 2003). Although this approach is adequate, cells are sometimes not well-preserved using PFA alone. To address this issue, we include a second fixative (picric acid- see above) and use the following method, which significantly increases the number of well-preserved cells. All steps are performed at RT.

1. Working in a fume hood, remove all the medium from a culture of the desired DIV and add 300 μ l of IF Fixative to the aperture of the dish, ensuring that all the cells are covered.
2. Incubate for 5 minutes.
3. Remove the IF Fixative and wash 3 times with 500 μ l of 1 x PBS.
4. Incubate the culture for 10 minutes in 500 μ l PN Blocking Solution.
5. Remove the blocking solution and add 200 μ l of PN Blocking Solution containing an appropriate dilution of the primary antibody. Incubate for 10 to 30 minutes depending on the antibody used.
6. Remove the primary antibody solution and rinse the culture 3 times with 500 μ l of PN Blocking Solution, incubating for 1 minute between each wash step.
7. Remove the blocking solution and add 200 μ l of PN Blocking Solution containing the appropriate type and concentration of labeled secondary antibody. Incubate for 10 to 30 minutes depending on the antibody.
8. Wash the dish 5 times with 500 μ l of PBS.
9. After the final wash, remove all of the PBS and quickly cover the culture with 200 μ l of Fluoromount-G (Electron Microscope Sciences 179840–25).
10. Gently cover the cells with a 25 mm circular coverslip (Electron Microscope Sciences 72223–01) and seal with slide sealant.

Results

3.1. A more efficient method of preparing primary mixed cerebellar cultures enriched in Purkinje Neurons

The current method for preparing primary mixed cerebellar cultures enriched for Purkinje neurons (PNs) has proven useful for the study of this cell type (Tabata et al., 2000). That said, the method is complex and often yields low numbers of fully-developed cells for

subsequent study. The first seven days are critical for the survival of these fragile cells, and typically about half of the PNs present initially die during this time period (Ghoumari, Dusart, El-Etr, Tronche, Sotelo, Schumacher et al., 2003). Moreover, our experience is that small changes in culture conditions can be quite detrimental to the growth of PNs. To address these issues, we have made a series of improvements to the culturing technique that has resulted in a substantial increase in the quality of the cultures and the survival of the PNs in them. Our efforts in this regard began with an extensive literature search to identify changes in tissue processing, culture conditions, and media additives that might improve the cultures. In the sections that follow, the degree to which the changes we adopted improved the cultures was estimated by measuring the total number of PNs in cultures at DIV 14 (based on staining with Calbindin D 28K, a PN marker), or the total number of transfected PNs (using pL7.mGFP), in at least three independent preparations.

3.1.1 Use of antibiotics—As described above, nucleofection of PNs is performed immediately prior to plating the cells. In the current nucleofection protocol (Wagner et al., 2011a), the HBBS during cerebella harvest and in the PN Recovery Medium post nucleofection are supplemented with antibiotics. The electric field generated during nucleofection transiently permabilizes the cells by the formation of small pores in the membrane. While these pores start to reseal after the removal of the electric field (van den Hoff, Moorman and Lamers, 1992; Van Bockstaele, Pede, Naessens, Coppennolle, Tendeloo, Verhasslet et al., 2008; Mo, Potter, Bertand, Holdebrand, Bruns and Weisz, 2010), it can take up to 20 minutes for the pores to fully close (Saulis, Venslauskas and Niktinis, 1991; Saulis and Saulé, 2012), allowing for direct entry of antibiotics into the cell. Because antibiotics can have a detrimental effect on cell growth and/or survival, we reasoned that a recovery period during which cells are not exposed to antibiotics might improve the cultures. Consistently, using antibiotic-free conditions prior to plating, and waiting until the first feeding step to add antibiotics to the media, resulted in a ~2.5-fold increase ($p=0.03$) in the total number of PNs and a ~2-fold increase ($p=0.04$) in the number of nucleofected cells (Table 2).

3.1.2 Papain treatment—The current method (Wagner et al., 2011a) uses a 30-minute papain digestion step at 33°C immediately after the cerebella have been harvested from the embryo. While the use of papain is acceptable when preparing cerebellar cultures from E18 embryos, it is not suitable when using cerebella from P0 or P1 pups (Tabata et al., 2000; Ahlemeyer and Baumgart-Vogt, 2005), suggesting that PNs are very sensitive to papain treatment. We sought, therefore, to further optimize this step. Given that elimination of the papain digestion step is not an option (because too few cells are obtained), we tested different temperatures and durations for papain digestion with regard to final cell yield. It was determined that a 10 minute incubation at RT yielded a ~4.3-fold increase ($p=0.01$) in cell survival over the original procedure. Additionally, this change led to a ~2.4-fold increase ($p<0.01$) in the number of cells that can be successfully nucleofected (Table 2).

3.1.3 Media additives—A search of the literature reveals many cytokines and growth factors that are neuroprotective and can aid in the survival of neurons in culture. For example, the standard PN culture medium used previously (Wagner et al., 2011a) contains

progesterone and T3, both of which are required for dendritic outgrowth and cell survival (Lindholm, Castrén, Tsoulfas, Kolbeck, Berzaghi, Leingärtner, et al., 1993). Interestingly, insulin-like growth factor I (IGFI), which is not present in the standard PN media, has been shown to aid PN survival (D'Mello, Galli, Ciotti and Calisano, 1993; Zhang, Ghetti and Lee, 1997; Tolbert and Clark, 2003; Vig, Subramony, D'Souza, Wei and Lopez, 2006) and to increase the number of PNs in primary rat cerebellar cultures by ~7-fold (Toress-Aleman, Pons and Santos-Benito, 1992). We decided, therefore, to see if this was also the case for primary mouse cerebellar cultures. Importantly, addition of IGFI resulted in a ~2.6-fold increase ($p=0.01$) in the total number of PNs and a ~2.3-fold increase ($p<0.01$) in the number of PNs that can be successfully nucleofected (Table 1). Of note, omission of progesterone, a standard PN media component, resulted in a 30% decrease in total PNs and a 50% decrease in the number of nucleofected PNs when compared to the original method. This observation highlights the importance of specific media components for PN survival (Table 2).

Individually, each of the changes in culturing conditions discussed above increased the total yield of PNs by ~2.5-fold, and increased the number of PNs that are successfully nucleofected by ~2-fold. Given this, we decided to combine all of the changes into one method to determine if there was a cumulative effect. To our satisfaction, the total yield of PNs was ~15-fold higher ($p<0.01$) than with original method. Moreover, while the efficiency of nucleofection decreased with the new culture method from 25% to 6%, the absolute number of healthy PNs successfully nucleofected per culture rose by ~3.4-fold increase ($p<0.01$).

3.2 Recovery of aggregated PN cultures

Purkinje neurons require other cell types to promote their growth and development, most notably granular neurons (Baptista, Hatten, Blazeski and Mason 1994). Perhaps related to this requirement, primary mixed cerebellar cultures often aggregate, although why some cultures do this and others do not is unclear. Interestingly, the resulting aggregates look similar to neurospheres of developing neurons (Flemming, He, Hao, Ketova, Pan, Wright et al., 2013). These aggregates make imaging extremely difficult because the aggregates are thick and loosely attached to the coverglass. To successfully recover aggregated cultures, it is critical that such cultures be identified early, as aggregated cultures older than DIV 5 cannot be recovered. It is worth noting that this is a qualitative method and not all cultures can be identified early enough for rescue. Significant clearing of cells on the dish within the first 4 to 5 days after plating is a good indication that aggregates will probably form. Experience and familiarization with culturing these cells will facilitate identifying these changes. While we have not determined precisely the efficacy of this method, ~ 30% of aggregated cultures can be restored to monolayer morphology.

3.3 Custom plasmids to specifically knockdown proteins in PNs in primary cultures

Prior to working with PNs, CMV promotor-driven versions of the myosin Va miRNA plasmids were expressed in melanocytes to determine knockdown efficacy by Western blotting of whole cell extracts using a myosin Va-specific antibody (data not shown). The three miRNAs that gave at least 75% knockdown in melanocytes were then used to create

the final *L7/Pcp2* promoter-driven, PN-specific miRNA plasmids (pKDMva-2, -3 and -4; see Table 1 for details). The expression of miRNA in PNs was marked indirectly by the expression of a reporter mGFP, which was also used as a cell volume marker. PNs were also nucleofected with an RFP-tagged ER marker to measure the percent of spines containing ER (see Wolfgang et al., 2011b for details). As a control, a scrambled miRNA not directed to any protein was used. Figure 3A shows a PN expressing the scrambled miRNA (marked by mGFP) and the RFP-ER marker. As anticipated, these cells appear indistinguishable from WT PNs with regard to ER targeting to spines (i.e. essentially all the spines seen with the volume marker contain a tubule of ER). Consistently, quantitation showed that 98% of spines in this control contained ER, a value very similar to that seen in WT PNs (99%; Wagner et al., 2011b). In contrast, Figure 3B shows a PN expressing the best of the three miRNAs against myosin Va, along with the RFP-ER marker. As hoped, the majority of the dendritic spines seen with the volume marker appear devoid of ER. This was borne out by quantitation, as 93% of spines were found to be devoid of ER, a value very close to that seen with dilute *lethal* PNs (99%; Wagner et al., 2011b). Of note, the data presented in Figure 3 corresponds to the miRNA plasmid against myosin Va that yielded the highest percentage of ER-free dendritic spines (pKDMVa-2; details in Table 1). While the other miRNA plasmids tested resulted in significant reductions in ER targeting, the reductions were smaller (~50%), presumably because the degree of protein knockdown was less. Together, these data demonstrate that our novel, miRNA-based method can be used successfully to knockdown proteins specifically in PNs.

Concluding remarks

Here we present an optimized method for culturing PNs in primary mixed cerebellar cultures. This optimized method yields at least 10-fold more PNs per cerebella than our previous method and is quicker to perform. In addition, we developed a novel miRNA-based approach to knockdown endogenous proteins in a PN-specific manner. These techniques allow preliminary data to be obtained in knockdown experiments prior to generating a knockout animal or, as demonstrated by our proof-of-principle experiment, can serve as a stand-alone approach when generating a knockout is not feasible or results in early embryonic lethality. The improvements we present here should substantially facilitate future studies of PNs *in vitro*.

References

- Ahlemeyer B, & Baumgart-Vogt E (2005). Optimized protocols for the simultaneous preparation of primary neuronal cultures of the neocortex, hippocampus and cerebellum from individual newborn (P0.5) C57Bl/6J mice. *Journal of Neuroscience Methods*, 149(2), 110–20. [PubMed: 16084598]
- Baptista ME, Hatten CA, Blazeski R, & Mason CA (1994). Cell-cell interactions influence survival and differentiation of purified purkinje cells in vitro. *Neuron*, 12(2), 243–260. [PubMed: 8110456]
- Cho RW, Park JM, Wolff SBE, Xu D, Hopf C, Kim J-A, ... Worley PF (2008). mGluR1/5-dependent long-term depression requires the regulated ectodomain cleavage of neuronal pentraxin NPR by TACE. *Neuron*, 57(6), 858–71. [PubMed: 18367087]
- Chung HJ, Steinberg JP, Haganir RL, & Linden DJ (2003). Requirement of AMPA receptor GluR2 phosphorylation for cerebellar long-term depression. *Science (New York, N.Y.)*, 300(5626), 1751–5.

- D'Mello SR, Galli C, Ciotti T, & Calissano P (1993). Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proceedings of the National Academy of Sciences*, 90(23), 10989–10993.
- De Zeeuw CI, Hansel C, Bian F, Koekkoek SKE, van Alphen AM, Linden DJ, & Oberdick J (1998). Expression of a Protein Kinase C Inhibitor in Purkinje Cells Blocks Cerebellar LTD and Adaptation of the Vestibulo-Ocular Reflex. *Neuron*, 20(3), 495–508. [PubMed: 9539124]
- Dekker-Ohno K, Hayasaka S, Takagishi Y, Oda S, Wakasugi N, Mikoshiba K, ... Yamamura H (1996). Endoplasmic reticulum is missing in dendritic spines of Purkinje cells of the ataxic mutant rat. *Brain Research*, 714(1–2), 226–230. [PubMed: 8861629]
- Dueñas AM, Goold R, & Giunti P (2006). Molecular pathogenesis of spinocerebellar ataxias. *Brain: A Journal of Neurology*, 129(Pt 6), 1357–70. [PubMed: 16613893]
- Eto M, Bock R, Brautigam DL, & Linden DJ (2002). Cerebellar Long-Term Synaptic Depression Requires PKC-Mediated Activation of CPI-17, a Myosin/Moesin Phosphatase Inhibitor. *Neuron*, 36(6), 1145–1158. [PubMed: 12495628]
- Fatemi SH, Halt AR, Realmuto G, Earle J, Kist DA, Thuras P, & Merz A (2002). Purkinje Cell Size Is Reduced in Cerebellum of Patients with Autism. *Cellular and Molecular Neurobiology*, 22(2), 171–175. [PubMed: 12363198]
- Fleming JT, He W, Hao C, Ketova T, Pan FC, Wright CCV, ... Chiang C (2013). The Purkinje neuron acts as a central regulator of spatially and functionally distinct cerebellar precursors. *Developmental Cell*, 27(3), 278–92. [PubMed: 24229643]
- Fournier KA, Hass CJ, Naik SK, Lodha N, & Cauraugh JH (2010). Motor coordination in autism spectrum disorders: a synthesis and meta-analysis. *Journal of Autism and Developmental Disorders*, 40(10), 1227–40. [PubMed: 20195737]
- Ghoumari AM, Dusart I, El-Etr M, Tronche F, Sotelo C, Schumacher M, & Baulieu E-E (2003). Mifepristone (RU486) protects Purkinje cells from cell death in organotypic slice cultures of postnatal rat and mouse cerebellum. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), 7953–8. [PubMed: 12810951]
- Goenawan H, & Hirai H (2012). Modulation of lentiviral vector tropism in cerebellar Purkinje cells in vivo by a lysosomal cysteine protease cathepsin K. *Journal of Neurovirology*, 18(6), 521–31. [PubMed: 23070819]
- Hansel C, Linden DJ, & D'Angelo E (2001). Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nature Neuroscience*, 4(5), 467–75. [PubMed: 11319554]
- Hirai H, & Launey T (2000). The Regulatory Connection between the Activity of Granule Cell NMDA Receptors and Dendritic Differentiation of Cerebellar Purkinje Cells. *J. Neurosci*, 20(14), 5217–5224. [PubMed: 10884305]
- Ito M, Sakurai M, & Tongroach P (1982). Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *The Journal of Physiology*, 324(1), 113–134. [PubMed: 7097592]
- Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, ... Mishina M (1995). Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluRδ2 mutant mice. *Cell*, 81(2), 245–252. [PubMed: 7736576]
- Leiner HC, Leiner AL, & Dow RS (1993). Cognitive and language functions of the human cerebellum. *Trends in Neurosciences*, 16(11), 444–447. [PubMed: 7507614]
- Leites M, Kovac J, Plomann M, & Linden DJ (2004). A unique PDZ ligand in PKCα confers induction of cerebellar long-term synaptic depression. *Neuron*, 44(4), 585–94. [PubMed: 15541307]
- Linden DJ (1996). A Protein Synthesis-Dependent Late Phase of Cerebellar Long-Term Depression. *Neuron*, 17(3), 483–490. [PubMed: 8816711]
- Linden DJ, Dickinson MH, Smeyne M, & Connor JA (1991). A long-term depression of AMPA currents in cultured cerebellar purkinje neurons. *Neuron*, 7(1), 81–89. [PubMed: 1676895]
- Lindholm D, Castrén E, Tsoulfas P, Kolbeck R, Berzaghi M, da P., Leingärtner A, ... Thoenen H (1993). Neurotrophin-3 induced by tri-iodothyronine in cerebellar granule cells promotes Purkinje cell differentiation. *The Journal of Cell Biology*, 122(2), 443–450. [PubMed: 8320266]

- Llano I, Dreessen J, Kano M, & Konnerth A (1991). Intradendritic release of calcium induced by glutamate in cerebellar purkinje cells. *Neuron*, 7(4), 577–583. [PubMed: 1681831]
- Llinas R (1985). Functional significance of the basic cerebellar circuit in motor coordination. In *Cerebellar functions* (pp. 170–185). Springer.
- Manto M-U (2005). The wide spectrum of spinocerebellar ataxias (SCAs). *Cerebellum* (London, England), 4(1), 2–6.
- Mauk MD, Medina JF, Nores WL, & Ohyama T (2000). Cerebellar function: Coordination, learning or timing? *Current Biology*, 10(14), R522–R525. [PubMed: 10898992]
- Ménasché G, Ho CH, Sanal O, Feldmann J, Tezcan I, Ersoy F, ... de Saint Basile G (2003). Griscelli syndrome restricted to hypopigmentation results from a melanophilin defect (GS3) or a MYO5A F-exon deletion (GS1). *The Journal of Clinical Investigation*, 112(3), 450–6. [PubMed: 12897212]
- Mercer JA, Seperack PK, Strobel MC, Copeland NG, & Jenkins NA (1991). Novel myosin heavy chain encoded by murine dilute coat colour locus. *Nature*, 349(6311), 709–713. [PubMed: 1996138]
- Miall RC, & Wolpert DM (1996). Forward Models for Physiological Motor Control. *Neural Networks*, 9(8), 1265–1279. [PubMed: 12662535]
- Miki T, Hirai H, & Takahashi T (2013). Activity-dependent neurotrophin signaling underlies developmental switch of Ca²⁺ channel subtypes mediating neurotransmitter release. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 33(48), 18755–63. [PubMed: 24285882]
- Miyata M, Finch EA, Khiroug L, Hashimoto K, Hayasaka S, Oda S-I, ... Kano M (2000). Local Calcium Release in Dendritic Spines Required for Long-Term Synaptic Depression. *Neuron*, 28(1), 233–244. [PubMed: 11086997]
- Mo D, Potter BA, Bertrand CA, Hildebrand JD, Bruns JR, & Weisz OA (2010). Nucleofection disrupts tight junction fence function to alter membrane polarity of renal epithelial cells. *American Journal of Physiology. Renal Physiology*, 299(5), F1178–84. [PubMed: 20702601]
- Piven J, Saliba K, Bailey J, & Arndt S (1997). An MRI study of autism: The cerebellum revisited. *Neurology*, 49(2), 546–551. [PubMed: 9270594]
- Sakamoto H, Mezaki Y, Shikimi H, Ukena K, & Tsutsui K (2003). Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. *Endocrinology*, 144(10), 4466–77. [PubMed: 12960093]
- Saulis G, & Saul R (2012). Size of the pores created by an electric pulse: microsecond vs millisecond pulses. *Biochimica et Biophysica Acta*, 1818(12), 3032–9. [PubMed: 22766475]
- Saulis G, Venslauskas MS, & Naktinis J (1991). Kinetics of pore resealing in cell membranes after electroporation. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, 321(1), 1–13.
- Shima Y, Kengaku M, Hirano T, Takeichi M, & Uemura T (2004). Regulation of dendritic maintenance and growth by a mammalian 7-pass transmembrane cadherin. *Developmental Cell*, 7(2), 205–16. [PubMed: 15296717]
- Soong B, & Paulson HL (2007). Spinocerebellar ataxias: an update. *Current Opinion in Neurology*, 20(4), 438–7540. [PubMed: 17620880]
- Tabata T, Sawada S, Araki K, Bono Y, Furuya S, & Kano M (2000). A reliable method for culture of dissociated mouse cerebellar cells enriched for Purkinje neurons. *Journal of Neuroscience Methods*, 104(1), 45–53. [PubMed: 11163410]
- Takagishi Y, Oda S, Hayasaka S, Dekker-Ohno K, Shikata T, Inouye M, & Yamamura H (1996). The dilute-lethal (dl) gene attacks a Ca²⁺ store in the dendritic spine of Purkinje cells in mice. *Neuroscience Letters*, 215(3), 169–172. [PubMed: 8899740]
- Takayama K, Torashima T, Horiuchi H, & Hirai H (2008). Purkinje-cell-preferential transduction by lentiviral vectors with the murine stem cell virus promoter. *Neuroscience Letters*, 443(1), 7–11. [PubMed: 18675313]
- Tanaka M, Yanagawa Y, & Hirashima N (2009). Transfer of small interfering RNA by single-cell electroporation in cerebellar cell cultures. *Journal of Neuroscience Methods*, 178(1), 80–6. [PubMed: 19114056]

- Tolbert DL, & Clark BR (2003). GDNF and IGF-I trophic factors delay hereditary Purkinje cell degeneration and the progression of gait ataxia. *Experimental Neurology*, 183(1), 205–219. [PubMed: 12957504]
- Torashima T, Okoyama S, Nishizaki T, & Hirai H (2006). In vivo transduction of murine cerebellar Purkinje cells by HIV-derived lentiviral vectors. *Brain Research*, 1082(1), 11–22. [PubMed: 16516872]
- Torashima T, Yamada N, Itoh M, Yamamoto A, & Hirai H (2006). Exposure of lentiviral vectors to subneutral pH shifts the tropism from Purkinje cell to Bergmann glia. *The European Journal of Neuroscience*, 24(2), 371–80. [PubMed: 16836635]
- Torres-Aleman I, Pons S, & Santos-Benito FF (1992). Survival of Purkinje Cells in Cerebellar Cultures is Increased by Insulin-like Growth Factor I. *European Journal of Neuroscience*, 4(9), 864–869. [PubMed: 12106309]
- Uesaka N, Mikuni T, Hashimoto K, Hirai H, Sakimura K, & Kano M (2012). Organotypic coculture preparation for the study of developmental synapse elimination in mammalian brain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 32(34), 11657–70. [PubMed: 22915109]
- Van Bockstaele F, Pede V, Naessens E, Van Coppenolle S, Van Tendeloo V, Verhasselt B, & Philippé J (2008). Efficient gene transfer in CLL by mRNA electroporation. *Leukemia*, 22(2), 323–9. [PubMed: 17972950]
- Van den Hoff MJ, Moorman AF, & Lamers WH (1992). Electroporation in “intracellular” buffer increases cell survival. *Nucleic Acids Research*, 20(11), 2902. [PubMed: 1614888]
- Vig PJS, Subramony SH, D’Souza DR, Wei J, & Lopez ME (2006). Intranasal administration of IGF-I improves behavior and Purkinje cell pathology in SCA1 mice. *Brain Research Bulletin*, 69(5), 573–9. [PubMed: 16647585]
- Wagner W, Brenowitz SD, & Hammer JA (2011a). Myosin-Va transports the endoplasmic reticulum into the dendritic spines of Purkinje neurons. *Nature Cell Biology*, 13(1), 40–8. [PubMed: 21151132]
- Wagner W, McCroskery S, & Hammer JA (2011b). An efficient method for the long-term and specific expression of exogenous cDNAs in cultured Purkinje neurons. *Journal of Neuroscience Methods*, 200(2), 95–105. [PubMed: 21708190]
- Wang VY, & Zoghbi HY (2001). Genetic regulation of cerebellar development. *Nature Reviews. Neuroscience*, 2(7),
- Yuzaki M, Forrest D, Curran T, & Connor JA (1996). Selective Activation of Calcium Permeability by Aspartate in Purkinje Cells. *Science*, 273(5278), 1112–1114. [PubMed: 8688099]
- Zhang W, Ghetti B, & Lee W-H (1997). Decreased IGF-I gene expression during the apoptosis of Purkinje cells in pcd mice. *Developmental Brain Research*, 98(2), 164–176. [PubMed: 9051257]

Figure 1: Guidance on harvesting embryonic mouse cerebella.

After anesthetizing with Isoflurane, euthanize the mouse by placing her face-down and performing a cervical dislocation, which involves squeezing at the base of the tail and behind the skull and swiftly pulling apart (A; see direction of arrow). Embryos are harvested by caesarian section, which involves opening the abdomen by making cuts along the indicated arrows (B). The two uterine horns on either side of the abdomen (black and white arrows) should be removed carefully (C). To be able to remove the brain, the head should be stabilized with Bonn scissors and forceps inserted into the orbits (D). A sagittal cut should be made while holding the head with forceps (arrow, E), being careful not to squeeze the forceps together. Once the brain is removed, the cerebellum should be excised from the frontal lobes. The cerebellum is denoted with an asterisk and should be cut along the indicated line (F).

Figure 2: Generating a PN-specific miRNA knockdown plasmid using pL7.mGFP.

A schematic of the pL7.mGFP plasmid (Wagner et al., 2011a) used to generate PN-specific miRNA knockdown plasmids (A). The full length *L7/Pcp2* promoter (red) is followed by mGFP (green). The fragment containing the miRNA sequence is subcloned into pL7.mGFP using the NotI and NheI restriction sites immediately after the *L7/Pcp2* promoter to generate a PN-specific, miRNA-mediated knockdown plasmid with a mGFP reporter (B).

Figure 3: PN-specific, miRNA-mediated knockdown of myosin Va.

(A) A wild type PN expressing a scrambled control miRNA and an RFP-ER marker exhibits robust targeting of ER to dendritic spines (scale bars 10 μ m and 5 μ m for the inset). (B) A wild type PN expressing a miRNA against myosin Va and the RFP-ER marker shows that dendritic spines are largely devoid of ER (scale bar 10 μ m and 5 μ m for the inset). (C) Quantitation of the percent of spines containing ER shows that PNs expressing the control, scrambled miRNA are essentially wild type as regards spine ER-targeting while PNs expressing a miRNA against myosin Va phenocopy *dilute-lethal* PNs (see text for further details). Micrographs were obtained using a Zeiss LSM 780 laser scanning confocal microscope equipped with a Zeiss 63 $\times$ /1.4 NA oil objective.

Table 1 –

Target sequences used to generate miRNA knockdown plasmids directed against myosin Va.

Target Sequence (5' to 3')	5' base number	Region within Protein	Plasmid name
TTCAGTTTCTGCAAACCTCAGA	3422	ORF; CC	pKDMva-1
GTTGACTGGCCGGATAGGCTC	4408	ORF; GTD	pKDMva-2
TAACCCTGGAATCAGATTGAC	4538	ORF; GTD	pKDMva-3
TTGACTACCTGCTTGATTAGC	5083	ORF; GTD	pKDMva-4
AACAGATGGCTTCTGCGTCAT	5319	ORF; GTD	pKDMva-5

ORF – Open Reading Frame; CC – Coiled-coil region; GTD – Globular Tail Domain

Table 2 –

Fold change relative to the original protocol as regards the total yield of PNs (“Total PNs”) and the total number of successfully transfected PNs (“Total Transfected PNs”) obtained with each individual improvement, and with all the improvements combined (calculated on a per cerebellum basis). Also shown is the overall percent transfection efficiency for each condition.

	Total PNs	Total Transfected PNs	Transfection Efficiency (%)
Original method	1	1	25
No Antibiotics until first feeding	2.5	2.2	21
10 min Papain digestion (RT)	4.3	2.4	42
IGF-I Supplementation	2.6	2.2	20
No Progesterone for 24 hrs	0.7	0.5	17
Combined	14.8	3.4	6