Video Article

Modeling Neuronal Death and Degeneration in Mouse Primary Cerebellar Granule Neurons

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URL: https://www.jove.com/video/55871
DOI: doi:10.3791/55871

Keywords: Neuroscience, Issue 129, Cerebellum, Cerebellar Granule Neurons (CGN), Adenovirus, Lentiviral Transduction, Excitotoxicity, N-methyl-D-aspartate (NMDA), Apoptosis, Potassium chloride (KCl), Camptothecin, Hydrogen peroxide (H_2O_2)

Date Published: 11/6/2017

Citation: Laaper, M., Haque, T., Slack, R.S., Jahani-Asl, A. Modeling Neuronal Death and Degeneration in Mouse Primary Cerebellar Granule Neurons. *J. Vis. Exp.* (129), e55871, doi:10.3791/55871 (2017).

Abstract

Cerebellar granule neurons (CGNs) are a commonly used neuronal model, forming an abundant homogeneous population in the cerebellum. In light of their post-natal development, abundance, and accessibility, CGNs are an ideal model to study neuronal processes, including neuronal development, neuronal migration, and physiological neuronal activity stimulation. In addition, CGN cultures provide an excellent model for studying different modes of cell death including excitotoxicity and apoptosis. Within a week in culture, CGNs express N-methyl-D-aspartate (NMDA) receptors, a specific ionotropic glutamate receptor with many critical functions in neuronal health and disease. The addition of low concentrations of NMDA in conjunction with membrane depolarization to rodent primary CGN cultures has been used to model physiological neuronal activity stimulation while the addition of high concentrations of NMDA can be employed to model excitotoxic neuronal injury. Here, a method of isolation and culturing of CGNs from 6 day old pups as well as genetic manipulation of CGNs by adenoviruses and lentiviruses are described. We also present optimized protocols on how to stimulate NMDA-induced excitotoxicity, low-potassium-induced apoptosis, oxidative stress and DNA damage following transduction of these neurons.

Video Link

The video component of this article can be found at https://www.jove.com/video/55871/

Introduction

Cerebellar granule neurons (CGNs) are well characterized in culture and have served as an effective model to study neuronal death and development [1,2,3,4,5,6]. The early expression of N-methyl-D-aspartate (NMDA) receptors in CGN cultures *in vitro* makes them an attractive model to study NMDA-induced signalling. Activation of these receptors with NMDA in conjunction with membrane depolarization is used to model physiological neuronal activity stimulation, and has allowed for research into the mechanisms of synaptic plasticity [7,8]. On the contrary, over-stimulation of these receptors by NMDA ligand can be used to model excitotoxicity, a major mechanism of neuronal loss in acute brain damage and neurodegenerative diseases [9]. One mechanism for the induction of excitotoxicity is through ATP starvation with reduced oxygen, as seen with acute neuronal injury. This results in membrane depolarization and elevated levels of glutamate release at the synapse. The subsequent overstimulation of the NMDA receptor by elevated glutamate results in excessive Ca^{2+} influx via these receptors, which in turn activates several pathways including Ca^{2+}-activated proteases, phospholipases, and endonucleases, resulting in the uncontrolled degradation of critical cellular components and cell death. Additionally, high intracellular Ca^{2+} leads to the generation of oxygen free radicals and mitochondrial damage [10,11].

While the majority of neuronal loss following NMDA-induced neuronal excitotoxicity is due to calcium influx and is Bax/Bak independent, other mechanisms of cell death cannot be excluded from this model. The appearance of both necrotic and apoptotic like cell death due to excitotoxicity is partially due to the generation of reactive oxygen species (ROS) and DNA damage caused by high intracellular Ca^{2+} levels [12]. DNA damage results in neuronal death through apoptotic mechanisms, being correlated with hallmarks of apoptotic cell death, such as the appearance of chromatin masses and apoptotic bodies. Induction of apoptosis is mediated through the release of cytochrome c from the mitochondria, and has been shown to be dependent on Bax/Bak oligomerization [13]. Bax/Bak oligomerization promotes pore formation in the outer mitochondrial membrane, resulting in cytochrome c release and the activation of pro-apoptotic regulators as seen with mild ischemic injury [14].

Generation of ROS is a significant issue in the brain due to the low endogenous levels of antioxidants, coupled with the large oxygen requirement for neuronal functioning [15]. When exposed to an ischemic event, nitric oxide synthase is upregulated , producing nitric oxide and increasing reactive oxygen species [14]. The increased concentration of oxygen radicals can result in DNA damage and indirectly cause energy starvation. High levels of DNA double-stranded breaks are remedied by the activation of poly ADP-ribose polymerase-1 (PARP-1), an eukaryotic chromatin-bound protein responsible for catalyzing the transfer of ADP-ribose units from NAD^+, a process integral to DNA repair [16]. However,

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with excessive damage due to oxidative stress, PARP-1 activation can cause energy starvation due to the increased drain on NAD^+, a necessary substrate for ATP production through oxidative phosphorylation. Ultimately, oxidative stress will trigger apoptosis in a Bax/Bak dependant manner leading to mitochondrial cytochrome c release, and has been shown to induce mitochondrial remodelling in CGNs [17].

Finally, changes in concentration of potassium chloride (KCl) in CGN cultures can be used to model low potassium/depolarization mediated apoptosis [18,19,20]. When exposed to low levels of K^+, CGNs undergo distinct physiological changes, resulting in reductions of both mitochondrial respiration and glycolysis, attributed to decreased cellular demand [21], as well as reduction in levels of nuclear factor-κB (NFκB) which regulates activities including inflammation and synaptic transmission [22]. This model is of particular interest for the study of cell death during neuronal development. The low K^+ environment more closely resembles physiological conditions, and causes hallmarks of cell death seen during neuronal development [23].

In summary, CGNs provide a longstanding model to investigate the underlying molecular mechanisms of neuronal death and degeneration. The following protocol will allow isolation and culturing of CGNs, expression or repression of a particular genetic pathway using viruses and the induction of neuronal death via different mechanisms representing neuronal injury and degeneration.

Protocol

This protocol is based on modifications of procedures that have been described previously [18,24,25,26,27]. This protocol is approved by the Animal Care Committee at McGill University.

1. Experimental Preparation

NOTE: The following stock solutions can be prepared and maintained until use.

1. **Dissection Solution**
    1. Dissolve 3.62 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 0.069 g of sodium phosphate ($NaH_2PO_4 \cdot H_2O$), and 1.306 g of D-(+)-Glucose in 500 mL of distilled H_2O. Then add 12.5 mL of HEPES Buffer, 450 µL of Phenol red solution, and 20 mL of BSA fraction V to the solution. Adjust to pH 7.4 using 1 N sodium hydroxide (NaOH).
    2. Sterilize with a 0.22 µm filter and store at 4 °C. This solution will remain stable for up to one week to 10 days.
2. **Trypsin**
    1. Prepare a stock solution at a concentration of 25 g/L of trypsin in 0.9% sodium chloride. Store the solution at -20 °C.
3. **Trypsin Inhibitor**
    1. Prepare a stock with a concentration of 10 mg/mL by dissolving 1 g of chicken egg white trypsin inhibitor in 100 mL of 1 mM HCl. Store this solution at -20 °C.
4. **DNase**
    1. Dissolve 100 mg of DNase 1 in 10 mL of sterile water to generate a 10 mg/mL stock. Store the solution at -20 °C.
5. **$MgSO_4$**
    1. Add 6.02 g of magnesium sulfate ($MgSO_4$) to 50 mL of distilled water (H_2O) to generate a 1 M stock. Store the solution at 4 °C.
6. **$CaCl_2$**
    1. Dissolve 0.735 g of calcium chloride ($CaCl_2 \cdot 2H_2O$) in 50 mL of distilled H_2O to a stock concentration of 100 mM. Store solution at 4 °C.
7. **KCl**
    1. Dissolve 3.7275 g of KCl in 50 mL of distilled H_2O to a stock concentration of 1 M. Store solution at 4 °C.
8. **D-(+)-Glucose**
    1. Dissolve 1.802 g of D-(+)-glucose in 50 mL of distilled H_2O to a stock concentration of 100 mM. Store solution at 4 °C.
9. **Cell Culture Media**
    1. Prepare cell culture media as follows: 5 mL of heat inactivated dialyzed Fetal Bovine Serum, 500 µL of 200 mM L-Glutamine, 100 µL of 50 mg/mL Gentamycin and 1 mL of 1.0 M KCL should be added to 45 mL of filter sterilized Eagle's minimal essential media (E-MEM) that is supplemented with 1.125 g/L D-Glucose to generate 50 mL of cerebellar granule neuron culture media. Store media at 4 °C.
10. **Cytosine Beta-D- Arabino Furanoside**
    1. Dissolve 48 mg of cytosine beta-D-arabino furanoside in 10 mL of growth media to a stock concentration of 20 mM. Store solution at -20 °C.
11. **Poly-D-Lysine**
    1. To generate a 2 mg/mL poly D-lysine stock, add 10 mL of sterile H_2O to 20 mg of poly D-lysine. Stock poly D-lysine should be stored at -80 °C in 100 µL aliquots.
12. NOTE: The following solutions should be prepared prior to dissection and maintained at 4 °C until use.
13. **$MgSO_4$ Supplemented Dissection Solution**
    1. Add 300 µL of stock $MgSO_4$ to 250 mL of dissection solution.
14. **Trypsin Dissection Solution**

1. Add 100 µL of stock trypsin and 12 µL of stock $MgSO_4$ to 10 mL of dissection solution.

15. **Trypsin Inhibitor Solution 1**
    1. Add 164 µL of stock trypsin inhibitor, 250 µL of stock DNase 1 and 12 µL of stock $MgSO_4$ to 10 mL of dissection solution.

16. **Trypsin Inhibitor Solution 2**
    1. Add 1040 µL of stock trypsin inhibitor, 750 µL of stock DNase 1 and 12 µL of stock MgSO4 to 10 mL of dissection solution.

17. **$CaCl_2$ supplemented dissection solution**
    1. Add 10 µL of stock $CaCl_2$ and 25 µL of stock $MgSO_4$ to 10 mL of dissection solution.

18. **Poly D-Lysine Coated Culture Dishes**
    1. To coat culture dishes, dilute 100 µL of stock poly D-lysine in 40 mL of sterile H_2O. For 35 mm Nunc culture dishes, add 2 mL of diluted poly D-Lysine solution. For 4 well plates, add 300 µL of diluted poly D-Lysine to each well.
    2. Let the poly D-lysine sit for 1 h before aspirating and washing each well with 4 mL or 600 µL (for 35 mm culture dishes or 4 well plates, respectively) of sterile H_2O. Then aspirate the H_2O and let the dishes air dry for 1 h.

2. Brain Extraction and Isolation of Cerebellum

1. Decapitate a 6-7 day old mouse pup using decapitation scissors. Perform the dissection of the brain in a sterile tissue culture hood.
2. **To remove the brain, grasp the head using a pair of forceps and cut through the skin toward the anterior of the head using microdissection scissors as demonstrated in Figure 1. Then cut through the skull bone using a new pair of scissors to minimize risk of contamination. Remove the skull covering the brain using forceps and tease out the brain using forceps or a spatula.**
    1. As needed, cut through the optic nerve to facilitate getting the brain out as a whole.
3. Place the brain in the dissection solution which is supplemented with $MgSO_4$. Keep the solution and the brain on ice.
4. Following brain removal, under a dissection microscope, dissect out the cerebellum from the brain while still in $MgSO_4$ supplemented dissection solution, and remove the meninges using fine forceps. Turn the cerebellum to its ventral side and insure removal of the choroid plexus.
5. Pool the cerebella into a 35-mm dish containing ~1 mL of $MgSO_4$ supplemented dissection solution.
6. Chop the tissue into small pieces and transfer into a 50 mL tube containing 30 mL of $MgSO_4$ buffered dissection solution.

3. Mouse Cerebellar Granule Neuron Isolation and Culturing

1. Centrifuge the 50 mL tube containing the chopped cerebral tissue for 5 min at 644 x g and 4 °C.
2. Remove the supernatant and add 10 mL of trypsin dissection solution. Then shake the tube at high speed for 15 min at 37 °C.
3. Add 10 mL of trypsin inhibitor solution 1 to the tube and rock gently for 2 min.
4. Centrifuge the tube for 5 min at 644 x g and 4 °C.
5. Remove the supernatant and add 2 mL of trypsin inhibitor solution 2, and then transfer to a 15 mL tube.
6. Triturate the tissue in the 15 mL tube until the solution becomes murky. Then let settle for 5 min.
7. Remove the clear supernatant and transfer the supernatant to a new tube containing 1 mL of $CaCl_2$ supplemented dissection solution.
8. Add another mL of trypsin inhibitor solution 2 to the bottom of the tube containing the pellet from step 3.6. Triturate again and let settle for 5 min. Remove the supernatant and add it to the tube containing the supernatant from step 3.7 (That is a repeat of steps 3.6-3.7). Repeat this process until most of the tissue is mechanically dissociated.
9. Add 0.3 mL of $CaCl_2$ supplemented dissection solution to the supernatant collection for every mL of supernatant.
10. Mix the contents of the tube and then centrifuge for 5 min at 644 x g.
11. Remove the supernatant and add 10 mL of fresh media to the pellet and mix.
12. Cells may then be counted and diluted to a concentration of 1.5×10^6 cells/mL. Please note that in general 10 million cells are expected from each dissected brain, dependent on trypsinization time and efficiency of dissection.Plate cells on previously made poly D-lysine plates. For 4-well plates, plate 0.5 mL, giving 7.5×10^5 cells per well. For 35-mm dishes, plate 4 mL, giving 6×10^6 cells per plate.
13. After 24 h, add cytosine-β-arabino furanoside (AraC) to the plates to reduce glial contamination. For each mL of media 0.5 µL of 20 mM AraC is required. Addition of AraC does not require a media change. If cells are to be maintained for 7-8 days, repeat this treatment on day 3.
14. Maintain cultures in a 5% CO_2 incubator at 37 °C and feed with 100 µL of 100 mM glucose added to the culture for every 2 mL of media every 2 days past 5 days. A complete media change is not required.

4. Lentivirus Packaging, Purification and Titration

NOTE: The protocol for the packaging, concentration, purification and titration of lentivirus has previously been described in detail without the use of a kit [28], including alternative methods for titration, such as flow cytometry [29]. Here we briefly present the protocol used in our lab for the production of lentivirus to study neuronal injury using speedy virus purification solution and a qPCR lentiviral titration kit.

1. Plate Hek293T cells in 10 cm culture dishes with Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% FBS. For obtaining a high viral titer, grow at least 5 plates of Hek293T cells for each transfection. Make sure on the day of transfection cells are 70% confluent. Change media three hours prior to transfection.
2. For each transfection, prepare two tubes. In tube 1 add 1.5 mL MEM-reduced serum media and 41 µL of transfection reagent, mix well. In tube 2, add 1.5 mL of MEM-reduced serum media and 6 µg of transfer plasmid, 6 µg of pCMV-dR8.2 vector (packaging plasmid), 3 µg pCMV-VSV-G vector (envelope plasmid), and 35 µL of p3000 enhancer reagent (supplied with lipofectamine). Mix well, and combine tubes 1 and 2, vortex and incubate for 15 min.

3. Remove 50% of the media from Hek293T plates and add DNA-lipid complexes to cells. Incubate for 6 h at 37 °C, 5% CO_2. Remove media and replace with 5 mL of fresh DMEM, incubate overnight.
4. At 24 h post-transfection, collect the first batch of viral supernatant from each plate. Keep the viral media at 4 °C and add 5 mL of fresh media into each plate of Hek293T cells. Incubate overnight.
5. 48 h post-transfection, collect the second batch of viral supernatant, combine it with the first batch and centrifuge combined viral supernatant for 10 min at 600 x g.
6. Filter the supernatant through a filter with 45 µm pore size.
7. Purify the virus using a speedy virus purification solution. For every 45 mL of viral supernatant, add 5 mL of lentiviral binding solution, mix, and centrifuge for 10 min at >5,000 x g and 4 °C.
8. Remove supernatant carefully so as not to disturb the pellet.
9. Add 20-40 µL of medium and re-suspend the pellet. Aliquot and store at -80 °C.
10. To obtain the lentiviral titer, a qPCR lentiviral titration kit is used. Dilute 2 µL of purified virus in 500 µL of PBS. Add 2 µL of the diluted virus to 18 µL of virus lysis buffer (provided in the kit).
11. Set up three different RT-q-PCR reactions in triplicates and label them as, viral lysate, positive control 1 (STD1), and positive control 2 (STD2). For each reaction add, 12.5 µL of 2x qPCR MasterMix, 2.5 µL of either viral lysate or STD1 (provided in the kit) or STD2 (provided in the kit), and 10 µL of reagent-mix (provided in the kit) in 0.2 mL PCR tubes.
12. Set the PCR program as follows: 20 min at 42 °C for reverse transcription, 10 min at 95 °C for enzyme activation, 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing/extension.
13. Calculate the viral titer using this formula:
    Titer of viral lysate = $5 \times 10^7/2^{3(Ctx-Ct1)/(Ct2-Ct1)}$.
    Ctx = average Ct value of unknown sample, Ct1 = average value of STD1, Ct2 = average value of STD2.
    NOTE: CGNs can be best transduced with lentiviruses or infected with adenoviruses depending on the mode of injury being studied. Addition of lentiviruses at an MOI of 2-3 to cell culture solution at the time of plating is used to study NMDA induced signalling at day 7 of the culture. This results in more than 80% transduction and is appropriate to pursue with biochemical analyses of these neurons (**Figure 2**). Addition of adenoviruses at day 5 of culture (at MOI 50) and studying NMDA induced signalling at day 7-8 is used for other techniques such as imaging. This treatment results in less than 10% infection of neurons, making it ideal for imaging and co-localization of proteins. Adenoviruses can be used at the time of plating to study DNA damage induced cell death by addition of camptothecin at day 2-3 or oxidative stress by addition of hydrogen peroxide. The presence of fluorescence proteins (GFP, RFP, CFP or YFP) tagged to the gene of interest can be used to estimate percent transduction and potential toxicity. With the recommended MOI, we see minimal toxicity and maximum transduction (**Figure 3**).

5. Modeling Neuronal Injury

1. To induce NMDA induced neuronal excitotoxicity, following 7 days *in vitro*, treat CGNs with 100 µM NMDA and 10 µM glycine for 1 h. Replace all the medium with conditioned medium from parallel cultures with no treatment. This concentration results in 50% cell death at 24 h post treatment (**Figure 3C**). Mitochondrial fragmentation is evident at 6-8 h post treatment (See Jahani-Asl *et al.*[26,27]).
2. To induce ROS-induced cell death (oxidative stress), treat neurons with 75-100 µM hydrogen peroxide (H_2O_2) and switch to conditioned media from parallel cultures following 5 min exposure to H_2O_2. Note, due to the instability of H_2O_2, optimize the concentration to a level that induces 50-70% cell death in culture following 24 h of treatment.
3. To induce DNA damage induced cell death, treat CGNs with 10 µM camptothecin. This concentration induces more than 50% cell death in 24 h. Mitochondrial fragmentation and early apoptotic signalling occurs 2-3 h after addition of camptothecin at this concentration (See Jahani-Asl *et al.*[18])
4. To induce low K+ induced neuronal apoptosis in CGNs, change media containing 25 mM K^+ to low potassium media with 5 mM K^+ following 7 days *in vitro*.

Representative Results

With careful dissection, the intact brain should be removed with minimal damage as seen in **Figure 1A-B**. Effort should be taken to minimize damage to the brain during removal, particularly damage to the cerebellum. Damaging the cerebellum makes for more difficult identification and complete removal of the meninges, and increases the likelihood of contamination of the neuronal culture. Once the meninges have been removed, the cerebellum can be dissected from the remaining tissue as seen in **Figure 1C** and prepared for dissociation.

Prior to plating, CGNs can be transduced with lentivirus or infected with adenovirus as described in **Figure 2**. We find maximum efficiency and minimal toxicity with MOI 50 for infection with adenovirus, and an MOI of 2-3 at day of plating for lentivirus (**Figure 3**).

Transduced healthy CGN cultures at 7 DIV are presented in **Figure 3A**. If large numbers of glia remain present in the culture, the concentration of AraC can be increased to ensure a pure neuronal population. Also glial cells take up the virus more easily than neurons and that becomes critical if the transductions are performed at day 5. The presence of neurons undergoing cell death can be assessed by the formation of pyknotic nuclei as seen in **Figure 3C**. All concentrations of the NMDA, H_2O_2, and Camptothecin are optimized to induce 50% cell death after 24 h. This allows for studying other early events that precede neuronal loss such as mitochondrial fragmentation.

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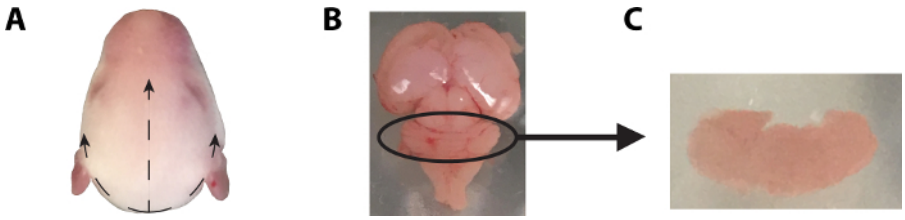

Figure 1: Removal of mouse brain and dissection of cerebellum. (**A**) To extract the brain of a 6-7 day old mouse, using a pair of forceps, grasp the head and cut the skin anteriorly along the dotted lines using a pair of microdissection scissors. Be careful to cut only the skin and connective tissue, too deep an incision may puncture the skull and damage the brain. These three incisions, straight along the midline, and two curving laterally, allow for the skin to be pushed back revealing the skull. Once exposed, the skull can be penetrated with the tip of the scissors, and cut anteriorly. Great care must be taken not to damage the cerebellum to facilitate identification and removal of meninges. Once cut, forceps may be used to peel back the skull, exposing the brain which may then be teased out into cool dissection solution using a pair of forceps or spatula. In order to remove the brain, the optic nerve may need to be severed. (**B**) Once removed from the skull, the meninges should be removed from the cerebellum using a pair of fine tipped forceps. (**C**) Using a pair of fine tipped forceps, the cerebellum is dissected from the remaining tissue and inspected to ensure complete removal of the meninges. Please click here to view a larger version of this figure.

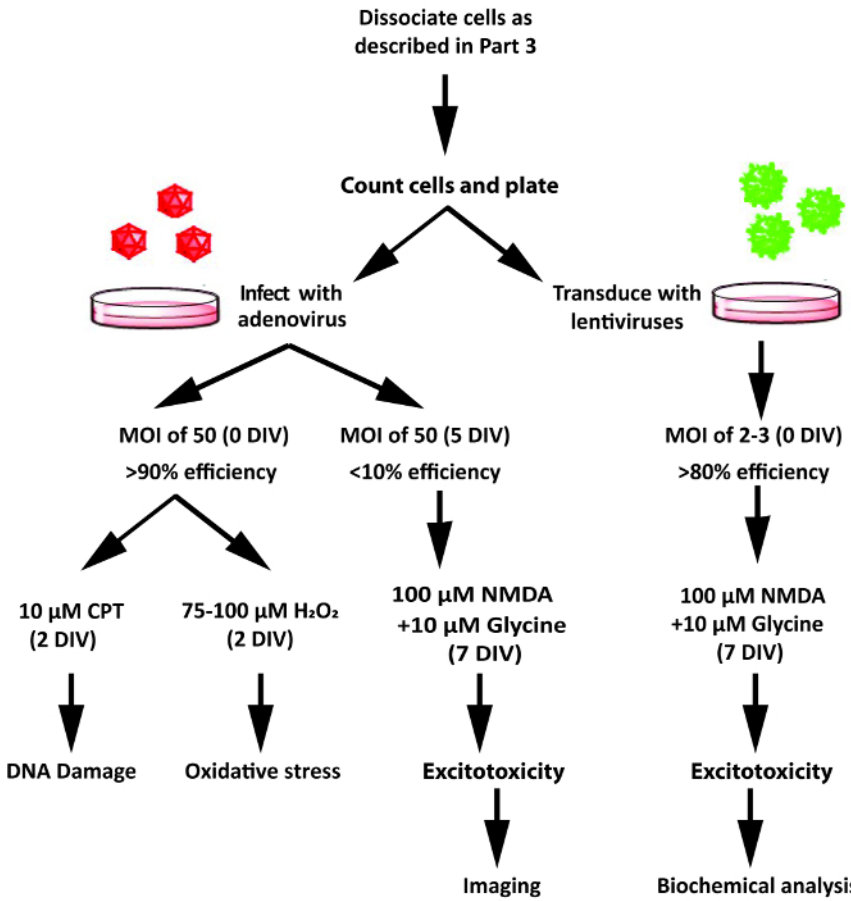

Figure 2: Modeling neuronal injury in cerebellar granule neurons. Isolated cerebella from day 6-7 mice are dissociated into single cells following the procedure presented in part 3. Following dissociation, cells are counted and resuspended in a volume of culture media to generate 1.5 x 10^6 cells/mL.For 35 mm dishes, 4 mL are plated, giving 6 x 10^6 cells per plate. For imaging slides, 0.5 mL is plated, giving 7.5 x 10^5 cells/well. CGNs can then be transduced with lentivirus or infected with adenovirus. Using adenovirus on the day of plating (0 days *in vitro* (DIV)) gives greater than 90% transfection efficiency and allows for the study of neuronal injury through oxidative stress and DNA damage. The addition of 10 µM camptothecin (CPT) will induce DNA damage, while 75-100 µM hydrogen peroxide (H_2O_2) will induce oxidative stress. The concentration of H_2O_2 must be optimized to induce 50% cell death after 24h. Infecting with adenoviruses at 5 DIV gives a lower transduction efficiency of less than 10%. At 7 DIV when NMDA receptors are enriched in the culture, Neurons can be treated with 100 µM NMDA and 10 µM glycine to induce excitotoxicity. This is ideal for subsequent imaging analysis or tracing a single neuron. Finally, transducing with lentivirus at 0 DIV, followed by treating with 100 µM NMDA and 10 µM glycine at 7 DIV, gives a sufficiently high transduction efficiency (>80%) to allow for biochemical analysis of the culture, including ChIP sequencing, examining protein expression, and performing live/dead assays. Please click here to view a larger version of this figure.

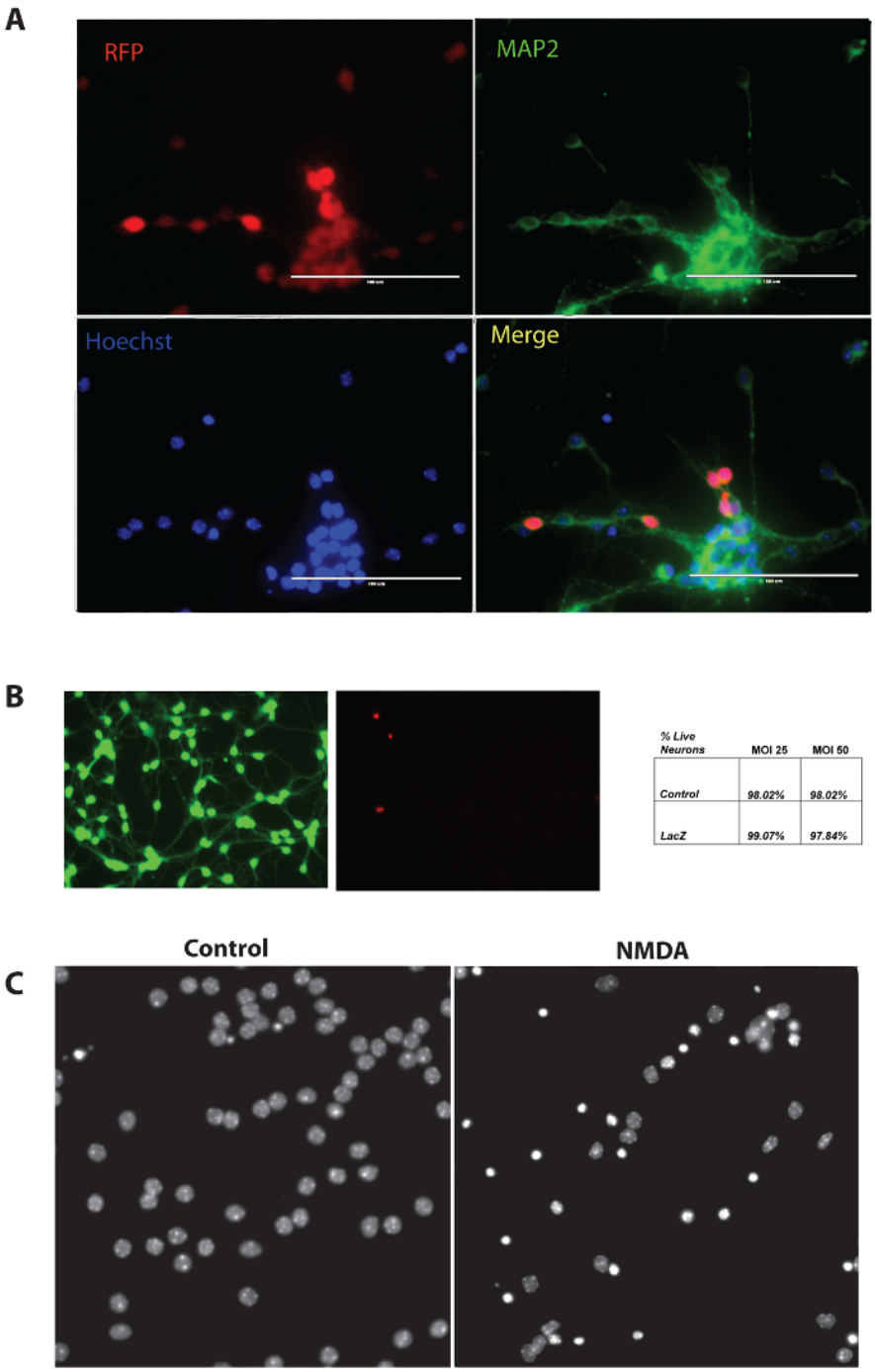

Figure 3: Cerebellar granule neurons. (**A**) Neurons were transduced with lentiviruses for RFP at an MOI of 3 at the time of plating, and fixed and stained at 7 days in vitro. Colocalization of RFP signal, MAP2, and Hoechst is shown to demonstrate healthy neurons that are fully transduced by lentiviruses. (**B**) The images represent live dead assay analyses of neurons infected with different MOI, to measure toxicity. CGNs infected with adenovirus expressing LacZ at an MOI between 25 and 50 allows for maximal efficiency while maintaining minimal toxicity. Less than a 1% difference in cell survival compared to control is seen when infecting at this MOI. (**C**) Hoechst staining of control CGNs and CGNs treated with NMDA to induce cell death. Note the formation of pyknotic nuclei with NMDA treatment. This is indicative of cell death, and can be seen in approximately 50% of the culture 24 h after treatment with 100 µM NMDA and 10 µM Glycine. Please click here to view a larger version of this figure.

Discussion

Here we provide a simple method for the culturing of primary mouse cerebellar granule neurons (CGNs), loss and gain of function studies, and modeling different mechanisms of cell death. Several factors affect the reproducibility of the results using this procedure which require close monitoring. These include the purity of the culture including the elimination of glial cells in the culture, the confluence of the culture, and

maintaining healthy cells. Introducing variability in these factors can bias the results and challenge the reproducibility. Below we describe how to minimize the factors that negatively affect the results.

To eliminate glial cells from the CGNs culture and establish a pure neuronal population, we use 10 µM of cytosine-β-arabino furanoside (AraC), a pyrimidine antimetabolite that kills proliferating cells by inhibiting DNA synthesis. If there is still a population of glial cells present, the concentration of AraC can be increased to 15 µM. This is also lot dependent. In addition, for cultures that are maintained for up to 7-8 days, a second AraC treatment can be performed on day 3. To obtain a pure neuronal population and avoid cell death, it is important to peel all the meninges off the cerebellum prior to cutting the tissue into pieces, followed by trypsinization. This should be done in a reasonably short period of time (not longer than 7-10 min per cerebellum). The presence of meninges in the neuronal culture results in unhealthy culture and eventually cell death. Another factor that could result in death of neurons prior to treatment is the trypsinization time. It is important that cells are not over-trypsinized. On the other hand, under-trypsinization requires further mechanical dissociation of the cells that may damage them or result in a low yield. Therefore, the trypsinization time requires optimization and it is lot dependent.

The next factor to be considered is the confluence of the cells at the time of use. Different confluency can generate variability in the results. This is particularly important depending on the readout in the experiments. With a low confluence state, neurons start to clump after 2-3 days. A too low or too high confluence state of the neurons impairs signalling. It is important to count the number of live cells for accurate plating.

Other methods of CGN cultures have been described, particularly looking at basic culturing procedures using a Papain Dissociation system kit [30] as well as post-culturing procedures and manipulation of CGN in order to study neuronal migration and morphology [31]. The protocol presented by Lee *et al.* describes the use of the Papain Dissociation System Kit. They recommend two methods to increase the purity of the isolated CGNs: running the cells through a Percoll gradient separation, as well as pre-plating on a poly-D-Lysine plate for 20 min. Both methods are to aid in the separation of CGNs from glial contaminants. This is recommended in case treatment with AraC alone is not sufficient to enrich for pure neuronal culture. Additionally, Lee uses 4-6 day old mouse pups, while this serves an effective model for examining neuronal differentiation; we find using CGNs from 6-7 day old mouse pups is more appropriate for the study of neuronal injury. Holubowska et al. presents a protocol for transfection of post mitotic cerebellar neurons using the calcium phosphate method *in vitro* and electroporation *in vivo*. These are excellent methods for genetic manipulation of neurons in which a single neuron can be traced. The transfection efficiency using calcium phosphate method can vary from 0.1-5%. Therefore, we find that for biochemical analyses including ChIP-Seq analyses, Co-immunoprecipitation, and immunoblotting, use of lentiviruses is more appropriate as it results in more than 80% transduction when transduced at the time of plating.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work is supported by Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research grants to A.J.-A.

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