Protocol

Cultures of Cerebellar Granule Neurons

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INTRODUCTION

Primary cultures of granule neurons from the post-natal rat cerebellum provide an excellent model system for molecular and cell biological studies of neuronal development and function. The cerebellar cortex, with its highly organized structure and few neuronal subtypes, offers a well-characterized neural circuitry. Many fundamental insights into the processes of neuronal apoptosis, migration, and differentiation in the mammalian central nervous system have come from investigating granule neurons in vitro. Granule neurons are the most abundant type of neurons in the brain. In addition to the sheer volume of granule neurons, the homogeneity of the population and the fact that they can be transfected with ease render them ideal for elucidating the molecular basis of neuronal development. This protocol for isolating granule neurons from post-natal rats is relatively straightforward and quick, making use of standard enzymatic and mechanical dissociation methods. In a serum-based medium containing an inhibitor of mitosis, cerebellar granule neurons can be maintained with high purity. Axons and dendrites can be clearly distinguished on the basis of morphology and markers. For even greater versatility, this protocol for culturing granule neurons can be combined with knockout or transgenic technologies, or used in cerebellar slice overlay assays.

RELATED INFORMATION

This protocol was adapted from methods originally developed in the work of Messer (1977), Gallo et al. (1982), Thangnipon et al. (1983), D'Mello et al. (1993) and Dudek et al. (1997).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

- <R><I>AraC (Cytosine-1- β -D-arabinofuranoside) solution
- Basal Medium Eagle (BME)
- <R>Culture medium for granule neurons
- <R>DNase (2 mg/mL) (0.5-mL aliquot for trituration; see Step 10)
- Glucose (1 M)
- <R>HHGN dissection solution
- <R>Poly-L-ornithine-coated plates (of desired size, depending on experiments to be performed; see Step 15)

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Rats, Long-Evans (post-natal day 6 [P6]) (one litter)
<!-- Trypan blue
<R><!-- Trypsin-DNase solution

Equipment

Centrifuge
<!-- CO₂ chamber
Forceps, fine (Dumont #5)
A second pair of forceps of any variety is also required.
Hemocytometer
Hood, laminar flow
Ice bucket and ice
Incubator preset to 37°C (5% CO₂)
Micropipettor with tips
Microscope, dissection
Pipettes (5-mL)
Scissors, surgical (large and small)
Timer for timing incubations
Tubes (Falcon, 50-mL)
Water bath preset to 37°C

METHOD

Isolation of Cerebella

1. Prepare poly-L-ornithine-coated plates and solutions.
2. Euthanize P6 Long-Evans rats using a CO₂ chamber.
To prevent having more than a few minutes lag time between the euthanasia and removal of the cerebella, consider doing this step in two parts.
3. Decapitate pups. To isolate the cerebella, begin by grasping a pup head at the nose and insert small surgical scissors into the foramen magnum (the aperture at the base of the skull through which the medulla and spinal cord extend). Proceed laterally, then anteriorly through the ear, and curve toward the midline. After reaching the midline from one side, reinsert scissors into the foramen magnum, and repeat the cutting procedure on the other side.
The two incisions should meet at the midline.
4. Using forceps, carefully lift the skull upward, and flip it back until the hindbrain is exposed (Fig. 1A). Make sure that, as the skull is lifted, the brain remains in the base of the skull.
5. If necessary, cut away any bone or other tissues that are obstructing the sides of the cerebellum. Extract the cerebellum with forceps (Fig. 1B), and place it into a dish containing at least 5 mL of HHGN dissection solution.
See Troubleshooting.
6. After isolation of the cerebella, use the dissection scope and fine forceps to remove the meninges, as well as any large blood vessels or extraneous brain regions that are stuck to the cerebella.
This step should be performed at a steady pace, with a plate of ice underneath the plate of cerebella. The meninges tend to come off most easily if you start by pulling away residual choroid plexus from the ventral side, where the fourth ventricle was located.

Dissociation and Culture of Cells

If you are working with two litters, an additional step of trituration is helpful; see Step 11.iv.

FIGURE 1. Dissection of rat cerebellum. (A) Posterior view of the brain of a post-natal day 6 rat pup. (From *top* to *bottom*) The regions exposed are the cerebral hemispheres, midbrain, cerebellum, and superior portion of the medulla. (Arrow) Cerebellum. (B) Positioning of forceps for removal of cerebellum.

7. Working in the tissue culture hood, decant the dish of cerebella into a 50-mL Falcon tube. Use a pipette to remove most of the HHGN solution, and then add at least 5 mL of fresh HHGN solution. Repeat this for a total of three washes.
8. After removing the last HHGN wash, add 5 mL of trypsin-DNase solution, being sure to first mix the solution by pipetting up and down. Place the tube in a 37°C water bath for 10 min. Gently swirl the cerebella every 5 min.
9. Wash three times with HHGN solution as in Step 7.
10. Prepare an aliquot of DNase for trituration by adding 250 μ L of DNase to 5 mL of BME in a separate Falcon tube. Mix by pipetting.
If you are working with two litters, you will need ~2 mL more of the DNase solution for additional trituration (Step 11.iv).
11. Remove the last HHGN wash, and add the diluted DNase solution from Step 10 to the cerebella. Dissociate by pipetting up and down with a 5-mL pipette as follows:
Avoiding bubbles is critical at this step.
 - i. For approximately the first 15 rounds of pipetting, do not press the pipette tip against the very bottom of the tube; rather, allow the cerebella to fall apart into smaller chunks.
See Troubleshooting.
 - ii. Continue to pipette up and down, but begin gently pressing the pipette tip against the bottom of the tube.
After approximately 10 more rounds of pipetting, you will find that the pieces of tissue are not becoming any smaller.
 - iii. Press the pipette tip more firmly against the bottom of the tube, and continue the trituration until clumps of tissue are no longer visible.
 - iv. To perform an additional trituration, transfer the cell suspension to ice for 5 min, allowing any remaining undetected clumps to sink to the bottom. Transfer the top half of the cell solution to a separate Falcon tube, and triturate the bottom again using fresh DNase solution.
12. Centrifuge cells at ~200g for 5 min at 4°C.
See Troubleshooting.
13. Use a pipette to remove the supernatant. Add 5 mL of culture medium for granule neurons, and pipette up and down until a homogeneous mixture is obtained. Add 10 mL more of culture medium incrementally, pipetting up and down after dispensing every 2-3 mL to ensure that the mixture remains homogeneous.
14. Place the cell suspension on ice, and count a small aliquot using a hemacytometer:

- i. Make a 1:10 dilution of cells by adding 100 μ L of the cell suspension to 650 μ L of culture medium and 250 μ L of trypan blue.
 - ii. Count healthy, spherical cells with a halo. Do not count cells that take up the trypan blue.
The expected yield is 10-15 million cells per cerebellum.
See Troubleshooting.
15. Plate cells at the desired density. For morphological assays in 24-well plates with coverslips, use 17-20 million cells per plate. For biochemical experiments in six-well plates, use 30 million cells per plate. Scale up for larger plates.
16. Maintain cells by adding AraC solution ~24 h after plating cells to a final concentration of 10 μ M. On day in vitro 3 (DIV3), add 1 M glucose to a final concentration of 25 mM.
See Troubleshooting.

TROUBLESHOOTING

Problem: Cerebella are broken upon removal.

[Step 5]

Solution: Consider the following:

1. To make sure that your incisions are not too deep, cut incrementally, removing the scissors and reinserting them after each small incision.
2. Pull away from the center as you cut, such that you are making an incision in the skull without damaging any of the soft tissue inside.
3. Do not extract the cerebellum until it is fully exposed, making sure you have cut away any obstructing tissue on the sides.

Problem: Cerebella do not fall apart easily after trypsinization.

[Step 11.i]

Solution: Trypsinize longer, for 15-20 min. If this does not help, make fresh trypsin-DNase aliquots, checking that the concentrations are accurate. When the trypsinization has worked properly, the cerebella should appear softened.

Problem: A viscous pellet is observed after centrifugation.

[Step 12]

Solution: The DNase may be spoiled or not concentrated enough. Add 50 μ L more of the 2-mg/mL aliquot, and further dissociate the cells.

Problem: Cells clumps are visible during counting.

[Step 14]

Solution: Apply more pressure when triturating, and observe the solution carefully for any residual clumps before centrifuging cells.

Problem: There is a low yield of healthy cells and a high rate of cell death.

[Steps 14 and 16]

Solution: There are many causes for this problem. Consider the following:

1. Most likely, the trypsinization or trituration was too harsh. Add trypsin for a shorter amount of time, and apply less pressure when dissociating the cells.
2. Make sure the culture preparation does not exceed 2 h.
3. If problems persist, prepare all the solutions and the ethanol-rinsed coverslips again.
4. A change in serum lot can have an adverse impact on the health of cultures.

Problem: Cells clump together after a few days in culture.

[Step 16]

Solution: Plating density is probably lower than expected from the count. Be sure not to count the unhealthy cells, which take up trypan blue.

Problem: Cells are not attached, or there are regions of the plate with no cells.

[Step 16]

Solution: Consider the following:

1. The coating may not have been even. Make sure the poly-L-ornithine solution covers the entire well, and, if using coverslips, ensure that they do not float up. (Press down with a pipette tip if they float.)
2. Make sure to wash the plates thoroughly with H₂O.

Problem: There are too many glia.

[Step 16]

Solution: Consider the following:

1. Remove meninges at a steady pace so that the time between cerebella extraction and trypsin treatment does not exceed 30 min.
2. Be certain pups are of the right age.
3. If the above factors are not the cause of the problem, prepare fresh AraC solution.
4. If glia levels remain high, increase AraC concentration to 15 μ M. If you are keeping the cells in culture for longer than 4-5 d, add a second dose of AraC on DIV3.

DISCUSSION

Cerebellar granule neurons have proven invaluable in uncovering the signaling pathways governing neuronal survival, migration, and differentiation. Studies of neuronal apoptosis have frequently relied on cultures of granule neurons, exploiting their responses to activity and growth factor deprivation as well as oxidative stress. These studies have led to the identification of key neuroprotective molecules including insulin-like growth factor 1, cyclic adenosine monophosphate, phosphatidylinositol-3 kinase, AKT, and myocyte enhancer factor 2 (D'Mello et al. 1993, 1997; Dudek et al. 1997; Mao et al. 1999; Li et al. 2001; Shalizi et al. 2003) and key mediators of neuronal cell death including cyclin-dependent kinase 1, c-Jun N-terminal kinase, and mammalian sterile 20-like kinase (Watson et al. 1998; Harris and Johnson 2001; Konishi et al. 2002; Becker and Bonni 2006; Lehtinen et al. 2006; for review, see Becker and Bonni 2004).

The classic paradigm for studying activity-dependent responses in granule neurons has been the use of membrane-depolarizing concentrations of extracellular potassium chloride (Gallo et al. 1987; Yan et al. 1994). Membrane depolarization activates voltage-sensitive calcium channels, leading to the entry of calcium into neurons and the activation of calcium-dependent signaling molecules, including Ca²⁺-calmodulin-dependent protein kinases and the phosphatase calcineurin (Sée et al. 2001; Linseman et al. 2003; Wayman et al. 2004; Sato et al. 2005; Suzuki et al. 2005). Calcium-dependent signaling pathways regulate the gene expression profile of cerebellar granule neurons (Kramer et al. 2003; Sato et al. 2005). An interesting example is the developmental switch that occurs in subunit composition for GABA_A and NMDA neurotransmitter receptors as granule neurons mature (Watanabe et al. 1992; Farrant et al. 1994; Mathews et al. 1994). Upon hyperpolarization, the α 6 subunits of the GABA_A receptor and the NR2C subunit of the NMDA receptor are up-regulated (Mellor et al. 1998; Suzuki et al. 2005; for review, see Nakanishi and Okazawa 2006).

Another realm in which granule neurons have occupied center stage is the study of neuronal migration. Granule neurons display a well-known maturation-dependent descent from the external to internal granular layer of the cerebellar cortex. They have been particularly useful in uncovering glial guidance mechanisms for neuronal migration, shedding light on both the cell biology of the migration process and the specific molecular pathways involved. Two crucial regulators of neuronal migration that were identified in studies of cerebellar granule neurons are the adhesion molecule astrotactin and members of the mPar6 α polarity complex (Rivas and Hatten 1995; Zheng et al. 1996; Solecki et al. 2004; for review, see Stitt et al. 1991; Solecki et al. 2006).

Most recently, granule neurons have been used to study the cell-intrinsic mechanisms underlying neuronal morphogenesis and connectivity. The transcription factors MEF2 and NeuroD, as well as the ubiquitin ligase Cdh1-APC and its target SnoN, have been identified as key regulators of morphological development in neurons (Gaudillière et al. 2004; Konishi et al. 2004; Shalizi et al. 2006; Stegmüller

et al. 2006). For these studies, granule neurons offer the benefit of having a highly stereotypical pattern of polarization with readily distinguishable axons and dendrites. In terms of understanding neuronal connectivity on a broader scale, perhaps the best asset of granule neurons is the relatively simple architecture of the cerebellar cortex, as this may facilitate translating molecular findings about individual cell types into functional consequences for a circuit.

In contrast to the ease of culturing granule neurons, Purkinje neurons—the post-synaptic targets of granule neurons—are notoriously challenging to isolate and maintain in culture. Although recent improvements in culture techniques may increase the survival and differentiation of Purkinje neurons in mixed cerebellar cultures (Furuya et al. 1998; Ito-Ishida et al. 2008), the overall number of Purkinje neurons in such cultures remains quite low. Purifying Purkinje neurons can require Percoll sedimentation and immunopanning technologies (Baptista et al. 1994), which are more expensive and labor-intensive than most neuronal culture protocols. In addition to this difficulty of culturing post-synaptic targets, cerebellar granule neurons develop post-natally, making it difficult to study granule neurons from the many knockout mice that die embryonically or at birth. However, the post-natal development of granule neurons can also be advantageous because the post-natal cerebellum is much easier to isolate than small regions of the embryonic cerebrum.

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