Aug 07, 2025

Primary Ventral Midbrain Neuronal Culture and Transfections

In 1 collection

DOI
https://dx.doi.org/10.17504/protocols.io.q26g7ny1qlwz/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.q26g7ny1qlwz/v1

Protocol Citation: Camila Pulido, Tim Ryan 2025. Primary Ventral Midbrain Neuronal Culture and Transfections. **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g7ny1qlwz/v1

Protocol status: Working
We use this protocol and it's working

Created: August 07, 2025

Last Modified: August 07, 2025

Protocol Integer ID: 224255

Keywords: ASAPCRN, Primary neuronal culture, Ventral midbrain, Dopaminergic neurons, Transfection, culture dopaminergic neurons from rat midbrain brain, primary ventral midbrain neuronal culture, transfections dopaminergic neuron, culture of primary vm neuron, dopaminergic neuron, rat midbrain brain, primary vm neuron, murine brain, neuronal function, difficult cells to culture, understanding of neuronal function

Funders Acknowledgements:
ASAP
Grant ID: 000580

Abstract

Dopaminergic neurons consist some of the most difficult cells to culture due to their scarcity, intricate arborization and intrinsic vulnerability. The culture of primary VM neurons from murine brains has advanced the understanding of neuronal function at the cellular and molecular level. This protocol presents an established method to isolate and culture dopaminergic neurons from rat midbrain brains along with a method to sparsely transfect dopaminergic neurons.

Guidelines

The animals should be used in accordance with protocols approved by national and institutional regulatory organizations.

Materials

Supplies

Media Supplies

- Neurobasal Plus Medium (Thermo Fisher, Cat. No. A3582901)
- Basal Media Eagle (BME) (Thermo Fisher, Cat. No. 21010046)
- Fetal Bovine Serum (FBS) (Atlanta Biologicals, Cat. No. S11550)
- N-21 Max (R&D Systems, Cat. No. AR008)
- Glutamax Supplement (Thermo Fisher, Cat. No. 35050061)
- Earle's Balanced Salt Solution (EBSS) (Thermo Fisher, Cat. No. 24010043)
- Kynurenic acid (Millipore Sigma, Cat. No. K3375)
- Papain (Worthington Biochemical, Cat. No. LK003178)
- PBS Cell Culture (Thermo Fisher, Cat. No. 10010031)
- GDNF (Sigma Aldrich, Cat. No. G1401-10UG)
- DMEM, high glucose (Thermo Fisher, Cat. No. 11965126)
- CNQX (Alomone Labs, Cat. No. C-141)
- Sodium Chloride (Millipore Sigma, Cat. No.S7653)
- Potassium Chloride (Millipore Sigma, Cat. No. P9333)
- Disodium Phosphate (Na_2HPO_4) (Millipore Sigma, Cat. No. 431478)
- Glucose (Millipore Sigma, Cat. No. G7021)
- HEPES (Millipore Sigma, Cat. No. H3375)
- NaOH (1N = 4 g of sodium hydroxide in water to make 100 mL)
- 12N HCl
- Ultra-Pure Water (Thermo Fisher, Cat. No. 10977-015)
- 2M Calcium Chloride (in H_2O) (RPI, Cat. No. C25100-50.0)

Other Supplies

- 0.22 µm syringe filters (VWR, Cat. No. 76479-044)
- 18 Gauge x 1-1/2'' needles (Thomas Scientific, Cat. No. 1145Q16)
- Oxygen Humidifier bottle
- Acetone (LabChem, Cat. No. LC104202)
- Cell strainers (40 µm) (Falcon, Ref No. 352340)
- Cloning Cylinders (6 mm OD) (VWR, Cat. No. 89083-360)
- Coverslips (22 mm X 22 mm, glass no. 1) (e.g. VWR, Cat. No. 16004-094)
- Ethanol (200 Proof) (Koptec, Cat. No. 6175)
- Grease (Millipore Sigma, Cat. No. Z273554-1EA)
- 35 mm dishes (D X H 35 X 10 mm) (Falcon, Cat. No. 351008)
- Poly-L-ornithine (Millipore Sigma, Cat. No. P3655-10MG)
- Wash-N-Dry Coverslip Racks (Millipore Sigma, Z688568-1EA)
- 95% oxygen and 5% CO_2 tank cylinder.

- Hemocytometer (like VWR, Cat. No. 15170-173)
- Coverslip holder (Millipore Sigma, Cat No. Z688568)
- SYLGARD 184 Silicone Elastomer Kit (Dow, Cat. No. 04019862)
- Carbon powder (Thermo Fisher, Cat. No. 033302)
- Glass petri dishes 60 × 20 mm (Millipore Sigma, Cat No. SLW1480/02D)
- Spinbar, Flea micro 5 × 2mm (VWR, Cat No. 58948-377)
- Sporicidin (Clontec, Cat No. 89176-480)

Stock Solutions

Heat Inactivating FBS:

FBS can be bought pre-heat inactivated but if not follow the below protocol to heat inactivate.

1. Thaw FBS in water.
2. Aliquot in to 20mL conical tubes.
3. Place in water bath at 56 °C for 00:30:00 .
4. Filter and aliquot into new 50 mL conical tubes.
5. Store at -20 °C .
6. When ready to use thaw at 4 °C .

Poly-L-Ornithine (1 mg/mL in H_2O, 100x):

1. To a 10 mg bottle add 10 mL of ultra-pure water.
2. Filter with a syringe filter and store in 100 µL aliquots at -20 °C .

GDNF [20 ug/mL] Stock Solution:

1. Dissolve 10 µg GDNF in 500 µL ultra-pure water.
2. Store as 10 µL aliquots at 20 °C .
3. Dilute 1:2000 in culture media for a final concentration of 10 ng/mL.

2X HEBS:

274 mM NaCl, 9.52 mM KCl, 1.42 mM $Na_2HPO_4.7H_2O$, 15mM D-glucose, 42 mM HEPES, 20 µM CNQX in H_2O to a ~ pH 7.10

1. Add 150 mL of ultra-pure water.
2. Add 3.2 g NaCl.

3. Add 142 mg of KCl.
4. Add 76 mg of $Na_2HPO_4 \cdot 7H_2O$.
5. Add 540 mg of D-glucose.
6. Add 2 g HEPES.
7. Add 400 µL of CNQX (10 mM stock).
8. Adjust pH to ~ pH 7.10 .
9. Add water to 200 mL .
10. Confirm pH is correct.
11. Filter and store in ~ 500 µL aliquots at -20 °C .

Note: When initially making HEBS try multiple pH's around pH 7.10 *to see which gives the optimal transfection efficiency.*

Culture Media Recipes

NeuroA media:

Makes 150 mL *to culture ~40 dishes; freshly prepare on the culture day.*

1. Add 90 mL Neurobasal Plus Medium.
2. Add 35 mL Basal Media Eagle (BME).
3. Add 15 mL heat-inactivated fetal bovine serum (FBS).
4. Add 3 mL N-21 Max.
5. Add 1.5 mL Glutamax.
6. Filter in a sterile bottle.

Papain solution:

Prepare 00:20:00 *before dissection.*

1. Add 5 mL EBSS solution to one vial of papain.
2. Add 5 µL kynurenic acid stock ([M] 0.5 Molarity (M) stock made by dissolving 946 mg in 10 mL of 1N NaOH)
3. Add 0.5 µL of 12N HCl at a time, until the solution color changes to salmon. DO NOT EXCEED 1.5 µL HCl.
4. Sterile filter the solution into a 15 mL conical tube whose cap has been pierced twice with an 18 gauge needle.

Preparing Coverslips

1. Using the coverslip holders place coverslips in a beaker.
2. Wash with acetone for 00:30:00, while shaking.
3. Wash with ethanol for 00:30:00, while shaking.
4. Rinse with ddH_20 twice
5. Dry and autoclave.

Animals

An example rat strain is the Sprague-Dawley strain from Charles River Labs (Charles River Strain Code: 400, RRID: RGD_734476). Animals should be used from P0-P1.

Equipment

- CO_2 Incubator (like VWR Symphony)
- Stereoscope (like ZEISS Stemi 2000)
- Microscope (like Olympus CK40)
- Student Scalpel Handle #3 Scalpel (like Fine Science Tools 91003-12)
- Surgical blade #10 (like VWR 72044-10)
- Surgical blade #11 (like VWR 72044-11)
- Dumont #5 Forceps (like Fine Science Tools 11252-00)
- Dumont #3 Forceps (like Fine Science Tools 11293-00)
- Dumont #5 Tweezers (like Roboz Surgical Store RS-4905)
- Operating Scissors (like Roboz Surgical Store RS-6828)
- BCL-1 hood (like Forma Scientific 1839 Laminar Flow Work Station)
- BCL-2 hood (like LABONCO Purifier Logic+ Class II, Type A2)
- Refrigerated benchtop Centrifuge (Beckman Coulter Allegra X-30R) with SX4400 swing-bucket rotor
- Hotplate Stirrer (like VWR Mini Hotplate Stirrer, 120 V; Cat No. 10153-308)

Safety warnings

Primary cells constitute a type-2 Biohazard and should be handled according to all national and institutional guidelines in a BSL-2 culture hood.

Before start

- On average, 1 rat pup yields approximately 20 dishes of VM neurons.
- NeuroA media should be prepared fresh and keep at Room temperature.
- Papain solution should be equilibrated to ~ 37 °C for 00:20:00
- Dissection tools should be kept in sporicidin.
- Dissection time should be about 00:05:00 per pup.
- Pipette solutions slowly on the side of the tubes to prevent bubbles.
- Incubators should be set to 5% CO_2 37 °C.

Preparing Dishes (Prepare day before) 10m

1 For each dish, place a coverslip in a p35.

2 Take an 100X poly-L-ornithine aliquot and dilute in 10 µL ultra-pure water (final conc. 10 µg/mL).

3 Add ~ 200 µL of diluted poly-L-ornithine to the center of each coverslip.

4 Place dishes in incubator Overnight

Preparing Papain Setup 10m

5 Prepare Papain Solution as detailed in Materials section.

6 Microwave 200 mL distilled water in a 400 mL beaker for 00:00:15 .

7 Alcohol-wipe the thermometer and place in the warm beaker.

8 Place beaker on a heating plate, the temperature should be maintained between 35 °C to 37 °C .

9 Start the oxygen (95% O_2 and 5% CO_2) connected to oxygen humidifier bottle.

10 Insert a sterile 18 gauge needle into the 15mL falcon tube's cap containing the papain solution, and connect needle to oxygen hose carrying humidified oxygen.

11 Place papain solution tube in the beaker pre-warmed with water and leave it equilibrate for ~ 00:20:00 , while oxygenating it.

Preparing for culture

10m

12 Prepare NeuroA media as detailed in Materials section.

13 Add 8 mL of PBS to a dissecting dish.

14 Add 4 mL of PBS to a p35.

15 Place in fridge until ready to dissect.

16 Prepare dissection hood and fill a bucket with ice.

17 Turn on centrifuge and setup to 17 °C .

18 Collect rat pups from your animal facility. You should collect one pup for every 20 dishes. Pups should be P0–P1.

Dissection of the ventral midbrain from rat pups

15m

19 *This section describes how to dissect the ventral midbrain from postnatal rodent brain. The VM is isolated using a slice cutting approach. Figure1 gives a schematic overview of individual steps.* ***This dissection method is adapted from Lautenschlager, et-al, 2018*** .

Citation

Lautenschläger J, Mosharov EV, Kanter E, Sulzer D, Kaminski Schierle GS (2018)
. An Easy-to-Implement Protocol for Preparing Postnatal Ventral Mesencephalic Cultures.

https://doi.org/10.3389/fncel.2018.00044 LINK

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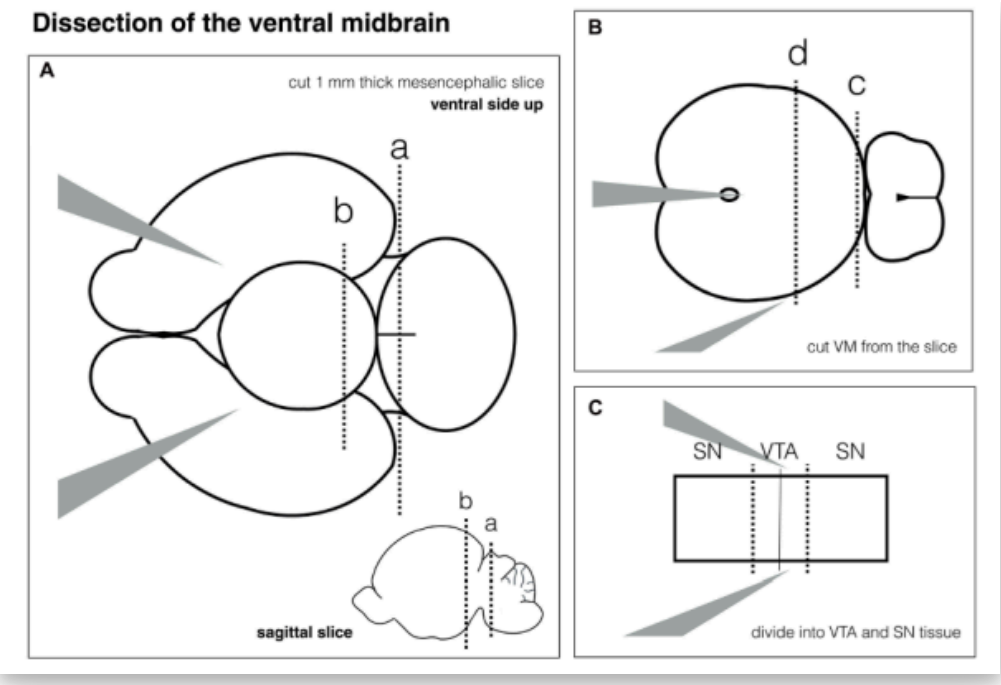

Figure 1. Schematic overview of individual steps for ventral midbrain (VM) dissection. (A) Dissection of mesencephalic slice from the whole brain. (B) Dissection of the VM. (C) Separation of Substantia
nigra (SN) and ventral tegmental area (VTA). ***This dissection method is adapted from Lautenschlager, et-al, 2018***

21 Perform the dissection on the cold marble. Marble is kept at -20 °C. Change out the marble every two pups.

22 Check that the papain tube has warmed up.

23 Decapitate rat pup using the scissors. Place rat carcass in a biohazard bag and dispose of according to your institution's policy.

24 Using tweezers stab through the eyes and use the sharp blade (#11) to cut through the skull.

25 While keeping tweezers in the eyes, using another pair of tweezers remove skull.

26 Use the scoop to remove the brain and place in dissecting dish filled with cold PBS with the ventral side facing up to see the midbrain flexur.

27 Hold the brain by inserting the fine forceps No. 3 into both hemispheres.

28 Gently remove the meninges over the hindbrain using the fine forceps No. 5.

29 Make a vertical cut with the scalpel (blade #10) at the level of the hindbrain. See Figure 1A, dotted line a.

30 Make another vertical cut cranial to the midbrain flexure – see Figure 1A, dotted line b.

31 You will have an approximately 1 mm thick coronal brain slice of the mesencephalon. The IV ventricle should be visible on the caudal side of the slice.

32 Put the fine forceps No. 3 into the ventricle to hold the slice – Figure 1B.

33 Make a vertical cut between midbrain and hindbrain – see Figure 1B, dotted line c.

34 Then make another cut half-way between the first cut and the ventricle – see Figure 1B, dotted line d.

35 Place the separation of substantia nigra and ventral tegmental area segment into the p35. Store on ice until all segments are dissected.

36 Once all VM segments are dissected, divide the VM into SN and VTA by cutting 1/3, 1/3, 1/3. See dotted lines in Figure 1C. Pieces can be mix for a mixed culture, otherwise separate them by region of interest.

Culture

1h

37 On the sterile hood, move the dissected midbrain pieces into the 15mL falcon tube with the Papain Solution and add a small sterile spinbar.

38 Place the Papain Solution tube back in the beaker, and keep oxygenating it.

39 Turn on Hotplate Stirrer to a minimum setting (VERY SLOW/GENTLE), and digest between 00:12:00 to 00:20:00 (check what is better for your preparition). Temperature MUST be maintained between 33 °C to 37 °C otherwise cells wont survive. 20m

40 During the enzymatic treatment:

40.1 Aspirate off the poly-o from the dishes coverslips.

40.2 Wash coverslips two times with 0.5 mL ultrapure water.

40.3 Add the cloning cylinders. Using heated forceps, place the cylinder in the grease and then place the cylinder in the center of the coverslip.

41 After digestion time is up, take out the spinbar and spin tissue at 300 x g, 17°C, 00:05:00 . 5m

42 After the spin is complete, aspirate off Papain Solution and add 1 mL of NeuroA media.

43 Using the P1000 pipettor, slowly pipette the cells up and down until fully dissociated. Make sure to not produce bubbles.

44 Place a cell strainer in a 50 mL falcon tube and pour the 1 mL cell suspension over the strainer.

45 Wash the 15 mL falcon tube that contained the cell suspension with 4 mL of NeuroA media. Add the 4 mL solution using a serological pipette over the strainer.

46 Take the strained cell suspension and place in a new 15 mL falcon tube and spin at 300 x g, 17°C, 00:10:00 . 10m

47 After second spin is completed, aspirate media and wash with 5 mL of NeuroA media. Resuspend the pellet and spin again at 300 x g, 17°C, 00:10:00 . 10m

48 After last spin, aspirate off the media and resuspend in NeuroA media. Our rule of thumb is to resuspend in 0.5 mL of NeuroA media per pup dissected.

49 Then add 10 µL to the hemocytometer and count the cells. You want ~12,500 cells/cylinder. Each cylinder holds 100 µL .

50 Dilute cells with NeuroA media to appropriate concentration.

51 Add 100 µL of NeuroA media with cells per cylinders.

52 Add 3 mL of NeuroA media to the outside of each dish.

53 Place dishes in the incubator.

Transfection 3h

54 *Transfections should be done DIV2 – DIV3. Recommend doing 40 dishes max at a time.*

55 Filter DMEM and equilibrate to Room temperature

56 For each dish rinse twice by replacing 60 µL cylinder media and replacing with 60 µL of equilibrated DMEM.

57 Return dishes to the incubator for 01:00:00 . 1h

58 Prepare DNA mix as bellow, but do not add HEBS. Incubate mix for 00:30:00 . 30m

59 After 01:00:00 , remove cells from the incubator.

60 Add HEBS dropwise, while vortexing, to the DNA mix. Immediately add 12 µL of DNA mix to each cylinder dish. Pipette quickly, do not add dropwise.

61 Return cells to the incubator for 01:00:00 . Do not open the incubator during this time. 1h

62 After 01:00:00 , remove cells from the incubator and remove 60 µL of cylinder's media.

63 Break off the cylinder with sterile tweezers to flood with NeuroA media.

64 Add 1.5 µL GDNF stock to every dish containing 3 mL NeuroA media to a final concentration of 10 ng/mL. 5m

65 Return to incubator until ready to image.

DNA Mixes (HEBS) 10m

66
- *These are starting suggestions for your transfection. Optimize as needed.*
- *These recipes are good for ten dishes, scale as appropriate.*
- *if targeting dopaminergic neurons, it is advised to use a TH promotor to drive expression in this selective population.*

67 Single Transfection: 6 µg of plasmid + 6 µL $CaCl_2$ + Ultrapure water to 60 µL . Incubate for 00:30:00 and add 60 µL HEBS dropwise, while vortexing, to the DNA mix.

68 Double Transfection: 4 µg of first plasmid + 5 µg of second plasmid + 6 µL $CaCl_2$ + Ultrapure water to 60 µL . Incubate for 00:30:00 and add

60 µL HEBS dropwise, while vortexing, to the DNA mix.

Protocol references

1. J. Lautenschläger, E. V Mosharov, E. Kanter, D. Sulzer, G. S. Kaminski Schierle, An easy-to-implement protocol for preparing postnatal ventral mesencephalic cultures. *Front. Cell. Neurosci.* **12**, 1–10 (2018). https://doi.org/10.3389/fncel.2018.00044

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Citations

Step 19

Lautenschläger J, Mosharov EV, Kanter E, Sulzer D, Kaminski Schierle GS. An Easy-to-Implement Protocol for Preparing Postnatal Ventral Mesencephalic Cultures.
https://doi.org/10.3389/fncel.2018.00044