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🌐 Vidyadhara et al., 2023 "Dopamine transporter...Parkinson's Disease" (L-DOPA treatment, IHC, HPLC, Microscopy Imaging and Analysis, Western Blotting, and FSCV)

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We use this protocol and it's working

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Abstract

This protocol details L-DOPA treatment, Immunohistochemistry, HPLC, and electron microscopy tissue preparation and settings and FSCV in publication "Dopamine transporter and synaptic vesicle sorting defects underlie auxilin-associated Parkinson's disease."

Attachments

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30KB

Materials

Reagents:

- 0.9% saline with 0.1 % heparin (50 µg of heparin in 1 ml distilled water)
- 2% EM grade paraformaldehyde (PFA) and 2% glutaraldehyde, prepared in 0.1M PB (prepared from 16% EM grade PFA, EMS sciences, catalog no.: 15710)
- 2.5% glutaraldehyde + 2% PFA + 0.1 M cacodylate buffer (borrow from EM facility)
- 0.1 M cacodylate buffer

> ⊗ Paraformaldehyde **Electron Microscopy Sciences Catalog #15710**

Buffers:

2X Sample Buffer (50 mL stock consisting of):

A	B
0.5 M Tris (pH 6.8)	12.5 mL (Final conc. of 125 mM)
10% SDS	20 mL (Final conc. of 4%)
20% glycerol	10 mL
Dithiothreitol (DTT, 100 mM)	0.77 g
Bromophenol blue (0.33 mg/mL)	16.5 mg
H_2O	7.5 mL

5X Sample Buffer (100 mL stock consisting of):

A	B
Tris pH 6.8, 1.0 M	31.25 mL (final conc. 312.5 mM)
SDS	10 g (final conc. 10%)
Glycerol	50 mL (final conc. 50%)
DTT (Mr 154.2)	3.855 g (final conc. 250 mM)
Bromophenol Blue	50 mg, leading to 0.5 mg/ml (0.05%)
H2O	to add up to 100 mL final volume

Filter using 0.2 μm syringe filter after preparation.
Aliquot in 10 mL and store at -20°C.

1.5M Tris (pH 8.8) – total 500 mL

90.86 g Tris

Adjust pH to 8.8 with HCl

Filter

0.5M Tris (pH 6.8) – total 500 mL

30.29 g Tris

Adjust pH to 6.8 with HCl

Filter

10X tank buffer (running buffer) – total 6 L

A	B
Tris	180 g
Glycine	864 g
SDS (add last)	60 g

To get 1X that contains 0.025M Tris, 0.192M Glycine, 0.1% SDS.

10x transfer buffer - total 1 L

A	B
Tris	30 g
Glycine	144 g

2 L transfer buffer (for one transfer tank (4 gels))

A	B
Transfer buffer	200 mL
Methanol	400 mL
DW	1400 mL

<u>10x TBS-T - total 2 L</u>

A	B
Tris	24.2 g
NaCl	174 g
pH with HCl to 8.0	11 mL
Tween-20	10 mL

To get 1X containing 10 mM Tris, 150 mM NaCl, and 0.05% Tween-20.

<u>Blocking Buffer</u>
- 5% nonfat dry Milk
- 5% Goat serum in TBST

Troubleshooting

L-DOPA treatment

1 Prepare the L-DOPA treatment solution by dissolving L-DOPA powder in 0.85% saline solution to achieve a concentration of ⚗ 15 mg/kg body weight.

2 Select adult mice for the study and divide them into pre-treatment and post-treatment groups.

3 Conduct behavior testing on the pre-treatment group one week before the L-DOPA treatment to establish a baseline for comparison.

4 On the day of the experiment, inject each mouse in the post-treatment group with a single dose of the L-DOPA treatment solution through the intraperitoneal route, at a dosage of ⚗ 15 mg/kg body weight and volume of ⚗ 10 ml/kg body weight.

5 Conduct behavior testing on the post-treatment group 20-25 minutes after the L-DOPA treatment to measure the effect of the treatment.

6 Use the pre-treatment group as a control to compare the behavioral differences before and after L-DOPA treatment.

Immunohistochemistry

7 Anesthetize the mice using isoflurane inhalation and perfuse intracardially with 0.9% heparinized saline followed by chilled 4% paraformaldehyde (PFA) in [M] 0.1 Molarity (M) phosphate buffer (PB).

8 Post-fix the brains in the same buffer for ⏱ 48:00:00 .   2d

9 Cryoprotect the brains in increasing grades of buffered sucrose (15 and 30%, prepared in [M] 0.1 Molarity (M) PB), at 🌡 4 °C .

10 Store the brains at 🌡 -80 °C until sectioning.

11 Perform serial sections of the brains (→|← 30 μm thick) coronally using a cryostat (Leica CM1850, Germany).

12 Collect the sections on gelatinized slides and store them at 🌡 -20 °C .

Immunohistochemistry: Immunoperoxidase staining

13 For immunoperoxidase staining, perform endogenous peroxidase quenching using 0.1% H_2O_2 in 70% methanol (⏱ 00:30:00). **30m**

14 Block with 3% bovine serum albumin (BSA) (⏱ 02:00:00) at 🌡 Room temperature . Incubate sections at 🌡 4 °C with primary antibody (⏱ 02:00:00 at 🌡 Room temperature , then ⏱ Overnight). **6h**

15 Incubate sections with biotin-conjugated secondary antibody at 🌡 Room temperature (1:200; 3-4 hours, Vector Laboratories, PK-6101). Perform tertiary labeling with the avidin-biotin complex solution at 🌡 Room temperature (1:100; 3-4 hours, Vector Laboratories, PK-6101).

16 Visualize staining using 3,3′-diaminobenzidine (Fluka, 32750) as a chromogen in a solution of [M] 0.1 Molarity (M) acetate imidazole buffer (pH 7.4) and H_2O_2 (0.1%).

Immunohistochemistry: Immunofluorescence staining

17 **Equilibration**: Rinse the sample with [M] 0.01 Molarity (M) PBS for ⏱ 00:05:00 , followed by rinsing with [M] 0.01 Molarity (M) PBS + 0.1% Tx for another ⏱ 00:05:00 . **10m**

18 **Penetration**: Apply [M] 0.01 Molarity (M) PBS + 0.5% Tx for 10-20 minutes (start with 15 minutes and adjust as needed), then wash 3 times.

19 **Neutralization**: Incubate with [M] 0.3 Molarity (M) Glycine in [M] 0.01 Molarity (M) PBS (without Tx) for ⏱ 00:20:00 , then wash 3 times. **20m**

20 **Blocking**: Incubate with 3% goat serum (GS) in working/wash buffer for 1.5 to 2 hours. Wash 2-3 times gently.

21 **Primary incubation:** Dilute the required concentration of primary antibody in 1% GS in working/wash buffer. Incubate for ⏱ 02:00:00 at 🌡 Room temperature and ⏱ Overnight at 🌡 4 °C . Wash 3-4 times.

4h

22 **Secondary incubation**: Dilute the secondary antibody in 1% GS in working/wash buffer at a 1:500 ratio. Incubate for 2-3 hours at 🌡 Room temperature . Wash 3-4 times with working/wash buffer and 2 more times with [M] 0.01 Molarity (M) PBS (without Tx).

23 Mount with DAPI.

24 Use 1X PBS with 0.1% Triton-X-100 as both washing and working buffer for both immunoperoxidase and immunofluorescence staining, except for phospho-antibodies where 1X Tris buffer saline can be used.

High-performance liquid chromatography (HPLC)

25 Anesthetize mice using isoflurane inhalation and sacrifice them by cervical dislocation.

26 Quickly remove and dissect the brains for dorsal striatum and snap freeze them. Store the tissues at 🌡 -80 °C until further use.

27 Homogenize the tissues using a handheld sonic tissue dismembrator in ⚗ 100-750 µL of [M] 0.1 Molarity (M) TCA containing [M] 0.01 Molarity (M) sodium acetate, [M] 0.1 millimolar (mM) EDTA, and 10.5% methanol (pH 3.8).

28 Spin the homogenates in a microcentrifuge at ⚙ 10000 x g, 00:20:00 and collect the supernatant for HPLC-ECD analysis.

20m

29 Estimate protein concentration using Pierce™ BCA Protein Assay Kit (Thermo Scientific).

30 Use the same buffer used for tissue homogenization as the HPLC mobile phase.

31   Perform HPLC using a Kinetix 2.6um C18 column (4.6 × 100 mm, Phenomenex, Torrance, CA USA).

Unbiased stereology to count midbrain dopaminergic neurons

32   Use the optical fractionator probe of the StereoInvestigator (Software Version 8.1, Micro-brightfield Inc., Colchester, USA) connected to a brightfield microscope to perform stereological quantification of tyrosine hydroxylase (TH) positive dopaminergic neurons.

33   Delineate the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) on every 6th TH+ve midbrain section using a 10X objective.

34   Identify the mounted thickness as ⊣⊢ 22.5 μm , which is also determined at every fifth counting site.

35   Imply a guard zone of ⊣⊢ 3.5 μm on either side, thus providing ⊣⊢ 15 μm of z-dimension to the optical dissector.

36   Count the neurons using a 40X objective with a regular grid interval of 22,500 μm² (x=150 μm, y=150 μm) and a counting frame size of 3600 μm² (x=60 μm, y=60 μm).

37   Begin the quantification at the first anterior appearance of TH+ve neurons in the midbrain structures to the caudal most part in each hemisphere separately, which is later summed to derive total numbers.

Electron Microscopy Tissue Preparation and Settings

38   Make sure that both freshly prepared saline and fixatives (2% PFA + 2% GA) are chilled.

39   Anesthetize the mouse with isoflurane inhalation in a desiccator.

40   Perform intracardial perfusion with chilled 0.9% heparinized saline for ~15-20 minutes (until the fluid that comes out of the heart become colorless) at a flow rate of 🧪 2 mL /minute.

41 Change the perfusion solution to a fixative, (2% PFA + 2% GA) (chilled) and flush it for 20-25 minutes (until tail, head and neck are stiff) at a flow rate of ⚗ **2 mL** /minute.

42 After intracardial perfusion, remove the whole brain from the skull and immerse in 2.5% glutaraldehyde + 2% PFA + **[M]** 0.1 Molarity (M) cacodylate buffer, 🕐 **02:00:00** at 🌡 **Room temperature** , followed by 🕐 **Overnight** incubation at 🌡 **4 °C** (immersion fixation).

4h

43 Next day, perform vibratome sectioning (⊣⊢ **200 μm** thick) of your area of interest*, using chilled **[M]** 0.1 Molarity (M) PBS (in the vibratome trough).

44 Immediately after sectioning, transfer the ⊣⊢ **200 μm** thick sections back to 2.5% glutaraldehyde + 2% PFA + **[M]** 0.1 Molarity (M) cacodylate buffer and allow it equilibrate for ~ 🕐 **00:30:00** at 🌡 **4 °C** .

30m

45 Wash the sections.

45.1 Wash the sections in **[M]** 0.1 Molarity (M) cacodylate buffer for 🕐 **00:02:00** . (1/3)

2m

45.2 Wash the sections in **[M]** 0.1 Molarity (M) cacodylate buffer for 🕐 **00:02:00** . (2/3)

2m

45.3 Wash the sections in **[M]** 0.1 Molarity (M) cacodylate buffer for 🕐 **00:02:00** . (3/3)

2m

45.4 Then, place the sections in the same buffer and deliver them to EM core for further processing.

46 EM imaging can be performed using FEI Tecnai G2 Spirit BioTwin Microscope.

47 Images are analysed using either FIJI (NIH) or iTEM software (ResAlta Research Technologies, USA).

Confocal/Fluorescence Microscopy and Image Analysis

48 Acquire fluorescent images using a laser scanning confocal microscope (LSM 800, Zeiss) with a 20X or 40X or 63X objectives or using a fluorescence slide scanner (VS200, Olympus) at 40X objective.

49 Ensure appropriate Z-depth is used.

50 Blind all images for genotype and age before subjecting to analysis using FIJI software from National Institute of Health (NIH).

51 After performing sum intensity projection, measure the expression intensity on an 8-bit or 16-bit image as the mean gray value on a scale of 0-255 or 0-65536, respectively.

52 To count microglial cells, threshold images using the 'otsu' algorithm and count cells larger than 75-pixel units for a given image using the 'analyze particles' function.

53 To count immunofluorescence labeled dopaminergic neurons, use a similar method with a size threshold of 25 μm^2 and above.

54 Count astroglial cells manually using the 'cell counter' function.

55 To count abnormal structures in the terminals, threshold images using the 'triangle' algorithm, followed by 'analyse particles' function. Count all the structures of size 5 μ^2 and above and the circularity between 0.3 to 1.

56 Count autophagic punctae manually using the 'cell counter' function, which can be further validated on thresholded images using 'triangle' algorithm, followed by 'analyse particles' function (size: 0.5 μ^2, circularity: 0.0 to 1).

57 Perform colocalization analysis using the "Coloc 2" function.

Western blotting

58 Homogenize the samples using an appropriate buffer and determine their protein concentration using a protein assay (such as the Bradford or BCA assay). Adjust the protein concentration of the samples to the desired amount using the buffer.

59 Heat the samples at 🌡 95 °C for ⏱ 00:10:00 if fresh, or 5 minutes if previously heated, to denature the proteins.

10m

60 Load precast or poured polyacrylamide gels with the desired concentration and number of wells. Ensure that the gel is completely polymerized before loading the samples.

61 Run the gels at 65 V through the stacking gel for about 30-45 minutes until the dye front reaches the top of the stacking gel. Then, increase the voltage to 120 V and run until the dye front reaches the bottom of the resolving gel or runs off the gel.

62 Incubate the PVDF membrane in 100% methanol for ⏱ 00:01:00 on a shaker to activate the membrane. Discard the methanol and rinse the membrane with distilled water.

1m

63 Incubate the activated membrane in distilled water for ⏱ 00:01:00 on a shaker to rehydrate the membrane.

1m

64 Soak the membrane in transfer buffer for 10-15 minutes prior to use to equilibrate the membrane with the buffer.

65 Assemble the transfer sandwich by placing the gel on top of the anode (black side), followed by a sponge soaked in transfer buffer, a piece of transfer paper, the PVDF membrane, another piece of transfer paper, a sponge soaked in transfer buffer, and finally the cathode (white side).

> **Note**
>
> Make sure the white side of the cassette is oriented towards the red electrode so the current passes through the gel to the membrane.

66 Transfer the proteins from the gel to the membrane by running the transfer at 🌡 4 °C at constant 400 mAmps for ⏱ 04:00:00 (⏱ Overnight) or at constant 100 V for ⏱ 01:00:00 , depending on the properties of the protein of interest.

6h

67 After transfer, incubate the blots in ⚗ 6 mL of blocking solution (such as 1X TBS + 5% non-fat milk + 5% goat serum) for ⏱ 01:00:00 at 🌡 Room temperature on a rocker to block nonspecific binding sites.

1h

68 Incubate the blots 🕐 Overnight on a rocker at 🌡 4 °C in the primary antibody diluted in 1X TBS + 5% non-fat milk + 5% goat serum.

> **Note**
>
> **Optional**: Add 🧪 5 μL of 10% SDS to the primary antibody solution.

1h

69 Wash the blots with 1X TBST (TBS + 0.1% Tween-20) to remove unbound primary antibody.

69.1 Wash the blots for 🕐 00:05:00 with 1X TBST. (1/3)

5m

69.2 Wash the blots for 🕐 00:05:00 with 1X TBST. (2/3)

5m

69.3 Wash the blots for 🕐 00:05:00 with 1X TBST. (3/3)

5m

70 Incubate the blots for 🕐 01:00:00 at 🌡 Room temperature on a rocker in the secondary antibody diluted in 1X TBST + 5% non-fat milk + 5% goat serum (optional: add 5 μL of 10% SDS to the secondary antibody solution).

1h

71 Wash the blots 3 times for 5 minutes each with 1X TBST to remove unbound secondary antibody.

71.1 Wash the blots for 🕐 00:05:00 each with 1X TBST. (1/3)

5m

71.2 Wash the blots for 🕐 00:05:00 each with 1X TBST. (2/3)

5m

71.3 Wash the blots for 🕐 00:05:00 each with 1X TBST. (3/3)

5m

71.4 Rinse the blots with TBS to remove residual Tween-20.

72 Image the blots using an appropriate detection method (such as chemiluminescence or fluorescence) and a suitable imaging system. Store the blots in TBS buffer or dry them and store them between filter papers in a light-protected bag.

Surgery and in vivo Fast Scanning Cyclic Voltammetry (FSCV)

73 Anesthetize mice using isoflurane (SomnoSuite Small Animal Anesthesia System, Kent Scientific; induction 2.5%, maintenance 0.8–1.4% in O2, 0.35 l/min).

74 Fix the head of the mouse on a stereotaxic frame (Kopf Instruments, Tujunga, CA).

75 Apply Puralube vet ointment on the eye to prevent cornea from drying out.

76 Use a stereotactic drill (0.8 mm) to perform a craniotomy (unilateral, right) to target the midbrain and dorsal striatum with the following coordinates (values are in mm from Bregma):

76.1 Midbrain: anteroposterior = -2.9, mediolateral=+1.0, dorsoventral=+4

76.2 Dorsal Striatum: anteroposterior = +1.2, mediolateral = +1.3, dorsoventral = +3.1

77 Place an Ag/AgCl reference electrode via a saline bridge under the skin.

78 Lower a 22G bipolar stimulating electrode (P1 Technologies, VA, USA) to target ventral midbrain (between 4-4.5mm). Adjust the exact depth for maximal dopamine release.

79 Lower a custom-built carbon fiber electrode (5 μm diameter, cut to ~150 μm length, Hexcel Corporation, CT, USA) to reach dorsal striatum for recording the evoked dopamine release.

80 Use diI-coated carbon-fiber electrodes to identify the electrode position in the dorsal striatum and the electrode track in the brain tissue to identify the position of the stimulation electrode.

81 Measure the evoked dopamine release using constant current (400μA), delivered using an Iso-Flex stimulus isolator triggered by a Master-9 pulse generator (AMPI, Jerusalem, Israel). A single burst stimulation consists of 30 pulses at 50Hz (0.6s).

82   Calibrate electrodes using a known concentration of dopamine in artificial cerebrospinal fluid (ACSF).

83   Use IGOR Pro for the data acquisition and analysis.

> **Note**
>
> Acquire code here: https://doi.org/10.1073/pnas.2013652117.

Protocol references

IGOR Pro: https://doi.org/10.1073/pnas.2013652117.