

Studying BDNF/TrkB signaling: transcriptome analysis from a limited number of purified adult or aged murine brain neurons

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Abstract

It is recognized by now that the basal ganglia contain some of the circuits most vulnerable to age-related effects. However, it is still unknown how these changes are regulated during aging. We have recently shown that loss of TrkB signalling in striatopallidal enkephalinergic (ENK⁺) neurons leads to age-dependent spontaneous hyperlocomotion, associated with reduced striatopallidal activation, demonstrating that BDNF-TrkB signalling in striatal ENK⁺ neurons contributes to the inhibitory control of locomotor behaviour exerted by the indirect pathway. Hence, we have established a unique mouse model that provides a rare example of an age-dependent locomotor defect. Identification of the genes and associated molecular pathways relevant to maintenance of locomotor control requires systematic, unbiased gene expression profiling of the aging striatal circuit from young adult and aged mouse brain, both in normal and TrkB-deficient conditions. For this purpose, we have chosen whole transcriptome analysis by RNA sequencing (RNA-Seq), that offers higher resolution than other methods. To achieve this we have established a protocol that allows for the isolation of fluorescently labeled neurons from adult (3 months) or aged (8 months) mouse brain for whole transcriptome analysis by RNA-Seq using a limited number (<200) of neurons. Neuronal subsets were genetically labeled *in vivo* with a fluorescent marker and isolated using a sucrose artificial cerebrospinal fluid (aCSF) solution and differential centrifugation before fluorescent activated cell sorting (FACS)-based purification. This was followed by direct cDNA synthesis using an optimized Smart-Seq method, resulting in the generation of robust libraries for Illumina sequencing. In contrast to previous methods used for neuronal gene profiling this protocol can be used for high-throughput gene expression profiling from limited numbers of adult or aged brain neurons at moderate costs. The whole protocol described here takes 3-4 days from neuronal purification to preparation of cDNA libraries ready for Illumina sequencing.

Key words

Neuronal purification, adult and aged murine brain, FACS-based purification, transcriptome analysis, RNA-Seq.

INTRODUCTION

Among risk factors associated with common neurological disorders affecting cognitive and motor functions, aging is the most significant [1]. It is also clear that the age-related changes are initially very subtle and affect specific neuronal circuits [2]. Unbiased studies of the aging brain circuits [3] require a systematic analysis of specific cell types forming the particular neuronal circuit, either as a whole cell population or as single cells, from young adult and aged mouse brain. For this purpose, quantitative transcriptome analysis, such as RNA sequencing, offers higher resolution than other methods [4]. However, it has proven a significant challenge to isolate adult or aged neurons under conditions that allow transcriptome analysis from single or limited numbers of neurons. Current protocols are restricted to the isolation of neurons from immature, juvenile/young adult brain [5, 6], and the best developed technology to analyse gene expression changes in specific neuronal populations from the adult mouse brain, requires a great number of cells (30,000 to 60,000 neurons). This can only be obtained from a high number of animals (4 to 5 animals for one biological replicate) [7], making experiments needing multiple comparison analysis unfeasible, and masking phenotypic heterogeneity of both cells and individuals. Here we describe an optimized version of a previously published method (Smart-Seq) [8][9] that allows purification of adult and aged CNS neurons and generation of sequencing libraries from as few as 200 neurons, permitting subsequent accurate quantification of gene expression changes from rare populations in individual mice.

Development/Overview of the protocol

We first developed a reliable method to purify genetically fluorescence-labeled neurons for transcriptome analysis by using previously established BAC-transgenic Cre line (BAC-Penk-Cre) crossed to a reporter line that expresses tdTomato fluorescent protein upon Cre-recombination in specific neuronal cell types [3, 10]. We then focused on the enkephalinergic medium spiny neurons (MSNs), as the BAC-transgenic line drives Cre expression in this subpopulation within the striatum [3]. The tdTomato reporter fluorescently labels enkephalinergic neurons red (Figure 1A). Adult (3

months old) and aged (8 months old) mice were used in the experiments described in this protocol. Mice were first transcardially perfused with a chilled sucrose artificial cerebrospinal fluid (aCSF) solution. This step is important to protect the brain as much as possible during further tissue dissociation and to clear it from blood. After perfusion, the brains were quickly removed and the region of interest (striatum) further dissected. This was followed by tissue dissociation that includes enzymatic digestion followed by trituration to obtain a single cell suspension. The cell suspension is then passed through a discontinuous gradient to eliminate excess debris. Cells were then labelled with LIVE/DEAD® Fixable Violet cell stain to assess live and dead cells by flow cytometry and then sorted to purify tdTomato positive neurons. Two hundred neurons were sorted in appropriate volumes according to the SMARTer® Ultra® Low RNA Kit instructions. This allowed us to obtain efficient cDNA synthesis from a very low number of adult and aged neurons, followed by robust library generations for Illumina sequencing. Below we describe the optimized SMART-Seq method to perform RNA-Seq from 200-sorted adult and aged neurons.

Applications and comparison with other methods

This recently established method is an excellent opportunity to identify age related molecular changes *in vivo* that associate with motor impairment. In particular, this method will allow us to initiate molecular dissection of signalling pathways activated by BDNF/TrkB and contributing to maintain locomotor activity control during aging. Generally, this method can be applied to study different neuronal populations from normal and/or diseased adult and aged mouse brain by using few purified neurons (200) from each single mouse brain. This now allows molecular characterization of mature (3 months old) and aged (8 months old) mouse CNS neuron subtypes that have been so far inaccessible. Previous methods were limited to the isolation of neurons from immature, juvenile/young adult mouse brain [5, 6], and/or required a remarkable number of cells for gene expression analysis [7].

MATERIALS

REAGENTS

- Pentobarbital sodium salt, Sigma-Aldrich, P3761
- Calcium chloride dihydrate, Sigma Aldrich, C3306-100G
- Glucose α -D-Glucose - anhydrous, Sigma Aldrich, 158968-500G
- Magnesium chloride hexahydrate, VWR, 436992S
- Potassium chloride, Fisher Scientific, 10355810
- Sodium bicarbonate, Sigma Aldrich, S8875-500G
- Sodium chloride, Fisher Scientific, 10316943
- Sodium dihydrogen phosphate monohydrate, Sigma Aldrich, S9638
- D-Sucrose, Fisher Scientific, 10638403
- RNaseZap, Ambion, AM9780
- DNA-OFF, Takara Bio, 9036
- Nuclease-free water, Ambion, AM9930
- StemPro Accutase cell dissociation reagent, Gibco, A11105-01
- Percoll, Sigma-Aldrich, P1644
- Hibernate A low fluorescence, BrainBits, HA-Lf
- Trypan blue 0.4%, Sigma-Aldrich, T8154
- LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, Molecular Probes, L34955
- SMARTer® Ultra® Low RNA Kit for Illumina sequencing, 100 reactions, Clontech, 634936
- Agencourt AMPure XP beads, Beckman Coulter, A63882
- Absolute ethanol, VWR, 20821.330
- Advantage 2 PCR kit, Clontech, 639206
- Nextera XT DNA sample preparation kit, 96 samples, Illumina, FC-131-1096

EQUIPMENT

- Needle (18G, pink) disposable, sterile for use with SZR -150 and SZR -160, 40 mm, 18 gauge, wall type thin, Microlance, Fisher Scientific, 10162534
- Needle (25G, orange) disposable, sterile for use with SZR -150 and SZR -160, 25 mm, 25 gauge, wall type regular, Microlance, Fisher Scientific, 12389169
- Surgical blades, Swann-Morton, No 11, non-sterile, carbon steel
- Syringe, 1 mL disposable sterile plastic concentric luer tip without needles 1 mL, Plastipak, Fisher Scientific, 10142104
- Forceps Dumont #5 straight, 110 mm length, Fine Science Tool (F.S.T), Carbon steel, 0.3 mm, 11251-10
- Forceps Dumont #7 curved, 115 mm length, Fine Science Tool (F.S.T), Carbon steel, 0.3 mm, 11271-10
- Dissecting scissors straight, 9.5 cm, Titanium, 0.3 mm, Fine Iris Scissors, 14094-11
- Dissecting scissors curved, 9.5 cm, Titanium, 0.3 mm, Fine Iris Scissors, 14095-11
- Perfusion pump, Cole-Parmer, Masterflex L/S Economy Drive, model 77202-50
- Tubing, silicone, HelixMark™, for peristaltic pumps, VWR, 228-1123
- Spatula 2350 Chattaway micro stainless steel 100 mm x 4 mm Fisher Scientific, 11523482
- Spatula 2123 lightweight, flat with rounded ends 150 mm x 30 mm, Fisher Scientific, 11786597
- Dissection board resin bonded cork 450 mm (w) x 20 mm (d) x 305 mm (h), Thermo Scientific Raymond Lamb, Fisher Scientific, 12628576
- Rotating wheel, Stuart rotator SB2 with the microtube holder SB3/1
- Vortex mixer
- Glass Pasteur pipette, Fisher FB50251
- 8-strip microcentrifuge tube, 0.2 mL, Starlab I1402-2908
- Microcentrifuge tube, 1.7 mL, low binding, Costar, #3207
- Centrifuge tube, 15 mL, Corning 430790

- Centrifuge tube, 50 mL, Corning 430829
- Teclu burner, Usbeck 1421, and laboratory lighter
- Fuel cartridge, Usbeck 1430
- Presept disinfectant tablets, Fisher 12368667
- Cell strainer, 40 μ m, BD Biosciences 352340
- Cell strainer, 100 μ m, BD Biosciences 352360
- Refrigerated benchtop centrifuge, Eppendorf 5417R, fixed angle rotor, F45-30-11
- Refrigerated benchtop centrifuge such as the Eppendorf 5810R with a swing bucket rotor, (or ultracentrifuge, Beckman, L8-M)
- Swinging bucket rotor (Beckman, SW41 Ti)
- Ultra-clear centrifuge tubes (Beckman, 344059)
- Haemocytometer
- Inverted phase contrast microscope (and 20X objective lens)
- BD FACSAria Fusion cell sorter, Becton Dickinson 656700, San Jose, CA, USA.
- Veriti 96-well thermal cycler (Applied Biosystems, 4375786)
- MagnaBot II magnetic separation device (Promega, V8351)
- Magnetic stand-96 (Ambion, AM10027)
- Axygen 96-well V-bottom plate (Axygen, P-96-450V-C)
- Semi-skirted 96-well PCR plate
- Adhesive PCR plate seal (Thermo Scientific, AB-0558)
- Filter tips (Axygen, 10 μ L, TF-300-L-R-S; 20 μ L, TF-20-L-R-S; 200 μ L, TF-200-L-R-S; 1,000 μ L, TF-1000-L-R-S)
- Qubit assay tubes, Invitrogen Q32856
- Qubit dsDNA high-sensitivity (HS) kit, Invitrogen Q32854
- Qubit 2.0 fluorometer, Invitrogen 32866
- Agilent 2100 Bioanalyzer, Agilent Technologies G2938C
- Agilent high-sensitivity DNA kit, Agilent Technologies 5067-4626

- HiSeq 2500 ultra-high-throughput sequencing system (Illumina)

REAGENT SETUP

Sucrose aCSF

1,000 mL

	(mg)
75 mM sucrose (MW 342.29)	25,670
87 mM NaCl (MW 58.44)	5,080
25 mM NaHCO ₃ (MW 84.01)	2,100
2.5 mM KCl (MW 74.54)	190
1.25 mM NaH ₂ PO ₄ ·H ₂ O (MW 137.99)	170
0.5 mM CaCl ₂ ·2H ₂ O (MW 147.01)	70
7 mM MgCl ₂ ·6H ₂ O (MW 203.30)	1,420
25 mM glucose (MW 180.16)	4,500

Mix and heat (~50°C) reagents in autoclaved H₂O until they are completely dissolved. Measure the pH with a pH meter and adjust it to 7.3-7.4 with 37% HCl. Filter the solution (do not autoclave).

Make 50 mL aliquots and keep it at -20°C.

Accutase

Thaw a new bottle of accutase at 4°C overnight (do not thaw it at 37°C). Make 5 mL aliquots and keep it at -20°C.

Percoll

Make 1 mL aliquots of Percoll and keep it at 4°C.

LIVE/DEAD® Fixable Violet Dead Cell Stain

Bring one vial of fluorescent dye (component A) and one vial of DMSO (component B) to room temperature (avoid light). Add 50 µL DMSO to a vial of fluorescent dye and mix by vortexing. Ensure that the dye is completely dissolved and spin down. Make 5 µL aliquots, seal the lid of each aliquot with parafilm and keep all aliquots at -20°C.

EQUIPMENT SETUP

Large-, medium- and small-diameter Pasteur pipettes

For medium- (~0.8 mm) and small- (~0.4 mm) diameter Pasteur pipettes, heat the open end of a Pasteur pipette with a Teclu burner until the opening is closed. Break the close end with tissue paper and use another Pasteur pipette to enlarge the opening to the desired size. The large- (~1.2 mm) diameter pipettes do correspond to the normal Pasteur pipette diameter, therefore require no manipulation. The diameter of the Pasteur pipettes can be confirmed using a microscope with a calibrated reticule. However, it is also possible to purchase Pasteur pipettes with different diameters. Clean all Pasteur pipettes by pipetting with RNaseZap 5 times then with nuclease-free water 5 times. Put the Pasteur pipettes in an autoclavable box and autoclave to sterilise them, or dry heat at 160°C.

PROCEDURE

Brain tissue dissociation (timing 1-2 hours)

1. A day before the experiment, bring an aliquot of sucrose aCSF and accutase from -20°C to 4°C and thaw them overnight; then place them on ice.
2. Transfer the mice to the procedure room at least 30 minutes before starting the procedure to allow them to habituate to the environment.
3. Prepare the apparatus (perfusion pump) and flush the tubing by running a few mL of sucrose aCSF through it. Set the speed of the perfusion pump to a pressure of 80 mmHg.
4. Set up all needed surgical tools.
5. Anesthetise the mouse by administering pentobarbital solution (according to manufacturer guidelines) via intraperitoneal injection.
6. Determine the depth of anesthesia via toe pinch-response method. If the animal is unresponsive continue to the next step.
7. Immobilize the animal on a proper surface (dissection board), make a small incision in the diaphragm with the curved dissecting scissors; continue along the rib cage on both sides. Lift the sternum to enter the thoracic cavity.
8. Remove any connective tissue around the heart. Hold the heart gently with sharp-end forceps.
9. Pass a 25G perfusion needle into the ascending aorta through the left ventricle.
(See Note 1).
10. Switch on the perfusion pump. Immediately make an incision into the right atrium of the animal heart with dissecting scissors to let the blood out and replaced by the chilled perfusion solution (sucrose aCSF). (See Note 2).
11. Use approximately 20 mL sucrose aCSF per animal.
12. Decapitate and quickly open the skull by using dissecting scissors, remove the brain and place it on an ice-cold metal plate for further dissection of the brain area of interest.

13. Mince the dissected brain area (e.g. striatum) with a surgical blade on an ice-cold metal plate. Transfer minced striatal tissue (typically 0.02 g of tissue for the striatum of one mouse) to a 1.5 mL microcentrifuge tube containing 1 mL ice-cold accutase cell dissociation reagent.
14. Digest the tissue by placing the microcentrifuge tube on a rotating wheel for 30 minutes at 4°C.
15. After 30 minutes incubation, centrifuge the dissociated striatal tissue at 450 *g*, at 4°C for 2 minutes. Discard the supernatant and resuspend the pellet in 1 mL ice-cold Hibernate A.
16. Dissociate the cells by pipetting (ten times) first with a large-diameter Pasteur pipette. Place the microcentrifuge tube on ice for 2 minutes to allow the large pieces of tissue to settle at the bottom of the tube. Transfer 600 µL cloudy suspension to a new pre-chilled 15 mL tube and place it on ice. Add 600 µL ice-cold Hibernate A into the original 1.5 mL microcentrifuge tube.
17. Repeat the dissociation step with the medium- and the small-diameter Pasteur pipettes. Pool the cloudy suspension from the second and third round with the suspension from the first round in the 15 mL tube. After the third round, add 750 µL (instead of 600 µL) ice-cold Hibernate A into the original microcentrifuge tube and mix by inverting the tube. Place the microcentrifuge tube on ice for 2 minutes and then transfer approximately 1,000 µL cloudy supernatant to the same 15 mL tube.
18. Pre-wet 100 µm and 40 µm cell strainers by using 1 mL Hibernate A. Remove any cell clumps remaining in the cell suspension by passing the cell suspension first through 100 µm cell strainer placed on top of an ice-cold 50 mL tube, then repeat with 40 µm cell strainer. Approximately 2.5 mL cell suspension can be obtained from one mouse.

Preparation of Percoll density gradient (timing 30 minutes to 1 hour)

19. Prepare three layers of Percoll gradient (volumes shown in the table below are **designed for the striatum of one mouse**). Adjust the volume of each reagent according to the number of samples).

Layer	Percoll (μL)	Hibernate A (μL)	1 M NaCl (μL)	Total (μL)
1 (bottom)	206.13	856.5	24.45	1,087.08
2 (middle)	162.63	900	19.13	1,081.75
3 (top)	120.08	942.5	14.88	1,077.45

- Pipette 1 mL of layer 1 (bottom) into a new ultra-clear centrifuge tube, gently and slowly followed by 1 mL of layer 2 (middle) and 3 (top), respectively. Keep the gradient at 4°C. (See Note 3).
20. Carefully apply the cell suspension on the top of the prepared Percoll density gradient. The cell suspension should float on top of the gradient. (See Note 4).
21. Centrifuge the gradient at 430 *g*, 4°C for 5 minutes. Make sure that the deceleration speed of the centrifuge is as slow as possible. We set acceleration at 'max' and deceleration at '0'.
22. Discard the top layer (approximately 1.5-2.5 mL), which contains mainly cellular debris (Figure 1B). Resuspend the remaining layers.
23. Centrifuge at 550 *g*, at 4°C for 5 minutes (acceleration set at 'max' and deceleration set at '0').
24. Discard supernatant, and resuspend the pellet in 1 mL ice-cold Hibernate A.

Viability staining (timing 40 minutes to 1 hour)

25. Transfer 10 μL of cell suspension into a microcentrifuge tube. Add 40 μL Hibernate A and 50 μL 0.4% Trypan blue and mix gently by pipetting. Pipette 10 μL of the mix to each of the two chambers of the haemocytometer (double chambers are most common for the Neubauer chamber cell counting). Use an inverted phase contrast microscope (with 20X objective lens), and count bright round cells within the central square in the middle of each of the two chambers (the central square is split in 25 squares of width 0,2 mm (**200 μm**); each of the 25 squares is subdivided in 16 small squares). Average the number of counts

obtained from the two central squares and calculate the number of live cells using the following formula:

$$\text{Concentration} = \frac{\text{Number of cells} \times 10.000}{\text{Number of square} \times \text{dilution}}$$

26. Thaw LIVE/DEAD fixable dead cell stain by leaving an aliquot at room temperature. Stain cells by adding 0.25 μL LIVE/DEAD fixable dead cell stain for every 1,000,000 cells. Incubate on ice (in the dark) for 30 minutes. Wash with 1 mL PBS and centrifuge at 550 g , 4°C for 5 minutes. Discard the supernatant and resuspend the cell pellet in 1 mL ice-cold Hibernate A.

Preparation of lysis buffer for collecting sorted cells (timing 30 minutes)

27. In the hood, prepare the reaction buffer stock solution by mixing 1 μL RNase inhibitor with 19 μL of dilution buffer (a component of the SMARTer® Ultra® Low RNA Kit), and scale up as needed.
28. Total volume of lysis buffer is 3.5 μL . This consists of 2.5 μL reaction buffer, 0.3 μL nuclease-free water added to each tube of an 8-strip 0.2 mL thin-walled PCR tube, and 0.7 μL volume containing 200 sorted cells. This is the number of cells we usually sort into each collection tube. We normally use 100 μm nozzle on a FACSAria Fusion Cell Sorter so each cell will be sorted in a 0.0035 μL volume.

FACS (timing 1-2 hours)

29. Viable neurons are sorted using a FACSAria Fusion Cell Sorter.
30. A number of gates are used to identify the cells of interest. Forward angle light scatter (FSC) and orthogonal light scatter (SSC) by cells were measured using a 488 nm laser and 488/10 nm band pass filters (Figure 2A). (See Note 5).
31. These cells are subjected to further gating to remove unwanted cell doublets by using width measurements from the light scattering pulses (Figure 2D-E).

32. Viable cells are identified from non-viable cells by exclusion of LIVE/DEAD violet intensity. LIVE/DEAD fluorescence is detected by excitation at 405 nm and measuring emission using a 450/50 nm (Figure 2F).
33. Expression of tdTomato **by** viable cells is detected by fluorescence using excitation at **561 nm** and measuring emission using a 582/15 nm band pass filter. (Figure 2G).
34. Viable tdTomato-positive cells (one to two hundred, depending on sample availability) are sorted on the basis of high fluorescence expression into a 0.2 mL thin-walled PCR tube containing 2.8 μ L lysis buffer. (Figure 3).
35. Immediately after reaching the desired number of cells, briefly spin the collection tube and quickly freeze on dry ice. (See Note 6-7).

First strand cDNA synthesis (timing 3-4 hours)

The protocol described below is optimized to prepare cDNA from 200-sorted neurons using the SMARTer® Ultra® Low RNA Kit for Illumina sequencing. It is critical to clean the hood, racks and pipettes with RNaseZap before next step. (See Note 8).

36. Thaw all reagents except reverse transcriptase and RNase inhibitor at room temperature.
37. Prepare the RT mix for all samples plus one additional sample by combining and mixing all reagents except reverse transcriptase and RNase inhibitor listed below in the table.

Reagents	Volume per one reaction (μ L)
5x first-strand buffer	2
100 mM DTT	0.25
10 mM dNTP mix	1
12 μ M SMARTer II A oligonucleotide	1
RNase inhibitor	0.25
SMARTScribe reverse transcriptase	1

38. Preset a thermal cycler at 72°C with heated lid (105°C) for 3 minutes.
39. Transfer 3.5 μ L cell lysate to a new 8-strip 0.2 mL thin-walled PCR tubes placed on pre-chilled IsoFreeze PCR rack. Add 1 μ L of 3' SMART CDS primer II A (12 μ M). Mix briefly by vortexing and spin down.

40. Incubate the samples at 72°C for 3 minutes and immediately put them back at 4°C in an isofreeze rack.
41. Add reverse transcriptase and RNase inhibitor to RT reaction mix while performing annealing of CDS primer in step 40 in order to jump start cDNA synthesis, thereby minimizing the cDNA synthesis bias.
42. Add 5.5 µL of the RT master mix into each 0.2 mL thin-walled tube. Mix gently by vortexing and spin down briefly to collect all contents.
43. Incubate the samples at 42°C with heated lid for 90 minutes. Terminate reaction by incubating the tubes at 70°C for 10 minutes.
44. While performing step 43 bring Agencourt AMPure XP beads to room temperature for at least 30 minutes before proceeding to next step.
45. To purify the first strand cDNA, mix the beads evenly by vortexing, add 25 µL Amgencourt AMPure XP beads to each sample and mix by pipetting for 10 times. Briefly spin down the contents in tubes.
46. Incubate samples with Agencourt AMPure XP beads at room temperature for 8 minutes. Then place the sample tubes on the MagnaBot II magnetic separation device for 5 minutes or longer until the solution is completely clear.
47. Discard the supernatant while the microcentrifuge tubes are on the magnetic separation device. Spin the microcentrifuge tube briefly to collect remaining liquid.
48. Place the sample tubes back on the MagnaBot II magnetic separation device for 2 minutes. Pipette out the residual liquid while the tubes are on the magnetic separation device.

cDNA amplification (timing 2 hours)

49. Prepare PCR master mix for all samples plus one additional reaction in the following order at room temperature.

Reagents	Volume per one reaction (µL)
10x first-strand buffer	5

10 mM dNTP mix	2
12 μ M IS PCR primer	2
Nuclease-free water	39
50x Advantage 2 polymerase mix	2

50. Add 50 μ L PCR master mix to each sample. Mix by vortexing and briefly spin down.

51. Transfer all samples from the clean room to general lab. Place the sample tubes in a preheated thermal cycler with heated lid using the following program:

Step	Temperature	Time
1	95°C	1 minute
2	95°C	15 seconds
	65°C	30 seconds
	68°C	6 minutes
3	Repeat step 2 for another 14 cycles (15 cycles in total)	
4	72°C	10 minutes
5	4°C	until collected

Purification of amplified cDNA (timing 30 minutes)

52. Bring Agencourt AMPure XP beads to room temperature for at least 30 minutes before use.

Mix the beads evenly by vortexing.

53. Transfer PCR product including the beads from step 51 to each well of Axygen 96-well V-bottom plate. Add 90 μ L Agencourt AMPure XP beads to the wells. Mix by pipetting 10 times. Incubate at room temperature for 8 minutes to allow the DNA binding to beads.

54. Place the 96-well plate on the Ambion magnetic stand-96 for 8 minutes or longer. Ensure that supernatant becomes clear of any beads. Pipette out the supernatant while the 96-well plate is still on the magnetic stand.

55. Add 200 μ L freshly prepared 80% ethanol to each sample. Try not to disturb the beads. Wait for 30 seconds and carefully discard supernatant.

56. Repeat step 55 for one more time.

57. Discard the supernatant. Ensure that no ethanol is left. Leave the 96-well plate on the magnetic stand at room temperature for 5 minutes or longer until first tiny cracks are seen in each pellet. (See Note 9).
58. Add 12 μ L purification buffer to each well to cover the beads. Remove the 96-well plate from the magnetic stand and incubate at room temperature for 2 minutes. Mix the pellet by pipetting up and down 10 times, and incubate the plate at RT for 2 minutes to elute DNA from beads.
59. Place the 96-well plate back on the magnetic stand for 1 minute. Collect only 10 μ L of the supernatant from each well into a new 96-well PCR plate. (See Note 10-11).

Quality check of the cDNA library (timing 1 hour)

60. Check the cDNA library size distribution on a Bioanalyzer using high sensitivity DNA chip from Agilent's High sensitivity DNA kit. A good cDNA library (Figure 4A, 4C) should peak at 1.5-2 kb without any short fragments (<500 bp).
61. Calculate the cDNA libraries concentration of each sample using Agilent Bioanalyzer software by setting up the DNA size ranging from 200 bp to 9,000 bp in size. Use the concentration indicated to determine the volume of DNA required (1 ng) for Illumina sequencing library preparation. (See Note 12).

Tagmentation and indexing (timing 2-3 hours)

62. Perform tagmentation with Nextera XT DNA sample preparation kit. Use 1 ng of cDNA library for tagmentation. Dilute the cDNA to have a final volume of 5 μ L and transfer to 96-well semi-skirted PCR plate.
63. Thaw amplicon tagment mix (ATM), tagment DNA (TD) buffer at room temperature. Mix by vortexing and briefly spin down.
64. Add 10 μ L TD buffer to each sample.

65. Add 5 μ L ATM to each sample, gently pipette 5 times to mix. Seal the 96-well plate with an adhesive PCR plate seal.
66. Centrifuge at 280 g, at 4°C for 30 seconds.
67. Place the 96-well plate in a preheated thermal cycler using the following program.

Step	Temperature	Time
1	55°C	5 minutes
2	10°C	Hold

68. Once the samples reach 10°C continue to the next step immediately.
69. Remove the adhesive PCR plate seal, add 5 μ L neutralize tagment (NT) buffer to each well. Gently pipette 5 times to mix.
70. Cover the 96-well plate with an adhesive PCR plate seal. Centrifuge at 280 g, at 4°C for 30 seconds.
71. Incubate the 96-well plate at room temperature for 5 minutes.
72. At room temperature, thaw the Nextera PCR master mix (NPM) and the index primers. Set up the PCR reaction by adding reagents in the following order:

Reagents	Volume per one reaction (μ L)
NPM	15
Index primer 1 (N7xx)*	5
Index primer 2 (N5xx)*	5

*Combination of the two index primers needs to be unique to each sample.

73. Add 25 μ L of the above master mix to sample mix. Mix by gently pipetting 5 times. Cover the 96-well plate with an adhesive PCR plate seal. Centrifuge at 280 g, at 4°C for 30 seconds
74. Perform PCR using the following program:

Step	Temperature	Time
1	72°C	3 minutes
2	95°C	30 seconds
3	95°C	10 seconds
	55°C	30 seconds

4	72°C	30 seconds
	Repeat step 3 for another 11 cycles (12 cycles in total)	
5	72°C	5 minutes
6	10°C	Hold

75. Perform the bead clean-up as outlined above (steps 52-56). Use 50 μ L Agencourt AMPure XP beads instead of 90 μ L.
76. Discard the supernatant. Ensure that no ethanol is left. Leave the 96-well plate on the magnetic stand at room temperature for 15 minutes.
77. Add 25 μ L resuspension buffer (RSB). Remove the plate from the magnetic stand and gently pipette up and down 10 times to mix the contents. Incubate at room temperature for 2 minutes. Place the 96-well plate on the magnetic stand for 2 minutes. Transfer approximately 23 μ L eluted indexed DNA to a new 96-well skirted PCR plate. (See *Note 13*).

Quality check of indexed cDNA library (timing 1 hour)

78. Check the average size of each library on a Bioanalyzer with high sensitivity DNA chip. A good indexed library (Figure 4B, 4D) has a broad peak from 300 to 800 bp. Obtain an average cDNA size by setting the Bioanalyzer to analyse any DNA ranging from 150 bp to 6,000 bp.
79. Measure the concentration of indexed cDNA for each sample by using Qubit dsDNA HS assay kit according the manufacturer's instruction (See *Note 14*).

Library pooling (timing 2 hours)

80. Calculate the molarity of each final library using the concentration (ng/ μ L) obtained with the Qubit and the average size of the library obtained on the Bioanalyzer. Dilute each library to get 2 nM solution.
81. Use equal nanomoles of each sample to pool. Make sure that each sample in the pool has a unique combination of N5xx and N7xx adapters. We usually pool seven samples in order

to aim for approximately 28.6 million reads for each sample assuming that the number of reads from one lane of flow cell is 200 million. For pooling fewer samples than 6 please check the Nextera DNA sample preparation guide (available at <http://www.illumina.com>) to determine the compatibility of each index.

82. Measure again the concentration and average size of each pool by Qubit and Bioanalyzer and if required adjust to 2 nM before sequencing.

Sequencing (1 to 2 days)

83. Perform single-end sequencing of the libraries according to the manufacturer's protocol and by using the TruSeq dual-index sequencing primers on either a HiSeq 2000, 2500 or MiSeq instrument; a 50-bp single-end sequencing on a HiSeq 2000 takes ~2 days, whereas that on a MiSeq takes less than 1 days. The degree of multiplexing per lane depends on the read depth obtained on each instrument.

Bioinformatic analysis (1 day)

84. Demultiplex sequencing data from pooled lanes. After a default quality-filtering step, record in fastq files representing raw data.
85. Align reads using Bowtie [11] against the murine transcriptome (mouse NCBI build37 Refseq transcripts). Discard non-uniquely mapped reads. Calculate the count number of reads for each transcript and reads per kilobase of a transcript per million mapped reads (RPKMs). Collapse overlapping Refseq transcripts to obtain the highest one expression value per gene loci.

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Author contribution

CV, developed the protocol, performed sample preparation for sorting, helped with the sample prep for Illumina Sequencing. ARN, helped to develop the protocol. AG, processed the samples for Illumina Sequencing. CV, AG and ARN, provided conceptual input. LM, performed dissection of specific brain areas prior tissue dissociation. PS and KC, operated the cell sorter. LM, designed the study and together with CN provided theoretical input, contributed to the experimental plans and supervised the project. LM, wrote the manuscript with comments and contributions from all authors.

Competing financial interests

The authors declare no competing financial interests.

NOTES

Note 1: Make sure that the end of a needle is in the left ventricle. Do not move the needle once it enters the left ventricle.

Note 2: If the perfusion is proceeding well, you should see the liver become pale, the lungs should not fill up with liquid, and liquid should not emanate from the nostrils. If this is not the case, it is most likely due to the perfusion needle not being completely in the left ventricle.

Note 3: Perform this step very gently and do not disturb the gradient once all three layers are placed into the tube. At this step, the three layers are not visually discernable.

Note 4: Gently apply the cell suspension; vigorous pipetting can affect the gradient separation.

Note 5: We found that setting up the gate on this plot is very important. Sorting neurons from incorrect gates (yellow and green in Figure 2A) results in high contamination of debris and, unlike the correct gate (pink, Figure 2A), RNA cannot be purified (compare Figure 2B, 2C).

Note 6: Frozen samples can be kept at -80°C for up to 6 months.

Note 7: Sorts were run at approximately 20 psi and using a 100 µm sort nozzle. Sort purity was validated by reanalysis of a sample of the sorted cells.

Note 8: To successfully generate cDNA libraries from these neurons, please make sure that total RNA is intact and free of contaminants. The present method is very sensitive to variations in pipette volumes etc. Please make sure that nothing is attached outside of the tips.

Note 9: Ensure that the beads are dry but avoid overdrying them.

Note 10: Collect only 10 μ L to reduce the risk of carryover the beads to next steps.

Note 11: Sample can be kept at -20°C.

Note 12: Sample can be kept at -20°C.

Note 13: Indexed cDNA libraries can be stored at -20°C for 3 months.

Note 14: Indexed cDNA libraries can be stored at -20°C for 3 months.

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Figure legends

Figure 1: tdTomato expression in a subtype of MSNs within the striatum. A) Fluorescent images showing Cre-mediated tdTomato expression (endogenous, red) in enkephalinergic striatal cells (enkephalin antibody, green) of a 3 months old striatal tissues obtained from crossing the BAC-Penk-Cre^{tg/+} to the Rosa26tdTomato^{T/+}. CPu, caudate putamen. Scale bar, 25 μ m. B) Percoll gradient of dissociated striatal cells from 3 months old mouse brain. Note the three layers within the tube. We discard the top layer and keep the two bottom layers for FACS.

Figure 2: Gating strategy for sorting. Dissociated striatal cells from 3 and 8 months old mouse brains were sorted using a FACS Aria Fusion cell sorter. (A) Representative plot between forward scatter area (FSC-A) and side scatter area (SSC-A). The correct gate is highlighted in pink while gates containing mainly debris are highlighted in yellow and green. (B-C) Representative electropherograms of RNA extracted from cells sorted from the correct gate (B, pink gate in A) or incorrect gate (C, green gate in A). Note the presence of 18S and 28S rRNA peaks denoting intact RNA (B), a feature that is absent in degraded RNA (C). (D-E) Representative plots used for selecting only single cells during the sort for forward scatter height (FSC-H) and forward scatter width (FSC-W) (D), and for side scatter height (SSC-H) and side scatter width (SSC-W) (E). (F) Histogram of LIVE/DEAD violet intensity, which determines cell viability. A gate for live cells was set to exclude the very brightly stained dead cells. Staining of dead cells is typically >50 fold higher than live cells. (G) Representative histogram for tdTomato intensity within the viable cell gate, which represents enkephalinergic neurons. Gates for positive and negative populations for tdTomato were set according to wild-type control.

Figure 3: tdTomato positive sorted neurons and enrichment of striatopallidal known markers. (A-D) Representative immunofluorescence of sorted (3 months old) live tdTomato+ cell (A), stained for NeuN (B, neuronal marker), and DAPI (C). D, is merged image of the A-C. (E) Quantitative RT-PCR of sorted tdTomato+ cells from BAC-Penk-Cre^{tg/+}; Rosa26-tdTomato^{T/+} mice, comparing gene expression in sorted ENK+ neurons to the total striatal cell population. The graph

shows pooled data from two mice. Values are mean \pm SD (n=2). Y axis is log2 of fold difference (- $\Delta\Delta Ct$). Note the enrichment of striatopallidal known markers (*Penk*, *Drd2*, *Adora2a*) and depletion of *Tac1*, a striatonigral marker.

Figure 4: Representative electropherograms of cDNA libraries. (A, C) Representative electropherograms of cDNA libraries obtained from two hundred sorted viable tdTomato-positive striatal neurons from 3 months old (A) and 8 months old (C) mice. The average size of cDNA is 1,409 bp (A) and 1,723 bp (C), respectively. (B, D) Representative electropherograms of the indexed cDNA library from the same samples shown in A, C, respectively. The average size of the indexed cDNA is 607 bp (B) and 703 (D), respectively, which is in the range of 300-800 bp.

Figure 1

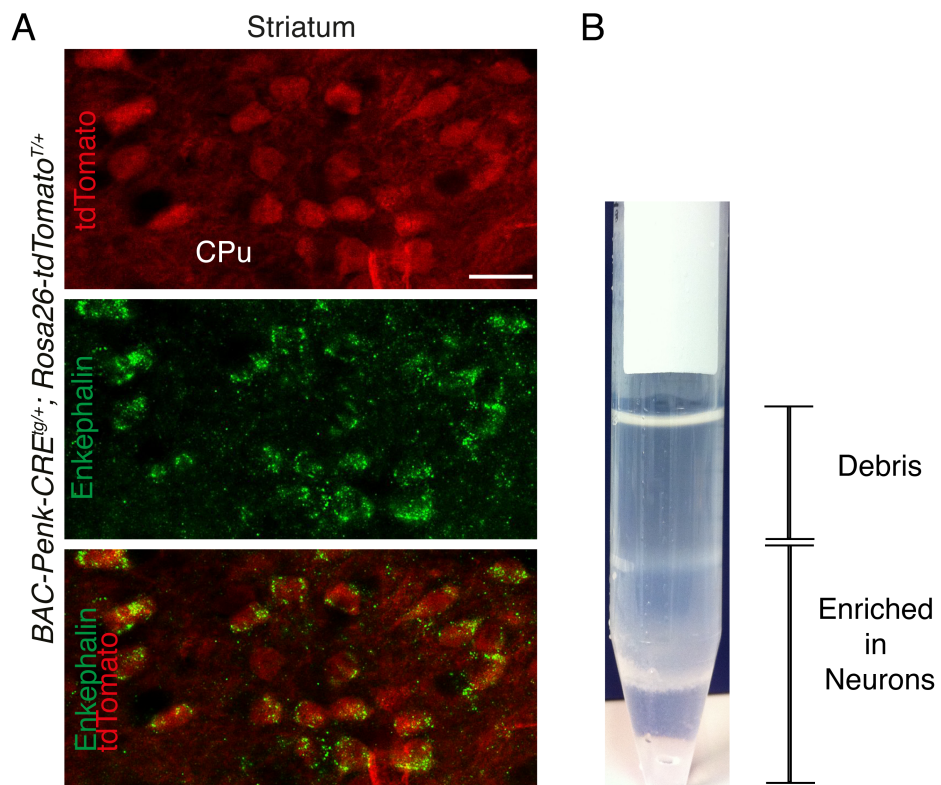
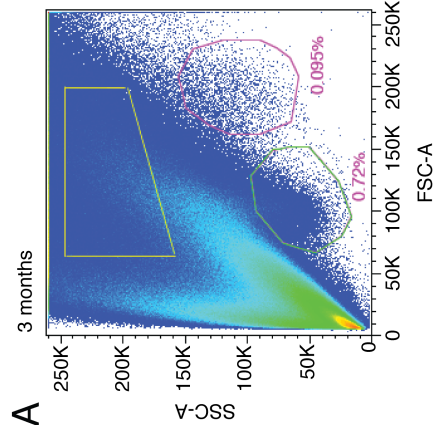
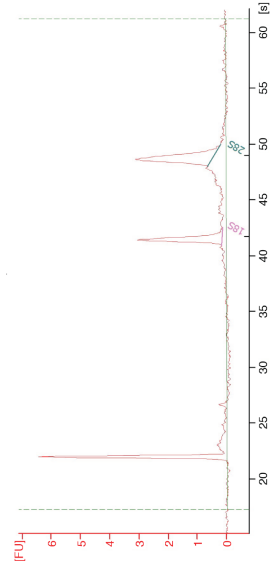


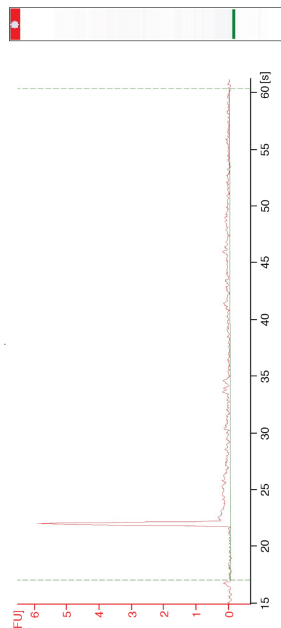
Figure 2



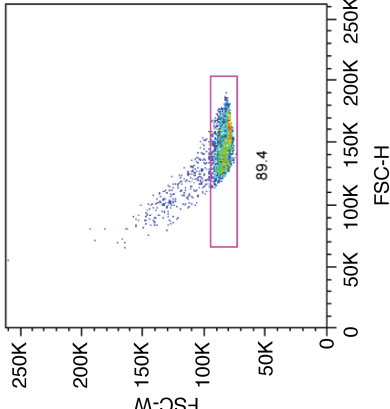
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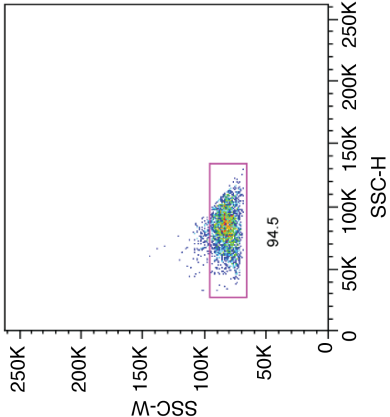
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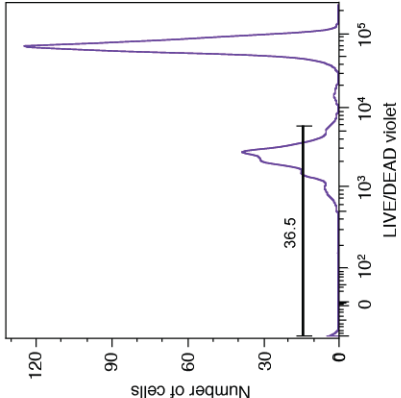
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E



F



G

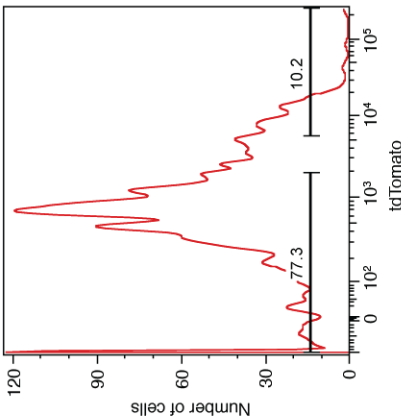


Figure 3

BAC-Penk-CRE^{tg/+}; Rosa26-tdTomato^{T/+}

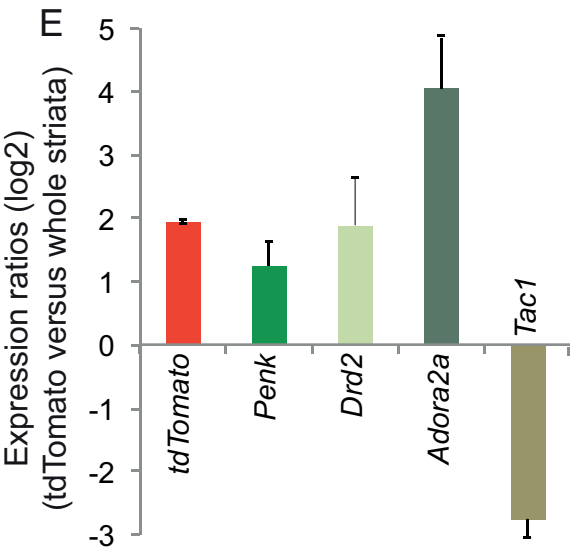
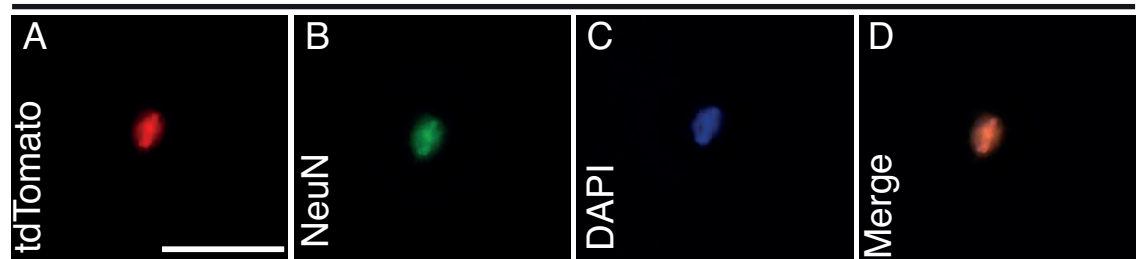


Figure 4

