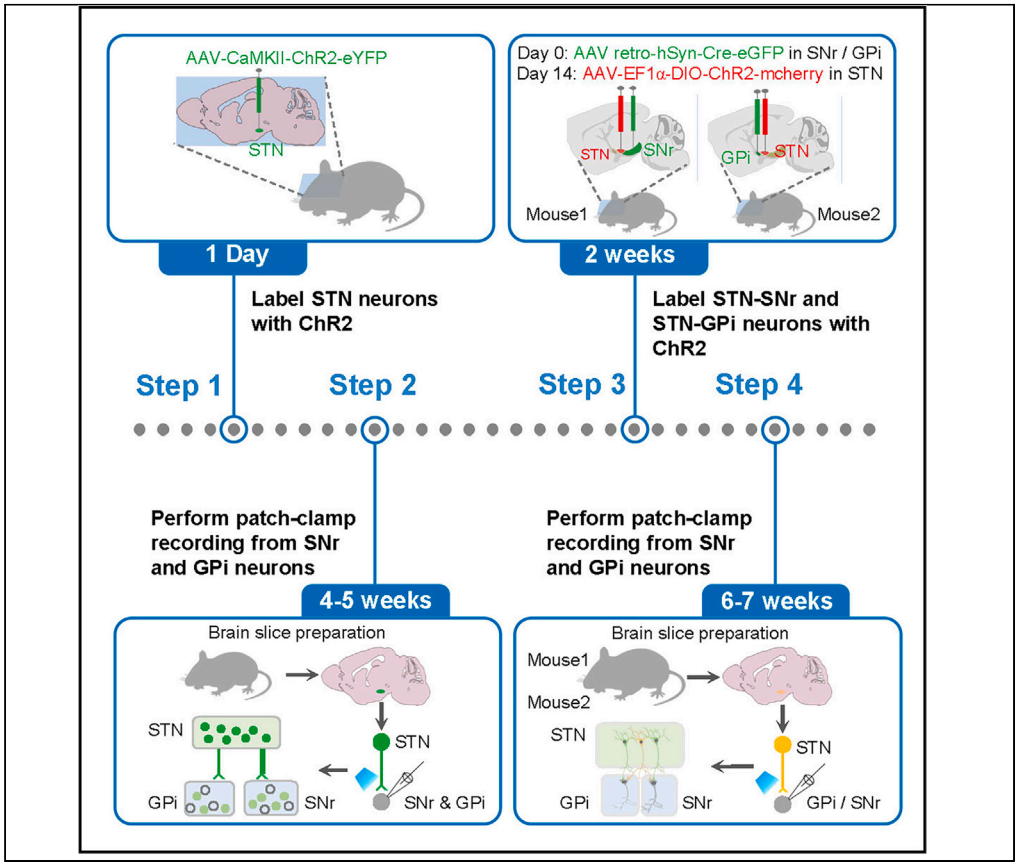
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to study projection-specific circuits in the basal ganglia of adult mice using viral vector tracing, optogenetics, and patch-clamp technique



Analysis of synaptic strength and plasticity provides functional insights of complicated neural circuits. Here, we describe steps for cell- and projection-specific optogenetic manipulation of divergent basal ganglia circuits using anterograde and retrograde viral vectors. We quantitatively analyze synaptic function of these circuits utilizing a patch-clamp technique. This protocol is applicable to probe potential circuit targets for treatment of brain diseases.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Ya-Wei Ji, Xiang-Ying Xu, Cui Yin, Chunyi Zhou, Cheng Xiao

chunyi.zhou@xzhmu.edu.cn (C.Z.)
xchengxj@xzhmu.edu.cn (C.X.)

Highlights

Cell-specific optogenetics enables quantitative analysis of divergent projections

Retrograde viral vectors reveal originating neurons of divergent projections

Patch-clamp and optogenetics allow probing circuit alterations in brain diseases

Ji et al., STAR Protocols 4, 102551
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102551

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol to study projection-specific circuits in the basal ganglia of adult mice using viral vector tracing, optogenetics, and patch-clamp technique

Ya-Wei Ji,[1,2,3,4,5] Xiang-Ying Xu,[1,2,3,4,5] Cui Yin,[1,2,3] Chunyi Zhou,[1,2,3,*] and Cheng Xiao[1,2,3,6,*]

[1]School of Anesthesiology, Xuzhou Medical University, Xuzhou, Jiangsu 221004, China

[2]Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical University, Xuzhou, Jiangsu 221004, China

[3]NMPA Key Laboratory for Research and Evaluation of Narcotic and Psychotropic Drugs, School of Anesthesiology, Xuzhou Medical University, Xuzhou, Jiangsu 221004, China

[4]These authors contributed equally

[5]Technical contact: jyw1994923@outlook.com; xiangyingxu001@163.com

[6]Lead contact

*Correspondence: chunyi.zhou@xzhmu.edu.cn (C.Z.), xchengxj@xzhmu.edu.cn (C.X.)
https://doi.org/10.1016/j.xpro.2023.102551

SUMMARY

Analysis of synaptic strength and plasticity provides functional insights of complicated neural circuits. Here, we describe steps for cell- and projection-specific optogenetic manipulation of divergent basal ganglia circuits using anterograde and retrograde viral vectors. We quantitatively analyze synaptic function of these circuits utilizing a patch-clamp technique. This protocol is applicable to probe potential circuit targets for treatment of brain diseases.

For complete details on the use and execution of this protocol, please refer to Ji et al.[1]

BEFORE YOU BEGIN

The combination of neuronal tracing and optogenetic techniques allows for the discovery of divergent neural circuits originated from a particular brain region playing similar or different roles in regulating behaviors. Few studies analyze synaptic strength and plasticity of the parallel and collateral projections in the divergent circuits. This protocol describes two sequential strategies to tackle this issue. We analyzed divergent circuits from the subthalamic nucleus (STN) to the substantia nigra pars reticulata (SNr) and globus pallidus interna (GPi). First, we performed brain slice patch-clamp technique to characterize synaptic strength and plasticity of the STN-SNr and STN-GPi projections in mice whose STN neurons are virally labeled with an optogenetic actuator, channelrhodopsin 2 (ChR2). Second, we combined retrograde viral vector and optogenetics to selectively label STN neurons sending projection to either the SNr or GPi and performed brain slice patch-clamp recording to characterize synaptic property of blue light-evoked postsynaptic currents from either GPi or SNr neurons, correspondingly, in these mice. The second strategy reveals that some STN neurons send collateral projections to both the SNr and GPi. This protocol is applicable, but not limited, to reveal synaptic architecture of neural circuits, precise targets of a drug, and detailed circuit pathophysiology of brain diseases.

The section below lists the Materials necessary to implement this protocol. The required equipment appears in the key resources table.

1. Prepare viral vectors.
   a. Purchase viral vectors (AAV serotype 2/9): AAV-CaMKII-ChR2-eYFP, AAV retro-hSyn-Cre-eGFP, AAV-EF1α-DIO-ChR2-mCherry.

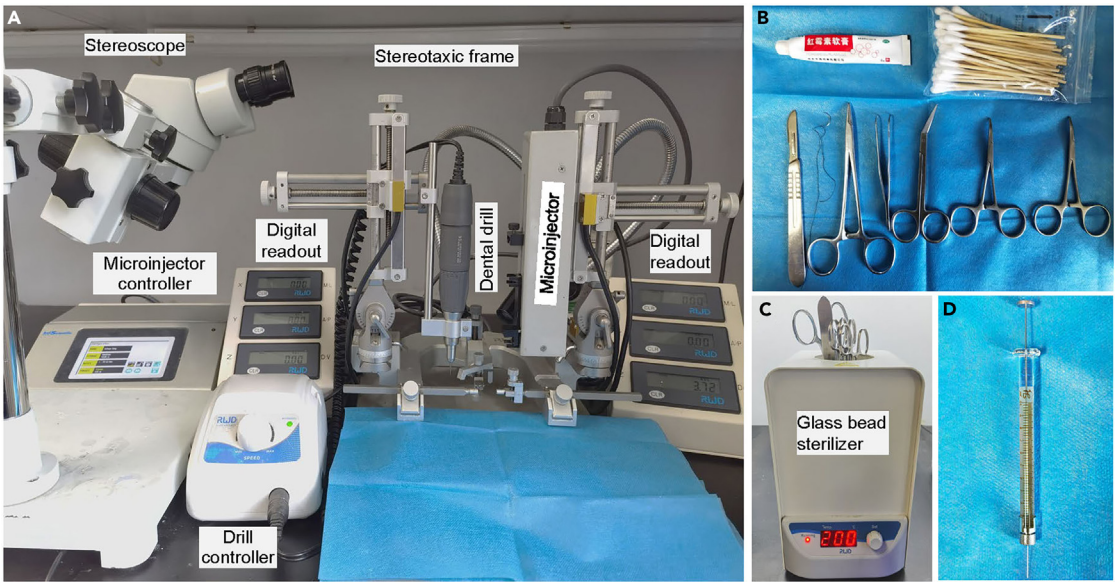


Figure 1. Surgical materials for microinjection

(A) A stereotaxic system for microinjection includes a stereotaxic frame with dual manipulators and digital readouts, a stereoscope and its support, a microinjector and its controller, a dental drill and its controller.

(B) Surgical tools.

(C) A glass bead sterilizer for sterilizing surgical tools.

(D) Hamilton syringe with a 33 gauge needle.

b. Aliquot the purchased viral vector into PCR tubes (3 μL each) and store the aliquots in a −80°C refrigerator.

Note: Aliquot the viral vector in a small volume to avoid repetitive thawing and freezing. Keep the thawed aliquot in 4°C refrigerator and use it within 1 week. For long-term storage and avoidance of waste, 3 μL may be a suitable volume.

2. **Prepare intracranial virus injection** (Figure 1).
   a. Order sodium pentobarbital for anesthesia, ophthalmic ointment, meloxicam
   b. Prepare hair shaver, adhesive tape.
   c. Prepare materials for sterilization: Disinfection bag, paper towel, cotton gauze, cotton swabs, forceps, scalpel, scalpel blade, surgery scissors, hemostats, saline, absorbable sutures, 75% ethanol.
   d. Set up a stereotaxic apparatus (see the key resources table).
3. Prepare materials and solutions for patch-clamp recordings.
   a. Supply materials: Crushed ice, 20 mL syringes, razor blade, superglue, beakers, hand-made brain slice incubator.
   b. Make physiological solutions.

This section lists the recipes of solutions used for patch-clamp recordings.

Recipe 1 for 10× stock solution for artificial cerebrospinal fluid (ACSF)

Reagent	Final concentration	Amount
NaCl	1250 mM	73.71 g
KCl	25 mM	1.864 g
$CaCl_2$	24 mM	2.664 g
$NaH_2PO_4 \cdot 2H_2O$	12 mM	1.873 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
$MgCl_2 \cdot 6H_2O$	12 mM	2.439 g
ddH_2O	N/A	>900 mL
Total	**N/A**	**1000 mL**

Note: Store the solution at 4°C. Discard it in two weeks.

Recipe 2 for 10× stock solution for modified ACSF (cutting solution):		
Reagent	Final concentration	Amount
NaCl	850 mM	49.72
KCl	25 mM	1.863
$CaCl_2$	5 mM	0.554
$NaH_2PO_4 \cdot 2H_2O$	12 mM	1.95
$MgCl_2 \cdot 6H_2O$	40 mM	8.132
ddH_2O	N/A	>900 mL
Total	**N/A**	**1000 mL**

Note: Store the solution at 4°C. Discard it in two weeks.

Alternatives: Other recipes for the cutting solution[2–4] may be tested to optimize the viability of brain slices and the success rate for patch-clamp recording.

Recipe 3 for 1×ACSF		
Reagent	Final concentration	Amount
NaCl	125 mM	100 mL ACSF stock solution
KCl	2.5 mM	
$CaCl_2$	2.4 mM	
$NaH_2PO_4 \cdot 2H_2O$	1.2 mM	
$MgCl_2 \cdot 6H_2O$	1.2 mM	
$Glucose \cdot H_2O$	10 mM	1.9817 g
$NaHCO_3$	26 mM	2.184 g
ddH_2O	N/A	>800 mL
Total	**N/A**	**1000 mL**

Note: Freshly make the solution before experiments. Discard it when finish the experiment on the same day.

Recipe 4 for 1× cutting solution		
Reagent	Final concentration	Amount
NaCl	85 mM	100 mL sACSF stock solution
KCl	2.5 mM	
$CaCl_2$	0.5 mM	
$NaH_2PO_4 \cdot 2H_2O$	1.2 mM	
$MgCl_2 \cdot 6H_2O$	4.0 mM	
$Glucose \cdot H_2O$	25 mM	4.9542 g
Sucrose	67.25 mM	23 g
$NaHCO_3$	24 mM	2.0162
ddH_2O	N/A	>800 mL
Total	**N/A**	**1000 mL**

Note: Make the solution one day before the experiment. Adjust the amount of sucrose according to the osmolality of the solution (310–320 mOsm). Store the cutting solution at 4°C. Discard it in 4 days.

Recipe 5 for internal solution

Reagent	Final concentration	Amount
K gluconate	135 mM	0.949 g
$CaCl_2$ (1 M)	0.5 mM	15 μL
EGTA	0.2 mM	0.0023 g
HEPES	10 mM	0.0715 g
Mg-ATP	3 mM	0.0524 g
GTP	0.1 mM	0.0015 g
ddH_2O	N/A	>25 mL
Total	**N/A**	**30 mL**

Note: Solve all reagents in ddH_2O to make 28 mL internal solution. Adjust pH to 7.25–7.35 with Tris-base and osmolality to 290–300 mOsm with sucrose. Set the volume to 30 mL with ddH_2O. Filter the internal solution with a sterilized 0.22 μm filter disc and aliquot the solution to 0.5 mL into EP tubes. Store the aliquots at −20°C. Keep the solution at 4°C during the experiment.

Institutional permissions

The care and use of animals and the experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee and the Office of Laboratory Animal Resources of Xuzhou Medical University under the Regulations for the Administration of Affairs Concerning Experimental Animals (1988) in China.

It is critical to obtain approval to perform animal experiments from corresponding institutional committees before experiments.

Preparation for intracranial virus injection

Timing: 2–4 weeks

Timing: 2–4 weeks (for step 4)

Timing: 1–2 h before experiment (for step 5)

This section describes animal care and material preparations for surgery prior to virus injection.

4. Purchase and take care mice for the experiment.
   a. Male C57BL/6 mice (12−14 weeks old) were purchased from the animal facility of the Xuzhou Medical University.
   b. The mice were group-housed (no more than 4 mice per cage) on a 12-h light/dark cycle with free access to food and water.
   c. After 2−4 weeks of adjustment to the environment, the mice were randomly allocated into the groups described in the following experiments.
5. Prepare materials for surgery (Figure 1).
   a. Clean, pack, and seal surgical tools in disinfection bags and autoclave the bags for half an hour, and store them in a clean cabinet.

Note: This step should be finished one day before the surgery.

b. Clean the area around the stereotaxic frame to perform the surgery and disinfect the area with 75% ethanol.
c. Spread sterilized paper towel beside the stereotaxic frame and put sterilized surgical tools on the paper tower.
d. Turn on the glass bead sterilizer for sterilization of the surgical tools during the surgery.
e. Mount dental drill and syringe pump onto the left and right manipulators of the stereotaxic frame, respectively.
f. Sterilize a 33 gauge injection needle three times with 75% ethanol and rinse it with sterilized Milli-Q water.
g. Load the injection needle onto a 10 μL Hamilton syringe and mount the syringe onto the syringe pump.
h. Check whether the syringe plunger is tight and the needle is not clogged before injection.
    i. Connect the injection needle with the syringe and push the plunger all the way to the end of the syringe.
    ii. Dip the needle into the sterilized saline and smoothly pull the plunger to check the level of saline.

Note: A small dead volume in front of syringe plunger means that the plunger is tight and the needle is not clogged.

i. Turn on the heating pad and set the temperature between 35°C and 37°C.
j. Dissolve 0.04 g sodium pentobarbital in 4 mL 0.9% sterilized saline for anesthesia.
k. Take the viral vector out from −80°C refrigerator, naturally thaw and place it on crushed ice, and protect it from light.

Preparation for patch-clamp recordings

Timing: 1–2 days before the experiment

Timing: 15–20 min (for step 6)

Timing: about 1 h (for step 7)

Timing: 5–10 min (for step 7a)

Timing: 5–10 min (for step 7b)

Timing: 20–30 min (for step 7c)

The section describes preparation of stock and working solutions for patch-clamp recording.

6. Prepare stock solutions.
    a. Make 1000 mL 10× stock solution of normal ACSF according to the recipe 1 and 1000 mL 10× stock solution of modified ACSF (cutting solution) for slice preparation according to the recipe 2.
    b. Store the stock solutions at 4°C in a refrigerator.

Note: Do not include sodium bicarbonate, sucrose, and glucose in the stock solutions to avoid precipitation and germination. Discard the stock solution in 2 weeks.

7. Prepare working solutions.
    a. Make 1000 mL normal ACSF according to the recipe 3.

i. Dissolve reagents other than those included in the 10× stock ACSF in 800 mL ddH_2O in a 1000 mL graduated flask.
ii. Add 100 mL 10× stock ACSF into a 100 mL measuring cylinder.
iii. Slowly add 100 mL stock solution into the flask while mixing.
iv. Add ddH_2O into the graduated flask to the 1000 mL indication line.

b. Make 1× cutting solution according to the recipe 4.
i. Dissolve reagents except those included in the 10× stock cutting solution in 800 mL ddH_2O in a 1000 mL graduated flask.
ii. Add 100 mL 10× stock cutting solution into a 100 mL measuring cylinder.
iii. Slowly add 100 mL stock solution into the flask while mixing.
iv. Add ddH_2O into the flask to the 1000 mL indication line.

⚠ CRITICAL: Do not dilute 10× stock solution first and add $NaHCO_3$ powder directly into the dilution.

Note: For optimal results, ACSF should be freshly made, and cutting solution should be stored at 4°C in a refrigerator no longer than 4 days.

c. Make internal solution.
i. Make 30 mL internal solution according to the recipe 5.
ii. Filter the solution with 0.22 μm filter disc, aliquot the solution into 0.5 mL, and store it at −20°C in a refrigerator.
iii. Use one freshly thawed aliquot in every experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV-CaMKII-hChR2(H134R)-YFP	BrainVTA (Wuhan, China)	Cat# PT-0296
AAV retro-hSyn-eGFP-Cre	OBiO Technology (Shanghai, China)	Cat# H4942
AAV-EF1α-DIO-ChR2-mCherry	OBiO Technology (Shanghai, China)	Cat# AG20297
Biological samples		
Mouse brain tissue	This manuscript	This manuscript
Chemicals, peptides, and recombinant proteins		
Tetrodotoxin	Tocris, Park Ellisville, MO, USA	Cat# 1078/1 CAS: 4368-28-9
4-Aminopyridine	Tocris, Park Ellisville, MO, USA	Cat# 0940/100 CAS: 504-24-5
6-Cyano-7-nitroquinoxaline-2, 3-dione disodium (CNQX)	Tocris, Park Ellisville, MO, USA	Cat# 1045/1 CAS: 479347-85-8
DL-2-amino-5-phosphonopentanoic acid (APV)	Tocris, Park Ellisville, MO, USA	Cat# 0105/10 CAS: 76326-31-3
Sodium pentobarbital	Sigma-Aldrich, St. Louis, MO, USA	Cat# P3761 CAS: 57-33-0
Ophthalmic ointment	Xinxiang Huaqing Pharmaceutical Co., Ltd, Xinxiang, Henan, China	H41020138
Meloxicam	Shanghai Aladdin Biochemical Technology Co., Ltd, Shanghai, China	Cat# M129228-100g CAS: 71125-38-7
Ethanol	Sinopharm Chemical Reagent Co. Ltd, Shanghai, China	Cat# 10009218 CAS: 64-17-5
Experimental models: Organisms/strains		
Mice	Animal facility of Xuzhou Medical University Jinan Pengyue Laboratory Animal Breeding Co. Ltd	C57 BL/6J, male, 3–6 months
Software and algorithms		
ImageJ	Schneider et al., 2012[5]	https://imagej.nih.gov/ij/
OpenEpi	Dean et al., 2013[6]	www.OpenEpi.com

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Ultra low temperature freezer	Thermo Fisher Scientific (Suzhou), Jiangsu, China	Cat# 902GP-ULTS
Finnpipette	Thermo Fisher Scientific (Suzhou), Jiangsu, China	Cat# 4651020N
Stereotaxic device	RWD Life Science Inc., Shenzhen, China	Cat# 68018
Dental drill	RWD Life Science Inc., Shenzhen, China	Cat# 78001
Drill bits (0.5 and 0.8 mm in diameter)	RWD Life Science Inc., Shenzhen, China	Cat# 78040 / 78042
Microinjection pump	KD Scientific Inc., Holliston, MA, USA	Cat# 78-8130
Stereoscope	RWD Life Science Inc., Shenzhen, China	Cat# 77001
Halogen cold light source	RWD Life Science Inc., Shenzhen, China	Cat# 76301
Forceps	RWD Life Science Inc., Shenzhen, China	Cat#F12019-10
Hemostats	RWD Life Science Inc., Shenzhen, China	Cat#F22006-12
Scalpel	RWD Life Science Inc., Shenzhen, China	Cat#S32003-12
Scalpel blade	RWD Life Science Inc., Shenzhen, China	Cat#S31010-01
Surgery scissors	RWD Life Science Inc., Shenzhen, China	Cat#S12003-09
Hair shaver	RWD Life Science Inc., Shenzhen, China	Cat# CP-5200
Dry glass bead sterilizer	RWD Life Science Inc., Shenzhen, China	Cat# RS3002
Microliter syringe (10 μL)	Hamilton, Bonaduz AG, Switzerland	Cat# SYR(10 μL)701RN
33 gauge Hamilton syringe needle	Hamilton, Reno, NV, USA	Cat# 91033
Autoclave	Zealway, Wilmington, DE, USA	G154TR
Heating pad	Thermo Fisher Scientific, Chino, CA, USA	Cat# 50-195-4000
Adhesive tape	Henkel, Düsseldorf, Germany	Cat# PSK6C
Surgical drapes	Thermo Fisher Scientific, Chino, CA, USA	Cat# 10-000-695
Disinfection bag	Thermo Fisher Scientific, Chino, CA, USA	Cat# 10-000-685
Cotton gauze	Thermo Fisher Scientific, Chino, CA, USA	Cat# 10-000-692
Cotton swabs	Yangzhou Yashen Technology Co., Ltd, Jiangsu, China	
Amplifier with dual headstages	Molecular Devices, San Jose, CA, USA	MultiClamp 700B
Analog-to-digital converter	Molecular Devices, San Jose, CA, USA	Digidata 1550B
pClamp 10.7 software	Molecular Devices, San Jose, CA, USA	pClamp 10.7
Upright microscope	Nikon, Japan	Eclipse FN-1
Flash 4.0 LTE CCD-camera	Hamamatsu, Hamamatsu city, Japan	Product # C11440-42U40
Dual motorized micromanipulator	Sutter Instrument, Novato, CA, USA	Product # MPC-325
TC-344B temperature controller with an inline heater	Warner Instruments, Holliston, MA, USA	Cat# W2 64-0101
Peristaltic pump	Baoding Longer Precision Pump Co., Ltd, Baoding, Hebei, China	BT100-2J
Micropipette puller	Sutter Instrument, Novato, CA, USA	Product # P-1000
Floating table	Jiangxi Liansheng Technology Co., Ltd., Nanchang, Jiangxi, China	Product # ZDT-P-MOT-F-012009-J
Faraday cage	DL Naturegene Life Sciences, Inc, Beijing, China	Product # DL-PBW
Borosilicate capillary glass	Sutter Instrument, Novato, CA, USA	Cat# BF-150-86-10
Vibratome	Leica, Nussloch, Germany	VT-1200
Self-contained ice flaker	Scotsman, Freshwater, UK	AF200
Osmometer	Gonotec, Berlin, Germany	Osmomat 3000 Standard
pH meter	Sanxin, Shanghai, China	MP512-01
Water bath	Shengwei Inc, Jintan, China	DK-8D
Carbogen (95% O_2/5% CO_2) tank	Xuzhou Teqi Gas Technology Co., Ltd, Xuzhou, Jiangsu, China	Cat# 22010
Carbon dioxide tank	Xuzhou Teqi Gas Technology Co., Ltd, Xuzhou, Jiangsu, China	Cat# 22013 CAS: 124-38-9
Nitrogen tank	Xuzhou Teqi Gas Technology Co., Ltd, Xuzhou, Jiangsu, China	Cat# 22006 CAS: 7727-37-9
Milli-Q water purification system	Merck Millipore, Billerica, MA, USA	Cat# ZLXS50020

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stainless steel brain slice anchor	Warner Instrument, Holliston, MA, USA	Cat# W1 64-1420
Graphite lubricant	Prime-Line, Malvern, AR, USA	Product # MP66780
LED light source	Plexon, Hong Kong, China	PlexBright 4 channel optogenetic controller
PlexBright controller software	Plexon, Hong Kong, China	Radiant
Power and energy meter	Thorlabs, Newton, NJ, USA	Item # PM100D
Patch cable	Inper, Hangzhou, China	Cat# MFO-F-W1.25-200-0.37-100
Cryostat	Leica, Nussloch, Germany	CM1950
Pre-cleaned glass slide	Jiangsu Shitai Experimental Equipment Co., Ltd, Jiangsu, China	Cat# 188105
Anti-fading mounting medium	Meilunbio, Dalian, China	Cat# MA0222-2

STEP-BY-STEP METHOD DETAILS

Intracranial injection of viral vectors

Timing: Surgery procedure takes about 1.5–2 h per mouse; histological assay takes 3–5 days

Timing: 20–45 min (for step 1)

Timing: 5–10 min (for step 2)

Timing: 15–20 min (for step 3)

Timing: 10–15 min/injection (for step 4)

Timing: 3–5 days (for step 5)

This section includes the procedure of virus injection and the verification of virus expression.

1. Anesthetize a mouse.
   a. Intraperitoneally inject sodium pentobarbital (40 mg/kg) into a mouse.

   Note: Check its response to noxious stimuli (e.g., tail pinch) to make sure that the mouse is under deep anesthesia. Otherwise, inject 10 mg/kg more sodium pentobarbital until the mouse does not respond to noxious stimuli. This step usually takes 20–45 min depending on how sensitive the mice are to the anesthetics, and deep anesthesia is able to be maintained for at least 2.5 h.

   Alternatives: Constant inhalation of isoflurane or sevoflurane can be used for the induction (5–10 min) and maintenance of general anesthesia in mice. The mice may recover within 5–10 min after termination of anesthetics.

   b. Remove the fur from the top of the skull with a shaver and clean the hair wastes with a piece of adhesive tape.
2. Mount the anesthetized mouse to the stereotaxic frame.
   a. Turn on the heating pad.
   b. Open the jaw of the mouse, place its incisors into the hole of the incisor bar of the stereotaxic frame, and stabilize the snout of the mouse with the nose clamp.
   c. Adjust the position of the incisor bar so that the ear bars reach the external auditory meatus of the mouse.
   d. Support the mouse head from beneath to place it in the middle of the head holder assembly.

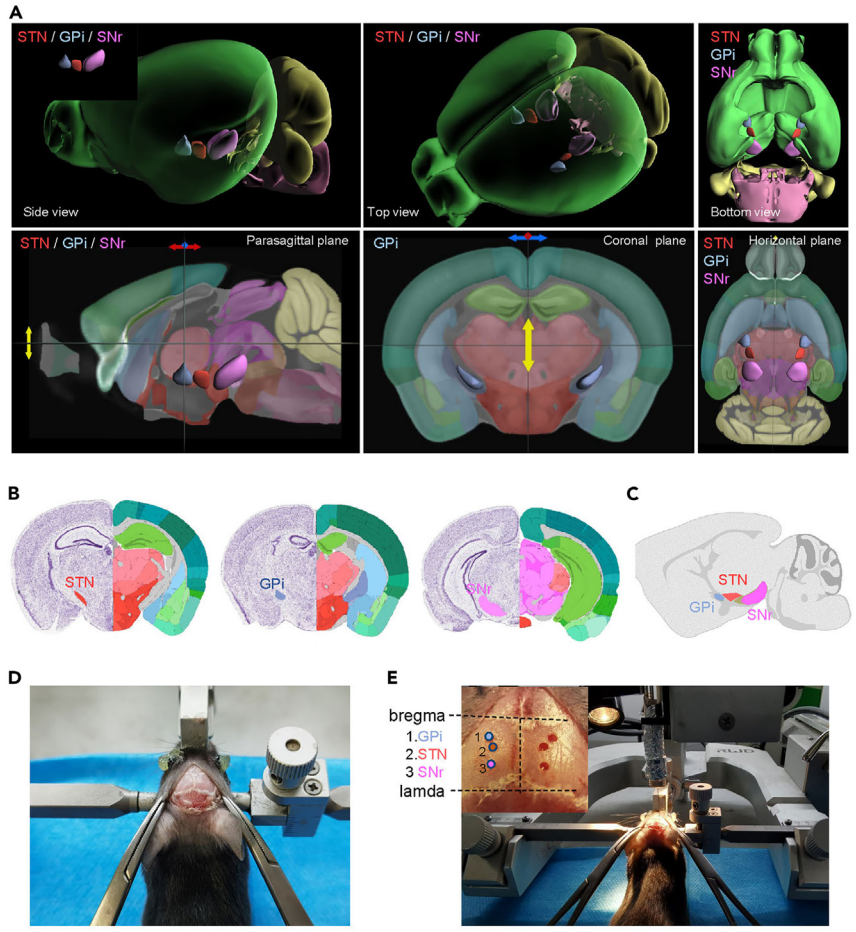


Figure 2. Determine the coordinates of the subthalamic nucleus, globus pallidus interna, and substantia nigra pars reticulata

(A) The location of the subthalamic nucleus (STN), globus pallidus interna (GPi), and substantia nigra pars reticulata (SNr) in different angle and planes.

(B and C) The STN, GPi, and SNr in coronal and parasagittal planes.

(D) Mount the mouse onto the stereotaxic frame. Cut open the skin and expose the skull.

(E) Move the tip of the drill bit to the bregma and zero the coordinates on the digital readouts of the manipulator. Mark the holes to be drilled according to the AP and ML coordinates of the STN, GPi, and SNr. Insert the injection needle into the STN.

Image credit for (A–C): Allen Adult Mouse Brain Reference Atlas.

i. Lock one ear bar to the position that it touches one ear of the mouse.
ii. Slide the other ear bar to the other ear.
iii. Lock the ear bar until the two ear bars hold the head tightly.

e. Pull out the tongue of the mouse out of the mouth from one side to prevent suffocation.
f. Use eye ointment to prevent dehydration of the cornea.

Note: The incisor bar and one of ear bars of the stereotaxic frame should be vertically adjustable.

3. Perform surgery (Figure 2) troubleshooting 1.
   a. Disinfect the scalp with cotton swabs soaked with 75% ethanol at least 3 time.
   b. Make an incision along the midline with a scalpel.
   c. Extend the incision and detach the skin from the skull with a pair of surgical scissors.

d. Expose the skull by moving the skin to either side with two hemostats.
e. Scrape off the connective tissue on the surface of the skull with a scalpel and wipe it off with cotton swabs.

Note: Wipe the skull with a cotton swab soaked with 3% hydrogen peroxide if the bregma and lambda are not clearly discernable. Use cotton swabs soaked with normal saline to clean up hydrogen peroxide.

f. Adjust the incisor bar vertically to position the bregma and lambda at the same vertical level.
g. Adjust the ear bars to level the two hemispheres.
h. Determine the coordinates by referring to the mouse brain atlas (STN: AP, −1.78 mm; ML, 1.50 mm; DV, 4.80 mm) (SNr: AP, −3.10 mm; ML, 1.50 mm; DV, 4.60 mm) or (GPi: AP, −1.30 mm; ML, 1.75 mm; DV, 4.70 mm) (Figures 2A–2C).[7]
i. Drill holes according to AP and ML coordinates.
   i. Position the tip of the drill bit to touch the bregma.
   ii. Zero the readout of the manipulator with the dental drill attached.
   iii. Move it to the target AP and ML coordinates on the skull.
   iv. Drill through the skull (Figures 2D and 2E).
j. Puncture the dura mater with a needle to prevent the glass pipette used for microinjection or the injection needle from breaking or bending.

Note: Mice older than 4 months are preferred because the mouse skull and brain stop growing thereafter and have less variability among individuals.

4. Perform virus microinjection troubleshooting 2.
   a. Connect the syringe to the syringe pump.
   b. Place a piece of parafilm (2 × 2 cm^2) disinfected with 75% ethanol on the skull of the mouse, and add 1 μL viral vector on it.
   c. Immerse the tip of the injection needle into the droplet of the viral vector, aspirate the vector into the syringe with the microinjection pump.

⚠ CRITICAL: After loading the viral vector, inject 50 nL viral vector to check whether there is a dead volume in the syringe.

   d. Position the tip of the injection needle to touch the bregma and zero the digital readout of the manipulator.
   e. Locate the tip of the needle to the target AP and ML coordinates and lower it gradually to the DV coordinate.
   f. Start virus injection at a speed between 50–80 nL/min, then retain the needle in the brain for 6–8 min after the injection.
   g. Gradually lift the injection needle up by 0.3 mm within 5 s and wait for at least 30 s.
   h. Repeat this procedure until the needle is lifted up out of the brain tissue.
   i. Wipe off the brain tissue on the tip of the needle immediately with a cotton swab soaked with normal saline.

Note: This is important to prevent the needle from being blocked by dried brain tissue. Additionally, an injection of 50 nL viral vector after wiping clean the needle may be required to confirm that the needle is not clogged during viral injection.

   j. Close the skin with sutures (4–0) and apply a thin layer of superglue to cover the wound.
   k. Place the mouse in a clean cage on a heating pad and return it to the housing area in the animal facility after the mouse wakes up.

l. Subcutaneously administer meloxicam (4 mg/kg) once per day up to 3 days for postoperative pain relief.

Note: Keep the skull and the brain tissue moist during injection to avoid scar formation in the surgery area around the injection needle, which may cause severe damage to brain tissue and blood vessels when the injection needle is removed.

'**a-c**' can be done before anesthetizing the mouse.

5. Perform histological assay.
   a. Sacrifice the mice 3 weeks after virus injection in a CO_2 chamber.
   b. Perform cardiac perfusion (see the detailed procedures in 'preparation for live brain slices') with 10 mL ice-cold phosphate buffered saline (PBS) and then 20 mL 4% paraformaldehyde (PFA) in PBS.
   c. Remove the mouse brain and post-fix it in 4% PFA for 12 h at 4°C.
   d. Dehydrate the mouse brain in 30% sucrose (in PBS) (for 2 days) for cryoprotection.
   e. Imbed the mouse brain in OCT and freeze it to −20°C in a refrigerator.
   f. Cut the mouse brain into 30 μm parasagittal sections with a cryostat.
   g. Mount brain sections onto glass slides.
   h. Coverslip the sections immersed in a mounting medium.
   i. Visualize fluorescence-labeled neurons in brain sections under a confocal microscope to confirm the location of the injection site and labeled neurons.
      i. Restriction of eYFP-labeled neurons in the STN confirms the correct injection site of AAV-CaMKII-ChR2-eYFP. This is important to assure that the blue light-evoked postsynaptic currents in SNr and GPi neurons are responses to glutamate released from STN projection neurons but not being contaminated by neurons outside the STN.
      ii. Restriction of mCherry-labeled neurons in the STN indicates that AAV-EF1α-DIO-ChR2-mCherry is injected into the STN in mice whose STN projection neurons were retrogradely transfected with Cre-recombinase by AAV retro-hSyn-Cre-eGFP injected into the SNr or GPi.
      iii. A clear dark area between labeled SNr and STN neurons is a sign that injection site of AAV retro-hSyn-Cre-eGFP for retrograde labeling of SNr-projecting STN neurons is correct and the eGFP-labeled STN neurons are unlikely labeled by the spread virus.
      iv. The fact that GPi and STN neurons, but not SNr neurons, are eGFP-labeled provides an evidence for correct injection site of AAV retro-hSyn-Cre-eGFP to label GPi-projecting STN neurons because if the virus spreads into the STN, it may retrogradely label SNr neurons. It is vice versa for AAV retro-hSyn-Cre-mCherry injection to label SNr-projecting STN neurons.
      v. In mice that STN-SNr or STN-GPi neurons are labeled, the presence of fluorescence-labeled fibers in the GPi or SNr, respectively, represents the existence of STN neurons projecting to both the SNr and GPi.

Patch-clamp recordings from brain slices

Timing: 8–10 h

Timing: 60–75 min (for step 6)

To verify that the virus was functional and to probe and measure the synaptic strength of the STN projections, we performed patch-clamp recordings on brain slices using a modified version of a protocol described previously.[1,3,8–10]

6. Prepare live brain slices.

a. Place a 250 mL beaker containing 200 mL cutting solution in a −80°C refrigerator for 12–15 min until ice flakes form, then go to step 'd'.
b. Prepare surgical tools, including scissors, hemostats, hook, forceps, disc filter, 20 mL syringe, filter paper, petri dish, brush, razor blade, and spatula (Figure 3A).
c. Install razor blade onto the blade holder of the vibratome.
    i. Split a razor blade from the middle into two halves and use one half for brain slice preparation.

Note: Scrape the wax off the blade, wipe the surface of the blade three times with cotton swab soaked with 75% ethanol, rinse the blade with deionized water, and paste a thin layer of graphite lubricant onto both sides of the blade with a cotton swab. These procedures are helpful to reduce the friction on the surface of the blade.

    ii. Attach it onto the knife holder of the vibratome and set the clearance angle of the knife holder to 18° (refer to the users' manual).
    iii. Turn on the vibratome.
    iv. Minimize the vertical vibration of the blade at the aid of a Vibrocheck device.
    v. Attach the ice tray and buffer tray, and fill crushed ice in the ice tray around the buffer tray.
d. Fill an ice-bucket with crushed ice, place the beaker filled with ice-cold cutting solution on the ice, and bubble the solution with carbogen (95% O_2 5% CO_2) for at least 10 min.
e. Fill a 250 mL beaker with 150 mL cutting solution, warm it up in a water bath at 32°C, put a slice incubator in the beaker, bubble the solution with carbogen (95% O_2 5% CO_2) for more than 10 min (Figure 3F), and go to step 'i'.
f. Perform cardiac perfusion (Figure 3B).
    i. Euthanize a mouse in a CO_2 chamber.
    ii. Open the chest of the mouse to expose the heart.
    iii. Stabilize the chest bone with a hemostat.
    iv. Clamp the descending iota with another hemostat.
    v. Hold the heart with a hook pricked into the right ventricle.
    vi. Open the right atrium with a pair of scissors.
    vii. Immediately inject 10 mL ice-cold cutting solution into the left ventricle within 20 s.

Note: Transcardiac perfusion removes blood to protect the brain from oxidative damage by the ferroheme and improve the viability of neurons. Successful perfusion makes the brain and the skin of the forelimbs look pale.

g. Prepare a brain block (Figures 3C–3E).
    i. Decapitate the mouse.
    ii. Make an incision on the skin of the skull.
    iii. Cut the midline or sagittal suture of the skull from the foramen magnum of the occipital bone to the olfactory bulb with a pair of scissors.
    iv. Open the skull with a pair of forceps.
    v. Remove the brain from the skull with a spatula.
    vi. Place the brain on a piece of filter paper soaked with the ice-cold carbogen-saturated cutting solution.
    vii. Trim off the temporal lobes on both sides and the brain stem caudal to the pons.
    viii. Split two hemispheres with a blade.
    ix. Glue the lateral side of the brain blocks down onto the specimen disc with the medial parts of the brain blocks facing up.
    x. Insert the specimen disc into the buffer tray, fill the buffer tray with ice-cold cutting solution, and bubble the solution with carbogen.

Note: Cutting parasagittal brain slices helps preserve neurons in the STN, SNr, GPi, and the substantia nigra pars compacta.

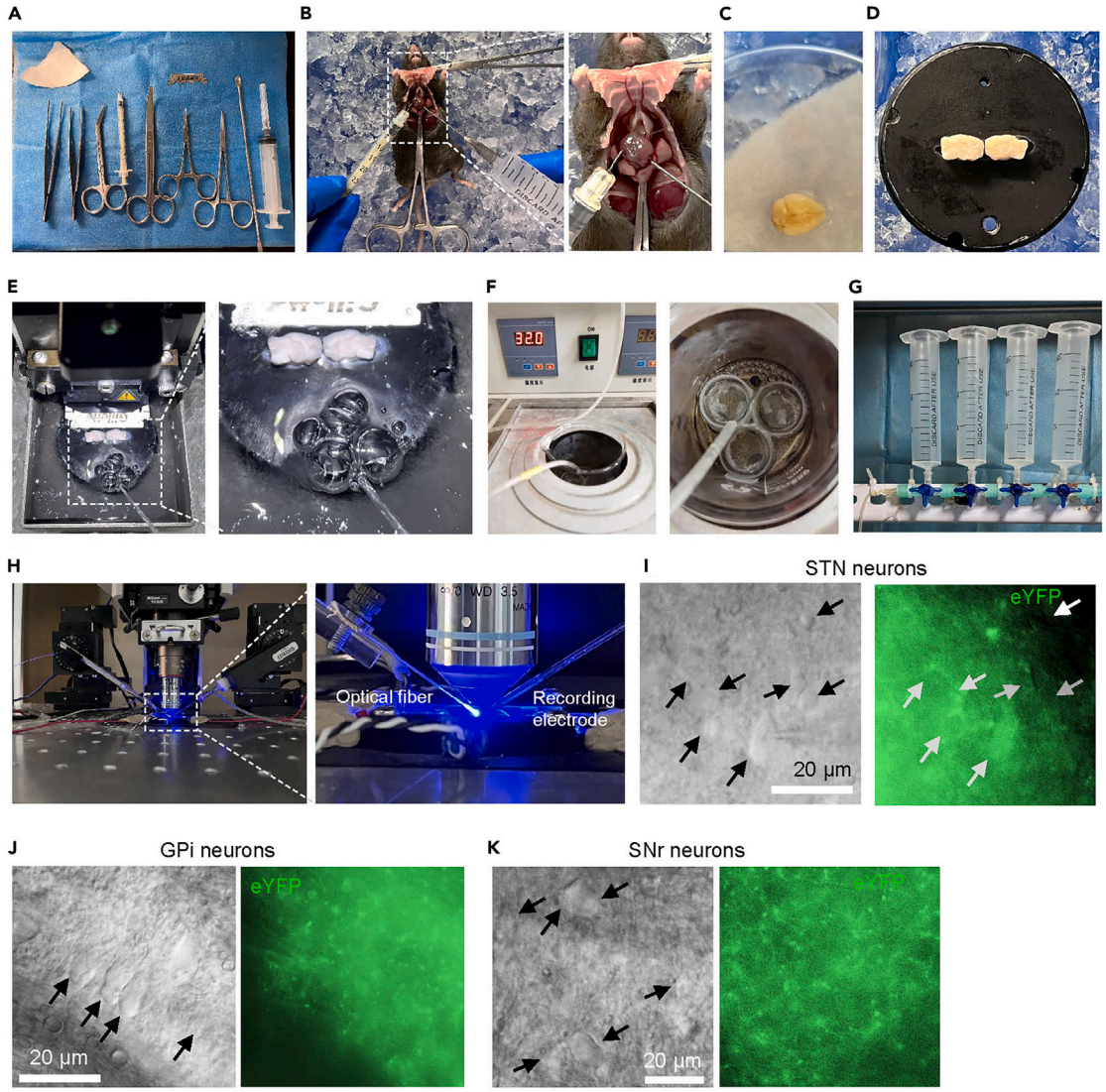


Figure 3. Procedures for brain slice preparation for patch-clamp recording

(A) Surgical tools for transcardiac perfusion.

(B) Put the euthanized mouse in supine on ice, open its chest, stabilize the chest bone with a hemostat, and clamp the descending iota with another hemostat. Open the right atrium and inject ice-cold cutting solution into the left ventricle.

(C) Remove the brain out of the skull and place it on a piece of filter paper soaked with carbogenated cutting solution.

(D) Trim the brain, split it into two brain blocks, and glue the brain block onto the specimen disc with the medial part facing up.

(E) Insert the disc in the buffer tray and fill the buffer tray with carbogenated cutting solution.

(F) Incubate brain slices at 32°C in cutting solution bubbled with carbogen.

(G) Home-made multichannel gravity-based perfusion system.

(H) Setup for patch-clamp recording and optogenetic stimulation.

(I) STN neurons under the DIC and green fluorescence illumination.

(J) GPi neurons in the DIC and green fluorescence illumination.

(K) SNr neurons in the DIC and green fluorescence illumination. Arrows in (I)-(K) point to live neurons. eYFP in right panels in (J, K) is located on puncta and fibers.

⚠ CRITICAL: The cutting solution has lower concentrations of sodium chloride and calcium, but higher concentrations of magnesium. It reduces the excitability of the brain tissue and protects neurons from excitotoxicity.

h. Slice the brain tissue (Figure 3E).

i. Set parameters of the vibratome: set a cutting window to cover the brain blocks, a vibration amplitude ranging between 1.9–2.2 mm, a blade travel speed between 0.02–0.12 mm/s, and a section thickness between 250–350 μm.
ii. Cut the brain blocks into slices.

Note: Adjust blade travel speed to high (0.12 mm/s) and low (0.02 mm/s) levels when the edge of the blade is outside and inside the target brain regions, respectively. It helps speed up the preparation of viable brain slices.

i. Incubate brain slices (Figure 3F).
   i. Transfer the brain slices into the slice incubator filled with 32°C cutting solution and incubate for 60–75 min.
   ii. Place a 250 mL beaker containing 150 mL ACSF in a water bath at 26°C and bubble the solution with carbogen (95% O_2 5% CO_2) for at least 10 min.
   iii. Transfer brain slices from 32°C cutting solution to 26°C ACSF and incubate these brain slices in 26°C ACSF until perform patch-clamp recording.

Note: A multi-compartment incubator with a wired bottom lifted by pillars is essential for sufficient carbogenation. In this setup, the barrier around the compartments prevents brain slices from being perturbed by strong bubbles.

7. Perform patch-clamp recordings from live brain slices troubleshooting 4.
   a. Adjust the parameter of the pipette puller to obtain patch electrodes with a resistance of 4–6 MΩ when filled with the internal solution.
   b. Turn on the PlexBright light source to deliver light to the brain slices through an optical fiber (200 μm in diameter; NA, 0.37) for optogenetic stimulation of STN neurons and their terminals in the SNr and GPi and start the Radiant software ready for the TTL control of the light source (Figure 3H).
   c. Turn on the fluorescent light source and CCD camera equipped on the upright microscope in the patch-clamp rig.
   d. Adjust the temperature controller to 32 ± 1°C and balance the flow rate (1.5–2 mL/min) and suction rate of the gravity perfusion system (Figure 3G).
   e. Transfer a brain slice into the recording chamber of the patch-clamp rig and stabilize the slice with a stainless steel anchor.

Note: The neuronal viability of the brain slices is better on the side facing up during brain slice preparation. This side should face up.

   f. Locate the 4× air objective (Plan Apochromat, NA 0.1, WD 30 mm) above the STN under the near infra-red illumination, then switch to 40× water immersion objective (ApoChromat, NIR, NA 0.80; WD 3.5 mm) to check the neuronal viability of the slice under infra-red illumination and to visualize virus expression under fluorescence illumination (Figure 3I).
   g. Select fusiform neurons with even light-gray color. Locate the tip of the optical fiber 200 μm away from the recorded neurons for optogenetic stimulation.
   h. Perform patch-clamp recordings from mice subjected to injection of AAV-CaMKII-ChR2-eYFP into the STN. Troubleshooting 5.
      i. Select a healthy STN neurons labeled by ChR2-eYFP (Figure 3I) and make whole-cell patch-clamp recordings of responses (inward currents and action potentials) to pulses of blue light (460 nm, 1 s constant or 1 ms at 5, 10, 20, 50 Hz, 2 mW) (Figure 3I) in the voltage-clamp and current-clamp modes.

Note: A weak positive pressure should be added to the recording pipette before the pipette enters the bath solution and approaches to the neurons to assure that the tip of the electrode is clean. This procedure facilitates gigaseal formation.

ii. Check ChR2-eYFP-labeled fibers in the SNr or GPi to confirm that ChR2-eYFP-labeled STN neurons project to these areas and choose a healthy SNr or GPi neuron among these fibers (Figures 3J and 3K).
iii. Make whole-cell patch-clamp recording of inward currents evoked by paired pulses (with intervals of 20, 50, and 100 ms) of blue light (1 ms, 1 mW) in the voltage-clamp mode.

Note: Under this recording condition (see recipes for ACSF and internal solution), excitatory and inhibitory postsynaptic currents recorded at the holding potential of −50 mV are inward and outward currents, respectively. Paired-pulse ratio and responsiveness to a train of light pulses at various inter-pulse intervals are helpful to characterize the short-term plasticity of synapses.

⚠ CRITICAL: If the recorded photocurrents are not blocked by the mixture of 1 μM tetrodotoxin and 0.3 mM 4-aminopyridine (4-AP), but blocked by 20 μM CNQX, it may support that SNr or/and GPi neurons are innervated by STN glutamatergic neurons via a monosynaptic machinery.[1] The amplitude of light-evoked currents represents the synaptic strength of the monosynaptic projection.

iv. Record spontaneous firing from SNr or GPi neurons in the whole-cell current-clamp mode, shine 1 s blue light (1 ms at 5, 10, 20, and 50 Hz, 2 mW), and examine the changes in the firing rate of the recorded neurons.[1]

i. Perform patch-clamp recordings from mice subjected to injection of AAV retro-hSyn-Cre-eGFP into the SNr or GPi and AAV-EF1α-DIO-ChR2-mCherry into the STN (Figure 4) troubleshooting 5.
   i. Select a healthy STN neurons labeled by ChR2-mCherry and make whole-cell patch-clamp recording of inward currents and action potentials evoked by pulses of blue light (1 s constant or 1 ms at 5, 10, 20, 50 Hz, 2 mW) in the voltage-clamp and current-clamp modes, respectively.
   ii. Check ChR2-mCherry-labeled fibers in the SNr or GPi to confirm that ChR2-mCherry-labeled STN neurons project to these areas and choose a healthy SNr or GPi neuron among these fibers.
   iii. Make whole-cell patch-clamp recordings of inward currents evoked by paired pulses (with intervals of 20, 50, and 100 ms) of blue light (1 ms, 1 mW) in the voltage-clamp mode.
   iv. Record spontaneous firing from SNr or GPi neurons in the whole-cell current-clamp mode, shine 1 s blue light (1 ms at 5, 10, 20, and 50 Hz, 2 mW), and examine the changes of the firing rate.

Note: A 1 ms blue light pulse at 0.5–1 mW should be used to evoke a monosynaptic response because a longer light pulse at higher intensity may cause a saturated response and may be unsuitable to study short-term synaptic plasticity. Series resistance should be monitored during recordings.

EXPECTED OUTCOMES

Evaluation of synaptic strength and plasticity of divergent projections

A repertoire of recently developed tools for neuroscience research can meet the demand to interrogate neural circuit at macro-, meso-, and micro-scales with an unprecedented precision. The combination of various viral vectors and transgenic mouse lines enables scientists to reveal cell- and projection-specific details in certain neural circuits. Optogenetic techniques allow for investigation of functions of a particular neural circuit in modulating animal behaviors, which may provide potential therapeutic strategy for neurological diseases. Whereas in most studies, patch-clamp techniques are used with optogenetics to confirm the existence of immediate synaptic transmission. Being

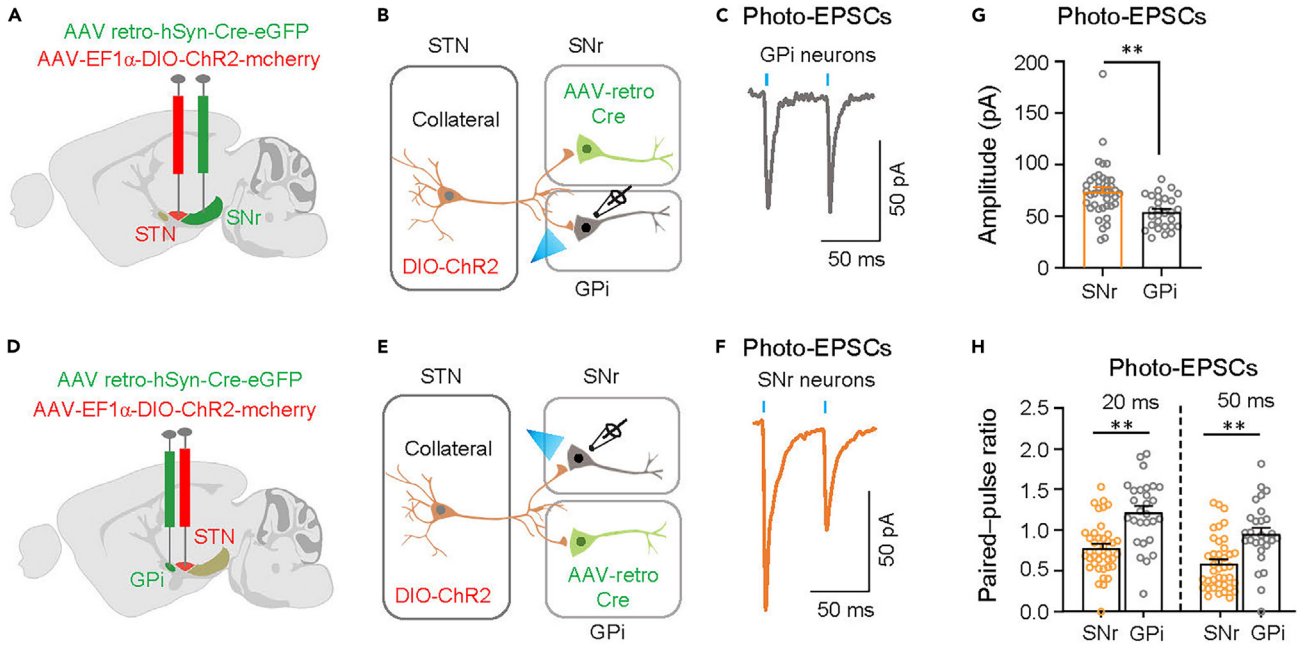


Figure 4. Synaptic function of projections from STN-SNr-GPi neurons to the GPi and SNr

(A) Combining retrograde viral vector and Cre-dependent optogenetics to label STN-SNr neurons.

(B and C) Diagram and trace of patch-clamp recording of photo-EPSCs from GPi neurons being innervated by STN-SNr-GPi neurons.

(D) Combining retrograde viral vector and Cre-dependent optogenetics to label STN-GPi neurons.

(E and F) Diagram and trace of patch-clamp recording of photo-EPSCs from SNr neurons being innervated by STN-SNr-GPi neurons.

(G) Summary of the amplitude of photo-EPSCs in SNr and GPi neurons innervated by STN-SNr-GPi neurons. $t = 3.48$, $df = 66$, $P = 0.0009$. SNr: $n = 40$; GPi: $n = 28$. Two-tailed *t*-test.

(H) Summary of the paired-pulse ratios of photo-EPSCs in SNr and GPi neurons innervated by STN-SNr-GPi neurons. $F_{(3,\ 132)} = 18.76$, $P < 0.0001$. 20 ms: $t = 5.04$, $P < 0.0001$. 50 ms: $t = 4.16$, $df = 132$, $P < 0.0001$. SNr: $n = 40$; GPi: $n = 28$. One-way ANOVA with Holm-Sidak's multiple comparisons test. Some of the data are derived from Figure 2D, G-K from the published paper.[1] The reuse of the data is licensed by Elsevier and Copyright Clearance Center.

complementary to previous studies, this protocol describes a procedure to evaluate the synaptic strength and plasticity of the projections from an upstream nucleus to different downstream nuclei.

Numerous morphological and functional studies have confirmed that different proportions of STN neurons project to both the SNr and GPi.[11,12] Although either electrical or optogenetic stimulation of the STN can evoke EPSCs in both SNr and GPi neurons,[13–16] these studies did not compare the magnitude of the STN-SNr and STN-GPi projections. Combining optogenetic labeling of STN neurons and patch-clamp recordings, we observed that the STN-SNr projection is much stronger than the STN-GPi projection; these projections significantly differ in short-term synaptic plasticity and responsiveness to stimulation.[1] These synaptic features may explain the difference in motor control patterns resulting from the regulation of the STN-SNr and STN-GPi projection.[1] That is, inhibition of the STN-SNr projection, but not the STN-GPi projection, facilitates locomotion; stimulation of the STN-GPi and STN-SNr projection induces transient and sustained inhibition of locomotion, respectively.[1] Additionally, delivering an anterograde transsynaptic viral vector carrying Cre (scAAV1-hSyn-Cre)[9,17] into the STN and Cre-dependent viral vector into the SNr or GPi may allow for reconstructing the anatomical architecture of the downstream SNr and GPi neurons of STN neurons. This approach may provide further neural circuit basis for the different roles of STN-SNr and STN-GPi projection in motor control.

In more complicated circuits, such as, the projections from GABAergic neurons in the ventral pallidum (VP) to GABAergic neurons and dopaminergic (DA) neurons in the ventral tegmental area (VTA), the combination of optogenetics and patch-clamp recording reveals that VP GABAergic neurons send much stronger innervations to VTA GABAergic neurons.[18,19] This circuit pattern conforms

the fact that stimulation of VP GABAergic neurons disinhibits VTA DA neurons promoting arousal in mice.[19]

Characterization of originating neurons of different projections with retrograde viral vectors

Some brain regions, including the STN, may be implicated in multiple behaviors. This heterogeneity may be associated with its divergent projections, which may differ in synaptic strength and plasticity, and origin as well. Viral vector-based retrograde neuronal tracers are valid tools to probe the originating neurons of these projections. In our recent study, we labeled SNr- and GPi-projecting STN neurons (STN-SNr and STN-GPi neurons) by injecting retrograde AAV[20] carrying eGFP or mCherry (AAV retro-hSyn-eGFP or AAV retro-hSyn-mCherry) into the SNr and GPi, respectively.[1] We observed that STN neurons projecting to the SNr distributed evenly throughout the STN, while those to the GPi are concentrated in the anterior part of the STN; STN-SNr neurons are more than STN-GPi neurons; there are some STN neurons projecting to both the SNr and GPi (STN-SNr-GPi neurons).[1] In this protocol, we combined retrograde viral tracing, optogenetics, and patch-clamp technique to evaluate the synaptic strength of the projections from STN-SNr-GPi neurons to the SNr and GPi (Figure 4). Optogenetic labeling of SNr-projecting STN neurons enables evaluation of the projection from STN-SNr-GPi neurons to GPi neurons (Figures 4A–4C), and vice versa (Figures 4D–4F). We observed that the projection from STN-SNr-GPi neurons to SNr neurons were stronger than that from STN-SNr-GPi neurons to GPi neurons (Figures 4G and 4H). Using this method, we revealed that the projections from STN-SNr-GPi neurons to the SNr and GPi are differentially modified in parkinsonian mice.[1] Including a neuronal tracer like biocytin or neurobiotin in the internal solution for patch-clamp recording may enable sophisticated post-hoc morphological assay of SNr and GPi neurons innervated by STN-SNr-GPi neurons.[11] These morphological information may clue strategies to study the potential contributions of these projections to motor deficits in parkinsonian mice.

The neuron types in the SNr and GPi are mostly GABAergic. For nuclei possess multiple neuron types, such as, the VTA, hypothalamus, and VP, etc, particular cell labeling strategies (including neurobiotin, biocytin, fluorescent dye, or cell-specific viral vector) and post-hoc immunostaining of a marker protein of a particular neuron type may be utilized to identify the recorded postsynaptic neurons. These methods may provide anatomical architecture of neural circuit with cell- and projection-specificity.

In summary, these two strategies are useful to characterize pathophysiological features in neurological diseases at the neuron, synapse, and neural circuit levels. These information may pave the foundation for the development of novel therapeutic strategies for brain diseases.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. 3–4 cells were recorded from each brain slice. If drugs for pharmacological characterization of the projection are applied, only one cell is recorded from each brain slice. In each experiment, we collect data from 4–5 mice (3–4 slices per mouse).
2. We discarded data acquired from cells showing a leakage current of > 100 pA and a series resistance (11–25 MΩ) changed by >20% during recording.
3. Calculate paired-pulse ratio by dividing the second photo-EPSC with the first photo-EPSC evoked by paired blue light pulses separated 20 and 50 ms apart.
4. SigmaPlot (version 14.0, SPSS Inc.) was used for statistical analysis. Data are expressed as the mean ± S.E.M in histograms.
   i. *Parametric tests* If the distribution of a parameter passes the normality test and the variations of the parameter between or among groups being compared conform the equal variance test, the comparison of the parameter between or among groups may be analyzed with the *t*-test or analysis of variance (ANOVA).

ii. *Non-parametric tests* If the data are not normally distributed and have unequal variance between or among groups, non-parametric analysis may be applied, such as, Mann-Whitney Rank Rum test or ANOVA Rank Sum test. Additionally, the data from different groups can be transferred into percentiles and the comparison of the distribution of the parameter between two groups can be performed using the Kolmogorov-Smirnov test.

LIMITATIONS

In this protocol, we combined viral vectors and optogenetics to implement cell- and projection-specific stimulation of the STN projections to the SNr and GPi. This protocol allows quantification of the synaptic strength of the STN-SNr and STN-GPi projections originated from STN neurons and collateral STN neurons. But this strategy does not allow interrogation of the STN-SNr and STN-GPi projections originated from STN neurons sending parallel projections only to the SNr and GPi, respectively. To fulfill this goal, the Flex Cre-off system may be applied to inactivate expression of ChR2 in STN-SNr-GPi neurons in mice subjected to injection of AAV-CaMKII-Cre off-ChR2-mCherry.

Optogenetic stimulation differ from electrical stimulation in recruiting synaptic inputs. Light pulses evoke neurotransmitter release by activating ChR2 channels on axonal fibers and terminals, while electrical pulses evoke neurotransmitter release by directly depolarizing axonal fibers and terminals. Electrical pulses can be as short as 100 μs, while the ChR2 channel opens and closes at millisecond time scale. Therefore, optogenetic technique and electrical stimulation may not regulate synaptic function through identical mechanisms. Cautions may be taken for the interpretation of the data.

TROUBLESHOOTING

Problem 1

It is difficult to target brain nuclei as small as the GPi and STN in intracranial microinjection. It is related to the step "intracranial injection of viral vectors-step 3".

Potential solutions

- Use 3–4 months old mice with the same gender, similar weight (mean ± 2 g), and similar pattern of bregma sutures on the skull.
- Adjust the bregma and lambda to the same vertical level, and so do the left and right hemispheres.
- Refer to the parasagittal planes in the mouse brain atlas[7] and set the coordinates according to the center point of the target area.
- Measure the distance between the bregma and lambda on the skull (Figure 2E), calculate a ratio by dividing this distance with that in the mouse brain atlas (4.2–4.3 mm),[7] and calibrate the target coordinates.
- Choose injection needle (33–35 gauge) with blunt tip instead of bevel tip.
- Check the syringe and needle before and after every injection about the smoothness of viral vector flow during suction and injection, and the straightness of the needle, as well.

Problem 2

It is difficult to restrict the viral vector in the target area. This is related to the step "intracranial injection of viral vectors- step 4".

Potential solutions

- Choose the volume of virus injected according to the size of the target area after several rounds of trials and errors.
- Wipe off the viral vector from the injection needle with a cotton swab soaked with sterilized saline to avoid virus expression along the track of the injection needle.

- Lower the injection needle to a depth 0.2 mm deeper than the target DV coordinate before virus injection, then lift it up to the DV coordinate. This procedure makes a pocket for virus injection and may facilitate the absorption and diffusion of the viral vector in the target area.
- Inject the viral vector at slow speed (50–80 nL/min) and retain the injection needle in place for at least 5 min to limit spreading of the virus along the track of the needle.
- Avoid of heating and cooling the syringe and plunger used for virus injection.
- Select particular serotypes of AAV (AAV1, AAV5 and AAV13) to reduce virus spreading.[21,22]

Problem 3

It is difficult to obtain good neuronal viability during brain slice preparation from adult mice. It is related to the step "patch-clamp recordings from brain slices-step 6".

Potential solutions

- Cool down the mouse brain after euthanasia. Expose the skull to ice, place the mouse body on ice, and perform transcardiac perfusion with ice-cold carbogen-saturated cutting solution.
- Reduce neurotoxicity with a modified cutting solution. Partially replace NaCl with sucrose to reduce driving force of voltage-gated sodium channels, lower calcium concentration, but increase magnesium concentration to block NMDA receptors. We used this recipe successfully prepared viable brain slices from Long-Evans rats aged up to 29 months.[23] Other modified cutting solutions, including NMDG-based and glycerol-based cutting solutions, deserve trying to optimize slice preparation.[2–4]
- Cutting parasagittal brain slices preserves GPi, STN, and SNr neurons better. These may be relevant to the orientation of major projections of these neurons which travel along the anterior-posterior axis. One may need to prepare coronal or parasagittal brain slices for the target area to determine which plane is better.
- Set a slow blade travel speed (0.02–0.04 mm/s) and a wide vibration range (>2 mm) when the blade goes through the target area.
- Incubate the slices in the cutting solution all the time and transfer the slices to normal ACSF 30 min before recording.

Problem 4

It is difficult to obtain gigaseal from neurons in brain slices of adult mice. It is related to the step "patch-clamp recordings from brain slices-step 7".

Potential solutions

- Select fusiform neurons with clear edges and smooth membrane surface.
- Optimize the parameters of the pipette puller to obtain electrodes suitable for neurons in target brain regions. With a smaller electrode, one may easily form gigaseal but may have difficulty in puncturing the membrane. One may balance these issues.
- Keep the tip of the electrode clean by adding a constant positive pressure in the electrode to push the intrapipette solution out to prevent contamination of the pipette tip from the debris. Release the positive pressure until a suction is made for gigaseal formation.
- Clean the debris on the surface of the neuron with intrapipette solution flowing out from the recording electrode before gigaseal formation.
- Move the electrode close to the neuron to form a dimple on the surface of the neuron, finely adjust the focus of the objective to confirm that the dimple is in the center of the neuron, and then make a weak suction.

Note: If the electrode and the surface of the neuron are clean and the electrode with a suitable size is in the right place, it is very easy to form gigaseal with a slight suction.

- It is important to keep the internal solution clean. First, clean the glass capillary with methanol and dry it overnight before use. Second, fill internal solution with a set of clean syringe and needle. Third, avoid touching the end of the electrode with fingers to prevent dust contamination.

Problem 5

Optogenetic stimulation readily evokes maximal activation of synaptic inputs and may not be suitable to characterize synaptic properties. This is related to the step "patch-clamp recordings from brain slices-step 7 (g, h)".

Potential solution

- To avoid maximally activating synaptic inputs from STN neurons to SNr and GPi neurons, we first recorded STN neurons and adjust the width (1 ms) and intensity (0.5 mW) of light to evoke single action potential by every light pulse.[1]
- We recorded light-evoked postsynaptic currents in SNr and GPi neurons and confirm that with these parameters, light-evoked postsynaptic currents were not maximal responses, and might be suitable to analyze synaptic plasticity in the STN projection to SNr and GPi neurons.

RESOURCE AVAILABILITY

Lead contact

Further information for resources and reagents should be directed to and will be fulfilled by the lead contact, Cheng Xiao (xchengxj@xzhmu.edu.cn).

Materials availability

This study did not generate new special reagents.

Data and code availability

Source data are provided with this protocol.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102551.

ACKNOWLEDGMENTS

All funding sources: this work was supported by the National Natural Science Foundation of China (81870891 [C.X.], 81971038 [C.Z.], 82071231 [C.X.], 82171235 [C.Z.], and 82271293 [C.X.]), the Fund for Jiangsu Province Specially-Appointed Professor (C.X., C.Z.), the Natural Science Foundation of Jiangsu Province (BK20211349, C.Z.), The Natural Science Foundation of the Jiangsu Higher Education Institutions of China (17KJA320007 [C.X.] and 18KJA320009 [C.Z.]), and the Leadership Program in Xuzhou Medical University (JBGS202203, C.X.).

AUTHOR CONTRIBUTIONS

C.X. and C.Z. designed and supervised this research. Y.-W.J. collected and analyzed the electrophysiological data. Y.-W.J., X.-Y.X., and C.Y. performed mouse survival surgeries and managed the mouse colony. C.X., Y.-W.J., and C.Z. collected the imaging data. Y.-W.J. and X.-Y.X. took pictures and drew diagrams for figures. C.X., C.Z., Y.-W.J., and X.-Y.X. wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ji, Y.W., Zhang, X., Fan, J.P., Gu, W.X., Shen, Z.L., Wu, H.C., Cui, G., Zhou, C., and Xiao, C. (2023). Differential remodeling of subthalamic projections to basal ganglia output nuclei and locomotor deficits in 6-OHDA-induced hemiparkinsonian mice. Cell Rep. *42*, 112178. https://doi.org/10.1016/j.celrep.2023.112178.

2. Ting, J.T., Daigle, T.L., Chen, Q., and Feng, G. (2014). Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. Methods Mol. Biol. *1183*, 221–242. https://doi.org/10.1007/978-1-4939-1096-0_14.

3. Xiao, C., Miwa, J.M., Henderson, B.J., Wang, Y., Deshpande, P., McKinney, S.L., and Lester, H.A. (2015). Nicotinic receptor subtype-selective circuit patterns in the subthalamic nucleus. J. Neurosci. *35*, 3734–3746. https://doi.org/10.1523/JNEUROSCI.3528-14.2015.

4. Ye, J.H., Zhang, J., Xiao, C., and Kong, J.Q. (2006). Patch-clamp studies in the CNS illustrate a simple new method for obtaining viable neurons in rat brain slices: glycerol replacement of NaCl protects CNS neurons. J. Neurosci. Methods *158*, 251–259. https://doi.org/10.1016/j.jneumeth.2006.06.006.

5. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675. https://doi.org/10.1038/nmeth.2089.

6. Dean, A.G., Sullivan, K.M., and Soe, M.M. (2013). OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 3.01. www.OpenEpi.com.

7. Paxinos, G., and Franklin, K.B.J. (2001). The Mouse Brain in Stereotaxic Coordinates, 2nd Edition (Academic Press).

8. Fan, J.P., Zhang, X., Han, Y., Ji, Y., Gu, W.X., Wu, H.C., Zhou, C., and Xiao, C. (2023). Subthalamic neurons interact with nigral dopaminergic neurons to regulate movement in mice. Acta Physiol. *237*, e13917. https://doi.org/10.1111/apha.13917.

9. Jia, T., Wang, Y.D., Chen, J., Zhang, X., Cao, J.L., Xiao, C., and Zhou, C. (2022). A nigro-subthalamo-parabrachial pathway modulates pain-like behaviors. Nat. Commun. *13*, 7756. https://doi.org/10.1038/s41467-022-35474-0.

10. Zhou, C., Gu, W., Wu, H., Yan, X., Deshpande, P., Xiao, C., and Lester, H.A. (2019). Bidirectional dopamine modulation of excitatory and inhibitory synaptic inputs to subthalamic neuron subsets containing alpha4beta2 or alpha7 nAChRs. Neuropharmacology *148*, 220–228. https://doi.org/10.1016/j.neuropharm.2019.01.015.

11. Sato, F., Parent, M., Levesque, M., and Parent, A. (2000). Axonal branching pattern of neurons of the subthalamic nucleus in primates. J. Comp. Neurol. *424*, 142–152. https://doi.org/10.1002/1096-9861(20000814)424:1<142::aid-cne10>3.0.co;2-8.

12. Van Der Kooy, D., and Hattori, T. (1980). Single subthalamic nucleus neurons project to both the globus pallidus and substantia nigra in rat. J. Comp. Neurol. *192*, 751–768. https://doi.org/10.1002/cne.901920409.

13. Ammari, R., Lopez, C., Bioulac, B., Garcia, L., and Hammond, C. (2010). Subthalamic nucleus evokes similar long lasting glutamatergic excitations in pallidal, entopeduncular and nigral neurons in the basal ganglia slice. Neuroscience *166*, 808–818. https://doi.org/10.1016/j.neuroscience.2010.01.011.

14. Fife, K.H., Gutierrez-Reed, N.A., Zell, V., Bailly, J., Lewis, C.M., Aron, A.R., and Hnasko, T.S. (2017). Causal role for the subthalamic nucleus in interrupting behavior. Elife *6*, e27689. https://doi.org/10.7554/eLife.27689.

15. Luan, Y., Tang, D., Wu, H., Gu, W., Wu, Y., Cao, J.L., Xiao, C., and Zhou, C. (2020). Reversal of hyperactive subthalamic circuits differentially mitigates pain hypersensitivity phenotypes in parkinsonian mice. Proc. Natl. Acad. Sci. USA *117*, 10045–10054. https://doi.org/10.1073/pnas.1916263117.

16. Shen, K.Z., and Johnson, S.W. (2006). Subthalamic stimulation evokes complex EPSCs in the rat substantia nigra pars reticulata *in vitro*. J. Physiol. *573*, 697–709. https://doi.org/10.1113/jphysiol.2006.110031.

17. Zingg, B., Chou, X.L., Zhang, Z.G., Mesik, L., Liang, F., Tao, H.W., and Zhang, L.I. (2017). AAV-Mediated Anterograde Transsynaptic Tagging: Mapping Corticocollicular Input-Defined Neural Pathways for Defense Behaviors. Neuron *93*, 33–47. https://doi.org/10.1016/j.neuron.2016.11.045.

18. Knowland, D., Lilascharoen, V., Pacia, C.P., Shin, S., Wang, E.H.J., and Lim, B.K. (2017). Distinct Ventral Pallidal Neural Populations Mediate Separate Symptoms of Depression. Cell *170*, 284–297.e18. https://doi.org/10.1016/j.cell.2017.06.015.

19. Li, Y.D., Luo, Y.J., Xu, W., Ge, J., Cherasse, Y., Wang, Y.Q., Lazarus, M., Qu, W.M., and Huang, Z.L. (2021). Ventral pallidal GABAergic neurons control wakefulness associated with motivation through the ventral tegmental pathway. Mol. Psychiatry *26*, 2912–2928. https://doi.org/10.1038/s41380-41020-00906-41380.

20. Tervo, D.G.R., Hwang, B.Y., Viswanathan, S., Gaj, T., Lavzin, M., Ritola, K.D., Lindo, S., Michael, S., Kuleshova, E., Ojala, D., et al. (2016). A designer AAV variant permits efficient retrograde access to projection neurons. Neuron *92*, 372–382. https://doi.org/10.1016/j.neuron.2016.09.021.

21. Han, Z., Luo, N., Wu, Y., Kou, J., Ma, W., Yang, X., Cai, Y., Ma, L., Han, L., Wang, X., et al. (2022). AAV13 enables precise targeting of local neural populations. Int. J. Mol. Sci. *23*, 12806. https://doi.org/10.3390/ijms232112806.

22. Xiao, C., Cho, J.R., Zhou, C., Treweek, J.B., Chan, K., McKinney, S.L., Yang, B., and Gradinaru, V. (2016). Cholinergic mesopontine signals govern locomotion and reward through dissociable midbrain pathways. Neuron *90*, 333–347. https://doi.org/10.1016/j.neuron.2016.03.028.

23. Fan, J.P., Geng, H.Z., Ji, Y.W., Jia, T., Treweek, J.B., Li, A.A., Zhou, C.Y., Gradinaru, V., and Xiao, C. (2022). Age-dependent alterations in key components of the nigrostriatal dopaminergic system and distinct motor phenotypes. Acta Pharmacol. Sin. *43*, 862–875. https://doi.org/10.1038/s41401-021-00713-5.