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Cell-attached and Whole-cell Patch-clamp Recordings of Dopamine Neurons in the Substantia Nigra Pars Compacta of Mouse Brain Slices

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[Abstract] The Substantia Nigra pars compacta (SNc) is a midbrain dopaminergic nucleus that plays a key role in modulating motor and cognitive functions. It is crucially involved in several disorders, particularly Parkinson's disease, which is characterized by a progressive loss of SNc dopaminergic cells. Electrophysiological studies on SNc neurons are of paramount importance to understand the role of dopaminergic transmission in health and disease. Here, we provide an extensive protocol to prepare SNc-containing mouse brain slices and record the electrical activity of dopaminergic cells. We describe all the necessary steps, including mouse transcardiac perfusion, brain extraction, slice cutting, and patch-clamp recordings.

Keywords: Acute brain slices, Transcardiac perfusion, Substantia Nigra, Dopaminergic neurons, Electrophysiology, Patch clamp, Mouse

[Background] The Substantia Nigra pars compacta (SNc) is a midbrain nucleus that provides a strong dopaminergic input to various areas of the brain, including the basal ganglia (BG) (Gerfen and Bolam, 2010). Dopaminergic axons originate from tyrosine hydroxylase (TH)-expressing neurons of the SNc. The neuromodulator dopamine (DA) released by synaptic terminals of these projections provides a vital contribution to the regulation of BG microcircuitry, which is crucially involved in modulating motor and reward functions (Zhai *et al*., 2019).

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. Dopaminergic neurons degenerate in PD, leading to a depletion of dopamine in the striatal circuitry and causing a detrimental imbalance between direct and indirect output pathways (Gerfen and Surmeier, 2011). This ultimately results in devastating symptoms such as persistent tremors, bradykinesia, rigidity, and often neuropsychiatric alterations (Surmeier *et al*., 2017). Thus, advancing our knowledge of dopaminergic neuronal functions in normal and pathological conditions is a task of great importance and a major goal for many neuroscience laboratories.

Although primary cultures are an interesting system to investigate the cellular physiology of dopaminergic neurons, a relatively low yield of about 5% in tyrosine hydroxylase (TH, a cytochemical marker of DA cells)-expressing neurons may represent a challenge for electrophysiological experiments (Choi *et al*., 2013). Moreover, it has been reported that cultured dopaminergic neurons lose their autonomous pacemaking activity (Masuko *et al*., 1992), which is crucial for the sustained release of dopamine and plays a fundamental role in both physiological and pathological conditions. On the other

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hand, *ex vivo* brain slices represent a reliable and reproducible preparation preserving specific features of dopaminergic neurons, including their autonomous pacemaking activity. Yet, a potential downside of this technique is the deafferentation caused by the cutting process used to prepare the sample. Alternative techniques such as *in vivo* extracellular single-unit recordings could be used to study TH^+ neurons (Farassat *et al.*, 2019); however, whole-cell recordings may be difficult to obtain *in vivo*, precluding the possibility to investigate intrinsic and synaptic properties of DA cells. Slice experiments therefore remain the gold-standard option for whole-cell recordings in DA neurons.

A great number of studies provide combinations of slice cutting and recording solutions that are specifically used for different brain regions (Bischofberger *et al.*, 2006) from young, adult, or aging mice (Ting *et al.*, 2014). Technical and manual precautions are obviously necessary in order to avoid, or at least minimize, various types of insults (mechanical, osmotic, metabolic, *etc.*) inflicted to the brain specimen during slice preparation. In particular, sucrose-based artificial cerebrospinal fluid (ACSF) has been used to prevent excessive intracellular influx of chloride and subsequent cell swelling and lysis (Aghajanian and Rasmussen, 1989). Here, we provide a sucrose-based cutting solution and slice preparation protocol tailored to SNc-containing brain slices. We describe all the necessary steps, including transcardiac perfusion, brain extraction, dissection, and patch-clamp recordings. We show how these methods can be used to study the pharmacological effects of acutely applied drugs as a tool to investigate potential novel targets for the treatment of neurodegenerative disorders such as PD (Regoni *et al.*, 2020).

Materials and Reagents

1. NaCl (Sigma-Aldrich, catalog number: 793566; store at room temperature (RT))
2. KCl (Sigma-Aldrich, catalog number: 409316; RT)
3. NaH_2PO_4 (Sigma-Aldrich, catalog number: S5011; RT)
4. $CaCl_2$ 1 M (Fluka, catalog number: 21114; store at 4°C)
5. $NaHCO_3$ (Sigma-Aldrich, catalog number: 792519; RT)
6. $MgCl_2$ (Sigma-Aldrich, catalog number: M8266; RT)
7. D-glucose (Sigma-Aldrich, catalog number: G5767; RT)
8. Sucrose (Sigma-Aldrich, catalog number: S9378; RT)
9. KH_2PO_4 (Sigma-Aldrich, catalog number: P9791; RT)
10. HEPES (Sigma-Aldrich, catalog number: H4034; RT)
11. EGTA (Sigma-Aldrich, catalog number: 03777; RT)
12. Na_2-ATP (Sigma-Aldrich, catalog number: A7699; store at -20°C)
13. Na-GTP(Sigma-Aldrich, catalog number: G8877; store at -20°C)
14. KOH (Fluka, catalog number: 319376; store at 4°C)
15. NaOH (Sigma-Aldrich, catalog number: S2770; store at 4°C)
16. Agarose (Fisher Scientific, catalog number: BP1356; RT)
17. Ketamine-HCl (Anesketin (100 mg/ml), Dechra; RT)

18. Xylazine (Rompun (20 mg/ml), Bayer; RT)
19. Ketamine/xylazine anaesthetic mixture (see Recipes)
20. Artificial cerebrospinal fluid (ACSF) 10× stock solution (w/o $CaCl_2$ and $MgCl_2$; see Recipes)
21. $MgCl_2$ 1 M stock solution (see Recipes)
22. Artificial cerebrospinal fluid (ACSF) 1× (see Recipes)
23. Cutting solution (see Recipes)
24. Internal solution (see Recipes)

Equipment

A. Equipment for brain slice preparation
1. VT1000S vibratome (Leica Microsystems, Wetzlar, Germany)
2. Osmometer 3320 (Advanced Instruments, Norwood, MA)
3. TW2 thermostatic water bath (Julabo, Seelbach, Germany)
4. Peristaltic pump with perfusion tubing line (Gilson Minipuls 3)
5. Paper filter disk (Millipore, catalog number: GSWPP04700)
6. Glass Petri dish (diameter: 10 cm; Merck, catalog number: BR455701)
7. Gas diffusing stone (Fisher Scientific, catalog number: 10686185)
8. 50-ml beaker
9. Stainless steel vibratome blades (Campden Instruments, catalog number: 752/1/SS)

B. Equipment for custom-made slice chamber
1. Plastic Petri dish (Thermo Fisher Scientific, catalog number: 150318)
2. Nylon mesh
3. Cyanoacrylate glue (*e.g.*, Super Attack; Loctite)
4. 100-ml beaker
5. Borosilicate glass capillaries (Sutter Instrument, catalog number: BF100-50-7.5 or equivalents)
6. Two plastic Pasteur pipettes
7. Assembly: The maintenance chamber is made up of a plastic Petri dish (diameter 3.5 cm), in which the bottom surface has been substituted with a soft nylon mesh (Figure 1A). The mesh is fixed to the dish rim with cyanoacrylate glue. The chamber is positioned inside a 100-ml beaker containing ACSF (70 ml) (Figure 1C). A glass capillary attached to a gas-impermeable tube is placed inside the chamber to supply oxygen (95% O_2 + 5% CO_2) (Figure 1D). The heads of two plastic Pasteur pipettes, one embedded into the other, are used to shim the chamber and hold the capillary inside the beaker (Figure 1B,C), to avoid bubble diffusion directly onto the slices. During preparation, individual slices are gently positioned on the mesh immediately after being cut away from the brain. After use, the maintenance chamber should be regularly disassembled for careful cleaning or replacement of its components.

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C. Dissection tools

1. Surgical scissors, sharp blunt (*e.g.*, Fine Science Tools (FST), catalog number: 14001-14)
2. Fine scissors, sharp 11.5 cm (*e.g.*, FST, catalog number: 14060-11)
3. Fine scissors, sharp 10.5 cm (*e.g.*, FST, catalog number: 14060-10)
4. Dumont #5, fine forceps (*e.g.*, FST, catalog number: 11254-20)
5. Dumont #3c, forceps (*e.g.*, FST, catalog number: 11231-20)
6. Blunt forceps (Aven Forceps, Straight Serrated Tips, catalog number: 18433)
7. Vannas spring scissors (*e.g.*, FST, catalog number: 15018-10)
8. Disposable sterile scalpel blade No.21
9. Spoon (similar to VWR, catalog number: USBE3317)
10. Flat spatula (similar to VWR, catalog number: RSGA038.185)
11. Cyanoacrylate glue ("superglue," *e.g.*, Super Attack, Loctite)

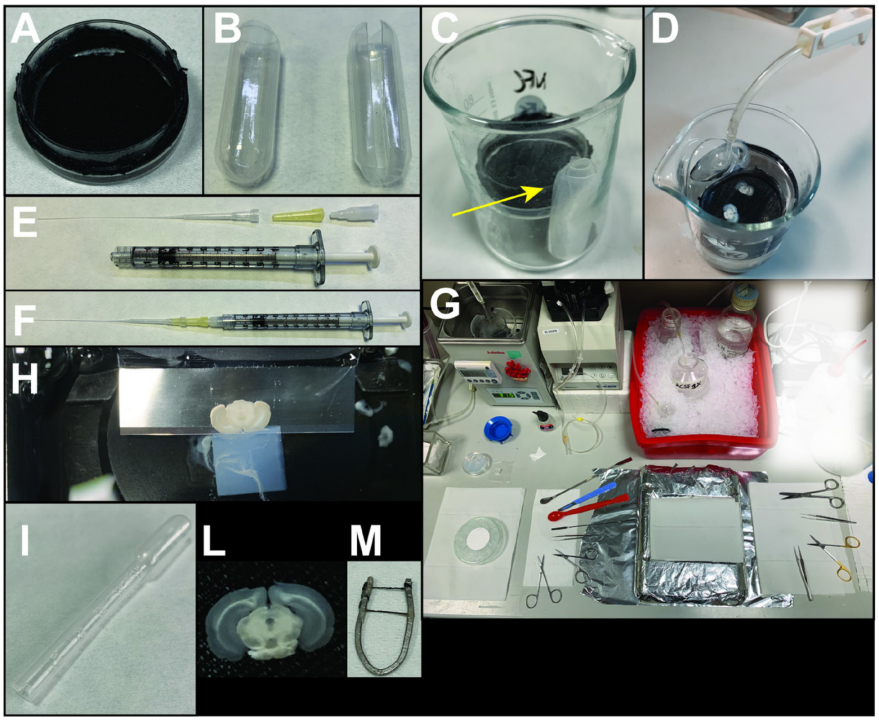

Figure 1. Representative images of the equipment required for SNc brain slice preparation. (A) Maintenance chamber. (B) Heads of plastic Pasteur pipettes used to create the envelope for the glass capillary oxygenator (arrow in C). (C, D) Custom-made slice chamber. (E-F) Components and assembled syringe for back-filling the electrode (see Note 4). (G) Preparation of the perfusion/slicing desk. (H) Coronal slice cut by the vibratome blade. Note the agarose cube positioned behind the brain specimen. (I) Plastic Pasteur pipette used to handle the brain slices. (L) Example of an SNc-containing brain slice. (M) U-shaped custom-made anchor (see Note 3).

D. Equipment for electrophysiology experiments (Figure 2)

1. BX51WI upright microscope (Olympus, Japan) equipped with infrared-differential interference contrast optics (IR-DIC)
2. IsoStation anti-vibration table (Newport, Irvine, CA)
3. Charge-coupled device (CCD) camera (*e.g.*, Hamamatsu, QImaging, Sentech, Teledyne, *etc.*)
4. MultiClamp 700B operational amplifier (Molecular Devices, Sunnyvale, CA)
5. Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA)
6. Personal computer
7. Mini 25 micromanipulator unit (Luigs & Neumann)
8. SM-5 electronic control (Luigs & Neumann)
9. Badcontroller V temperature controller (Luigs & Neumann)
10. PC-10 electrode puller (Narishige, Japan)
11. 1-ml BD Luer-Lok™ insulin syringe without needle (BD, catalog number: 329651)
12. Microloader tips (Eppendorf, catalog number: 5242956003)
13. Syringe-driven filter unit (PVDF, 0.22 µm; SLGVR04NL Millex)
14. Home-made brain U-shaped anchor (see Note 3)
15. Borosilicate glass capillaries with filament (O.D.: 1.5 mm, I.D.:1.10 mm; 7.5 cm length) (Sutter Instrument, catalog number: BF150-110-7.5HP)

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Figure 2. Patch-clamp setup

Software

1. pClamp 10 (Molecular Devices, https://www.moleculardevices.com)
2. GraphPad Prism (La Jolla, CA, https://www.graphpad.com/scientific-software/prism/)

Procedure

A. Prepare fresh ACSF 1× (see Recipe section below).

B. Setup instruments and solutions for brain slice preparation (see Note 2 and Figure 1G).
   1. Fill the slice maintenance chamber with ACSF, deliver 95% O_2 + 5% CO_2 through a small glass capillary (same as those used to pull patch clamp pipettes, Figure 1D), and place the chamber in the thermostatically controlled water bath at 32°C.

2. Place a glass bottle containing ~20 ml ACSF in a tray filled with ice and oxygenate (95% O_2 + 5% CO_2) the solution using a gas-diffusing stone for at least 20 min (see Note 1).
3. Mount a perfusion tubing onto the peristaltic pump. Place the suction end into the oxygenated ice-cold ACSF and mount a 25 G needle at the opposite end.
4. Fill the vibratome tray with ice. Mount the vibratome buffer chamber (which will receive the specimen plate; see Section F).
5. Take 200 ml sucrose-containing cutting solution (kept at 4°C) and fill the vibratome buffer chamber. Oxygenate the cutting solution with 95% O_2 + 5% CO_2.
6. Prepare a 10-cm glass Petri dish filled with ice and capped with its own lid. Place a paper filter disk over the lid and wet the filter with 2-3 ml ice-cold cutting solution.
7. Prepare a 2% agarose cube (~1 cm^3 in volume; see Note 6).

C. Animal anesthesia
1. Use a stock ketamine/xylazine mixture (see Recipes section below), adjusting the volume of injection according to the mouse weight (in our experiments, we injected ~300 µl in 25- to 30-day-old mice of both genders).
2. Perform an intraperitoneal injection of the anesthetic solution, then put the mouse back in the cage.
3. Carefully monitor the status of anesthesia before proceeding with transcardiac perfusion. Correctly anesthetized mice should be completely unresponsive to paw pinching. Do not proceed with cardiac perfusion until the animal is completely anesthetized.

D. Transcardiac perfusion
1. Set the speed of the peristaltic pump so that 1-2 drops per second of ice-cold ACSF flow through the 25 G needle (if using a Gilson minipuls 3, a speed of 4.5 is recommended). Before continuing, be sure that no bubbles are present in the perfusion tubing line.
2. Place the mouse (ventral side facing up) on a polystyrene tray covered with aluminum foil.
3. To facilitate the surgical procedure, fix the mouse limbs to the tray using syringe needles.
4. Pour a few ml 70% ethanol on the mouse abdomen to prevent fur dispersion.
5. Using forceps (*e.g.*, Dumont #3c), lift the skin of the mouse at the level of the liver and use fine scissors to perform a V-shaped cut through the abdominal wall, starting from beneath the rib cage to the collarbone to expose the liver and the diaphragm (Figure 3A).
6. Carefully separate the liver from the diaphragm using blunt forceps.
7. Using sharp scissors, make a small incision at the center of the diaphragm and cut from side to side to expose the pleural cavity.
8. While lifting the sternum, cut the sternopericardial ligament to separate the heart from the bone.
9. Holding the heart with blunt forceps, insert the perfusion needle into the left ventricle (Figure 3A).

10. Once the needle is in place, cut the right atrium of the heart with Vannas spring scissors (avoid touching the descending aorta).
11. Increase the speed of the peristaltic pump to 5 ml/min. The animal will be correctly perfused when the liver and limbs have turned pale white (taking approximately 20-30 s starting from the moment the perfusion speed is raised).

E. Brain collection

1. Remove the head of the mouse using surgical scissors. Place the head onto the ice-filled glass Petri dish.
2. Expose the skull by cutting the mouse skin from the neck to the nose tip. Remove skin and muscle residues using fine forceps (Dumont #5) (Figure 3B).
3. Use fine scissors to cut the skull at the level of the lambda and remove the cerebellum and occipital bone (Figure 3B).
4. Use fine scissors to carefully cut along the lateral part of the skull starting from the most posterior part of the parietal bone to the eye socket. Subsequently, cut the nasal bone from side to side at the level of the anterior frontal bone suture (Figure 3B).
5. Using fine forceps, peel away the dorsal part of the skull. If performed correctly, minimal strength is sufficient to detach the skull and expose the brain.
6. Insert a small flat spatula at the level of the olfactory bulbs and separate the brain from the base of the skull in the rostro-caudal direction. Cut the cranial nerves in the most ventral part, and carefully detach the anterior part of the brain.
7. Trim any residual connection of the brain with the skull and delicately drop the brain into a 50-ml beaker filled with oxygenated, ice-cold ACSF.

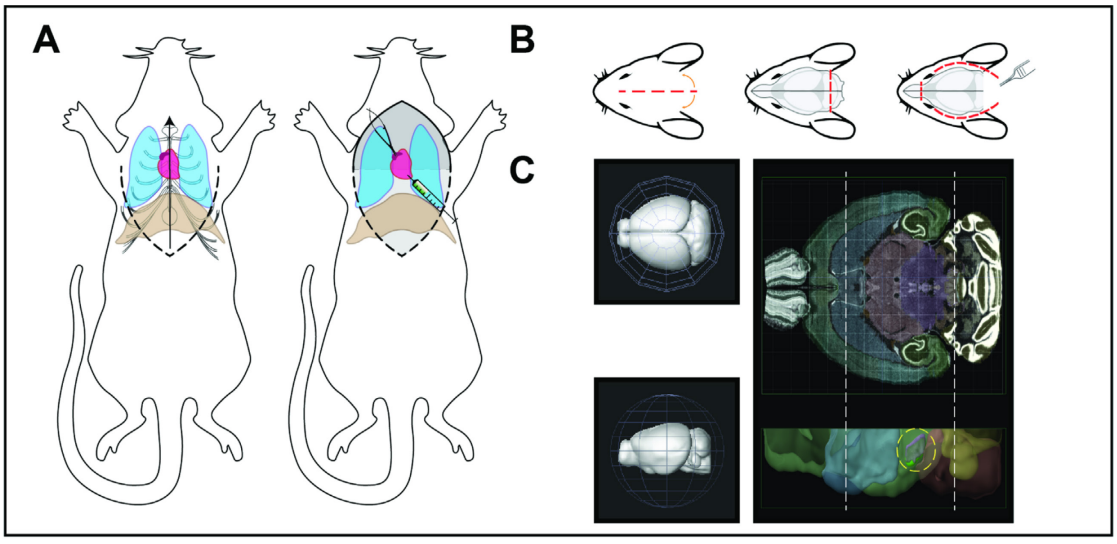
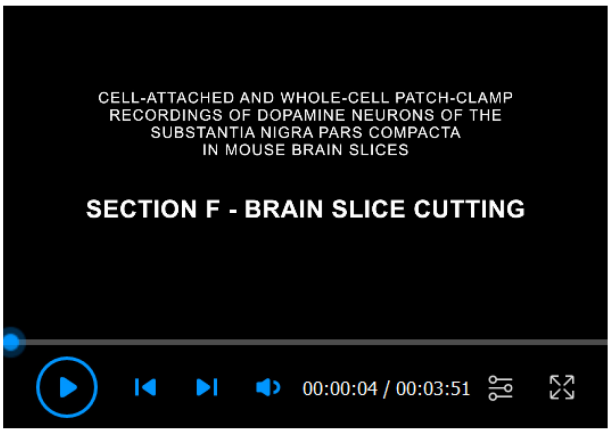


Figure 3. Representation of the essential steps for brain slice preparation. (A) Schematic images of mouse transcardiac perfusion. Left, a V-shaped cut in the abdomen skin is represented by the dashed lines. Right, needle insertion into the left ventricle and subsequent

cut in the right atrium with Vannas scissors. (B) Scheme of skull cuts prior to brain explant. Red dashed lines represent cutting trajectories as described in section E (brain collection). (C) 3D dorso-ventral and antero-posterior views of the mouse brain (Allen Institute; Brain Explorer 2). White dashed lines represent coronal cuts, both anterior (at the level of the striatum) and posterior (to remove the residual cerebellum). The yellow dashed circle highlights the SN.

F. Brain slice cutting (see Video 1)



Video 1. Brain slice cutting procedure. Preparation of SNc coronal slices using a vibratome and slice collection in a mantainance chamber.

1. Pick up the brain from the ACSF solution using a spoon and lay it onto the ice-filled glass Petri dish (with the ventral part touching the filter paper disk).
2. Using a scalpel, perform a coronal cut at the level of the striatum and a second coronal incision to remove the residual cerebellum (Figure 3C). Be careful not to cut the midbrain region containing the SNc.
3. Pour 1-2 drops superglue at the center of the vibratome specimen plate and spread to form a thin layer. The layer surface should be large enough to host the brain block and the agarose cube.
4. Lift the brain with a spatula and carefully remove the residual ACSF using absorbent paper.
5. Lay the brain onto the glue on the vibratome specimen plate, with the posterior coronal plane facing up.
6. Glue the 2% agarose cube close to the cortices (*i.e.*, behind the brain block). This will help to keep the brain steady and firm throughout the cutting process (Figure 1H).
7. Insert and lock the specimen plate into the vibratome buffer chamber with the ventral side of the brain facing the blade (such that the SNc will be closer to the blade; Figure 1H).
8. Set the vibratome frequency at approximately 85 Hz, slice thickness at 250 µm, and advance speed at 0.15 mm/s. Start cutting the tissue.
9. Discard all slices that do not contain the SNc.

10. Approximately 4-5 slices containing the SNc will be obtained with these cutting settings (Figure 1L).

    Note: We advise using Sylvius aqueduct and hippocampal formation as supplementary elements to clearly define the anteroposterior coronal plane (Figure 4).

11. Transfer the brain slices containing the SNc into the maintenance chamber at 32°C and allow them to recover for 30 min. Cover the chamber with a plastic lid. To handle the slices, use a plastic Pasteur pipette (Figure 1I) previously cut at 2/3 of its length or a fine brush (the use of a brush requires particular attention to avoid pinching the tissue with the bristles).

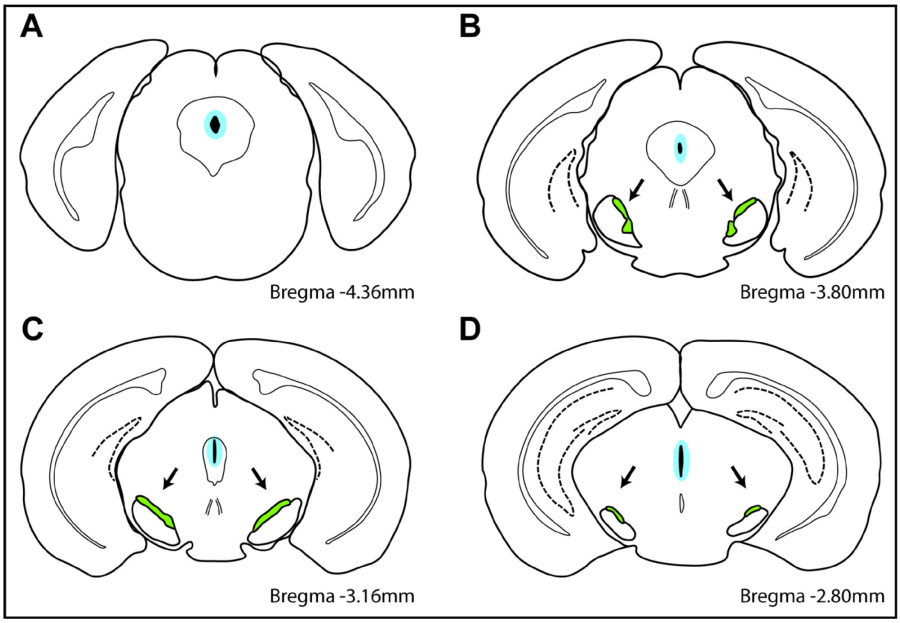


Figure 4. Schematic representation of coronal sections containing the SNc. Arrows point to SNc areas (green) through different cutting planes. Light blue outlines the Sylvius aqueduct.

In this section, we will discuss the experimental setup used in a recent study published by our lab (Regoni *et al.*, 2020).

G. Cell-attached and whole-cell patch-clamp electrophysiology

1. Turn on the perfusion system of the electrophysiology setup so that oxygenated ACSF will continuously flow in the recording chamber at a rate of 2-3 ml/min. Turn on a suction system (vacuum- or peristaltic pump-driven) to maintain a constant level of liquid in the recording chamber.
2. Turn on the bath temperature controller and set it to 32°C. Wait until the ACSF temperature in the bath has reached the desired level.
3. If multiple drug concentrations are to be tested, prepare the proper volume of oxygenated ACSF supplemented with the different drug concentrations. In our custom-made perfusion setup, we used three 50-ml syringes connected to a Luer-lok 3-way manifold system that could be easily switched on/off according to the experimental protocol.

4. Carefully position an SNc-containing slice in the recording chamber and fix it to the chamber bottom using the U-shaped anchor (Figure 1M).
5. Visually identify the SNc using a 4× objective (Figure 5A). As previously mentioned, use the Sylvius aqueduct and hippocampal formation as supplementary elements to clearly define the antero-posterior coronal plane of the brain slice.
6. Switch to a larger objective (40×) to visually identify dopaminergic neurons. These cells are packed next to each other within a relatively narrow area at the edge of the SNr. As shown in Figure 5A, dopaminergic neurons display an ovoidal, elongated soma (longer axis of about 20 µm) and are easily recognizable.
7. Fill a patch-clamp pipette with the internal solution, remove any air bubbles, and load it on the electrode holder (see Note 4 and Figure 1E, F).
8. Apply slight positive pressure to the pipette tip before moving toward the brain slice.
9. Using the micromanipulator, gently move the pipette toward the cell, while continuously monitoring the pipette tip resistance. Upon touching the cell membrane, the pipette resistance progressively increases, and a small indentation of the membrane beneath the pipette tip becomes visible.
10. Quickly remove the positive pressure.
11. Carefully apply slight and continuous negative pressure until the resistance increases to at least 1 GΩ (giga-seal), reaching a cell-attached configuration.
12. Dopaminergic cells of the SNc spontaneously fire action potentials in a pace-making fashion. Cell firing will be stable approximately 2 min after obtaining a cell-attached configuration. Under our conditions, the DA neuronal spontaneous firing pattern is mostly regular, with a mean firing frequency of 1.75 ± 0.18 Hz in WT animals (Regoni *et al.*, 2020) (Figure 5C).
13. Pharmacological studies on drugs able to modify the firing pattern of these cells can be performed by acutely switching the perfusion from control to drug-supplemented ACSF at different concentrations.
14. Finally, move into whole-cell patch clamp configuration by quickly applying brief negative pressure to the pipette to open the cell membrane (“break-in”).
15. At this stage, current-clamp experiments can be performed to univocally identify the firing pattern of DA neurons, as shown in Figure 5C (see also Figure 5D and Note 5). This step is necessary because different types of cells in the SNc are able to fire action potentials spontaneously. Cell recognition may be ambiguous in the cell-attached configuration; thus, whole-cell recordings are required to better detect membrane potential responses to hyperpolarizing current steps (namely, a prominent and fast I_h-mediated depolarizing sag that is typical of DA cells). Firing and electrical membrane parameters of SNc TH^+ neurons are summarized in Table 1.

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Table 1. Firing and electrical membrane parameters of SNc TH⁺ neurons

Input resistance (MΩ)	Spontaneous firing frequency (Hz)	AP amplitude (mV)	AP threshold (mV)	AHP amplitude (mV)	AP halfwidth (ms)	Sag amplitude (mV)
120 ± 10	1.75 ± 0.18	60 ± 1.7	-37 ± 0.7	21 ± 1.2	1.73 ± 0.05	44 ± 5

Data are given as the mean ± S.E.M. AP, action potential; AHP, afterhyperpolarization.

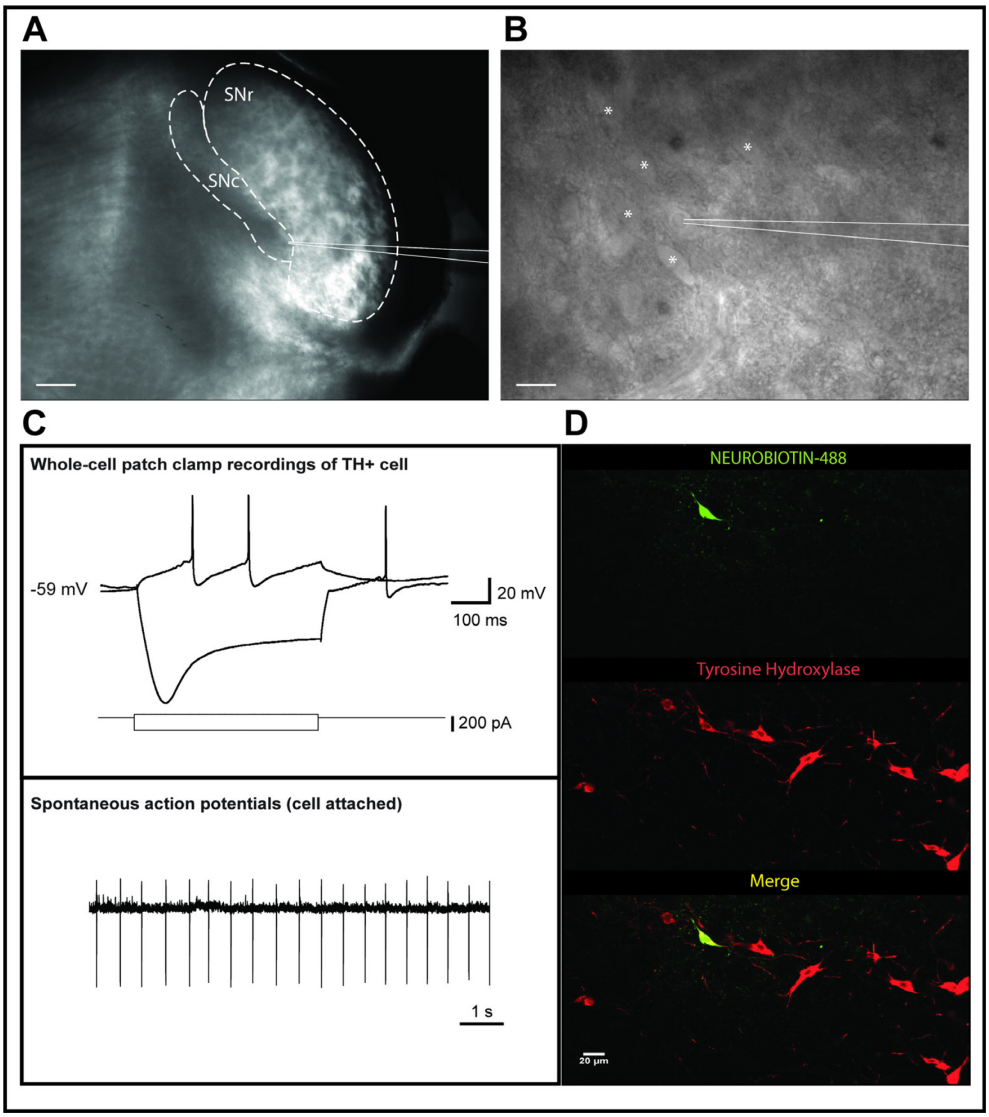

Figure 5. Electrophysiological recordings in dopaminergic neurons of the SNc. (A) Brightfield microphotograph showing an SNc-containing brain slice (4× objective; scale bar: 200 µm). (B) Example of a visually identified TH⁺ cell approached by a patch-clamp pipette (outlined by white lines). Asterisks mark other putative dopaminergic neurons (note the ovoidal shape of cell bodies; scale bar: 20 µm). (C) Example of patch-clamp whole-cell recordings in current-clamp mode from an SNc DA neuron in an acute slice. The upper traces show membrane potential hyper- and depolarizing responses to intracellular injection of negative and positive current steps, respectively (-200/+50 pA, 500 ms). Traces in the lower panel show cell-attached

recordings of spontaneous regular action potential firing in a different SNc DA neuron. (D) Confocal images showing one TH^+ neuron, previously filled with Neurobiotin-488 (green) during a whole-cell recording, surrounded by other TH^+ (red) neurons in the SNc.

Data analysis

Data analysis details can be found in the Materials and Methods section of Regoni *et al.* (2020). Under our conditions, the DA neuronal spontaneous firing pattern is regular and the signal-to-noise ratio of cell-attached recordings is high. Thus, the firing frequency can be easily assessed by eye counting the number of action potentials within a given time interval (*e.g.*, 20 s).

Notes

1. To oxygenate physiological solutions, we use gas-diffusing stones or glass capillaries attached to gas-impermeable tubing connected to a source (*e.g.*, tank or pipe system) of 95% O_2 + 5% CO_2 gas mixture.
2. A key aspect for good brain slice preparation is to be as rapid and precise as possible. During transcardiac perfusion and brain collection, being fast will reduce the risk of hypoxia-induced cell death. Moreover, it is important to be manually accurate to avoid excessive mechanical stress on the brain sample during the dissection process.
3. The U-shaped anchor is made up of a flat platinum wire with 2-3 nylon threads crossing through the two arms of the U, as shown in Figure 1M. The nylon is fixed to the platinum wire with superglue.
4. To easily load the patch-clamp electrode with a clean intracellular solution, we use a custom-made syringe made up of the following components: a 1-ml BD Luer-Lok™ insulin syringe without a needle; a microloader tip; a syringe-driven filter unit; and a 200-µl micropipette tip.
   To build the system: (1) attach one side of the filter unit to the Luer-Lok™ syringe; (2) cut the 200-µl tip at 2/3 of its length and insert it into the other side of the filter unit; and (3) insert the 200-µl tip into the microloader tip.
5. A further control step may be performed to univocally identify the patched cell. As shown in Figure 5D, the recording internal solution containing 1.5 mg/ml Neruobiotin-488 Tracer (Vector Laboratories, catalog number: SP-1125-2) was added. The dye diffused into the DA cell soma during the whole-cell recording. Subsequently, the brain slice was collected, fixed in 4% PFA, and cryopreserved. Sections of 14-µm thickness were obtained using a Leica cryostat (Leica CM1850), collected onto Superfrost slides, and air-dried overnight before storage at -80°C until staining. Immunostaining using a monoclonal antibody against tyrosine-hydroxylase (1:500; Mab318, Millipore) was performed to molecularly identity the patched neuron. Leica SP5 equipped with a 40× objective (Germany) was used to acquire the images.

6. To prepare a 2% agarose cube, add 1 g agarose to 50 ml water. Heat the solution in a microwave oven until the agarose is completely dissolved, then pour it into a 10-cm Petri dish. Once the agarose solution becomes solid, store at 4°C. Use a surgical knife to cut small cubes (approx. 1 cm^3 in volume).

Recipes

Always use high-quality water with a resistivity of at least 18.2 MΩ·cm (*e.g.*, Millipore Milli-Q) for patch-clamp experiment solutions.

1. Ketamine/xylazine anesthetic mixture
   500 µl ketamine-HCl
   250 µl xylazine
   5.25 ml 0.9% (w/v) NaCl
2. Artificial cerebrospinal fluid (ACSF)
   125 mM NaCl
   3.5 mM KCl
   1.25 mM NaH_2PO_4
   2 mM $CaCl_2$
   25 mM $NaHCO_3$
   1 mM $MgCl_2$
   11 mM D-glucose
   Saturated with 95% O_2 and 5% CO_2 (pH 7.3)
3. Cutting solution
   50 mM sucrose
   125 mM NaCl
   2.5 mM KCl
   1.25 mM NaH_2PO_4
   0.1 mM $CaCl_2$
   25 mM $NaHCO_3$
   6.2 mM $MgCl_2$
   2.5 mM D-glucose
   Saturated with 95% O_2 and 5% CO_2
4. Internal solution
   10 mM NaCl
   124 mM KH_2PO_4
   10 mM HEPES
   0.5 mM EGTA
   2 mM $MgCl_2$

2 mM Na_2-ATP

0.02 mM Na-GTP

Adjust pH to 7.2 with KOH

Preparation of stock and "ready-to-use" solutions:

a. $MgCl_2$ 1 M stock solution (100 ml)
   9.52 g $MgCl_2$
   Filter and store at 4°C
b. Artificial cerebrospinal fluid (ACSF) 10× stock solution (1 L; w/o $CaCl_2$ and $MgCl_2$)
   73.05 g NaCl
   21 g $NaHCO_3$
   2.6 g KCl
   1.72 g NaH_2PO_4
   1.98 g glucose
   Filter and store at 4°C
c. ACSF 1× (1 L)
   1 ml $MgCl_2$ 1 M
   2 ml $CaCl_2$ 1 M
   Add ~700 ml water
   Add 100 ml 10× ACSF
   Add water to adjust volume to 1 L
   Check osmolarity. Correct value must be 300 ± 2.5 mOsm
d. Cutting solution 1× (1 L)
   7.305 g NaCl
   2.1 g $NaHCO_3$
   186 mg KCl
   172 mg NaH_2PO_4
   450 mg glucose
   17.11 g sucrose
   6.2 ml $MgCl_2$ 1 M
   100 µl $CaCl_2$ 1 M
   Filter and store at 4°C up to 1 week
e. Internal solution (50 ml)
   844 mg KH_2PO_4
   29 mg NaCl
   120 mg HEPES
   9 mg EGTA
   100 µl $MgCl_2$
   55 mg Na_2-ATP

0.5 mg Na-GTP
Adjust pH to 7.2 with KOH
Check osmolarity. Correct value must be 290 ± 2.5 mOsm
Aliquot 1 ml in 50 capped tubes and store at -20°C

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Competing interests

There are no conflicts of interest or competing interests.

Ethics

Mice were maintained and bred at the Animal Facility of the San Raffaele Scientific Institute in compliance with institutional guidelines and international laws (EU Directive 2010/63/EU EEC Council Directive 86/609, OJL 358, 1, December 12, 1987, NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All experiments were conducted with the aim of minimizing the number of sacrificed animals.

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