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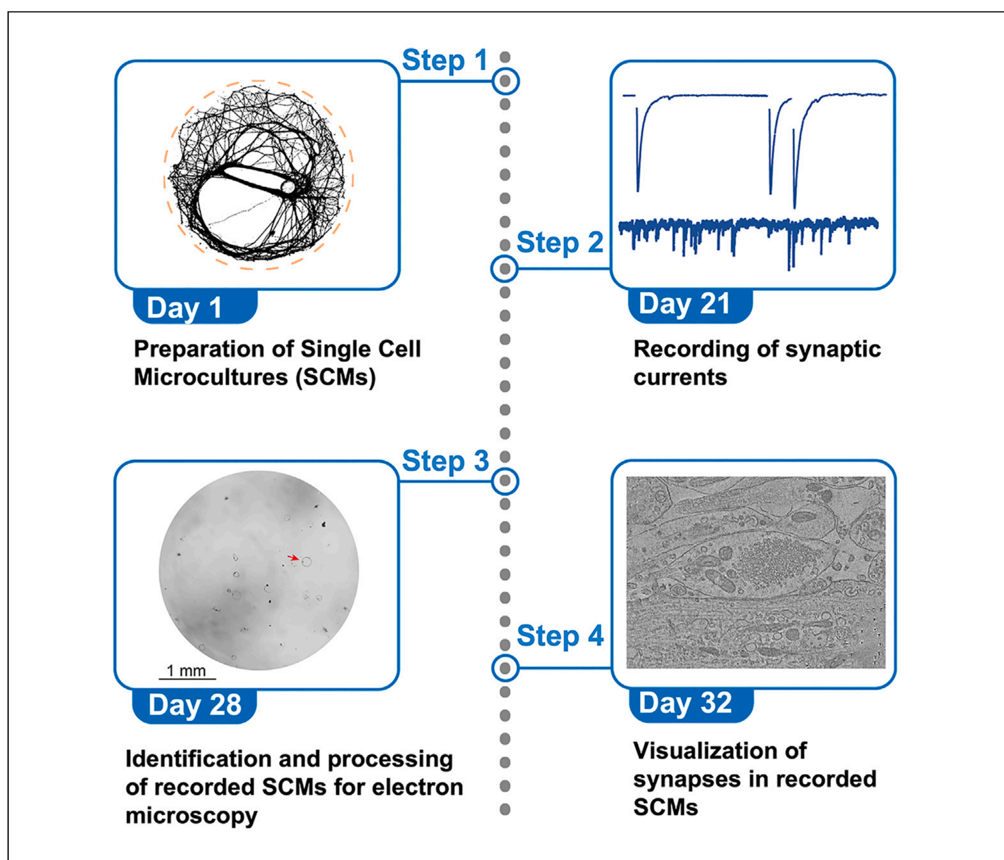
Protocol to correlate electron microscopy with electrophysiology in single-cell autaptic microcultures



Pablo Martínez San Segundo, Anna Priscil·la Pérez González, Cecilia D. Velasco, Artur Llobet

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Highlights

Technique to establish cholinergic single-cell autaptic microcultures (SCMs)

Steps to identify the characteristic neurotransmission features of SCMs

Guidance on associating neurotransmitter release with the ultrastructure of autapses

Single-cell microcultures (SCMs) form a monosynaptic circuit that allows stimulation and recording of postsynaptic responses using a single electrode. Here, we present a protocol to establish autaptic cultures from rat superior cervical ganglion neurons. We describe the steps for preparing SCMs, recording synaptic currents, and identifying and processing the recorded neurons for electron microscopy. We then detail procedures for visualizing synapses. This protocol is illustrated by correlating evoked and spontaneous neurotransmitter release with the ultrastructural features of synapses recorded.

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

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Protocol to correlate electron microscopy with electrophysiology in single-cell autaptic microcultures

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SUMMARY

Single-cell microcultures (SCMs) form a monosynaptic circuit that allows stimulation and recording of postsynaptic responses using a single electrode. Here, we present a protocol to establish autaptic cultures from rat superior cervical ganglion neurons. We describe the steps for preparing SCMs, recording synaptic currents, and identifying and processing the recorded neurons for electron microscopy. We then detail procedures for visualizing synapses. This protocol is illustrated by correlating evoked and spontaneous neurotransmitter release with the ultrastructural features of synapses recorded.

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

BEFORE YOU BEGIN

The protocol below describes the specific steps to carry out a correlative electrophysiology and electron microscopy experiment of a single cell autaptic neuronal culture. There are three sequential steps: (i) preparation of a single cell microculture (SCM) from rat superior cervical ganglion neurons, (ii) recording of synaptic activity, (iii) processing of recorded neurons for electron microscopy. Expected outcomes are based on the identification of the characteristic ultrastructure of synapses associated to synaptic responses. It is necessary to have access to a fully equipped tissue culture room, a patch clamp recording set-up and, to a transmission electron microscope. The application of the protocol here described can be found in previously published works.[1–3] To learn more about the use of SCMs to the study of neurotransmission see.[4–6]

Although the method is illustrated for a specific type of neuron, it can be applied to any single cell neuronal culture. Superior cervical ganglion neurons display simpler trophic requirements than central nervous system neurons, which allows them to form functional cholinergic synapses in the absence of glia.[5]

The superior cervical ganglion contains adrenergic neurons embedded in a dense meshwork of connective tissue. To successfully complete the protocol, it is important to practice manual steps before doing the procedures here described, particularly, those related to the generation of collagen microdots.

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As the growth of SCMs lacks glial support, the nature and characteristics of the neuronal substrate is key. Best results are obtained with collagen prepared in the laboratory, which should be ready before starting the protocol here described.

The presence of Ciliary Neurotrophic Factor (CNTF) in the culture medium induces a phenotypic change of adrenergic neurons to cholinergic. The expression of functional acetylcholine receptors in autapses is typically observed after two weeks in culture and can be certified by electrophysiological recording. It is important to have a 10 mM stock solution of hexamethonium, which is a blocker of ganglionic neuronal acetylcholine receptors, to confirm the presence of cholinergic neurotransmission. The specific steps that must be carried out before beginning the procedure are described below.

Institutional permissions

The method of neuronal culture here described requires the permission of the relevant regulatory committees on animal use. This protocol was approved by the Department of Environment from the Generalitat de Catalunya (Spain).

Preparation of glass pipettes for mechanical dissociation of ganglia

Timing: 30 min

Mechanical dissociation of superior cervical ganglia is achieved by repetitive suction and expulsion of the ganglia through a modified glass Pasteur pipette.

1. Reduce the size of the tip opening using a Bunsen burner before sterilizing the pipettes in the autoclave. Decrease the diameter of the tip from the original size of 1.2 $\pm$ 0.15 mm to large, medium, and small openings, corresponding to approximate reductions of the tip size by 50%, 66% and 75%, respectively.

Preparation of rat tail collagen

Timing: 5 days

Day 1 (4–5 h)

All materials used must be previously sterilized. Keep sterile conditions during the whole procedure because collagen cannot be sterilized after its preparation. Scissors, forceps, hemostats, and water must be autoclaved.

2. Extraction of collagen fibers.
   a. Collect tails from adult rats (~4–6 rats) and rinse in 95% ethanol.
   b. Clamp tails at its proximal end using a hemostat. Place a second hemostat to ~ 1 cm from the first one. Fracture the tail by bending it sharply with the second hemostat. The proximal segment of the tail is held only by tendons. Slide off the tendons slowly. Cut the pieces of tendon that dangle from the remainder portion of the tail dropping them into a sterile Petri dish.
   Repeat step b) with all tails until all tendons are removed.
   c. Wash tendons three times in sterile water while teasing apart their fibrils with forceps.
   d. Collect approximately 4 g of tendons and place them in a sterile conical flask with 150 mL of 3% (v/v) glacial acetic acid (~0.5 N).
   e. Stir the solution at the minimum possible speed at 4°C for 24 h to dissolve the tendons.

⚠ CRITICAL: As tendons dissolve the solution becomes denser, slowing down the stir bar. Increase gradually the speed of the stirring magnet. Keep in mind that if stirred for too long or too vigorously, tendon proteins other than collagen could start dissolving.

Day 2 (4 h)

f. When most of the tendons are dissolved collect the solution and centrifuge at 20000 g for 75 min.
g. Dialysis of collagen. Collect the supernatant and transfer it (avoiding bubbles) to a dialysis membrane, which has been previously boiled in 1 mM EDTA for 10 min and washed 5 times in sterile water.
h. Place the membrane in a 5 L glass beaker and dialyze it against 3 L of cold filtered-sterile 0.1× MEM, pH 4.0 without bicarbonate. Leave at 4°C.

Day 3 (30 min)

i. Replace the 3 L of cold filtered-sterile 0.1× MEM, pH 4.0 without bicarbonate medium with the same volume of newly prepared 0.1× MEM.

Day 4 (30 min)

j. Repeat step i).

Day 5 (3 h)

k. Collect the collagen and add penicillin/streptomycin (1% v/v final concentration) (100× stock) to 1×.
l. Distribute the collagen in 5 mL aliquots and store at 4°C. Label the vials with the preparation date.

⚠ CRITICAL: Collagen must be diluted in a medium with a pH around 8, for instance RPMI-1640 10×. This dilution is done immediately before spraying the collagen dots on the day of the culture. It is necessary to test the optimal dilution as it changes from batch to batch. Do not use collagen older than 4 months.

Practicing the establishment of collagen microdots

Timing: 2 h

3. Practice the establishment of collagen microislands.
   a. Immerse 60 thermanox coverslips in a glass beaker containing 50 mL of 70% (v/v) ethanol during 5 min.
   b. Under the vertical laminar flow hood, take coverslips individually, rinse with sterile MilliQ water and distribute them in 12 well-plates.
   c. Leave the coverslips dry under the UV light.
   d. Take 1 mL of RPMI-1640 10× and mix it with 1 mL of prepared collagen. The 1:1 dilution is the first condition to be tested.
   e. Place the diluted collagen in a sterile perfume atomizer.
   f. Spray the collagen dots (see details in the autaptic cultures section) and immediately put 1 mL of PBS or culture media previously warmed in the incubator.
   g. Leave them in the incubator for 10 min and check the appearance of microdots (Figure 1).

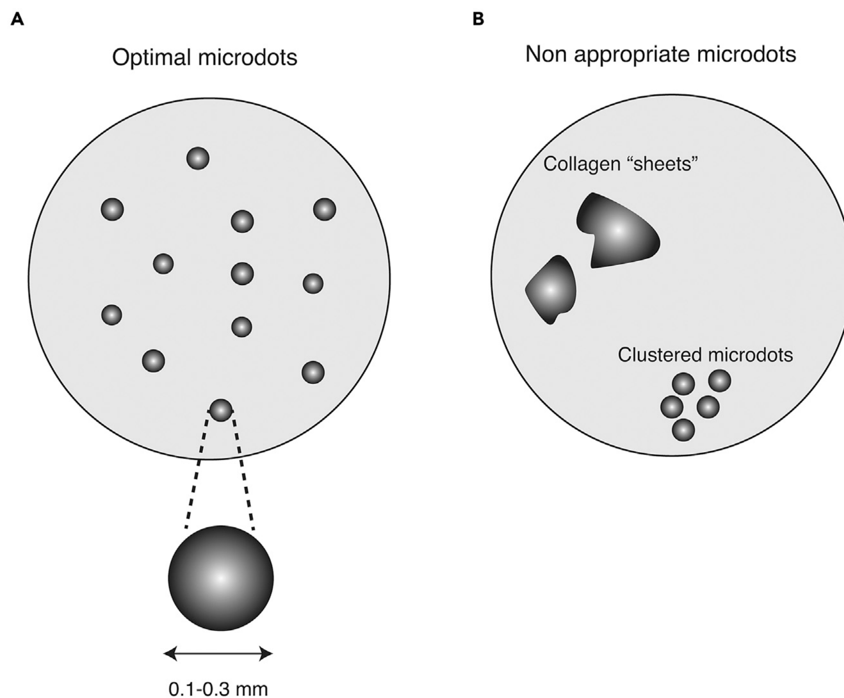

Figure 1. Testing the establishment of collagen microdots in a coverslip
(A) Optimal distribution of microdots on a coverslip, which should have a diameter ranging from 0.1 to 0.3 mm.
(B) If collagen is too diluted, there is a tendency to form "sheets" of collagen. If spraying is performed too close to the coverslip, microdots can appear cluttered and display a non-homogeneous distribution.

h. Repeat steps d-g by increasing the amount of collagen up to 600 μL and decreasing the volume of medium accordingly (final volume of 2 mL), until the desired profile and distribution of collagen dots is achieved (Figure 1). It is recommendable to increase the amount of collagen if the dots easily detach. If the collagen concentration is too high, the atomizer will clog.

Preparation of solutions for patch-clamp recording

Timing: 2 h

4. Preparation of external recording solution.
   a. Add: sodium chloride, potassium chloride, HEPES hemisodium salt and magnesium chloride (see materials and equipment) to a beaker with 800 mL sterile double distilled water (ddH2O) and stir until reagents are fully dissolved.
   b. Titrate pH to 7.4 using 1 M NaOH.
   c. Add the necessary volume of ddH20 to reach 290 mOsm (~1 L of external solution is reached).
   d. Transfer the solution to a sterile bottle indicating pH, osmolality and preparation date.
   e. Keep the stock solution at 4°C. Discard solutions older than 1 month.
5. Preparation of internal recording solution.
   a. Add all components (see materials and equipment) to a beaker with 40 mL sterile ddH2O and stir until reagents are fully dissolved, keeping the beaker on ice.
   b. Titrate pH to 7.2 using 1 M KOH.
   c. Add the necessary volume of ddH20 to reach an osmolality of 290 mOsm (~50 mL of internal solution is reached).
   d. Distribute the internal solution in 1 mL aliquots. Keep vials at −20°C.

Preparation of electrodes and recording chamber

Timing: 6 h

6. Prepare electrodes and recording chambers.
   a. Prepare recording electrodes by immersion of silver wires in bleach for 30 min. The thicknesses of the patch and reference electrodes are 0.25 and 0.5 mm, respectively.
   b. Solder the reference electrode to a socket connector contact with a size of 2.5 mm.
   c. Attach the reference electrode prepared as indicated to the recording chamber (RC-25F) using an epoxy adhesive and wait ~6 h until the epoxy adhesive hardens.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM/F-12 with glutamine, without HEPES	Gibco, Paisley, Scotland, UK	11320-033
Fetal bovine serum	Biological Industries, Israel	04-007-14
Rat serum	Supplied by Animal Facilities	
Mouse nerve growth factor, NGF-7S	Alomone Labs, Israel	N-130
Rat ciliary neurotrophic factor, CNTF	Alomone Labs, Israel	C-245
Penicillin/streptomycin	Sigma-Aldrich, St. Louis, MO	P0781
Agarose II-A	Sigma-Aldrich, St. Louis, MO	A9918
Collagenase type IA	Sigma-Aldrich, St. Louis, MO	C9891
Trypsin EDTA 0.05%	Gibco, Paisley, Scotland, UK	25300-62
Minimum essential medium Eagle, MEM	Sigma-Aldrich, St. Louis, MO	M0644
RPMI-1640 medium 10×	Sigma-Aldrich, St. Louis, MO	R1383
Ethylenediaminetetraacetic acid, EDTA	Sigma-Aldrich, St. Louis, MO	E-9884
Sodium chloride	Merck, Darmstadt, Germany	CAS# 7647-14-5
Potassium chloride	Merck, Darmstadt, Germany	CAS# 7447-40-7
Magnesium chloride solution	Merck, Darmstadt, Germany	CAS# 7786-30-3
HEPES hemisodium salt	Merck, Darmstadt, Germany	CAS# 103404-87-1
Calcium chloride solution	Merck, Darmstadt, Germany	CAS# 10043-52-4
Glucose	Merck, Darmstadt, Germany	CAS# 50-99-7
Potassium gluconate	Merck, Darmstadt, Germany	CAS# 299-27-4
Sodium guanosine 5-triphosphate	Merck, Darmstadt, Germany	CAS# 36051-31-7
Disodium adenosine 5′-triphosphate	Merck, Darmstadt, Germany	CAS# 34369-07-8
HEPES	Merck, Darmstadt, Germany	CAS# 7365-45-9
Sodium cacodylate 0.2 M (pH 7.6)	Electron Microscopy Sciences	11650
25% glutaraldehyde (GA) solution	Electron Microscopy Sciences	16216
Potassium ferricyanide	Electron Microscopy Sciences	20150
4% osmium tetroxide (OsO_4) solution, 2 mL/vial	Electron Microscopy Sciences	19150
Tannic acid	Electron Microscopy Sciences	21700
Propylene oxide solution	Electron Microscopy Sciences	20410
Embed-812 resin	Electron Microscopy Sciences	14900
Araldite-502	Electron Microscopy Sciences	10900
DDSA special	Electron Microscopy Sciences	13710
DMP-30	Electron Microscopy Sciences	13600
Ethanol absolute ≥99.5%	VWR Chemicals	CAS# 64-17-5
Epoxy adhesive	RS Components, UK	132-605
Other		
Glass Pasteur pipettes 150–59 mm	Deltalabs, Barcelona, Spain	701
Dialysis tubing cellulose membrane	Sigma-Aldrich, St. Louis, MO	D9527-100FT

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dumont #55 biology tweezers, 11 cm long, straight, 0.05 × 0.01 mm tips	World Precision Instruments Inc, Sarasota, FL	14099
Vannas scissors, 8 cm long, straight, 0.015 × 0.015 mm, super fine	World Precision Instruments Inc, Sarasota, FL	501778
McPherson Vannas scissors, 12 cm long, curved.	World Precision Instruments Inc, Sarasota, FL	503364
Borosilicate glass capillaries (without filament), 1.2 mm outer diameter, 0.69 mm inner diameter; 100 mm length	Harvard Apparatus, UK	Model GC120F-10 Order #30-0044
Micropipette puller	Sutter Instrument, Novato, CA	Model P-97
Trough filament, 3.0 mm wide	Sutter Instrument, Novato, CA	FT330B
Silver wire 0.25 mm diameter	Advent, UK	AG548511
Silver wire 0.50 mm diameter	Advent, UK	AG548609
Socket solder contact 2.5 mm 22–26 AWG	Bulgin Limited, Switzerland	SA3347/1
Circular solder contact 2.5 mm 22–26 AWG	Bulgin Limited, Switzerland	SA3348/1
Crimp contact 2 mm diameter	Phoenix Contact, Germany	SF-7QP2000
Water hydraulic micromanipulator	Narishige, Japan	model MHW-3
CV-4 headstage, gain X1/100	Axon Instruments, US	
Pipette holder	Molecular Devices, UK	Model 1-HL-U
RC-25F recording chamber	Warner Instruments, Hamden, US	Cat# 64-0233
P-1 platform chamber	Warner Instruments, Hamden, US	Cat# W4 64-0277
Series 20 stage adapters, 11 cm diameter	Warner Instruments, Hamden, US	Cat# 64-2411
Suction tube, Series 20 classic design, right hand	Warner Instruments, Hamden, US	Cat# W4 64-1400
Tygon S3 E-3603 flexible tube	Saint Gobain Life Sciences	12355909
Cover glasses 24 × 50 mm, thickness #1	VWR, Germany	Cat# 631-0146
Cover glasses 15 mm diameter, thickness #1	VWR, Germany	Cat# 631-15798
High-vacuum grease	Dow Corning, Sigma- Aldrich, St. Louis, MO	Cat# Z273554-1EA
Axopatch-1D patch-clamp amplifier	Molecular Devices, San Jose, CA	
ITC-18 board	InstruTech Corp, Port Washington, NY	
Thermanox coverslips (diameter 15 mm)	Thermo Scientific	174969
Surgical blade	Swan-Morton	0211
12-well culture plate	Sarstedt	83.3921
Glass microbeaker 2.5 mL	Electron Microscopy Sciences	60982
BEEM embedding capsules size 3	Electron Microscopy Sciences	69910-01
BEEM embedding capsule holder for size 3	Electron Microscopy Sciences	69917-01
Forceps, Rochester-Pean 7.25″	World Precision Instruments Inc	501707
Nugent utility forceps, angled tip	World Precision Instruments Inc	504473
Laboratory stove at 60°C		
Vapor pressure osmometer	Wescor Inc, USA	Model 5520
Liquid N_2 container		
Vegetable Oil		

MATERIALS AND EQUIPMENT

Pre-plating medium

Reagent	Final concentration	Amount
DMEM/F-12 with glutamine, without HEPES		8 mL
Fetal Bovine Serum (FBS)	20% v/v	2 mL
TOTAL		10 mL

Culture medium

Reagent	Final concentration	Amount
DMEM/F-12 with glutamine, without HEPES		23,6 mL
Fetal Bovine Serum (FBS)	2,5% v/v	625 µL
Rat serum (RS)	2,5% v/v	625 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Mouse NGF-7S	5 nM	* 125 µL
Rat CNTF (added after the 1st change of medium)	2 nM	* 50 µL
Penicillin/Streptomycin (P/S)	0.50%	125 µL
TOTAL		25 mL

*Diluted from a stock solution of 1 µM stored at −80°C. The addition of NGF and CNTF is done after equilibration of the culture media.

External recording solution		
Reagent (MW = molecular weight in g/mol)	Final concentration	Amount
Sodium chloride (NaCl, MW = 58.3)	130 mM	7.58 g
Potasium chloride (KCl, MW = 74.6)	5 mM	373 mg
HEPES hemisodium salt (MW = 249.3)	10 mM	2.493 g
Magnesium chloride ($MgCl_2$, stock 1M)	2 mM	2 mL
Glucose (MW = 180)	10 mM	* 1.8 g
Calcium chloride ($CaCl_2$, stock 1M)	2 mM	* 2 mL
ddH_2O	Add for total volume of 1 L	
TOTAL		1 L

Note: recording solution can be stored at 4°C for one month.
*Glucose and $CaCl_2$ are added on the day of the experiment.

Internal recording solution		
Reagent (MW = molecular weight in g/mol)	Final concentration	Amount
Potassium gluconate (Kglu, MW = 234.3)	130 mM	1.52 g
HEPES (MW = 238.3)	10 mM	119.15 mg
Magnesium chloride ($MgCl_2$, stock 1M)	4 mM	0.2 mL
Ethylene glycol-bis(2-aminoethylether)-N-N,N′,N′-tetraacetic acid (EGTA, MW = 380.4)	1 mM	19.02 mg
Disodium adenosine 5′-triphosphate (Na_2-ATP, MW = 551.1)	3 mM	82.6 mg
Sodium guanosine 5′-triphosphate (Na-GTP, MW = 523.2)	1 mM	340.08 mg
ddH_2O	Add for total volume of 50 mL	
TOTAL		50 mL

Note: Internal recording solution can be stored at −20°C for six months.

Puller settings				
Step	Heat	Pull	Velocity	Time
1	*298	120	7	200
2	295	120	9	200
3	295	120	9	200
4	295	120	9	200
5	300		80	110

*Parameters adjusted for a though 3 mm filament with a ramp test value of 303 to obtain micropipettes with a series resistance of ~ 5 MΩ.

Solution buffers

- Sodium cacodylate 0.1 M: add 50 mL Sodium cacodylate 0.2 M in 50 mL ddH_2O.
- Sodium cacodylate 0.05 M: add 10 mL Sodium cacodylate 0.2 M in 30 mL ddH_2O.

Note: Prepare the solution before use.

Fixative solution		
Reagent	Final concentration	Amount
Glutaraldehyde 25%	1.8% (v/v)	3.6 mL
Sodium cacodylate (pH 7.6) 0.1 M	0.1 M	46.4 mL
Total	N/A	**50 mL**
Note: Osmolality must always be checked, and it should be between 290–320 mOsm. It is possible to adjust it with ddH2O.		

⚠ CRITICAL: Glutaraldehyde is a toxic strong irritant and should be handled inside the fume hood wearing the appropriate protective gear including gloves and the lab coat. All waste should be placed in chemical fixative waste bottles.

1% OsO_4 and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate		
Reagent	Final concentration	Amount
Aqueous 4% OsO_4	1% (v/v)	2 mL
Potassium ferricyanide	1.5% (w/v)	0.12 g
Sodium cacodylate (pH 7.6) 0.1 M	0.1 M	6 mL
Total	N/A	**8 mL**
Note: Prepare it fresh before use. This recipe is for the processing of 4 samples using 1 vial of 4% OsO_4.		

⚠ CRITICAL: OsO_4 is highly volatile and reactive. Handle it under the fume hood, using double gloves, the lab coat and the FFP3 mask. All materials containing OsO_4 must always be previously treated with vegetable oil to inactivate OsO_4. Seal tightly afterwards and place in a container specific for toxic residues.

Tannic acid in 0.05 M sodium cacodylate		
Reagent	Final concentration	Amount
Tannic acid	1% (w/v)	0.01 g
Sodium cacodylate (pH 7.6) 0.05 M	0.05 M	10 mL
Total	N/A	**20 mL**
Note: Vortex quickly as it clumps easily. Wear double gloves and mask.		

EtOH dilutions

- Ethanol 90% (v/v): add 90 mL Ethanol 100% to 10 mL ddH_2O.
- Ethanol 70% (v/v): add 70 mL Ethanol 100% to 30 mL ddH_2O.

Note: Prepare the solution before use.

Epon resin		
Reagent	Final concentration	Amount
Embed-812 resin	N/A	8.3 mL
Araldite-502	N/A	5 mL
DDSA Special	N/A	18.3 mL
DMP-30	N/A	0.6 mL
Total	N/A	**50 mL**
Note: Vortex quickly after adding all the compounds and then leave it to settle for at least 30 min.		

Epon resin and propylene oxide mix		
Reagent	Final concentration	Amount
Propylene oxide	1:1	4 mL
Epon resin	1:1	4 mL
Total	N/A	8 mL

⚠ CRITICAL: Propylene oxide is very toxic and volatile. Careful work under the fume hood is required. Wear a lab coat, double gloves and FFP3 mask. Propylene oxide will dissolve most laboratory plastic, hence EM-grade plasticware or glass should be used for handling this solution.

STEP-BY-STEP METHOD DETAILS

Autaptic cultures

Timing: 18–21 days

This section describes the procedure to establish an autaptic culture of cholinergic neurons from rat superior cervical ganglia. Mature functional synapses are achieved in a time window of 18–21 days.

Day 1 (4–5 h)

1. Preparation of coverslips and substrates.
   a. Immerse 24 thermanox coverslips in a glass beaker containing 50 mL of 70% (v/v) ethanol.
   b. Sonicate for 15 min.
   c. Under the vertical laminar flow hood, take coverslips individually, rinse with sterile MilliQ water and distribute them in 12 well-plates.
   d. Leave the coverslips dry under the UV light.
   e. Warm the 0.15% w/v agarose suspension in the microwave until achieving dissolution.
   f. Place the agarose solution in the previously sterilized coverslips and remove it before it becomes solid. This manipulation ensures the creation of a thin layer of agarose that provides a non-permissive substrate for neuronal growth.
   g. Leave the coverslips under the UV light for at least 1 h for proper jellification and sterilization.

⚠ CRITICAL: Check that rat tail collagen (see the 'before you begin' section) is in optimal conditions. Collagen must present an appropriate viscosity and turbidity. When aliquots are stored for more than 4 months, or are subject to temperature alterations, they lose their properties.

2. Preparation of culture media.
   a. Place 25 mL of culture medium in a 25 cm^3 flask with filter cap (see the 'materials and equipment' section) and leave it in the incubator at 37°C during 1 h to equilibrate temperature and pH.

   Note: For seeding neurons, culture medium containing 0.25% penicillin/streptomycin is used but, for subsequent medium changes the concentration of antibiotics doubles to 0.50%.

   b. Take another 25 cm^3 flask with filter cap and place 10 mL of pre-plating medium. Leave it in the incubator at 37°C.

⚠ CRITICAL: The % of CO_2 selected in the incubator must be adjusted to guarantee a pH between 7.2 and 7.4 of the culture medium. Typical values range between 5 and 8% CO_2.

3. Extraction of the superior cervical ganglion.
   a. Albino Sprague-Dawley rats (P0-P2) are chilled on ice for 15 min to achieve anesthesia.

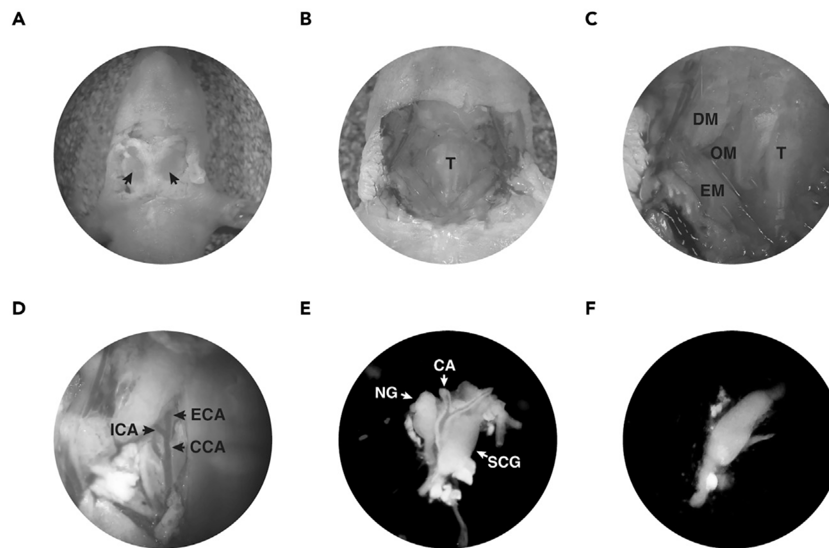

Figure 2. Dissection and extraction of the superior cervical ganglion from rat pups

(A) Image showing the pup facing upwards after removing the skin from the ventral area of the neck. The salivary glands are exposed (arrows).

(B) Removing the glandular tissue allows the visualization of the trachea (T) and (C) the bilateral sternocleidomastoid (EM), digastric (DM) and omohyoid (OM) muscles (magnification from B).

(D) View of the common carotid artery (CCA) and its external (ECA) and internal (ICA) bifurcations after removal of the EM and OM.

(E) Representative example of the resulting structure obtained from cutting the carotid artery (CA) at its internal, external, and common ends. The superior cervical ganglion (SCG) is found in the dorsal part of the bifurcation of the carotid artery. The nodose ganglion (NG) is adjacent to the exterior carotid artery.

(F) SCG separated from the carotid artery before removing the connective tissue that recovers it.

b. The rat pup is secured with needles to expose its ventral part and maintained at a temperature between 4°C and 8°C.
c. A superficial incision is made at the level of the trachea. Skin is removed revealing the salivary glands (Figure 2A).
d. Salivary glands are removed allowing visualization of the trachea and the sternocleidomastoid muscles on both sites (Figures 2B and 2C).
e. Gently clean the surrounding tissue to reach the carotid artery, which is identified by its characteristic Y-shape (Figure 2D).
f. The SCG is located beneath the bifurcation of the artery. The SCG and the adjacent segment of the carotid artery are removed together (Figure 2E).
g. Transfer immediately the portion of the artery and the SCG to a petri dish with ice-cold PBS.
h. Repeat steps c-g to remove the contralateral SCG. Use all the litter, which typically contains 8–15 pups.
i. Use Dumont #55 tweezers to separate the SCG from the carotid artery (Figure 2F).
j. Transfer SCGs to a petri dish containing sterile ice-cold PBS.
k. Use thin tweezers to remove the capsule of the SCG. An excess of connective tissue can lead to an abundant presence of fibroblasts in the culture.

Note: Extraction of rat superior cervical ganglion (SCG) is performed in a horizontal laminar flow hood at room temperature using a stereo microscope. Details of the surgical procedure are shown in Figure 2 and can also be found elsewhere.[7] Surgical material for this procedure is kept under UV light for at least 30 min prior to initiating the dissection.

4. Tissue dissociation (sequential enzymatic and mechanical treatment).

a. Enzymatic treatment. Transfer the isolated SCGs to a 1.5 mL vial containing 2.5 mg/mL collagenase solution. Place in an incubator (37°C and 8% CO_2, 10 min).
b. Carefully remove the collagenase solution without perturbing the SCGs and add 1 mL of 0.05% trypsin-EDTA solution. Place at 37°C for 20 min in an incubator.
c. Remove the trypsin-EDTA solution and replace it with 1 mL of pre-plating medium to ensure inactivation of remaining enzymes.
d. Mechanical treatment. Using sterilized glass Pasteur pipettes (see the 'before you begin' section), repeatedly aspirate and eject the ganglia through the tip.

Note: Successful dissociation of the ganglia is achieved by the sequential use of large, medium, and small tip pipettes.

i. Begin using a large tip pipette.
ii. Change pipette when the tissue passes through its opening without friction.

e. End the procedure when the medium has a turbulent aspect without visible pieces of tissue. The time required for mechanical dissociation typically ranges between 1 and 3 min.

⚠ CRITICAL: It Is important to avoid the formation of bubbles during mechanical dissociation. It is helpful to suction media without any ganglia before starting the disaggregation of the tissue: This will prevent the tissue from sticking inside the pipette.

5. Pre-plating period.
   a. Place pre-plating medium (10 mL) containing the cell suspension in a 10 cm-diameter-culture dish and place in the incubator.

Note: The duration of the pre-plating period solely depends on the desired outcome. The longer it is, the fewer the number of glial cells in the final culture. A pre-plating period of 30 min will provide 10% of SCMs, resulting in the majority of autaptic cultures containing glial cells. The optimal pre-plating period to obtain a predominant population of SCMs is 1 h.

Note: During the pre-plating time prepare all the items (RPMI medium, perfume atomizer and collagen), required to generate the substrate microdots (also known as microislands).

⚠ CRITICAL: Pre-plating periods longer than 1 h may have a negative effect on neuronal development.

6. Seeding of neurons.
   a. Collect the medium containing the neuronal suspension from the 10 cm-diameter-culture dish and place it in a 15 mL tube. Centrifuge at 2000 g for 2 min.
   b. Remove the supernatant and resuspend the pellet in 1 mL culture medium.
   c. Resuspended cells are forced to pass three times through a sterile 25G hypodermic needle. The aim is to enable a better isolation of the neurons preventing the clusters that these neurons tend to form.
   d. Count the total number of cells in a Neubauer chamber.
   e. Place the required volume of cells in preheated and pH balanced cultured medium, to reach a final concentration between 2500 and 3000 cells/mL.
   f. Mix in the perfume atomizer the previously tested amounts of RPMI 10× and collagen. (see the 'before you begin' section)
   g. Discard the first spraying.
   h. Use two hands. Hold the atomizer in one hand and with the other hand position the agarose-coated coverslips horizontally to the atomizer. Spray the collagen solution.
   i. Immediately spray the second plate and start seeding the neurons (1 mL per well) before the collagen dots dry out.

j. Let the neurons settle in the incubator.

⚠ CRITICAL: As mentioned in the 'before you begin' section, it is highly important to practice the spraying step to learn how to obtain the optimal shape and size of collagen microdots. This is a critical step to ensure reproducibility of the culture. The goal is to achieve micro-islands of diameters ranging from 0.1 to 0.3 mm (Figure 1).

Day 2 (1.5 h)

7. Maintaining neurons in culture.
   a. Prepare culture medium in sterile conditions. Place DMEM/F-12 containing fetal bovine serum, rat serum and penicillin/streptomycin. Notice that for seeding cells the concentration of penicillin/streptomycin is of 0.25%. In subsequent medium changes the concentration is increased to 0.50%.
   b. Leave the culture medium in the incubator to equilibrate temperature and pH for at least 1 h.
   c. Add 5 nM of NGF and 2 nM of CNTF.
   d. Replace medium of each well (containing 1 mL) by removing 400 μL and adding 600 μL of the freshly prepared cultured medium.
   e. The first medium change should take place 24–48 h after seeding.
   f. Repeat the previous steps of this section 3 times per week.

Note: Autaptic cultures begin to display cholinergic neurotransmission after 15 days *in vitro*. Experiments must initiate after 18 days *in vitro*, when synapses are mature. Dendrites show a robust and thick aspect under the microscope at this point (Figure 3).

⚠ CRITICAL: Always check the pH of the culture medium visually. An orange color is appropriate. Avoid violet tonalities that indicate a high pH.

Electrophysiological recordings

⏲ Timing: 1 day

The use of autaptic cultures allows to record synaptic currents using a single electrode in the whole-cell configuration of the patch-clamp technique. This is an advantageous experimental setup in comparison to the recoding of synaptic currents in mass cultures since neurons are randomly connected and two electrodes (one for stimulation and one for recording) are needed. Whole-cell voltage clamp recordings of SCMs therefore display both, pre- and postsynaptic currents. The presence of functional autaptic synapses is identified by the generation of excitatory postsynaptic currents (EPSCs) that appear immediately (3–10 ms) after the sodium current associated to the generation of an action potential.

Day 21

Preparation of solutions for patch-clamp recording

⏲ Timing: 30 min

8. Preparation of the external recording solution.
   a. On the day of the experiment add glucose and $CaCl_2$ to 100 mL of external solution (see before you begin section) to reach a final concentration of 10 mM and 2 mM, respectively. Final osmolality should be of ~300 mOsm after the addition of glucose and $CaCl_2$.
   b. Leave the solution containing glucose and $CaCl_2$ at room temperature (~22°C). Return the stock solution to the fridge.

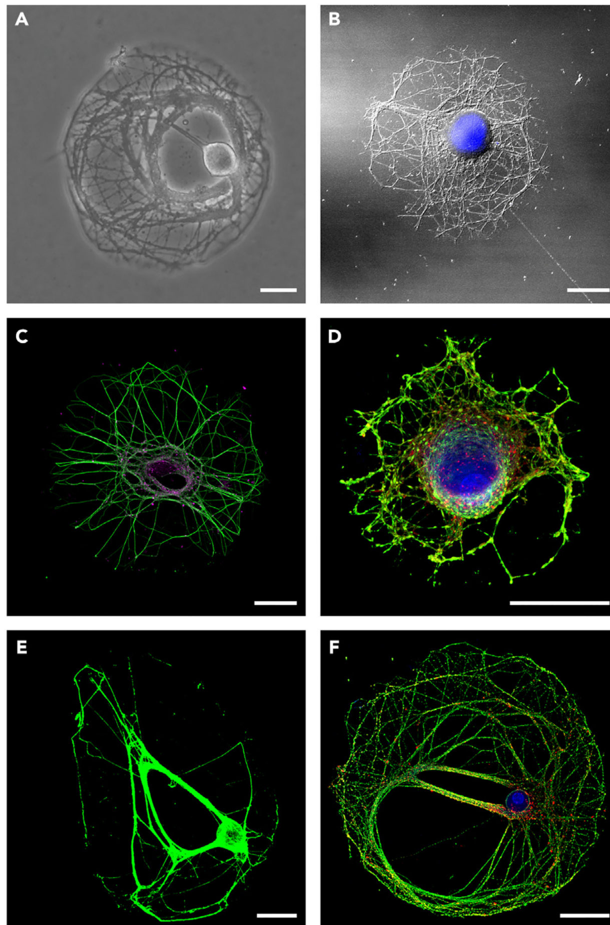

Figure 3. Representative examples of single cell microcultures (SCMs)
(A) Transmitted light image of a SCM displaying a characteristic appearance.
(B) Transmitted light image of a SCM showing the nucleus stained with DAPI (blue).
(C) SCM stained with acetylated-tubulin (green) and synapsin-1 (magenta).
(D) Example of a SCMs stained with alpha-tubulin (green), synapsin-1 (red) and DAPI (blue).
(E) Autaptic neuron stained for the presence of the alpha2delta-1, an auxiliary voltage-gated calcium channel subunit.
(F) SCM stained with synapsin-1 (red), alpha-tubulin (green) and DAPI (blue). Scale bar 50 μm.

Note: During electrophysiological recordings, neurons are continuously perfused with external recording solution. Always prepare a volume that allows the performance of planned experiments. Usually, 100 mL allow to record during half a day.

9. Preparation of the internal recording solution.
   a. Thaw a vial containing the internal solution (see before you begin section) and flow through a 0.2 μm filter.
   b. Keep the internal solution at 4°C while recording to avoid the degradation of nucleotides.

Preparation of patch pipettes

Timing: 30 min

10. Pull borosilicate glass capillaries (1.2 mm outer diameter, 0.69 mm inner diameter) in a horizontal forging micropipette puller (Figure 4A).
    a. Use a 5 steps protocol for pulling the patch pipettes.

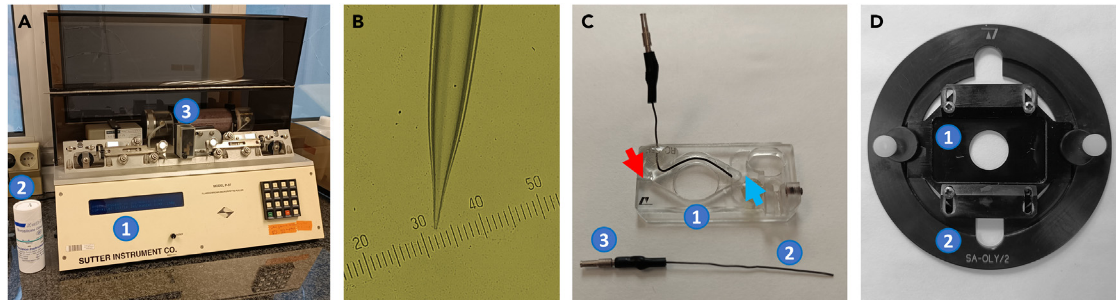

Figure 4. Micropipette puller and recording chamber

(A) Overview of the micropipette puller (1), the borosilicate glass capillaries (2) used to prepare patch pipettes and the humidity-control chamber (3). Detailed methods of how to install a through filament and how to choose the appropriate parameter settings (heat, pull, velocity and time) to prepare micropipettes can be found in.[8]

(B) Zoom on a patch pipette tip. For experiments in whole-cell configuration, outwardly convex tips are preferred because they hold pressure more optimally. Notice that the pipette tip is ~1 μm width.

(C) The RC-25F chamber contains an inlet (red arrow) and an outlet (blue arrow) to facilitate the perfusion with external recording solution. Chlorinated reference electrode (2) soldered to a socket connector contact (3). Notice that the reference electrode is glued to the recording chamber (1).

(D) P-1 chamber platform (1) mounted on a Series 20 stage adapter (2).

b. Visualize in a microforge the shape of the pipette tip. Typical series resistances of pipettes used for recordings are 4–5 MΩ when filled with internal solution.

Note: The trough filament of the pipette puller must be replaced occasionally. After installing a trough filament, a new ramp test must be run to establish the appropriate heat settings. The different parameters (heat, pull, velocity and time) must be adjusted after each filament change.

⚠ CRITICAL: An optimal shape of the micropipette tip is crucial for obtaining the gigaseal on the neuronal membrane and for obtaining reliable whole-cell patch clamp recordings (Figure 4B).

Setting up the instruments and the perfusion system

⏲ Timing: 30 min

11. Take the RC-25 recording chamber with a glued reference electrode and the P-1 chamber platform (Figures 4C and 4D).
12. Maintenance of the patch clamp amplifier headstage, pipette holder and micromanipulator.
    a. Place the headstage above the recording stage to position a patch pipette using an electrode holder (Figure 5A). The pipette contains the internal recording solution and a chlorinated silver electrode that is in contact with the electrode holder.

Note: Renew the electrode regularly. Dichlorination can be seen by a change from black to silver.

    b. Check the movement of the patch pipette in the X, Y and Z axes controlled by the micromanipulator (Figure 5B).
    c. Work with smooth pipette movements to obtain successful electrophysiological recordings.

⚠ CRITICAL: Maintaining the micromanipulator in good conditions and without drift is critical for successful recordings. For a water micromanipulator, add ddH20 with a 1 mL syringe to fill up drop by drop the 3-axis water micromanipulator avoiding the formation of bubbles. Micromanipulator maintenance must be done regularly, i.e. once per month.

Figure 5. Representative images of a patch-clamp setup

(A) Main components: camera to visualize cells (1), microscope (2), manual XY translation platform (3), bath perfusion reservoirs (4), headstage (5) and micromanipulator (6).

(B) Zoom on the recording chamber (1) and the reference electrode (2) connected through a wire (3) to the headstage (4). Perfusion is achieved by the continuous delivery of solution from a manifold connected to the chamber inlet (5) and a suction needle connected to the vacuum system (6).

(C) Rig containing the amplifier (1), ADC converter (2) and a low pass Bessel filter (3).

(D) Zoom on the headstage (1), positioner (2) and, electrode holder (3) connected to a patch pipette (4). Notice the tubing connected to the holder to exert the negative pressure necessary to establish the gigaseal (5).

(E) General overview of the patch-clamp set up. Notice the position of a 1 mL syringe (1) to deliver negative pressure (shown in a close-up image).

(F) Zoom of the two parts of the micromanipulator: three-axis water micromanipulator (1) and positioner (2).

Preparing the neurons for patching

Timing: 30 min

13. Perfusion and microscope system.
    a. Fill the reservoir of the bath perfusion (Figure 5A) with external solution. Flow 2–4 mL of external solution through the tubing to displace air and avoid the formation of bubbles during perfusion.
    b. Place a 24 × 50 mm cover glass of thickness #1 onto the P-1 chamber platform.
    c. Coat the internal edges of the circular aperture of the RC-25F chamber with high vacuum grease.
    d. Open in sterile conditions, i.e., under the vertical flow hood, the 12 well dish containing the SCMs.
    e. Using tweezers, pick one thermanox coverslip containing SCMs and place on a piece of laboratory film.
    f. Press the RC-25F bath recording chamber on the top of the coverslip.
    g. Move the laboratory film and the RC-25F chamber to non-sterile conditions.

h. Add 200 μL of external recording solution to the mounted coverslip to avoid dryness.
i. Transfer the RC-25F recording chamber on the top of the cover glass of the P-1 platform and seal using the screws.
j. Mount the P-1 platform onto a Series 20 stage adapter (Figure 4D) to complete the assembly and place it on the inverted microscope. Connect the chamber to the perfusion system. To add external solution to the bath recording chamber, connect a manifold to the inlet and a right-hand suction tube to the outlet. This connection allows a continuous exchange of external solution. Adjust the flow to ~1 mL/min. Avoid overflow or dryness (Figure 5B).
k. Recording of SCMs can be carried out with air objectives, such as Olympus LCPlanFI 40×/0.6, or LCPlanFI 60×/0.7, or oil-immersion objectives, as for example, Olympus Plan Apo 60×, 1.45 NA. The choice of a particular objective is not critical, as long as it allows proper micromanipulation and visualization of recorded cells.
l. Inspect the culture and select a SCM for recording.

Note: The morphology of each SCM is unique (Figure 3), which is a key advantage to correlate electrophysiology with morphology.

14. Electrophysiological recording equipment.
    a. To measure changes in current amplitude during voltage-clamp recordings, connect the headstage to the patch-clamp amplifier (Figure 5C). Depending on the amplifier it is necessary to use an Analog-to-Digital Converter (ADC). The software required to carry out recordings can be commercial or open access, i.e., WCP software (https://spider.science.strath.ac.uk/sipbs/software_ses.htm), created by Dr. John Dempster, University of Strathclyde, UK) ,or, mafPC (https://www.xufriedman.org/mafpc), developed by Matthew Xu-Friedman University of Buffalo, US) and based on IgorPro software .

Patching the microcultures

Timing: 4 h (for steps 15 and 16)

Timing: 1 day (for steps 17 and 18)

15. Patch-clamp an autaptic neuron in the whole-cell configuration (Figure 6).
    a. Fill a glass pipette with ~3 μL of internal solution.
    b. Place the pipette in the electrode holder and attach it to the headstage (Figure 5D).
    c. Connect the lateral nozzle of the electron holder to a suction tubing that allows the delivery of the negative pressure (Figures 5D and 5E) necessary to establish the gigaseal.
    d. Using the positioner and the micromanipulator (Figure 5F) move the tip of the patch pipette to contact the membrane of the selected SCM.
    e. Apply a small amount of negative pressure using the mouth or a syringe and seal the tip of the patch pipette with a membrane resistance >1 GΩ (gigaseal).
    f. Apply negative pressure to establish the whole-cell configuration.
16. Recording of synaptic currents.
    a. Check whether the neuron presents functional autapses. Apply a 1–2 ms depolarizing step driving membrane potential to 0 mV and observe the presence of excitatory postsynaptic currents (EPSCs, Figure 7).

Note: The presence of functional autaptic synapses is identified by the generation of EPSCs appearing immediately after (5–10 ms) the generation of the sodium current (Figure 7A).

    b. Run protocols established in the laboratory to investigate neurotransmission. It is possible to record synaptic strength, measure the functional size of the readily releasable pool of synaptic vesicles (RRP) or evaluate short-term synaptic plasticity by delivering paired-pulsed

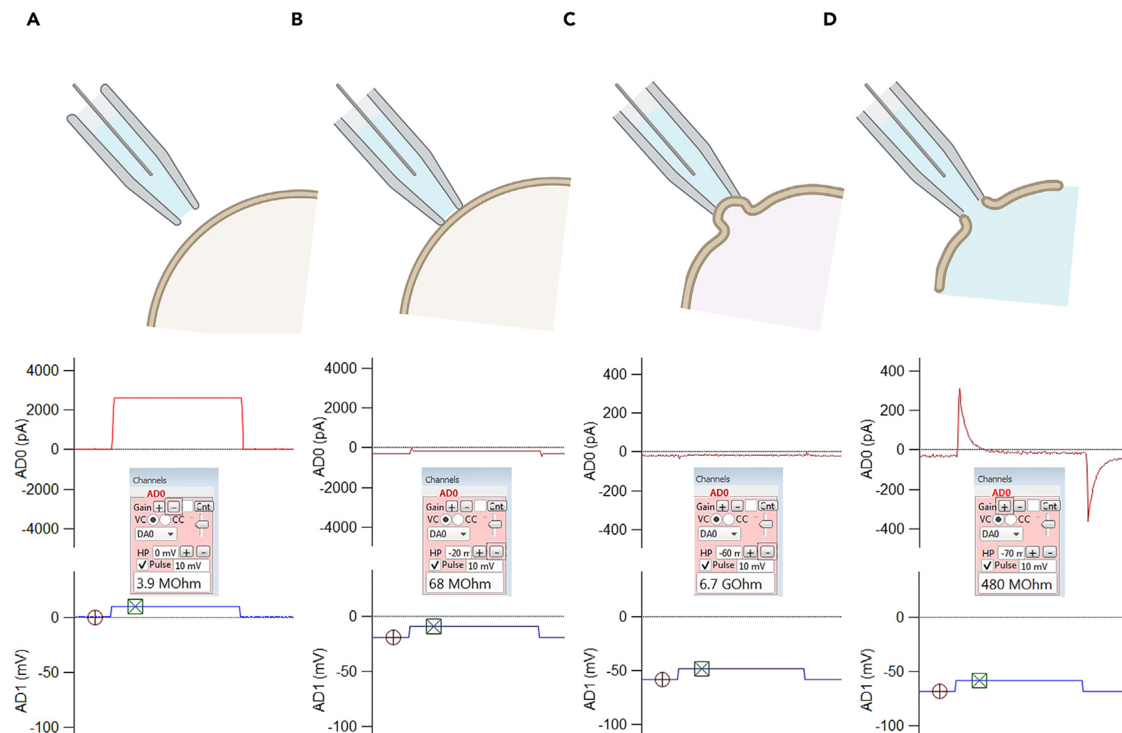

Figure 6. Steps to patch clamp an autaptic neuron in a single cell microculture (SCM)

(A) bath, (B, C) cell-attached and (D) whole-cell. (A) Up, a patch pipette filled with internal recording solution is placed in the bath. Gentle positive pressure is applied to its tip to avoid a possible pipette obstruction caused by cell debris. Down, visualization of the membrane current in response to the application of a 10 mV voltage step. During this stage, voltage membrane is set to 0 mV.

(B) Proceed carefully with the micromanipulator to set the patch pipette in juxtaposition to the neuron membrane. Immediately after touching the cell membrane with the tip, remove the positive pressure while a brief suction is applied. Set a negative membrane potential, for example to −20 mV.

(C) Set the membrane potential to −60 mV and wait until membrane resistance increases >1 GΩ.

(D) Up, achieve the whole-cell configuration by rupturing the membrane under the pipette with a brief suction. Holding potential is set to −70 mV in this example. Down, the successful establishment of the whole-cell configuration is certified by the appearance of slow capacitive transients.

stimulations given at different time intervals. It is also possible to record spontaneous neurotransmitter release (Figure 8). The choice of stimulation protocols will depend on the type of questions asked. For example, if there is an interest in spontaneous neurotransmitter release, it is not necessary to subject the cell to successive stimulations to investigate short-term plasticity.

c. Retract the recording electrode.
d. Take an image of the recorded cell using the camera attached to the microscope stage (Figure 5A) using 10× and a 40× objectives. Having low and high magnification images of the recorded SCM facilitates its identification in the successive steps of the protocol.
e. Disconnect the perfusion system and take the P1 platform and the RC-25 chamber containing the coverslip out from the microscope stage. Take it to the fume hood.
f. Disassemble the RC-25 chamber and pick the thermanox coverslip using tweezers. Immerse the coverslip in fixative solution following the details of the electron microscopy section (steps 21 and 22).

Note: After primary fixation, the coverslips can be stored in 0.1 M Sodium Cacodylate buffer until all the samples have been recorded by electrophysiology (see "Pause point" after step 22).

Day 22

17. Repeat steps 8 to 16 on a daily basis.

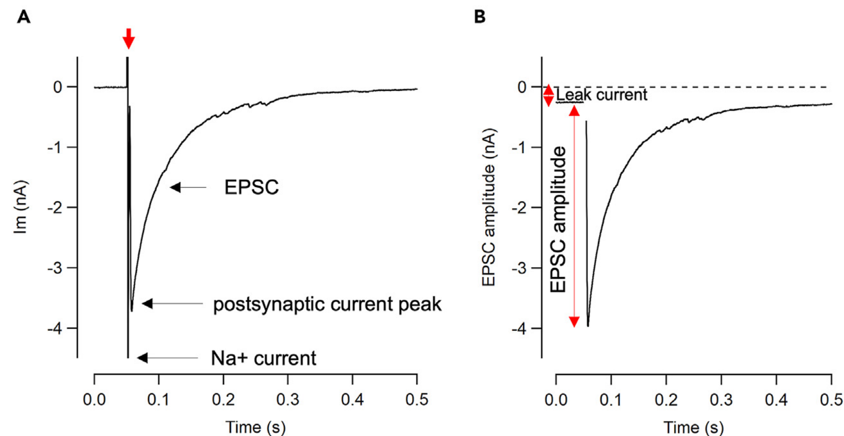

Figure 7. Voltage-clamp recording of autaptic currents in cholinergic Single Cell Microcultures (SCMs)
(A) Sodium current generated by the depolarizing step from −70 mV to 0 mV (red arrow). The inward current initiates an action potential that propagates through the axon. Upon arrival to synapses the stimulus evokes an autaptic excitatory postsynaptic current (EPSC). In SCMs, the postsynaptic current is caused by the entry of Na^{+} and Ca^{2+} ions through cholinergic postsynaptic receptors.
(B) Synaptic strength is assessed by measuring EPSC amplitude, which is obtained by subtracting the peak of the current from basal leak. The sodium current has been blanked for illustration purposes.

18. Finish when all coverslips of the culture have been recorded.

Note: An estimated time of 3 days is required to record a culture batch.

Day 25

Analysis of voltage clamp recordings
The timing required for the analysis of the recordings obtained will depend on the number of cells evaluated and the complexity of the analysis. An average period of 3 days is considered and will typically range between 2 and 7 days.

19. Analysis of evoked neurotransmission.
    a. Synaptic strength is evaluated by measuring EPSC amplitude. The value is obtained by subtracting the maximum current value of the evoked response from the basal leak current (Figure 8A).

Note: Neurons with leak currents greater than 20% of the resulting EPSC, or greater than 500 pA, are discarded, since it indicates poor sealing and poor voltage maintenance during voltage clamp.

    b. RRP size is estimated using trains of high-frequency stimulation delivered at 14–20 Hz. The plot of the cumulative amplitude of EPSCs as a function of experimental time allows the fitting of a linear function to the steady state phase of depression.[9] Extrapolation of this line to the ordinate axis provides an estimate of the RRP size (Figure 8B).
    c. Paired pulse ratio (PPR) is determined by measuring the relationship between $EPSC_2/EPSC_1$. Two consecutive stimulations are given at different time intervals (from 50 ms up to 1–2s) to calculate PPR. Values of PPR<1 are indicative of depression (Figures 8C and 8D), whilst values larger than 1 (PPR>1) are indicative of facilitation.
20. Analysis of spontaneous neurotransmitter release.
    a. The analysis of miniature excitatory postsynaptic currents (mEPSCs) is evaluated by measuring the mEPSC amplitude and its frequency. Automated detection is suggested, for example using Igor Pro procedures such as neuromatic.[10]

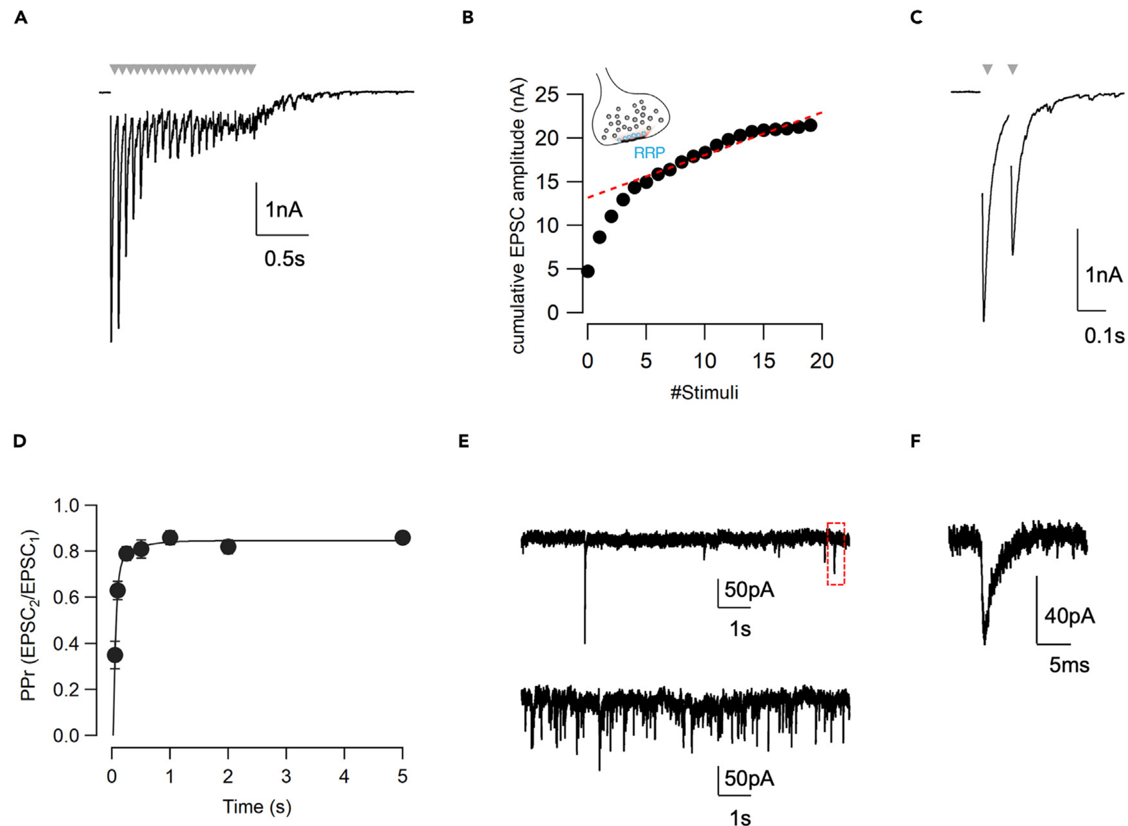

Figure 8. Analysis of neurotransmitter release in single cell microcultures (SCMs)

(A) EPSCs evoked by the delivery of a train of 20 stimuli at 14 Hz (gray arrows). Notice the depression of synaptic responses after 5–6 stimuli until reaching a steady state.

(B) Cumulative analysis of the EPSCs evoked in (A). A linear function (red dotted line) is fitted to the steady state of the depression. Extrapolation of this line with the ordinate axis provides an estimate value of readily releasable pool of synaptic vesicles (RRP, red arrow).

(C) Illustrative example of a paired pulse stimulation (gray arrows) delivered at a time interval of 100 ms. Notice that the amplitude of the 2nd EPSC is smaller than the amplitude of the 1st, indicating depression. In this example paired pulse ratio (PPR = $EPSC_2/EPSC_1$) is 0.59.

(D) Recovery from paired pulse depression as a function of the time interval between pulses.

(E) Illustrative examples of miniature excitatory postsynaptic events (mEPSCs) recorded during 10 s in two different SCMs. The number of mEPSCs divided by the time of recording provides an estimate of the frequency of spontaneous neurotransmitter release. Notice the variability in mEPSC frequency in the two SCMs.

(F) Magnification of the mEPSC squared in (E).

b. Calculate the frequency of spontaneous neurotransmitter release in SCMs. Typically occurs at a low frequency, below 1 Hz.

Note: Automatic identification of mEPSCs must be manually revised to discard possible errors.

Note: Spontaneous neurotransmitter release is a stochastic process, therefore, high variability in mEPSC frequency is usually observed (Figure 8E). The longer is the overall recording time of, the more reliable are the data obtained.

Electron microscopy

Timing: 5 days

This section describes the steps to prepare the recorded autaptic neurons for transmission electron microscopy (TEM). The purpose of this process is to correlate the electrophysiology of a specific

neuron with its cellular structure. The following steps describe the preparation of 4 coverslips selected after the analysis of recordings obtained.

Day 28

21. Place a coverslip containing the recorded SCM in the fixation solution containing 1.8% glutaraldehyde prepared in 0.1 M Sodium Cacodylate buffer. Use two sequential steps:
    a. 20 min fixation at RT.
    b. 20 min fixation at 4°C.
22. Wash three times at RT with 0.1 M Sodium Cacodylate for at least 5 min while gently agitating.

Note: Incubations with different solutions are carried out by adding a volume of 2 mL/well in 12-well plates. All the procedure is performed under the fume hood.

Note: Fixation is very important. It is carried out immediately after recording (step 16). High osmolality solutions may lead to artifactual images. The fixative solution used has an osmolality of ~320 mOsm.

Pause point: The samples can be stored in 0.1 M Sodium Cacodylate at 4°C for 1–2 weeks.

23. Use the analysis data to select 4 coverslips containing the SCMs that more appropriately describe the phenotype that aims to be investigated. These are the coverslips that will be used to obtain electron microscopy images correlative to electrophysiology.
24. Incubate the 4 coverslips in freshly made 1% Osmium tetroxide / 1.5% potassium ferricyanide for 1 h at 4°C in the darkness without agitation.

Note: From this point onwards, samples must be kept in the darkness by covering the 12-well plates with aluminum foil.

25. Transfer the samples into a new 12-well plate removing as much OsO_4 as possible and wash them twice with 0.1 M Sodium Cacodylate at RT while gently agitating in an orbital shaker for at least 5 min.

Note: Before discarding OsO_4 in a container specific for toxic residues, all materials containing OsO_4 must be treated with vegetable oil. The inactivation of OsO_4 becomes obvious by the change of oil color from transparent to intensely dark. Tightly seal all remnants of inactivated OsO_4.

26. Incubate the coverslips with freshly made 1% tannic acid / 0.05 M Sodium Cacodylate buffer at RT for 45 min without agitation.
27. Wash the samples twice with 0.05 M Sodium Cacodylate at RT for at least 5 min while gently agitating.
28. Dehydrate the samples by incubating them in increasing concentrations of ethanol prepared in ddH2O for 5 min at RT while agitating.
    a. Wash once with ddH2O.
    b. Wash twice with 70% ethanol.
    c. Wash twice with 90% ethanol.
    d. Wash twice with 100% ethanol.

Note: At this time, autaptic neurons are stained black and can be easily located under the stereo microscope (Figure 9A).

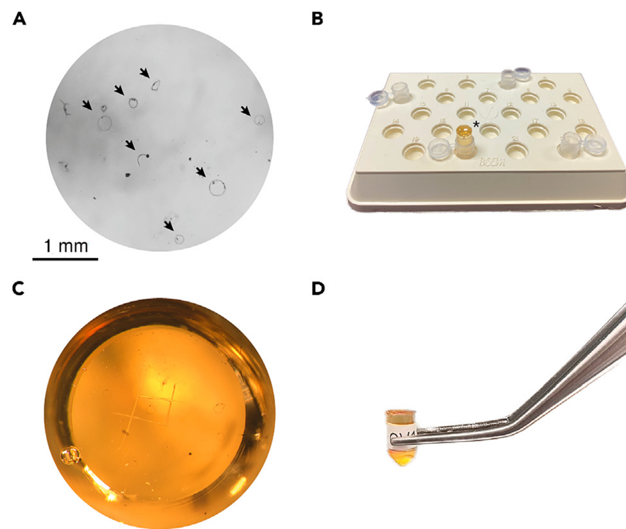

Figure 9. Sample preparation for electron microscopy of single cell microcultures (SCMs) correlative to electrophysiology

(A) Image of SCMs (arrows) stained with OsO_4 visualized under the stereo microscope.

(B) Set up for preparing embedding capsules. Notice how empty capsules are used as a support for the resin blocks on which coverslips will be mounted. Asterisk indicates the resin stub.

(C) Thermanox coverslip mounted onto the resin block visualized under the stereo microscope. The recorded neuron is positioned on the middle (square).

(D) Resin block after separating the thermanox coverslip with liquid N_2. The recorded neuron is contained into the resin block ready to be sectioned and imaged.

Pause point: It is possible to stop the protocol here and leave the cells overnight in 70% ethanol at 4°C.

Day 29

29. Using a scalpel blade, draw a square around the recorded neuron under the stereo microscope. To identify the specific neuron within the coverslip, use the micrograph that had been previously taken after the electrophysiological recording.
30. Epon resin embedding.
    a. Transfer the coverslips to glass microbeakers.
    b. Immerse the cells in 2 mL of 1:1 Epon resin and propylene oxide for 30 min at RT.
    c. Remove as much as possible of the previous mix and transfer the coverslips into new wells containing fresh 100% Epon resin. Incubate for at least 1 h at RT.
    d. Incubate the cells for an additional hour or overnight in 100% fresh Epon resin.

Note: Since propylene oxide dissolves most laboratory plastic, glass microbeakers are placed inside the 12-well plate. To extract propylene oxide from the bottle and mix it with the resin, a previously 1 mL-marked glass pipette is used.

Note: Propylene oxide is highly toxic. Although it provides the best results, alternatives methods of resin infiltration could be assayed.

31. Prepare the embedding capsule holder before mounting (Figure 9B).
    a. Spatially distribute empty embedding capsules in the capsule holder.
    b. Separate the previously polymerized resin blocks from their capsule and place them into the new empty embedding capsules so only half of the block is inside.

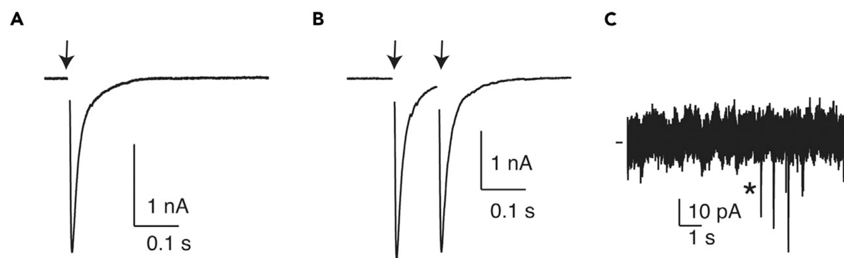

Figure 10. Characteristics of cholinergic neurotransmission in a Single Cell Microculture (SCM)

(A) Excitatory postsynaptic current (EPSC) obtained in response to a 2 ms depolarization from −60 mV to 0 mV.

(B) Paired pulse plasticity assayed by delivering two stimuli at a time interval of 100 ms. Notice the presence of a slight depression in the second EPSC.

(C) Spontaneous neurotransmitter release. Miniature excitatory postsynaptic currents (mEPSCs, asterisk) occurred at a frequency below 1 Hz.

32. Mount the coverslips facing downwards on top of the resin blocks. Pick the coverslip with tweezers and make sure that the recorded neuron is placed on the middle of the block (Figure 9C).

Note: The square surrounding the neuron of interest carved with the razor blade can be seen by looking at the coverslip against the light. Avoid bubble formation.

33. Polymerize the resin by heating in an oven at 60°C for 2 days.

Day 31

34. Separate the resin blocks from the thermanox coverslips by immersion in liquid N_2.
    a. Place enough liquid N_2 in an appropriate container to immerse the resin block.
    b. Hold the resin block with the 7.25″ forceps and immerse it into the liquid N_2 for 12 s.
    c. Immediately after, separate the coverslip from the resin block with a clean move using the Nugent utility forceps.

Note: Make sure that there are no remains of thermanox on the resin block.

35. Visualize the EM block under the stereomicroscope to verify that the recorded neuron remains on it ready to be processed (Figure 9D).

Day 32

36. Proceed with the ultra-thin sectioning of Epon resin blocks in 90 nm sections. Stain the samples with uranyl acetate and lead citrate to increase the level of contrast in the final images.
37. Obtain images at the transmission electron microscope.

Note: Technical steps related to the sectioning and visualization of recorded neurons by transmission electron microscopy are out of the scope of this protocol as they require detailed know-how and expert technical support.

EXPECTED OUTCOMES

The protocol here described allows to use transmission electron microscopy to observe the ultrastructure of synapses present in autaptic neuronal cultures. Figure 10 shows an example of the typical neurotransmission features of a SCM.

Cells can be fixed and processed for electron microscopy upon characterizing synaptic transmission present in the culture. Figure 10 illustrates control features of neurotransmitter release[1–6,9,10] and displays the measurement of synaptic strength (Figure 10A), short-term plasticity (Figure 10B) and spontaneous neurotransmitter release (Figure 10C). It is strongly recommended to choose cells with synaptic transmission representative of the condition studied and to use protocols that specifically address questions asked. The goal is to associate the characteristic morphological features of synapses studied to the quantitative analysis of neurotransmitter release.

The round shape of the microdot is obvious by low magnification at the transmission electron microscope (Figure 11A). Increase the magnification but, keeping a panoramic view, look for presynaptic terminals. They are characterized by the presence of vesicle clusters (Figure 11B). Increase magnification and certify the identification of a synapse (Figure 11C). Several strategies can be used to distinguish axosomatic from axodendritic synapses. Numerous microtubules are present in dendrites and axons but, not in the cell body. There is the possibility of looking for the same synapse in contiguous sections, thus allowing to obtain a 3-D reconstruction. For this purpose, the ultrathin sections must be mounted on specific grids. By combining visualization at low and high magnification the same synapse can be identified using reference points such as portions of axons and dendrites or large intracellular organelles such as mitochondria. The examples illustrated in Figure 11C1–C5 show serial sections through a synapse. The alignment of serial sections has to be done with specific software, such as TrakEM2[11] or IMOD.[12] Excellent results can be obtained using two to three times thicker sections and visualize them by electron tomography.[1]

Multiple synapses can be observed throughout the sections of the SCM. Common hallmarks are a central synaptic vesicle cluster surrounded by organelles, which are mainly endosomes and mitochondria. Axodendritic synapses tend to be smaller than axosomatic synapses.

The long duration of the method here presented is compensated by the wealth of information provided by a representative, single cell. The consistent ultrastructural features of the many synaptic contacts present in a SCM allow to faithfully define the morphological bases of the synaptic transmission characterized electrophysiologically.

LIMITATIONS

The low number of neurons processed for electron microscopy is the main limitation of the protocol. The neurons selected must display a synaptic transmission representative of the phenotype under study. For example, if a drug that enhances short-term depression is investigated, it is first necessary to know the best experimental conditions that cause the observed effect. The protocol presented is laborious and it is not recommended for screening, or, carrying out the initial characterization of a phenotype.

TROUBLESHOOTING

Problem 1

Microdots detach from the agarose-coated coverslips.

Potential solution

- Check the collagen dilution that you are using to establish the microdots. If the collagen concentration is too low, the dots will easily detach (see 'before you begin' section, step 3, and Figure 1). It is highly recommendable to check the optimal working-dilution of collagen every month.
- Check that collagen (see 'before you begin' section, step 2) is in optimal condition. Collagen aliquots stored for more than 4 months or subjected to temperature alterations lose their properties. If aliquots are older than 4 months, prepare a new batch of rat tail collagen.

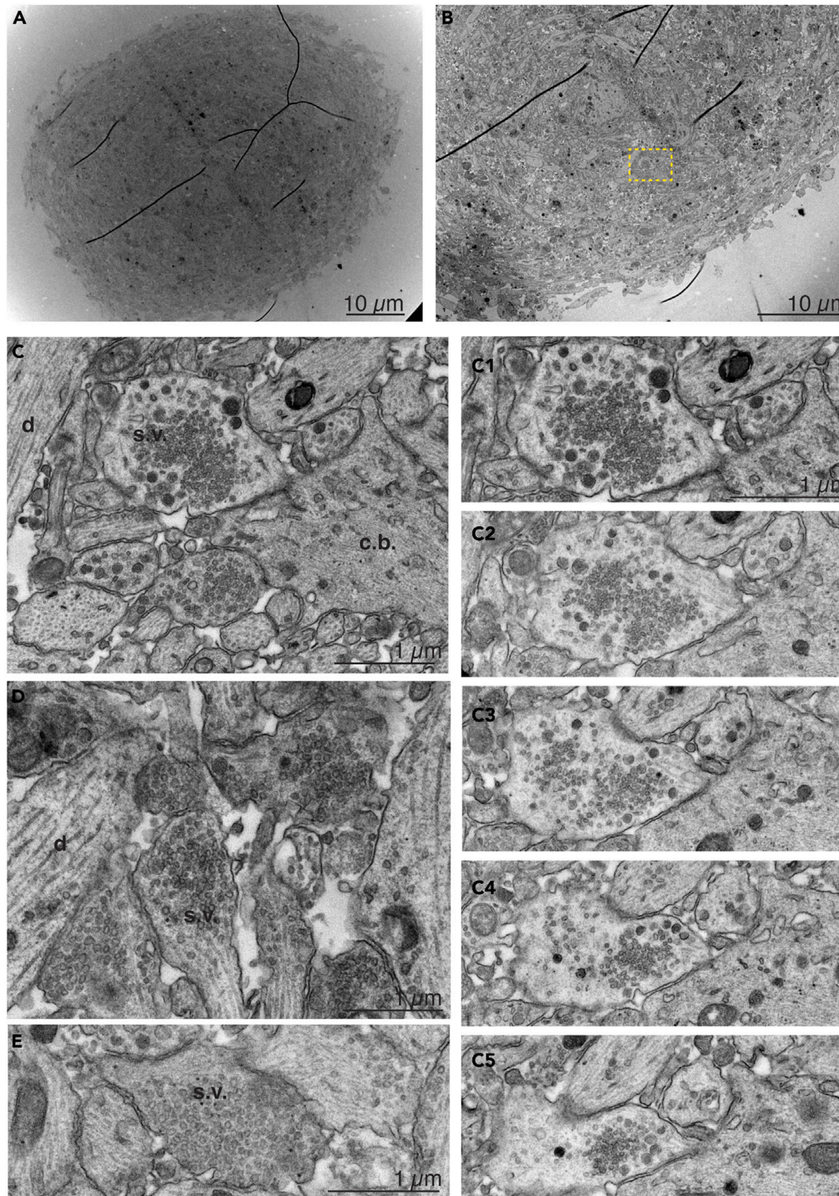

Figure 11. Transmission electron microscopy images obtained of a recorded neuron

(A) Low magnification image of the Single Cell Microculture (SCM).

(B) Inspection of the section reveals putative synapses that are identifiable by the presence of synaptic vesicle clusters.

(C) The characteristic structure of presynaptic terminals can be observed in the region boxed in B). Two synapses are established on the cell body (c.b.). Dendritic processes (d) and synaptic vesicles (s.v) can be identified. A single synapse (C1) can be identified in serial sections (C2-C5).

(D and E) Other synapses are observed in the SCM studied but, all of them display a comparable ultrastructure.

Problem 2

Neurons growth in small clusters and autaptic cultures are not achieved.

Potential solution

- Be sure to count the correct number of neurons that you are seeding. We found optimal results at a final concentration of 2500 cells/mL.

- The enzymatic treatment is not working properly (step 4). Make new stocks of 2.5 mg/mL collagenase solution and 0.05% trypsin-EDTA solution. Collagenase activity decreases over time. Prepare a new stock every three months.
- Mechanical disaggregation of the tissue must be improved (see 'before you begin' section, step 1). Check the tip opening of glass Pasteur pipettes.

Problem 3

Glial cells are growing in the autaptic cultures.

Potential solution

- Improve the removal of connective tissue from the isolated superior cervical ganglia (step 3, Figure 2).
- Extend the pre-plating period between 5 and 10 min (step 5). If the pre-plating period is too short, the percentage of glial cells increases. P0 rat pups provide less non-neuronal cells than P2 rat pups.

Problem 4

Mature autaptic neurons do not display an elaborated neuritic tree.

Potential solution

- Check the stock of NGF. Avoid repeated freeze-thaw cycles. Do not use stocks stored for more than 6 months at −80°C.
- Check the lot of NGF since a certain variability among manufacturers or batches may occur.

Problem 5

Autaptic neurons do not display cholinergic neurotransmission properties (Figures 7 and 8), after more than 2 weeks in culture.

Potential solution

- Check the stock of CNTF. Avoid repeated freeze-thaw cycles. Do not use stocks stored for more than 6 months at −80°C.
- Repeat step 6 to learn how to establish collagen microdots. The presence of a high density of microdots in the same coverslip is desirable since autaptic neurons grow better in the vicinity of other SCMs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Artur Llobet (allobet@ub.edu).

Technical contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the technical contact, Pablo Martínez San Segundo (pablomartinezs@ub.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

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AUTHOR CONTRIBUTIONS

M.S.S.P., P.G.A.P., V.C.D., and L.A. have contributed to developing the method described here. The steps presented are a result of teamwork where all authors have been implicated in the stages of the project. All authors wrote and reviewed the final version of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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