Sparse postnatal labeling and quantification of superficial cortical cell synapses in the mouse neocortex

We present a strategy for measuring the density of presynaptic boutons of superficial neuronal cells in the mouse neocortex. First, we show how to sparsely label individual postmitotic cells by neonatal pial-surface electroporation. Then, we present a custom-made code that allows quantification of the density of presynaptic boutons along axonal processes. Although we applied this strategy to somatostatin-positive cells, a major population of cortical interneurons, the same approach can be adjusted to target other types of neuronal cells.

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

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Highlights
Sparse labeling of cortical cells by neonatal pial-surface electroporation
Labeling of presynaptic boutons with Synaptophysin-EGFP fusion protein
MATLAB script for axon tracing and automatic bouton detection and segmentation
Protocol
Sparse postnatal labeling and quantification of superficial cortical cell synapses in the mouse neocortex

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https://doi.org/10.1016/j.xpro.2022.101837

SUMMARY
We present a strategy for measuring the density of presynaptic boutons of superficial neuronal cells in the mouse neocortex. First, we show how to sparsely label individual postmitotic cells by neonatal pial-surface electroporation. Then, we present a custom-made code that allows quantification of the density of presynaptic boutons along axonal processes. Although we applied this strategy to somatostatin-positive cells, a major population of cortical interneurons, the same approach can be adjusted to target other types of neuronal cells.

For complete details on the use and execution of this protocol, please refer to Gesuita et al. (2022).1

BEFORE YOU BEGIN
In this protocol, we target one of the largest populations of cortical inhibitory cells, the somatostatin-positive (SST⁺) interneurons. The specificity is given by the use of the SST-IRES-Cre mouse line, where the expression of the CRE recombinase is under the control of the same promoter of Sst. Nevertheless, the same strategy can be used to label other types of neuronal cells, by using different CRE diver mouse lines.

To quantify the density of SST⁺ presynaptic boutons, we first perform pial-surface electroporation in the somatosensory cortex of neonatal mice.2–5 This strategy allows for mosaic labeling of a handful of superficial cortical postmitotic SST⁺ cells. Specifically, we electroporate SSTCre/+ pups at postnatal day (P) 1–2 with a CRE-dependent plasmid carrying a tdTomato and the mouse presynaptic protein synaptophysin (mSyp) fused with an enhanced green fluorescent protein (EGFP): pCALNL-tdTomato-2A-mSyp::EGFP (Addgene #191213). This approach labels individual SST⁺ cells in red and their presynaptic boutons in green. Nevertheless, other plasmids can be co-electroporated, allowing for the labeling of presynaptic boutons and any additional manipulation of choice. For example, in our published study, we co-electroporated a CRE-dependent plasmid carrying a Cas9 protein and specific guide RNAs to perform cell autonomous knock-out of a gene of interest.1

Neonatal electroporation allows fast and sparse labeling of cells within a day, permitting their study during early phases of development. It is more difficult to obtain the same by using viral vectors that have much slower onset of expression.6 Moreover, this technique leads to very sparse labeling of cells which allows the full reconstruction of their axo-dendritic profile, something much more difficult to obtain with a virus-based method.
Brains are then collected at the desired age (in our protocol we tested both P10 and P15) and sliced using a vibratome. The few labeled cells can then be individually imaged using confocal fluorescent microscopy and processed through our analysis code.

**Institutional permissions**
Animal experiments were approved by the Cantonal Veterinary Office of Zurich and the University of Zurich. Any reproduction of this protocol needs the acquisition of permissions from the relevant institutions.

**Purification of the plasmid**

**Timing:** 4 days

The final concentration of the electroporated plasmid is 3,500 ng/μL; therefore, it is necessary to purify and concentrate the construct pCALNL-tdTomato-2A-mSyp::EGFP (Addgene #191213) using a maxiprep. In this protocol, we use the QIAGEN Plasmid Plus Maxi Kit, which includes an extra step for efficient endotoxin removal. This is advisable when using a plasmid in vivo. Nevertheless, any equivalent kit or procedure can be used.

1. Next to a Bunsen burner flame, transform the plasmid into DH5α competent cells as follows:
   a. Take one tube of 50 μL of DH5α competent cells from the −80°C freezer and keep it on ice.
   b. Let the cells thaw on ice for 5–10 min.
   c. Add 1 μL containing 1 pg-100 ng of plasmid DNA to the cells, gently flick the tube 2–3 times and incubate on ice for 30 min.
   d. Turn on the water bath and set it to 42°C.
   e. Heat shock the cells for 30 s at 42°C.
   f. Move the tube on ice for 2 min.
   g. Add 500 μL of room temperature (20°C–25°C) Miller’s LB Broth (LB) to the cells and incubate in a shaker (200–300 rpm) at 37°C for 1 h.
   h. Take two LB-agar plates with ampicillin 100 μg/mL:
      - **Note:** The antibiotic is chosen according to the resistance gene expressed by the plasmid.
      i. Label the bottom with the name of the plasmid and either “low” or “high”.
      ii. Warm the plates at 37°C for a few minutes.
   i. Transfer 50 μL of cells to the center of the “low” plate and the rest to the center of the “high” plate.
      - **Note:** Using two plates with different amounts of cells ensures nicely separated colonies independently of the efficiency of the transformation.
   j. Sterilize a metal or glass spreader over the flame and cool it down by touching the periphery of the LB-Agar plate.
   k. Spread the cells evenly over the agar, close the lid, and put the plates upside down into an incubator at 37°C overnight (12–16 h).

2. On the following day (day 2), next to a flame:
   a. Pick one medium size colony with a sterile 10–200 μL tip.
   b. Put it into a round-bottom tube with 3–5 mL of LB with ampicillin 100 μg/mL.
   c. Incubate in a shaker (200–250 rpm) at 37°C overnight (12–16 h).
      - **Note:** The antibiotic is chosen according to the resistance gene expressed by the plasmid.

3. On day 3, remove the tube from the incubator:
   a. The LB should be cloudy. If not, incubate longer: it should get cloudy within a few hours.
b. Leave the tubes at 4°C.
c. In the evening, next to a flame, gently flick the tube 4–5 times and incubate 200 μL of LB culture into 200 mL of LB with ampicillin 100 μg/mL (use a 1 L flask).
d. Incubate in a shaker (200–250 rpm) at 37°C for 12–16 h.

4. On day 4, proceed as described in the protocol of the QIAGEN Plasmid Plus Maxi Kit (https://www.qiagen.com/no/resources/download.aspx?id=def7a04d-9d1a-439e-acfc-8d4efc66aef8&lang=en), or equivalent kit.

Note: 200 mL of cell culture are usually enough for two maxipreps.

5. When eluting the purified plasmid (in the last step of the kit), reduce the elution buffer volume to 50 μL to concentrate the plasmid as much as possible. Measure plasmid concentration and purity with a Nanodrop: 260 nm/280 nm and 260 nm/230 nm ratio values should be close to 2.
6. Store the plasmid at −20°C.

Preparation of the injection micro-syringes

© Timing: 30 min

Prepare injection micro-syringes by pulling PCR glass micropipettes (Drummond 5-000-1001-X10). These are capillary pipettes that come with thin steel plungers.

7. Set the following program on the Flaming Brown Micropipette Puller (Model P-87): Heat = 850, Pull = leave this blank, Vel. = 55, Time = 250.
**Note:** Pulling parameters should be adjusted when using a different puller machine to obtain micro-syringes similar to the one depicted in Figure 1A.

8. Place a glass micropipette on the metallic indentations and lock it in place by tightening the screws. Then, start the program. By placing the micropipette exactly in the middle, you can obtain two identical micro-syringes.

9. Check the pulled tip under a microscope equipped with an ocular micrometer and cut where the inner opening diameter is around 30–40 μm using fine forceps.

10. Grind the tip creating a sharp 35° angled needle by using a micro grinder (NARISHIGE EG-45).

\[\text{CRITICAL: It is very important to sharpen the micro-syringe needle for easy and good penetration through the skull.}\]

11. Clean the micropipette tip by spraying it with ethanol 70%. Check that the needle is sharp and without debris under a dissecting microscope. Store in a clean container, such as a Petri dish with a strip of reusable adhesive (e.g., UHU Patafix) to keep the tip intact by raising and immobilizing it.

12. Cut the metal plunger in half by using a nipper, and bend the cut end into the shape of an umbrella handle (Figure 1A). Clean the plungers with ethanol 70%. Store in a clean container (e.g., a Petri dish).

**Preparation of the working space**

\[\text{Timing: 1 h}\]

13. Prepare neurogel as follows:
   a. Add Milli-Q® water, propylene glycol, NaOH, and parabens into a 200 mL beaker (follow the recipe in the "materials and equipment" section). Then, add a magnetic stirring bar and start stirring.
   b. Add Carbopol powder slowly while stirring vigorously.
   c. Cover with parafilm or tin foil and wait until the solution is fully jellified (15–20 min); usually, you can consider the gel ready when the stirrer bar gets trapped into the viscous gel.

14. Prepare the plasmid DNA solution and keep it on ice.

\[\text{CRITICAL: Prepare the DNA solution right before use. Moreover, always prepare a fresh tiny stock of trans-cyclohexane-1,2-diol (TCHD) 10 mg/mL in PBS 1× every time; TCHD is stable when kept on ice. TCHD is an amphipathic molecule that increases the permeability of the nuclear pore complex barrier and allows the plasmid to enter the nucleus and be transcribed.}\]

15. Sterilize the surgical tools (a pair of fine sharp spring scissors and a pair of forceps) through autoclaving or a hot bead sterilizer.

16. Prepare sterile Petri dishes to lay the surgical tools during the procedure.
17. Clean all the surfaces with 70% ethanol.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| DH5α competent cells | New England Biolabs | Cat#C2987H |
| **Chemicals, peptides, and recombinant proteins** | | |
| Agar | Sigma-Merck | Cat#05039 |
| Ampicillin | Sigma-Merck | Cat#A9518 |

(Continued on next page)
### Critical commercial assays

**Qiagen Plasmid Plus Maxi Kit**

Qiagen

Cat#12963

### Experimental models: Organisms/strains

- **Mus musculus, SST-ires-Cre (Ssttm2.1(cre)Zjh/J), P1-P15, no distinction between females and males.**
  - The Jackson Laboratories
  - Cat#013044; RRID:IMSR_JAX:013044

### Recombinant DNA

- **pCALNL-TdTomato-2A-mSyp::EGFP**
  - Gesuita et al. 2022
  - Addgene Cat#191213

### Software and algorithms

- **Matlab 2019a or later versions**
  - MathWorks
  - https://www.mathworks.com/

- **RStudio 1.2.1335**
  - RStudio Team
  - http://www.rstudio.com/

- **Custom-made Matlab script**
  - This study
  - https://doi.org/10.5281/zenodo.6980507

### Other

- **1L Flasks**
  - N/A

- **3D printed whole-body mouse mold**
  - Ho et al. 7

- **Bunsen burner**
  - FALC INSTRUMENTS
  - Cat#140205010

- **Cover slips**
  - Menzel-Glaser
  - Cat#BB02400600A113MN20

- **ECM 399 Electroporation System**
  - BTX
  - Cat#45-0000

- **Eppendorf PCR clean tubes**
  - Eppendorf
  - Cat#0030 123.328

- **Falcon® 14 mL Round Bottom Tubes**
  - Falcon
  - Cat#352059

- **Heating pad**
  - N/A

- **Insulin syringe (30G x 1/2")**
  - B Braun
  - Cat#91511255

- **Isopropyl alcohol**
  - N/A

- **Lancet**
  - N/A

- **Magnetic Stirrer**
  - N/A

- **Microgrinder**
  - NARISHIGE
  - EG-45

- **Micropipette Puller**
  - Sutter Instrument
  - Flaming/Brown Model P-87

- **Microscope with ocular micrometer**
  - N/A

- **Nail polish**
  - N/A

- **Nanodrop**
  - Thermo Scientific
  - Nanodrop 1000

- **Nipper**
  - N/A

- **PCR glass micropipettes**
  - Drummond
  - Cat#5-000-1001-X10

- **Peristaltic pump**
  - N/A

- **Petri dishes**
  - Thermo Scientific
  - ST101VR20/C

(Continued on next page)
MATERIALS AND EQUIPMENT

**Neurogel**

| Reagent                      | Final concentration | Amount  |
|------------------------------|---------------------|---------|
| Carbopol 940                 | 1%                  | 0.5 g   |
| NaOH 10 M                    | 0.1 M               | 0.5 mL  |
| Propylene glycol             | 20%                 | 10 mL   |
| Parabens                     | 0.1%                | 50 µL   |
| Milli-Q® water               | N/A                 | 35 mL   |
| Total                        |                     | 50 mL   |

Note: Prepare the neurogel following the instructions from the “preparation of the working space” section.

Note: Store at 4°C for up to 1 week.

**Plasmid DNA solution**

| Reagent                      | Final concentration | Amount  |
|------------------------------|---------------------|---------|
| plasmid DNA 7,000 ng/µL      | 3,500 ng/µL         | 5 µL    |
| Fast green 1%                | 0.05%               | 0.5 µL  |
| TCHD 10 mg/mL                | 1 mg/mL             | 1 µL    |
| PBS 1×                       | N/A                 | 3.5 µL  |
| Total                        | N/A                 | 10 µL   |

Note: Always prepare a fresh tiny stock of TCHD 10 mg/mL in PBS 1×, right before use, and keep it on ice.

Note: Prepare the plasmid DNA solution fresh, right before use, and keep it on ice.

Software minimal requirements

- Be sure to have MATLAB 2019a or later versions.
- Be sure to have R Studio with ggplot2 library installed. If not, visit [https://ggplot2.tidyverse.org/](https://ggplot2.tidyverse.org/)

**STEP-BY-STEP METHOD DETAILS**

**Pre-surgical analgesia**

© Timing: 40 min
In this step, SST\(^{Cre/+}\) P1-P2 mouse pups are removed from their home cage and injected with analgesic treatment.

**Note:** Pups can be genotyped for SST-Cre as described here: https://www.jax.org/Protocol?stockNumber=013044&protocolID=38270. However, when breeding a SST\(^{Cre/Cre}\) mouse to a C57BL/6J wild-type mouse, all pups in the litter will be SST\(^{Cre/+}\) and no genotyping is needed. If other mouse lines are used, the respective genotyping protocol should be followed.

**Note:** Analgesic treatments should be adjusted according to the relevant governmental regulations on animal experimentation.

1. Remove the parents from the home cage and temporarily place them in a separate clean cage.
2. Move the P1-P2 pups to a small box with some of the bedding from the home cage.
3. Place the parents back in the home cage and bring the pups to a separate surgery room.
4. Place half of the box on the heating pad at 37°C to allow the pups to move away from the warmth.
5. Weigh the animal and document the weight.
6. 30 min before the surgery, inject Buprenorphine (0.1 mg/kg) (as analgesic) via subcutaneous (s.c.) injection in the neck with an insulin syringe (30G × 1/2") by pulling up the skin to create space for the inserted needle.

### Neonatal pial-surface electroporation

**Timing:** 15–20 min per pup

In this step, P1-P2 pups are anesthetized using isoflurane and then neonatal pial-surface electroporation is performed. The electroporation protocol is adapted from previously published protocols.2–5

7. Load the micro-syringe with plasmid DNA solution.

**Note:** To easily load the micro-syringe, put a drop of plasmid DNA solution on a piece of parafilm and tap it with the back of the micro-syringe; DNA solution will slowly enter by capillarity. Then, insert the plunger.

8. Anesthetize and maintain the anesthesia as described below:
   a. Turn on the oxygen extractor and wait for the flow to stabilize (1–2 min).
   b. Set the oxygen flow to 1 L/min.
   c. Place the pup into the induction chamber (oxygen 1 L/min, isoflurane 4%).
   d. After 3.5 min, test anesthesia with a gentle foot pinch. If the animal is not anesthetized (i.e., it retracts its paw), wait another 30 s and check it again. Wait for loss of motor response.
   e. Once anesthetized, transfer the pup to the heating pad and place the snout into a 3D-printed whole-body mouse mold (we use a mold from a previously published protocol7). Keep the mouse under anesthesia (oxygen 1 L/min, isoflurane 4%).

**Note:** A detailed description of the protocol for isoflurane-induced anesthesia in pups has been previously published by Ho and colleagues.7 Alternative anesthesia protocols, such as anesthesia by hypothermia4 and analgesic treatments can be used according to the relevant governmental regulations.

9. Inject Meloxicam (5 mg/kg) (as additional analgesic) subcutaneously in the neck with an insulin syringe (30G × 1/2")

10. If necessary, mark the pup by cutting toes or by other less invasive means, such as toe tattoo.
11. Wipe the skin over the skull with Chlorhexidine soap solution and apply Emla® cream 5% (Lidocaine/Prilocaine) generously.

12. Put a drop of neurogel on both the positive and the negative electrodes (we used platinum tweezertrodes electrodes of 5 mm diameter from BTX, Cat#45-0489; the positive electrode is the one with the blue screw, as depicted in Figure 1C). Use a 1–2 mm thick layer of neurogel covering the entire electrode surface.

13. Two minutes after the Emla® cream 5% application, use spring scissors and cut a small window (1.5 × 1.5 mm) into the skin covering the skull. Cut only three sides of the window, and use the forceps to flip the skin and expose the skull (Figure 1B).

14. Deliver the plasmid DNA above the pia surface by using a previously prepared sharpened glass micropipette.
   a. Hold the capillary between the thumb and the middle finger, keep it tilted at 30°–45° and puncture the skull without penetrating the brain.

   Note: The skull is very soft, in particular at the coronal suture. Since the dura covers the inner surface of the skull, this meningeal layer is easily penetrated when puncturing the skull.

   b. Inject around 0.5 μL (the capillaries are graduated and have a tick every 1 μL) by pressing the plunger with your index finger.

   Note: Since the pia is tightly connected to the brain surface, if you do not puncture the brain you will be injecting right above the pia surface. When injecting above the cortical somatosensory area, you should be able to see the blue plasmid DNA solution spread evenly over a circular surface of around 2.5–3 mm in diameter. If you don’t see this, you are probably injecting too deep inside the brain and not in the meningeal space (Figure 1B).

   Optional: Since the skin is quite thin and transparent, it is possible to directly puncture the skin and the skull together, without cutting a window first. We prefer this strategy as it is less invasive and reduces the stress on the parents. However, it is advisable to begin making a window to better understand the anatomy and get experienced injecting in the meningeal space.

15. Place the negative 5 mm electrode over the injection window (the positive electrode is on the opposite side, under the jaw) and apply two trains of ten 50 millisecond-long 99 V pulses, with a 950 millisecond-long interval using a standard electroporator (ECM399, BTX); wait 3 s between the two trains (Figure 1C).

   Note: Before performing neonatal pial-surface electroporation in the first pup, check once that the electrodes are working. To do so, make a bridge of neurogel between the electrodes and deliver a train of pulses; if you see bubbles forming, the electrodes are working correctly.

   △ CRITICAL: Be sure to have a gentle, but firm, contact between the head, the neurogel and the electrodes. When delivering a train of pulses, you should be able to see some small bubbles popping inside the neurogel under the negative electrode.

16. Wipe the residues of neurogel off the head.

17. Flip the skin back in position and seal with a small drop of tissue glue (e.g., Vetbond glue).

18. Put the animal in a warm recovery cage (a small box with home cage bedding to ensure the pups smell like the home cage and are accepted back by their parents) which is half placed on the heating pad at 37°C (to allow the animals to move away from the heated side).

19. Repeat with a new pup until the entire litter has been electroporated; then, return the pups to the parents.
   a. Place the pups back in their home cage and observe if the parents correctly nest them.
   b. If the mother does not take care of the pups after surgery, check the section troubleshooting 1.
c. Follow up with analgesic treatment according to your local animal experimentation guidelines.

Brain slicing and imaging

Timming: 2 days

In this step, pups are perfusion-fixed. Then, the brain is extracted and sliced with a vibratome. Finally, the sections are imaged.

20. Perfuse-fix the pups at the required age; in our protocol we tested both P10 and P15 (for a detailed protocol on adult animals, please see Wu et al., 2018):

a. Transfer the pups to a dedicated experimental room with a hood for fixative perfusion.

b. Deeply anesthetize one pup by injecting a solution of Ketamine (65 mg/kg) and Xylazine (13 mg/kg) intraperitoneally using an insulin syringe (30G x 1/2”).

c. Test anesthesia with a gentle foot pinch and wait for the loss of motor response.

d. Open the thorax and the diaphragm by using fine scissors.

e. Expose the heart, place a small needle (26G) connected to a peristaltic pump (or equivalent pumping system) into the left ventricle and cut a small hole in the right atrium.

f. Inject 5 mL of PBS 1 x to remove the circulating blood followed by 10 mL of Paraformaldehyde 4% in PBS 1 x (PFA 4%).

g. Dissect the brain and post-fix it in PFA 4% at 4°C overnight (12–16 h).

h. Move the brain to PBS 1 x and store at 4°C until slicing (within a few weeks).

Note: While storing at 4°C, protect the brains from light to prevent fluorescence loss.

21. Slice the brains with a vibratome (Leica). Check the vibratome manual for more details on best practices:

a. To cut coronal sections, trim the cerebellum with a sharp blade and glue the cut posterior side of the brain on a vibratome metal block using superglue, with the olfactory bulbs facing up.

b. Submerge the block and the brain in PBS 1 x.

c. Cut 80 μm-thick coronal sections (speed = 0.3 mm/s, amplitude = 1 mm) and transfer them on microscope slides with a tape frame on the borders working as a spacer (if you electroporated the somatosensory area, collect sections matching images from position 153 to position 297 of the Allen Brain reference Atlas https://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas).

d. Add a few drops of Fluoromount-G™ mounting medium with DAPI, cover with a coverslip, and let the slides dry flat overnight (12–16 h) at 4°C.

22. On the following day, start imaging fluorescent cells using a confocal microscope. We used a Leica SP8 with an HC PL APO CS2 40x water immersion objective, Z-stacks of 0.5 μm, with a resolution of 1600 x 1600 pixels. Both synaptophysin::EGFP and tdTomato channels have to be imaged. An example of a labeled cell is reported in Figure 1D. If there appear to be no cells labeled, check the section troubleshooting 2.

Note: It is advisable to immobilize the coverslip by sealing it with some nail polish on the sides. If imaging takes several days it is advisable to store slides vertically at 4°C to avoid the coverslip from slowly compressing the tissue.

Image processing, manual tracing and presynaptic boutons counting

Timming: 1.5–2 h per cell
In this step, tdTomato axons are traced and synaptophysin::EGFP boutons are counted through a custom-made MATLAB script. This script consists of 4 MATLAB files that have to be run sequentially (Figure 2):

**extractConfocalData.m**: this script uses the Bio-formats library provided by the Open Microscopy Environment consortium to read the confocal picture and converts it into a .mat file. You can check the list of supported formats here: https://docs.openmicroscopy.org/bio-formats/6.10.0/supported-formats.html. The output is a MATLAB format picture.

**filterAndSegment.m**: this script filters the synaptophysin::EGFP channel using a Wiener filter followed by a bilateral filter. Then, both synaptophysin::EGFP and tdTomato channels are thresholded using Otsu’s method. Finally, synaptophysin::EGFP puncta are segmented using a watershed algorithm. The output is a MATLAB file that can be used for axon tracing.

**manualTracing.m**: this script allows the user to trace cell axons by clicking some anchor points on the process of interest. The script uses these points to find the shortest path that goes through the tdTomato axon using the fast marching method. The path is computed both from source to sink and from sink to source to improve the centerline tracing. Then, the script calculates synaptophysin::EGFP puncta sizes by finding puncta profiles falling onto 3D centerline profiles (Figure 3). Finally, the script computes the path distance. The output is a MATLAB table containing the length of the traced axons and the sizes of all detected boutons.

**convertMATtoTXT.m**: this script converts the MATLAB tables into .txt tables ready to be analyzed through RStudio (see “quantification and statistical analysis”).

**Note**: Computing time depends on the size of the confocal picture and the computational power of your machine. In the following protocol, we report waiting times when processing pictures of 1.5 GB on a machine equipped with Intel® Xeon® Silver 4114 CPU @ 2.20 GHz 2.20 GHz (2 processors) and 512 GB of RAM.

23. Open a web browser and go to the website https://github.com/argunsah/punctaDensity (or to https://doi.org/10.5281/zenodo.6980507).
24. Download the code (press “Code” and select “Download ZIP”), and extract the punctaDensity-main.zip folder.
25. Open MATLAB. Go to the Home tab > Preferences > General > MAT-Files and make sure that “MATLAB Version 7.3 or later (save -v7.3)” is selected.
26. Change the current MATLAB folder to the punctaDensity-main folder; from now on, all newly generated files will be saved here.
27. Run extractConfocalData.m file (Figure 4A):
   a. Select the folder containing your confocal images.
   b. Select the confocal images you want to analyze.
   c. Press “Add”.
   d. Your selected images will appear in the window on the right.
   e. Press “Done”.
   f. Wait until all selected confocal files are converted into MATLAB format images (these files have the same names as the confocal picture, but .mat extension).

**Note**: This could take up to 5 min per confocal picture.

28. Run filterAndSegment.m file:
   a. Select MATLAB format images from step 27f similarly to steps 27a–e.
   b. Wait until all files are processed and new MATLAB files are created (these files end with “.info.mat”).
Figure 2. Pipeline of the computational analysis
Note: This could take up to 15–20 min per confocal image.

Note: In case the script gives an error, check the section troubleshooting 3.

29. Run `manualTracing.m` file:
   a. Select files ending with "_.info.mat" from step 28b similarly to steps 27a–e.
   b. When an image appears (it takes up to 2 min), adjust the image contrast using the "Adjust Contrast" window. Do not press "Adjust Data" when you are done. Just simply close the window (Figure 4B).

Note: The image is displayed as a grayscale max intensity projection of both channels merged; this is to facilitate the manual tracing. Nevertheless, only the green channel will be considered for presynaptic bouton counting.

   c. To trace the axons, use two keyboard buttons ("z" for zooming in, "x" for zooming out) and two mouse buttons ("left click" to locate a point, "right click" to conclude the tracing).
   d. Choose an axon and left-click at the tip of a process. Keep clicking more points along the process. When you are done, right-click and wait for the red tracing to be shown on the image (Figure 4C).

Note: The more left-clicked points, the longer it will take to trace the process.

Note: If there are multiple tangled processes it is necessary to left-click several points along the axon you want to trace.
e. In the “Tracing Quality” window press “Yes” if the tracing of the axon looks fine. Press “No” to discard it and start again the tracing (Figure 4C).

f. Press “Done” when you have completed tracing all the processes you want to trace. A MATLAB table containing the length of the axons and the sizes of all detected boutons will be created (this file ends with "_infoNums_#date#.mat"). #data# suffix is added to help the user track when the analysis has been performed. A final image with all traced axons labeled in red will be saved for reference (this file ends with "_figure.png") (Figure 4D).

g. Wait for the next image to be loaded and repeat the tracing procedure.

30. Run convertMATtoTXT.m file:
   a. Select files ending with "_infoNums_#date#.mat" from step 29f similarly to steps 27a–e.
b. A .txt file will be created for further analysis using R language (these files end with "_info-Nums##_date##_newFormat.txt") (see Table 1).

**Note:** In case the script gives an error, check the section troubleshooting 4.

### EXPECTED OUTCOMES

Successful electroporation allows the labeling of around 20 individual superficial SST\(^+\) cells. This number can change when using different CRE-driver mouse lines. An example of a labeled cell is depicted in Figure 1D.

After the complete tracing of axonal processes using our MATLAB custom-made pipeline, a .txt table will be produced as depicted in Table 1. This table will provide the length of each traced axon and the size of all detected presynaptic boutons; these data can be further analyzed as indicated in the following section.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Our MATLAB code produces a single .txt file per analyzed cell (the output file from step 30). This file is structured as indicated in Table 1. Based on our experience, between 10 to 15 cells coming from at least 3 animals are sufficient for the exploration of the phenotype.\(^1\)

We include a simple code written in RStudio that can be used to analyze these files and quantify synaptic density in two different conditions (e.g., control vs treatment). To run it, you need to split the .txt files from condition 1 and condition 2 into two separate folders. Open RStudio, create a new R Markdown file, and paste the following chunks.

**Chunk 1:** Specify the paths to the folders containing .txt files from condition 1 and condition 2. This chunk creates a list of all files to be analyzed.

```r
---
name: 'Table 1. Example of the output of the MATLAB code: every column is a single traced process'
---

| Process 1 | Process 2 | Process 3 | Process 4 | Process 5 | Process 6 | ... | Process n |
|-----------|-----------|-----------|-----------|-----------|-----------|-----|-----------|
| Process length | 300.09 | 26.25 | 47.54 | 20.52 | 20.77 | 152.24 | ... | 82.96 |
| Puncta 1 size | 0.59 | 1.98 | 4.36 | 1.98 | 1.98 | 4.36 | ... | 1.98 |
| Puncta 2 size | 1.78 | 1.19 | 1.39 | 0.40 | 6.14 | 1.19 | ... | 0.40 |
| Puncta 3 size | 1.98 | 1.98 | 2.57 | 3.37 | 3.17 | 2.97 | ... | 1.98 |
| Puncta 4 size | 1.98 | NaN | 2.38 | 2.57 | 1.98 | 2.57 | ... | 2.97 |
| Puncta 5 size | 2.57 | NaN | 4.75 | 1.39 | 0.20 | 5.15 | ... | 1.98 |
| Puncta 6 size | 2.38 | NaN | 2.77 | 0.59 | 5.54 | 2.38 | ... | 2.77 |
| Puncta 7 size | 2.38 | NaN | 0.99 | 0.20 | 2.77 | 3.17 | ... | 2.18 |
| ... | ... | ... | ... | ... | ... | ... | ... | ... |
| Puncta n size | 1.98 | NaN | NaN | NaN | NaN | 2.77 | ... | 2.57 |

The value in bold in the first row is the length of the process (\(\mu m\)), while the other values in the same column are the size (\(\mu m\)) of every detected puncta along that process. The matrix has as many rows as the largest number of puncta detected for a process, therefore, empty rows are filled with "NaN".

---

\(^1\) We include a simple code written in RStudio that can be used to analyze these files and quantify synaptic density in two different conditions (e.g., control vs treatment). To run it, you need to split the .txt files from condition 1 and condition 2 into two separate folders. Open RStudio, create a new R Markdown file, and paste the following chunks.

```r
```
**Chunk 2:** This chunk combines the individual files into a single data frame named "out".

```r
folder <- c(condition1, condition2)
...
```

```
```r
out <- data.frame(
  File = character(),
  Condition = character(),
  Cell_ID = character(),
  Process_ID = character(),
  Process_Length = numeric(),
  Puncta_Size = numeric(),
  stringsAsFactors = FALSE
)
z <- 1
for(i in 1:length(folder)) {
  df <- read.table(file = folder[i], header = FALSE)
  for(c in 1:ncol(df)) {
    for(r in 2:nrow(df)) {
      out[z,1] <- folder[i] # File name
      out[z,2] <- ifelse(folder[i] %in% condition1, "condition1", "condition2")
      out[z,3] <- i # Cell_ID
      out[z,4] <- paste(i,c,sep = "_") # Process_ID
      out[z,5] <- df[1,c]# Process_Length
      out[z,6] <- df[r,c] # Puncta_Size
      z <- z+1
    }
  }
}
out <- subset(out,!out$Puncta_Size=="NaN")
...
```

**Chunk 3:** This chunk plots a histogram showing the frequency of different puncta sizes. Puncta smaller than 0.5 μm are then removed from the total count as they are much smaller than the average size of a presynaptic bouton (around 2 μm) and look as a background signal. There might also exist big puncta, which correspond to clusters of boutons too close to be distinguished and segmented by the MATLAB script. This code classifies them as outliers according to the interquartile range criterion. To be able to estimate the number of puncta composing each cluster, the code divides each outlier by the mean size
of detected puncta (calculated without outliers). Finally, the code adds an extra column to the “out” data frame with the number of presynaptic boutons composing each puncta.

```
```{r}
# plot the histogram of puncta sizes
hist(out$Puncta_Size, breaks = "Scott")
# remove puncta smaller than 0.5 micrometers
out <- subset(out, out$Puncta_Size > 0.5)
# find the outliers
outliers <- boxplot.stats(out$Puncta_Size)$out
# calculate the mean size of a puncta without outliers
mean <- mean(out$Puncta_Size[!out$Puncta_Size %in% outliers])
# add the number of distinct puncta
# for the outliers, the number is calculated as puncta size/mean puncta size
out$Number <- ifelse(out$Puncta_Size %in% outliers, out$Puncta_Size/mean, 1)
...
```

Chunk 4: This chunk calculates the puncta density of each traced process and produces a boxplot per cell with the densities of each traced process (Figure 5A).

```
```{r}
df <- out[, c("Process_ID","Number")]
df <- aggregate(Number ~ Process_ID, df, sum)
out <- out[,1:5]
out <- unique(out)
out$Number <- df[match(out$Process_ID, df$Process_ID),2]
out$Density <- out$Number/out$Process_Length
# plot
out$Cell_ID <- factor(out$Cell_ID, levels=unique(out$Cell_ID))
library(ggplot2)
ggplot(out, aes(x = Cell_ID, y=Density, fill = Condition)) +
geom_boxplot() 
...
```

Chunk 5: This chunk plots total puncta density per cell calculated as the total number of puncta per cell divided by the sum of the lengths of all traced processes per cell (Figure 5B).

```
```{r echo=FALSE}
plot <- aggregate(out[,5:6],by=list(out$Cell_ID),FUN=sum, na.rm=TRUE)
LIMITATIONS

Neonatal pial-surface electroporation allows sparse labeling of superficial cortical cells that can be individually imaged and quantified at a single cell resolution. However, it is possible to observe some variability within the group of electroporated neurons, with some cells showing strong expression of the plasmid and other cells being faintly labeled. Weaker labeling that still allows the reconstruction of the cells is not a problem when estimating the density of presynaptic boutons. Nevertheless, puncta size measuring can be affected by the weaker expression of the plasmid.

When sectioning the brain, some cells may be cut in half. Nevertheless, you should be able to find both halves on two consecutive sections; if you do not need to reconstruct the whole cell, single axons can still be localized, traced and analyzed.

Neonatal pial-surface electroporation is more efficient when performed at P1. When using SST<sup>Cre/+</sup> P1 pups you can easily label around 15–20 cells per brain. The efficiency drops when electroporating at P2, resulting in around 5–10 labeled cells per brain. Unfortunately, neonatal pial-surface electroporation does not typically work at later stages. Nevertheless, one could achieve later expression of a gene by including an inducible cassette in the plasmid and electroporate it at P1 and P2.<sup>4</sup>

---

```r
colnames(plot)[colnames(plot) == "Group.1"] <- "Cell_ID"
a <- unique(out[,1:3])
plot$Condition <- a[match(a$Cell_ID,plot$Cell_ID),2]
plot$Density <- plot$Number/plot$Process_Length
#plot
ggplot(plot, aes(x = Condition, y =Density, fill = Condition)) + geom_boxplot()
```

---

Figure 5. Boxplots generated by the R Studio script

(A) Each boxplot represents a cell; each data point is the presynaptic bouton density of every traced process.

(B) Each boxplot represents one condition; each data point is the total number of presynaptic boutons divided by the total length of traced processes per cell.
Finally, in our previous study we were able to find cells labeled by neonatal electroporation until P20, but we did not check at later stages.

**TROUBLESHOOTING**

**Problem 1**
The mother does not take care of the pups after surgery (step 19b).

**Potential solution**

- Check first for any external source of stress to the animals (noisy animal facility, light-dark cycle not working correctly, etc.).
- If possible, avoid using first-litter mothers since the chances of pup cannibalism are higher.
- To avoid additional stress, do not take pups one by one from the parents: always remove the parents and place them in a new cage while taking the litter.
- It might be helpful to take only half of the litter so that the parents will never experience being separated from all their pups.
- Before returning the litter to the parents, leave them for at least half an hour in the recovery cage with some bedding from the parental cage. It is better that the parental cage has not been cleaned in the few days preceding the surgery.
- When giving the pups back to the parents, place them outside the nest and observe if the parents correctly place them back in the nest.

**Problem 2**
There appear to be no cells labeled (step 22).

**Potential solution**

- Since only a few cells are labeled per brain, be sure to have checked carefully all brain sections by eye with an epifluorescence microscope using a 10× or 20× objective.
- When using a different CRE-driver mouse line, be sure that the CRE is already expressed at P1–P2.
- Be sure to use the correct parameters of the electroporator.
- Neonatal pial-surface electroporation is more efficient at P1 compared to P2. However, isoflurane anesthesia works better at P2. Depending on the anesthesia protocol allowed by your local animal experimentation guidelines, it is preferable to use P1 over P2.
- Be sure that you inject between the skull and the pia; when doing so, you should be able to see the blue plasmid DNA solution spreading evenly over a circular surface of around 2.5–3 mm in diameter (Figure 1B).
- Be sure that you are positioning the electrodes correctly, with the negative one over the injection side. Be aware of any residue of neurogel that could make a bridge between the two electrodes.

**Problem 3**
When running `filterAndSegment.m` the following error message appears: `Undefined variable “cube” or class “cube”` (step 28).

**Potential solution**

- Go to the Home tab > Preferences > General > MAT-Files and make sure that “MATLAB Version 7.3 or later (save -v7.3)” is selected.

**Problem 4**
When running `convertMAToTXT.m` the following error message appears: `Undefined function or variable ‘writematrix’` (step 30).
Potential solution

- Be sure you are using MATLAB 2019a or a later version.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Theofanis Karayannis (karayannis@hifo.uzh.ch).

Materials availability
Plasmids generated in this study have been deposited to Addgene: pCALNL-tdTomato-2A-mSy-p::EGFP Cat#191213.

Data and code availability
The original code for synaptic bouton counting has been deposited at https://doi.org/10.5281/zenodo.6980507.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

We are grateful to all members of the Karayannis laboratory for stimulating discussions. Imaging was performed with equipment maintained by the Center for Microscopy and Image Analysis, University of Zurich. L.G. was supported by the Forschungskredit of the University of Zurich, grant no. [FK-20-029]. Research in the T.K. lab was supported by grants from the European Research Council (ERC, 679175) and the Swiss National Science Foundation (SNSF, 31003A_170037). The graphical abstract and part of Figure 1 were created with BioRender.com.

AUTHOR CONTRIBUTIONS

L.G. performed plasmid cloning, electroporation, imaging, and data analysis and wrote the manuscript. A.O.A. developed the MATLAB code and wrote the manuscript. T.K. acquired the funding and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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