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ARTICLE *in* NATURE PROTOCOLS · JANUARY 2015

Impact Factor: 9.67 · DOI: 10.1038/nprot.2015.001 · Source: PubMed

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In vivo rapid gene delivery into postmitotic neocortical neurons using iontoporation

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Published online 4 December 2014; doi:10.1038/nprot.2015.001

This protocol describes a method for directing the expression of genes of interest into postmitotic neocortical neurons *in vivo*. Microinjection of a DNA plasmid-amphiphilic molecule mix into the neocortex followed by delivery of an *ad hoc* electric pulse protocol during the first few days of life in mice allows rapid, focal and efficient expression of genes in postmitotic neurons. Compared with other gene delivery techniques such as *in utero* electroporation and viral infection, this method allows rapid (12 h), focal (50–200 μm), mosaic-like (50 to several hundred neurons) targeting of postmitotic neurons within existing circuits. This 'iontoporation' protocol, which can be completed within ~20 min per mouse, allows straightforward assessment of genetic constructs in postmitotic cortical neurons and subsequent genetic, histological and physiological investigations of gene function.

INTRODUCTION

The ability to rapidly and accurately target the expression of a gene of interest to specific cell types during neuronal development is crucial to investigating the genetic programs underlying the acquisition of neuronal identity and cell diversity. Here we describe a protocol for directly reprogramming postmitotic neurons in the neocortex of early postnatal mice[1].

Techniques used to deliver genetic material into neurons *in vivo*

The two main techniques used to deliver genetic material into neurons *in vivo* are *in utero* electroporation and viral infection. Electroporation uses application of an electrical field to transiently create pores in the cytoplasmic membrane, allowing exogenous plasmid DNA to enter into the cytoplasm of the cell[2], and it can also be used *in vitro* in slices[3]. Once in the cytoplasm, plasmid DNA is rapidly degraded, unless it can access the nucleus[4]. For this reason, the efficacy of electroporation is essentially limited to mitotic cells[5–8], which lack a nuclear envelope during metaphase and anaphase, allowing the plasmid to be trapped within the nucleus as the nuclear membrane forms back toward the end of telophase. As a consequence of this limitation, electroporation must be performed at the time and site of birth of neurons, which, in the neocortex, implies injecting the plasmid into the lateral ventricle of embryos *in utero* and directing the electric field toward the ventricular wall, where progenitors are located. By using this approach with inducible (e.g., tamoxifen-inducible CreER^{T2}) systems, or with promoters or enhancers that confine expression to postmitotic neurons, expression of a gene of interest can be restricted to the progeny of these neurons (i.e., to postmitotic neurons[9]). Although single postmitotic neurons can be electroporated *in vivo* using specific protocols in which, presumably, application of large focal currents allows access to the nucleus[10–15], electroporation of a large number of postmitotic neurons, which is usually required for genetic, histological, and functional or behavioral analyses, has been more challenging. Accordingly, focal electroporation in distinct brain regions, mostly using invasive electrodes, generally only leads to very few (1–10) transfected neurons[16–18], although higher numbers have been reported in regions such as the hippocampus, where progenitors are present even in adulthood[5,8] (summarized in **Table 1**; refs. 1,11,13,17–28).

Because of these limitations, the most common technique to target postmitotic neurons is viral delivery of a genetic construct. For brain delivery, lentivirus, adenovirus and adeno-associated viruses are most often used[29]. Virus infection is based on the recognition of cell surface proteins that interact with viral proteins, and it allows the insertion of the genetic material[30]. Cell type–specific expression can be obtained by using type-specific promoters or by the expression of chimeric receptor proteins on the neuronal surface. The latter can be used to define 'starter cells' and to trace the input-output connectivity of an infected neuron when using trans-synaptically transported viruses such as pseudorabies virus[31,32]. Although viruses are widely used in neuroscience research and their production is now well standardized, their use has several limitations such as potential toxicity, the need for dedicated facilities and equipment, time required for production (usually 2–3 weeks), as well as slow onset of expression, which normally starts within 2–3 d at the earliest[33].

Protocol development

To circumvent the main limitations of both electroporation and viral gene delivery in targeting postmitotic neurons, we recently developed an electrochemical gene delivery technique, which we termed iontoporation; it enables rapid (<12 h) targeted gene expression of large numbers (10–500 or more) of postmitotic cortical neurons (**Figs. 1**, **2** and **Table 2**). Iontoporation combines the physical principle of an *ad hoc* electric field protocol applied to the cellular membrane, which creates pores in the cytoplasmic membrane[2], with trans-cyclohexane-1,2-diol (TCHD), an amphipathic molecule that collapses the selectivity of the nuclear pore complex, allowing the passive diffusion of macromolecules such as plasmid DNA into the nucleus[34,35]. Thus, the combined effects of an *ad hoc* electrical field, which allows exogenous DNA to enter into the cytoplasm and presumably even the nucleus, and TCHD, which contributes to an increase in the permeability of the nuclear pore complex barrier, allow the plasmid to enter into the nucleus, where it has access to the transcriptional machinery of

TABLE 1 | Summary of intraparenchymal electroporation studies targeting postmitotic neurons.

Reference	Species	Age	Region targeted	Electrode type	No. of transfected neurons
Inoue *et al.*[19]	Mouse	Adult	Hindbrain	Invasive	Not quantified (NQ)
Wei *et al.*[20]	Mouse and rat	Adult	Cingulate cortex	Invasive	NQ, >100?
Konishi *et al.*[21]	Rat	P3	Cerebellum	Invasive	NQ, ~50–100?
Akenaya *et al.*[22]	Rat	Adult	Neocortex and hippocampus	Invasive	NQ
Guo *et al.*[23]	Rat	Adult	Medulla	Invasive	NQ, ~5–10?
Nagayama *et al.*[24]	Mouse	Adult	Cerebellum, olfactory bulb and neocortex	Invasive	~80
Nevian and Helmchen[25]	Rat	Adult	Neocortex	Invasive	~1–10
Wang *et al.*[26]	Hamster	Adult	Hypothalamus	Invasive	NQ, >100?
Kitamura *et al.*[13], Judkewitz *et al.*[11]	Mouse and rat	Adult	Neocortex and cerebellum	Invasive	~1–10
Fan *et al.*[27]	Rat	Adult	Cingulate cortex	Invasive	NQ, ~10–100?
De Vry *et al.*[28]	Mouse	Adult	Dentate gyrus	Invasive	>100
Molotkov *et al.*[17]	Rat	P2	Neocortex and striatum	Surface	NQ, ~10–50?
De la Rossa *et al.*[1]	Mouse	P1	Neocortex	Surface	>100
Ohmura *et al.*[18]	Mouse and cat	Adult	Thalamus	Invasive	~1–10

the host cell. Precise targeting of postmitotic neurons is done by stereotaxic microinjection, and iontoporated neurons show strong expression 12 h after injection when using a gene driven by a strong promoter, such as the ubiquitin or *CAG* promoter, enabling stable expression over several weeks at least (**Fig. 2a**,**b**)[1]. Control over the number of cells expressing the transgene is performed by adjusting the injection volume (**Fig. 2c**), allowing layer-specific gene delivery with small volumes of injection (50 nl) and *post hoc* confirmation of the injection site[1]. As neocortical neurons are born sequentially from progenitors located along the ventricular wall, late-born superficial layer neurons may be labeled even after early intraventricular injection and electroporation, owing to successive rounds of progenitor division. Iontoporation is therefore particularly suitable for the specific targeting of early-born, deep cortical layer neurons, as it avoids 'residual' gene expression in later-born superficial neurons. Furthermore, by using plasmid constructs in which gene expression is controlled by cell type–specific promoters and enhancers, transcription can be limited to cell types of interest, such as γ-aminobutyric acid (GABA)ergic interneurons (*Dlx5/6* promoter[36]) or astrocytes (glial fibrillary acidic protein (*GFAP*) promoter, modified from Addgene plasmid no. 27055), the latter being difficult to target via classical electroporation (**Fig. 2d**,**e**). Although iontoporation works best during the first 3 d of life, tamoxifen-inducible plasmids[37] can be used for expression at later time points, including in adulthood[1]. Also, in principle, the use of shRNA-encoding constructs offers the possibility to downregulate targeted transcripts within iontoporated neurons. Finally, direct injection of the plasmid into

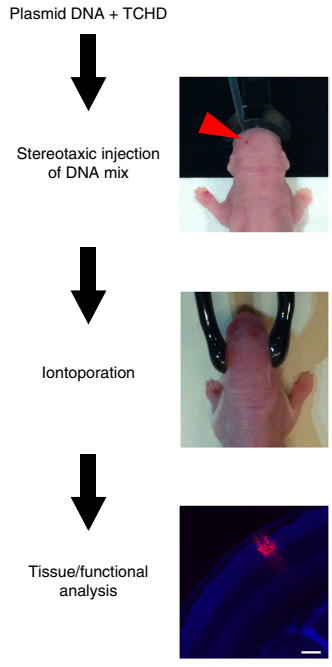

Figure 1 | Schematic summary of the iontoporation protocol. After preparation of the plasmid-TCHD solution (Steps 1–7), the mix is injected stereotaxically into the target area (Steps 8–17), and the iontoporation is performed (Steps 18 and 19; red arrowhead indicates injection site). Expression of the plasmid can be detected as early as 12 h after the procedure. Scale bar, 200 μm.

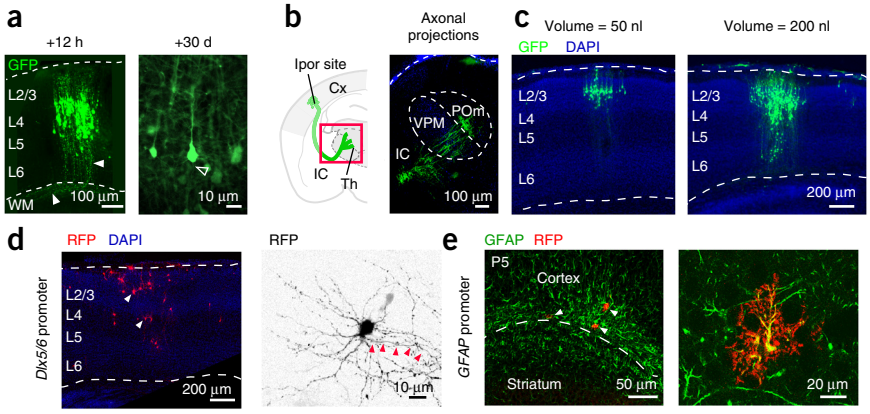

Figure 2 | Targeting postmitotic neurons with iontoporation. (**a**) Example of GFP expression 12 h (left, arrowheads indicate subcortically extending axons) and 30 d (right; empty arrowhead: axon) after iontoporation of a CAG-IRES-GFP plasmid. L1–6, layers 1–6; WM, white matter. (**b**) Axonal projections from iontoporated cortical neurons are visible several hundreds of microns away, in the thalamus (red box in left image shows location of the axons shown on the right, coronal section, axonal projections and iontoporation site are shown in green). Cx, cortex; IC, internal capsule; Ipor, iontoporation; POm, posteromedial nucleus of the thalamus; Th, thalamus; VPM, ventral posteromedial nucleus of the thalamus. (**c**) Dose-dependent labeling of cortical neurons (here in layers 2/3). Tens of neurons are transfected when injecting 50 nl of the DNA mix, whereas hundreds of neurons or more can be transfected with larger volumes of injection. (**d**,**e**) Injecting a plasmid with a cell type–specific promoter or enhancer allows cell type–specific expression. (**d**) The use of a *Dlx5/6* promoter–containing plasmid allows specific expression in inhibitory interneurons[38] (arrowheads in low magnification, left). Right, higher magnification from a different section showing neuronal processes, including the axon (arrowheads). (**e**) The use of a *GFAP* promoter–containing plasmid allows specific expression in astrocytes (arrowheads in low-magnification left image). The relatively low number of RFP-expressing astrocytes may reflect both promoter efficiency and the relatively low number of these cells in the early postnatal cortex. Labeled astrocytes were not found when using a regular *CAG* promoter. Right, higher magnification from a different section showing typical astrocytic morphology. Dashed lines indicate superficial and deep limits of the neocortex.

postnatal pups is advantageous in that it spares nursing mothers, which can be reused for other litters.

By using this strategy, we have been able to directly re-program select subtypes of cortical neurons by overexpression of a single transcription factor, leading to acquisition of novel type-specific molecular, morphological, electrophysiological and circuit features in reprogrammed neurons within otherwise intact postnatal cortical circuits[1]. By allowing the mosaic expression of select transgenes within neurons of existing cortical circuits, iontoporation is thus well suited to investigate gene-circuit interactions and molecular controls over developmental circuit assembly.

TABLE 2 | Comparison of electroporation, viral vectors and iontoporation for gene delivery into neurons.

Parameter	*In utero* electroporation	Virus	Iontoporation
Speed of expression	Hours	Days	Hours
Maximal plasmid size	12 kb	4–5 kb (for adeno-associated virus)	12 kb
Spatial accuracy of expression	+	+++	+++
Adequate for targeting small number of cells	–	+	++
Ease of use	++	+	+++
Cost	+	++	+
Mother spared by intervention	No	Yes	Yes
Toxicity	No	Possible	No
Selective targeting of postmitotic neurons	Yes[a]	Yes	Yes
Neurons accessible in all brain structures	No (must have access to progenitors)	Yes	?
Time of intervention	Prenatal	Usually adult	Early postnatal
Yield (positive mice per attempts)	50–80%	80–100%	30–50% (for 50-nl injections; increases with higher volumes)

[a]Possible with CreER^{T2} constructs, or with promoters or enhancers specific to postmitotic neurons. +++, high; ++, intermediate; +, low; –, absent.

MATERIALS

REAGENTS

- Plasmid DNA of interest (maximum size tested: 12 kb). A pCAG_IRES_GFP plasmid (Addgene, no. 11159) can be used as a control. The final concentration of the plasmid needs to be ≥5 µg/µl (see point 3)
- LB broth medium (Sigma-Aldrich, cat. no. L3022)
- LB broth agar (Sigma-Aldrich, cat. no. L2897)
- Competent *Escherichia coli* XL1 blue (Stratagene, cat. no. 200236)
- Agarose (Sigma-Aldrich, cat. no. A5093)
- Qiagen EndoFree plasmid maxi kit (Qiagen, cat. no. 12362)
- Qiagen plasmid maxi kit (Qiagen, cat. no. 12162)
- Isopropanol, molecular biology grade, 100% (Sigma-Aldrich, cat. no. 59300)
- Ethanol, molecular biology grade, 100% (Fluka, cat. no. 02860)
- H_2O, DNase and RNase free (Invitrogen, cat. no. 10977-023)
- Trans-cyclohexane-1,2-diol (TCHD; Sigma-Aldrich, cat. no. 141712)
- Tamoxifen (Sigma-Aldrich, cat. no. T5648)
- Corn oil (Sigma-Aldrich, cat. no. C8267)
- PBS, 10× (Sigma-Aldrich, cat. no. D8662)
- Fast Green (Sigma-Aldrich, cat. no. F7258)
- Neurogel (pharmaceutical preparation: Carbopol 940 1%, NaOH 0.4%, propylene glycol 20%, parabens 0.1% (all wt/vol) and H_2O)
- Mice. The procedure has been applied on CD1 and C57BL/6 mice; other strains may be used, but adjustments in stimulation parameters (see point 19) may be needed. The iontoporation procedure *per se* tends to be more successful with C57BL/6 pups, but CD1 mothers usually take better care of their pups. When using wild-type mice, we thus preferred CD1 mice **! CAUTION** Experiments using live rodents must conform to all relevant governmental and institutional regulations.

EQUIPMENT

- Bacteria incubator, 37 °C with shaker
- Petri dishes (Nunclon, cat. no. 150350)
- Growing flasks (for bacteria)
- Centrifuge ≥10,000*g* (Eppendorf, cat. no. 5415D)
- Thermoblock (Labnet, cat. no. D1100)
- Agarose electrophoresis equipment (Apelex, cat. no. 311000)
- NanoDrop spectrophotometer (Thermo Scientific, cat. no. nd-1000)
- Nanoject II nanoinjector (Drummond, cat. no. 3-000-205A)
- Mouse stereotaxic frame with mouse/neonatal rat stereotaxic adaptor (Harvard Apparatus, cat. no. 724794)
- Electroporator CUY21SC (Nepa Gene, cat. no. CUY21SC)
- Glass micropipette puller (Sutter Instrument, cat. no. P-97)
- Glass capillaries (Drummond, cat. no. 3-000-203-G/X)
- Platinum electrode paddles, 5 mm in diameter (Nepa Gene, cat. no. CUY650-5)
- Heating pad, 37 °C (Harvard Apparatus, cat. no. 340925)
- Surgical stereomicroscope, 40× (Leica, cat. no. M50)
- Glass micropipette beveler (Sutter Instrument, cat. no. BV-10)
- Digital rat stereotaxic instrument (Stoelting, cat. no. 51900)
- Micro-scissors (Harvard Apparatus, cat. no. 728503)
- Micro-forceps (Harvard Apparatus, cat. no. 522110)

REAGENT SETUP

Fast Green Dissolve Fast Green in 1× PBS to a final concentration of 0.01% (wt/vol). ▲ **CRITICAL** Freshly prepare the solution.

Trans-cyclohexane-1,2-diol Dissolve TCHD in 1× PBS to a final concentration of 10 mg/ml. ▲ **CRITICAL** Freshly prepare the solution.

Tamoxifen Prepare a 10 mg/ml tamoxifen (corn oil, 0.1% (vol/vol) ethanol) solution (optional if using $CreER^{T2}$-inducible plasmid). For 10 ml of solution, use 100 mg of tamoxifen, 100 ml of 100% ethanol and 9.9 ml of corn oil. Dilute the tamoxifen in ethanol and then add the corn oil. ▲ **CRITICAL** Freshly prepare the solution.

PROCEDURE

Plasmid preparation ● TIMING 3 d for plasmid cloning, 30 min for plasmid-TCHD preparation

1| Clone the desired gene using standard cloning techniques[38,39]. Plasmids up to 12 kb have been iontoporated successfully alone or in combination with other plasmids.

▲ **CRITICAL STEP** Ensure that the plasmid selected has a promoter that can drive strong expression in the host neurons (e.g., *CAG-IRES-GFP* drives strong expression in cortical neurons[1]).

? TROUBLESHOOTING

2| Purify the plasmid using a commercially available plasmid purification kit. We use the Qiagen maxiprep endotoxin-free kit, although purifications with the Qiagen maxiprep kit have resulted in successful iontoporation as well. Purify the plasmid according to the manufacturer's instructions, and elute it in DNase/RNase-free water.

3| Measure the DNA concentration and purity. The concentration of the plasmid after purification needs to be ≥5 µg/µl. A NanoDrop spectrophotometer can be used; purity of the plasmid is optimal with 260 nm/280 nm and 260 nm/230 nm ratio values close to 2. If these ratios are <1.5, do not use that plasmid for iontoporation.

4| Mix 5 µl (5 µg/µl) of the plasmid with 4 µl of 0.01% (wt/vol) Fast Green in 1× PBS and 1 µl of 10 mg/ml TCHD in 1× PBS. The final concentration of the mix is 2.5 µg/µl plasmid, 0.004% (wt/vol) Fast Green and 1 mg/ml TCHD. TCHD is stable when kept on ice; we have not observed a decrease in labeling efficiency in successive pups throughout the course of a surgical session. Increasing the TCHD concentration does not appear to improve the transfection efficiency.

Pipette preparation ● TIMING 20 min per pipette, usually done in batches ahead of time

5| Pull a glass capillary with the glass micropipette puller. Two 30-µm-tip glass micropipettes will be obtained with each original capillary.

6| Bevel the tip of the micropipette by using the glass micropipette beveler. The angle of the tip must be at 45°.

▲ **CRITICAL STEP** For a good penetration and to avoid brain damage, it is very important that the tip of the glass micropipette be beveled at 45° and that the tip be sharp. Unbeveled tips can damage the cortex during the injection.

7| Fill the glass 30-µm micropipette with 10 µl of plasmid mix. The micropipette should be previously mounted on the nanoinjector. Use the surgical microscope to do this.

Surgery preparation ● TIMING 15 min for mice preparation

8| Separate the mother from the postnatal day 1 (P1) pups. Keep the mother in a distinct cage throughout the procedure, together with a plastic glove to get her used to the smell once she is returned to her pups.

▲ **CRITICAL STEP** Keep the mother in a calm place.

? TROUBLESHOOTING

Surgery ● TIMING 12–15 min per pup

9| Put the pup on ice for 4 min in order to anesthetize the pup by hypothermia.

▲ **CRITICAL STEP** The time on ice must be adjusted experimentally when using different ages and strains. Four minutes is the proper time for C57BL/6 pups.

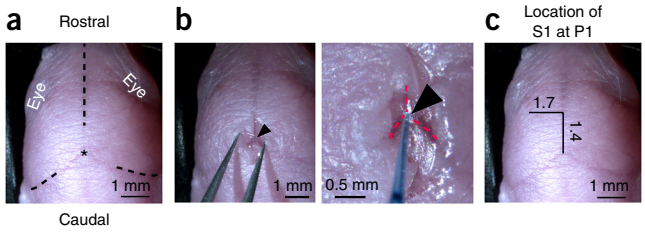

Figure 3 | Defining the stereotaxic coordinates of the target site. (**a**) The vascular lambda (asterisk) is identified as the point at which the sagittal sinus (vertical dashed line) and the transverse sinuses (horizontal dashed lines) converge. It serves as the point of origin for *x-y* stereotaxic coordinates. (**b**) The vascular lambda (arrowhead) is revealed by a small skin incision, on top of which the pipette tip is placed, and coordinates are zeroed. (**c**) The pipette tip is next moved to the desired coordinates using the stereotaxic frame system, and a small skin incision is performed above the target. Coordinates (in mm) for the primary somatosensory cortex (S1) in P1 CD1 mice are indicated.

10| Keep the Cunningham plate of the stereotaxic frame cold (~4 °C, by putting a few dry-ice pellets in 70% (vol/vol) ethanol in the well at the back of the plate).

▲ **CRITICAL STEP** If the plate is too cold or wet, or if it is not cold enough, the pups will either not wake up (if too cold) or wake up too early (if too warm).

? TROUBLESHOOTING

11| Place the pup on the Cunningham plate, open its mouth and fix its snout with the incisor adaptor. Gently fix the head with the rubber pad ends of the ear bars. Further details can be found in Cetin *et al.*[40].

12| Looking at the fixed head from above, identify the vascular lambda (sinus confluent), where the transverse and sagittal sinuses unite (asterisk in **Fig. 3a**). This will serve as the point of origin for stereotaxic *x-y* coordinates.

13| Make a small (1 mm) incision of the skin overlying this point using micro-scissors (**Fig. 3b**) and place the filled pipette on top. Calculate all stereotaxic coordinates from this point to define the injection site (**Fig. 3**).

14| Displace the micropipette to the site of injection using the digital coordinates according to a mouse brain atlas or according to your own age- or strain-specific coordinates[41]. Coordinates for the primary somatosensory cortex at P1 in C57BL/6 mice are 1.5 mm anteriorly and 1.2 mm laterally to the vascular lambda (**Fig. 3c**). For P1 CD1 mice, values are 1.4 anteriorly and 1.7 mm laterally. Make a small (1 mm) incision of the skin overlying the target as described above.

▲ **CRITICAL STEP** Precise injections require some training (e.g., by injecting a dye such as cholera toxin B). It is important to have a mouse atlas and to adapt the coordinates to the mouse's age and strain. Slowly enter into the cortex with the micropipette, to the desired calculated depth (e.g., 100 µm for L4 at P1 for C57 mice and 150 µm for CD1 mice).

15| Inject 10 nl of the plasmid every 10 s, not faster. Injection of 200 nl will result in large iontoporation areas, whereas 50 nl or less will result in a very focal iontoporation.

▲ **CRITICAL STEP** While injecting the plasmid, it is important that the pup be well anesthetized. Any movement will result in cortical damage and plasmid leakage.

? TROUBLESHOOTING

16| Wait 5 min after the last pulse of injection is done. Do not go faster than this; this is a crucial step that allows the plasmid-TCHD mix to diffuse and avoids sucking out the mix when removing the micropipette.

17| Remove the micropipette very slowly, avoiding any unwanted movement.

18| Put the 5-mm electrode paddles on each side of the head, applying a small amount of conducting gel (Neurogel), which should remain strictly under the paddles in order to avoid bridging the two electrodes (**Fig. 1**).
▲ **CRITICAL STEP** Proper positioning of the electrode paddles, conductive gel placement and skin insulation are particularly important when small volumes are injected in order to allow most of the current to flow through the injected area.

19| Give two trains of ten pulses (1 Hz, 50 ms duration; Nepa Gene CUY21SC settings: time on 50 ms, time off 950 ms, voltage 99 V, 10×), with a 3-s pause between the two trains. These settings have been determined by applying increasing voltages to distinct pups after injection of the plasmid-TCHD mixture and charting the effective current delivered by the device. Positive electroporations in this calibration phase were identified by screening for cortical GFP expression in whole-mount brains, typically corresponding to several hundred positive neurons. The amplitude of the current that passes across the electrodes at the last pulse must be at least 50 mA for the iontoporation to succeed. Current values <50 mA will result in unsuccessful iontoporations, and higher values carry the risk of injuring the brain. Note that the current increases with each pulse (for a given voltage), perhaps reflecting an increase in conductance as a result of an increase in temperature. Voltage pulses should be adjusted to stay within this range of currents for cortical electroporations, but they may need adjustments when investigating other brain regions or different species or ages.
? TROUBLESHOOTING

20| Place the pup on the warm pad at 37 °C. It should fully recover in <10 min. Repeat Steps 9–20 for additional pups.
? TROUBLESHOOTING

21| Once all the pups are iontoporated and have recovered, put them back into their cage and wait for a few minutes for them to settle down before bringing back their mother. As skin incisions are only 1 mm long and the pups usually do not display signs of discomfort or pain (e.g., body twisting, panting, scratching and absence of suckling) after surgery, we do not recommend the routine use of opioids or local anesthetics because their use in newborn mice carries a risk of dosage-related toxicity. If an occasional pup shows signs of discomfort, it should be euthanized, but repetitive occurrences should prompt a review of the surgical procedure (large incision? Blunt pipette tip?).
? TROUBLESHOOTING

22| If you are using a CreER^{T2} tamoxifen-inducible plasmid, inject as follows. For pups younger than P5, use 50 µl of the tamoxifen–corn oil solution described in Reagent Setup (0.5 mg of tamoxifen). Between P5 and P10, use 100 µl of the solution, for adult mice use 500 µl and for pregnant mice use 1 ml. Plasmid expression is induced within ~24 h.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	No expression of the plasmid; plasmid works well in other cell types but not in cells of interest	Inefficient cloning; promoter is not adequate for the target neurons	Test plasmid expression in an adequate *in vitro* culture system or by *in utero* electroporation Ensure that your detection system is adequate, using control plasmids Ensure that the open reading frame is functional by sequencing Test other promoters
		The current passing through the injection site is <50 mA; the angle of the electric field is not perpendicular to the injection site; the electric field does not pass through the injection site	Re-position paddles to adjust the direction of the electric field; DNA flows toward the positive electrode. Dry the skin between paddles; gel should not bridge the two electrodes on the skin surface. Make sure that a thin (<1 mm) gel layer is present between the paddles and the skin

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
8,21	Mother disrupts the nest or does not take care of the pups after surgery	The mother is stressed by an unfamiliar environment (e.g., noises, unusual smells and movement)	Habituate the mother to the new environment a few hours before the surgery Put the mother in a new cage (as opposed to taking the pups one by one away for surgery) Put a glove used for surgery into the mother's cage to familiarize her with the smell Be extremely gentle when handling the mother and the pups The room where the mother is kept should be dark, quiet (e.g., no music) and without distinctive smells Use experienced mothers (i.e., not first-litter mothers) Consider changing the mouse strain. CD1 mice are typically better mothers than C57 mice Do not put the mother back with the pups too soon after surgery (i.e., wait 30 min after awakening of all the pups)
		Blood is present on the pups when the mother is re-introduced	Gently wash the pups to remove traces of blood. Dry them after washing Minimize the skin incision size
10,15	Pup wakes up during surgery	Inefficient anesthesia	The time to sleep on ice for a P1 C57 pup is ~4 min when the stereotaxic Cunningham plate is at 4 °C. Adjust these parameters as required
10,15, 19,20,21	The pups do not survive the surgery	The time left on ice is too long; the Cunningham plate is too cold; the current applied is too high; the pup is not immediately placed on a warm pad after surgery	Reduce the time on ice; do not apply voltages >100 V; place the pup on a warm pad; oxygen administration may help recovery

● **TIMING**

Steps 1–4, plasmid preparation: plasmid cloning, 3 d; plasmid-TCHD preparation, 30 min
Steps 5–7, pipette preparation: 20 min per pipette, usually done in batches ahead of time
Step 8, surgery preparation: 15 min
Steps 9–22, surgery: 12–15 min per pup
The whole surgical procedure, from onset of anesthesia to the waking up of the pups, should be performed within ~15–20 min or less to minimize anesthetic complications (pup waking up) and the total duration of separation between the mother and her litter.

ANTICIPATED RESULTS

Over the past few years, there has been a marked expansion of the use of electroporation-related techniques to express specific genetic constructs into specific target regions in the brain, which, together with the use of type-specific and inducible promoters (including in combination with transgenic mice), allows virtually any cell type to be specifically targeted. The current protocol is designed for early postnatal targeting of neocortical neurons in postnatal mice, and it may require adjustments when working in other brain regions, at other ages or in different species. Iontoporation allows gene expression within a few hours, thus providing a useful tool to study genetic controls over acquisition of cell identity during neuronal differentiation. In combination with other labeling techniques (e.g., retroviral vectors) in which the electroporated construct can be used to define 'starter cells'[32,33], it can also provide a powerful tool for dissecting cortical circuit assembly with cellular resolution. It is crucial that all the steps described in the protocol above be carefully performed to ensure maximal efficiency. When the procedure is correctly performed, ~30–50% of the pups in the litter will express the transgene (iontoporation at P1), although some variability will be present when first training on the procedure. Examples of successful, typical iontoporations are presented in **Figure 2**. This protocol was designed for cortical iontoporation on P1 pups of C57 or CD1 strains. Other brain areas were not tested and might need adjustments.

ACKNOWLEDGMENTS We thank the members of our laboratory for helpful discussions. We also thank A. Benoit and F. Smets for technical assistance, L. Frangeul for help with the manuscript and L. Telley for photomicrograph image acquisition. Work in the Jabaudon laboratory is supported by the Swiss National Science Foundation, the Leenaards Foundation, the Brain and Behavior Foundation and the Synapsy Foundation.

AUTHOR CONTRIBUTIONS A.D.L.R. and D.J. developed the protocol and wrote the manuscript; A.D.L.R. performed the experiments.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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