Journal of Visualized Experiments

In vivo direct reprogramming of resident glial cells into interneurons by intracerebral injection of viral vectors

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59465R3
Full Title:	In vivo direct reprogramming of resident glial cells into interneurons by intracerebral injection of viral vectors
Keywords:	Direct conversion, whole-cell patch clamp, FLEX vector system, Cre-inducible system, parvalbumin, functional assessment, slice recordings
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lund, Skåne, Sweden

1 **TITLE:**
2 In Vivo Direct Reprogramming of Resident Glial Cells into Interneurons by Intracerebral
3 Injection of Viral Vectors
4
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17
18 **KEYWORDS:**
19 direct conversion, whole-cell patch clamp, FLEX vector system, Cre-inducible system, slice-
20 recordings, parvalbumin
21
22 **SHORT ABSTRACT:**
23 This protocol aims at generating directly reprogrammed interneurons in vivo, using an AAV-
24 based viral system in the brain and a FLEX synapsin-driven GFP reporter, which allows for cell
25 identification and further analysis in vivo.
26
27 **LONG ABSTRACT:**
28 Converting resident glia in the brain into functional and subtype-specific neurons in vivo
29 provides a step forward towards the development of alternative cell replacement therapies
30 while also creating tools to study cell fate in situ. To date, it has been possible to obtain
31 neurons via in vivo reprogramming, but the precise phenotype of these neurons or how they
32 mature has not been analyzed in detail. In this protocol, we describe a more efficient
33 conversion and cell-specific identification of the in vivo reprogrammed neurons, using an AAV-
34 based viral vector system. We also provide a protocol for functional assessment of the
35 reprogrammed cells' neuronal maturation. By injecting flip-excision (FLEX) vectors,
36 reprogramming and synapsin-driven reporter genes are delivered to specific cell types in the
37 brain that serve as the target for cell reprogramming. This technique allows for the easy
38 identification of newly reprogrammed neurons. Results show that the obtained reprogrammed
39 neurons functionally mature over time, receive synaptic contacts and show electrophysiological
40 properties of different types of interneurons. Using the transcription factors Ascl1, Lmx1a and
41 Nurr1, the majority of the reprogrammed cells have properties of fast-spiking, parvalbumin-
42 containing interneurons.
43
44 **INTRODUCTION:**

45 The overall goal of this method is to efficiently convert brain resident glia in vivo into functional
46 and subtype-specific neurons such as parvalbumin-expressing interneurons. This provides a
47 step forward towards the development of an alternative cell replacement therapy for brain
48 diseases without the need for an exogenous cell source. It also creates a tool to study cell fate
49 switches in situ.
50
51 The brain has only a limited capacity for generating new neurons. Therefore, in neurological
52 diseases, there is a need of exogenous cell sources for brain repair. For this, different sources of
53 cells have been subjected to intense research over the years, including cells from primary
54 tissue, stem cell-derived cells and reprogrammed cells[1-3]. Direct reprogramming of resident
55 brain cells into neurons is a recent approach that could provide an attractive method for brain
56 repair as it uses the patient's own cells for generating novel neurons inside the brain. To date,
57 several reports have shown in vivo reprogramming through viral vector delivery in the brain[4-9]
58 in different brain regions such as the cortex, spinal cord, striatum and the midbrain[5,10,11], as well
59 as in intact and lesioned brain[5,8,11,12]. Both inhibitory and excitatory neurons have been
60 obtained [4,8], but the precise phenotype or functionality of these cells has not yet been analyzed
61 in detail.
62
63 In this protocol, we describe a more efficient reprogramming and cell-specific identification of
64 in vivo reprogrammed neurons. We provide a protocol for functional assessment of the
65 neuronal maturation and characterization of the phenotype based on functionality and
66 immunohistochemical traits.
67
68 We used a Cre-inducible AAV vector and a GFP reporter to identify the in vivo reprogrammed
69 neurons. This choice of viral vectors has the advantage of infecting both dividing and non-
70 dividing cells of the brain, increasing the number of targeted cells, as an alternative to the use
71 of retrovirus[7,8]. A neuron-specific synapsin-driven FLEX reporter (GFP), enabled us to specifically
72 detect the newly generated neurons. Previous studies have used subtype-specific promoters for
73 in vivo reprogramming[7,9], that also allow the expression of reprogramming genes and reporters
74 in specific cell types. However, that method requires further identification of reprogrammed
75 neurons by postmortem analysis of the co-expression of reporter and neuronal markers. The
76 use of a neuron-specific reporter, such as the one described herein, allows for a direct
77 identification. This provides a direct proof of a successful conversion and allows a live cell
78 identification that is required for patch-clamp electrophysiology.
79
80 **PROTOCOL:**
81 All experimental procedures were carried out under the European Union Directive
82 (2010/63/EU) and approved by the ethical committee for the use of laboratory animals at Lund
83 University and the Swedish Department of Agriculture (Jordbruksverket). Mice are housed in a
84 12 h light/dark cycle with ad libitum access to food and water.
85
86 **1.   Viral vectors**
87

1.1   Cloning of AAV vectors

1.1.1   To create Cre-inducible AAV5 vectors, insert cDNA for GFP, Ascl1, Lmx1a and NR4A2 (Nurr1) in a reverse orientation flanked by two pairs of heterotypical, antiparallel LoxP flip-excision (FLEX) sequences (**Table of Materials**). For the cDNA insertion, use a backbone like pAAV-Cba-FLEX or one with a similar structure, containing FLEX sequences and a chicken beta actin (CBA) promoter.

1.1.2   Insert each single cDNA (e.g., Ascl1) in the backbone by polymerase chain reaction (PCR) and restriction enzymes. Express GFP under the control of a synapsin promoter and *Ascl1*, *Lmx1a*, *Nurr1* under the control of a ubiquitously expressed CBA promoter.

NOTE: Ensure to have endotoxin free DNA using specific Endotoxin-Free Plasmid DNA Isolation Kits.

1.1.3   Perform sequencing and restriction analysis on the constructs before use, in order to check the success of the cloning step.

1.2   AAV5 viral vector production

CAUTION: Refer to local biosafety guidelines when handling adeno-associated virus (AAV). In Sweden, the AAV used in this protocol requires Biosafety Level 2 (BSL-2).

1.2.1   Seed HEK293T cells with standard culture media (DMEM + Glutamax + 10% FBS + penicillin (100 U/mL) streptomycin (100 μg/mL), see **Table of Materials**) in T175 flasks at a density of 3×10^6 cells per flask. Account for 5 flasks per batch of AAV and plan for 6 batches at a time.

1.2.2   When the cells reach 50-70 % confluency, prepare the following mix for transfection.

1.2.2.1 In a 15 mL centrifuge tube, add equimolar amounts of vector plasmid and pDG series helper plasmid (pDP5, pDP6), with a total amount of 72 μg per 175 cm^2 flask.

1.2.2.2 Add Tris-EDTA buffer (TE buffer, 10 mM Tris-HCl, 1 mM EDTA) to a final volume of 144 μL.

1.2.2.3 Add ultrapure water so that the total volume becomes 1296 μL and mix.

1.2.2.4 Add 144 μL of 2.5 M CaCl$_2$ and mix. Add 177 μL of HEPES Buffered Saline (HBS) (1.5 mM Na$_2$HPO$_4$,140 mM NaCl, 50 mM HEPES) to the DNA solution and mix immediately by vortexing.

1.2.2.5 Incubate at room temperature (RT) for exactly 60 s.

1.2.3   Replace the medium in the flasks with fresh medium and add transfection mix (1.8 mL).

132

133   1.2.4   Three days after transfection, harvest the cells by pouring off thr medium from the
134   flasks into a disposable container for waste and add 5 mL of harvest buffer (EDTA added to
135   Phosphate-Buffered Saline, (see **Table of Materials**, DPBS) to a concentration of 5 mM) in each
136   flask to allow cell detachment.

137

138   1.2.5   Pour the cell solution in a 50 mL centrifuge tube. Add another 4 mL of DPBS to each flask
139   to rinse the remaining cells and pool with first cell solution. Centrifuge the harvested cells at
140   1,000 x g for 5 min at 4 °C.

141

142   1.2.6   After centrifugation, remove the supernatant and dissolve the pellets in 15 mL of lysis
143   buffer (50 mM Tris-HCl pH 8.5 ,150 mM NaCl, 1 mM $MgCl_2$) by vortexing.

144

145   1.2.7   Collect the lysate in 50 mL centrifuge tubes. Freeze them in CO_2-ice/ethanol bath for 15
146   min and store in a -20 °C freezer. Thaw the harvested cell lysate in a 37 °C water bath before
147   use.

148

149   **1.3**   **AAV5 viral vector purification**

150

151   1.3.1   Perform AAV Purification by Iodixanol Gradient Ultracentrifugation[13] and use
152   ultracentrifuge sealing tubes with centrifugation at 350,000 x g for 1 h and 45 min at RT.

153

154   1.3.2   Use a 10 mL syringe with an 18G needle and insert about 2 mm below the 40/60% phase
155   border with the bevel facing upwards in order to extract the AAV-containing phase. Make sure
156   to stop before reaching the protein band after 5-6 mL has been extracted.

157

158   1.3.3   Store the gradient extracts in autoclaved glass bottles at 4 °C. Avoid storing times longer
159   than overnight.

160

161   1.3.4   Dilute the Iodixanol gradient extract 3-fold by slowly pipetting in 12 mL of IE buffer (20
162   mM Tris-HCl pH 8.0, 15 mM NaCl) while swirling.

163

164   1.3.5   Purify and concentrate the diluted Iodixanol gradient through an anion exchange filter.
165   Push it through slowly with a speed no faster than 1 drop/s. Push 3 mL of IE buffer slowly
166   through the filter to wash it.

167

168   1.3.6   Elute into a centrifugal filter unit with 1-2 mL of elution buffer (20 mM Tris-HCl pH
169   8.0;250 mM NaCl). Add DPBS to the device to a final volume of 4 mL. Centrifuge at 2,000 x g at
170   RT until less than 0.5 mL is left in the filter. Remove the liquid from the bottom of the tube,
171   refill with 4 mL of DPBS and centrifuge again. Repeat this step two more times. Make sure that
172   the volume of concentrated vector on the filter is about 200 μL after the last centrifugation
173   step.

174

175   1.3.7   Remove the 200 μL concentrated vector using a pipette and push the concentrate

176 through a 0.22 µm filter to further concentrate it. Aliquot 200 µL into a 9 mm glass vial with
177 interlocked insert (**Table of Materials**).
178
179 NOTE: The AAV5 vectors stocks can be stored in -80 °C freezers for long storage, or at 4 °C if
180 used within 2 weeks.
181
182 **1.4   AAV5 viral vector titer determination**
183
184 1.4.1   Determine AAV5 titer using standard quantitative polymerase chain Reaction (qPCR)
185 with primers for Inverted Terminal Repeat (*ITR*) sequence and a 5´FAM / 3´BHQ1 probe for ITR
186 sequence (**Table of Materials**). Use a standard curve achieved with known amounts of ITR
187 containing plasmid. Each AAV vector should have a range of $1E^{+14} - 1E^{+15}$ genome copies per
188 milliliter if ITR sequence is used for titer determination.
189
190 NOTE: A successful AAV5 virus production gives stocks with titers in the range of $3E^{+13} - 7E^{+13}$
191 units per mL.
192
193 **2.   Injection of reprogramming factors into the brain**
194
195 **2.1   Animal setup, stereotaxic placement and drilling**
196
197 NOTE: This protocol focuses on the use of Ascl1, Lmx1a and Nurr1 (ALN) for the reprogramming
198 of NG2 glia into interneurons. In our experience, interneurons of a similar phenotype can be
199 obtained using other factor combinations[11].
200
201 2.1.1   Prior to surgery, prepare the viral mix containing the vectors Cba-FLEX-Ascl1, Cba-FLEX-
202 Lmx1a, Cba-FLEX-Nurr1 and the reporter vector Syn-FLEX-GFP. Add each one of the stocks
203 prepared in section 1 to the final mix, so that the final viral solution has 5% of each one of the
204 reprogramming factors (Ascl1, Lmx1a and Nurr1) and 10% of the reporter construct (5% A, 5%
205 L, 5% N and 10% GFP).
206
207 NOTE: The AAV5 vector mixes can be stored at 4 °C and kept for future use in vivo.
208
209 2.1.2   Anaesthetize the mouse using 2% isoflurane in a mix of air and nitrous oxide (N_2O) at a
210 4:1 ratio. Monitor the animal's breathing by observing the movements of the diaphragm.
211 During the surgery, maintain anesthesia using 1-1.5 % isoflurane.
212
213 NOTE: The mouse model hereby described consists of a mouse strain that specifically expresses
214 Cre in NG2 glia. In vivo reprogramming can be achieved using different mouse strains that
215 express Cre in other populations of glial cells (e.g., astrocytes[14]).
216
217 2.1.3   Once the animal is completely sedated (e.g., complete muscle relaxation and no
218 response to a pinch in the foot pad), shave the area around the incision site and bring the
219 animal to the stereotaxic frame.

220

221   2.1.4   To maintain animal's body temperature during surgery, attach a heating pad to the base
222   of the stereotaxic frame. Place the mouse on a clean, dry paper towel.

223

224   2.1.5   Carefully place the mouse's head into the ear bars. If correctly placed, no lateral
225   movement of the head should be observed. Set the left ear bar to 4 mm, prior to starting
226   placing the mouse in the stereotaxic frame.

227

228   2.1.6   Secure the tooth bar in place, and then tighten the nose bar. Make sure that the head
229   does not move in any dimension and point straight ahead (the midline being perpendicular to
230   the plane of the ear bars). Apply ophthalmic ointment for eye protection.

231

232   2.1.7   Before the beginning of surgery, administer the appropriate analgesia (e.g., 0.05 mg/kg
233   of Buprenorphine, sub-cutaneous).

234

235   2.1.8   Clean the incision area with a cotton gauze or a wipe soaked with 70% EtOH, without
236   approaching the eye area.

237

238   NOTE: For intracerebral viral injection, a 5 or 10 µL syringe adapted with a pulled glass capillary
239   is used. Glass capillaries are pulled using a micropipette puller, resulting in a capillary with a
240   very fine tip, which will minimize the invasiveness of the procedure. To adapt the capillary into
241   the syringe, use a piece of rubber tubing over the connection between the needle of the syringe
242   and the glass capillary and melt it using a heat source (e.g., a lighter). A tight connection
243   between the two pieces will make sure there are no fluid leaks during the injection. Before
244   starting the surgery, test this by filling the syringe with saline and pushing the liquid out of the
245   syringe.

246

247   2.1.9   Make an incision of approximately 0.5-0.8 cm along the midline of the head. Cut
248   through both cutaneous and sub-cutaneous layers, with a scalpel.

249

250   2.1.10 Widen the skin flaps on each side of the incision. Clean the incision of any blood and
251   scrape back the subcutaneous layers with a cotton bud.

252

253   2.1.11 Move the M/L-D/V arm of the stereotaxic frame into place (above the animal) and
254   secure it.

255

256   NOTE: The stereotaxic frame allows adjustment of the syringe along the Anterior/Posterior
257   (A/P, Y axis), Medial/Lateral (M/L, X axis) and Dorsal/Ventral (D/V, Z axis) axis.

258

259   2.1.12 Move the syringe along the different axis of the stereotaxic frame, to bring the tip of the
260   glass capillary just above bregma (the junction point where the different skull plates meet).

261

262   2.1.13 Make sure the capillary tip is perfectly straight in both A/P and M/L planes. In case of an
263   ambiguous bregma, take the average of the lateral and midline sutures.

264

265 2.1.14 When the tip of the capillary is correctly placed above bregma, reset both the M/L and
266 A/P values to 0.0 on the digital coordinate counter.

267

268 2.1.15 To make sure the head of the animal is in a perfectly flat position, use the digital
269 coordinate counter to measure the D/V coordinate value, when the A/P arm is at +2.0 and -2.0
270 (M/L = 0.0), as well as when M/L arm is at +2.0 and -2.0 (A/P = 0.0). Adjust the height of tooth
271 bar and ear bars accordingly.

272

273 2.1.16 Move the syringe to the desired coordinates for injection of viral vectors in the striatum
274 (A/P = +1.0; M/L = -2.0, relative to bregma).

275

276 2.1.17 Raise the syringe slightly and, by looking at the injection site through the microscope,
277 drill a hole using a dental drill at the injection coordinates. Start to drill at the site, working in
278 circular and gentle manner.

279

280 NOTE: Do not put too much downward pressure, as the drill-bit should be sharp enough to go
281 through the bone without additional force. Avoid long, sustained drilling as this creates heat.

282

283 2.1.18 At the end of the drilling, check that the dura mater remains intact and exposed for
284 injection.

285

286 **2.2   Syringe setup preparation**

287

288 **2.2.2**   Place a piece of cotton gauze over the open incision and flush the syringe with saline
289 solution.

290

291 **2.2.3**   After flushing, take up an air bubble of 1-2 µL, followed by 1 µL of solution containing
292 the viral vectors, avoiding any unintentional bubbles. Make sure that the viral solution can
293 easily be visualized below the air bubble, whilst being injected.

294

295 **2.3   Viral injection**

296

297 2.3.1   Remove the cotton gauze from over the incision site, and lower the syringe using the
298 D/V arm of the stereotaxic frame. As the surface of the skull is approached, carefully look
299 through the microscope and measure the D/V level of dura mater – it should bulge slightly
300 under gentle pressure.

301

302 2.3.2   While touching the dura mater with the tip of the capillary, set the D/V coordinate to
303 0.0.

304

305 2.3.3   Lower the syringe, progressing slowly to the desired depth (D/V = -2.7, relative to dura
306 mater). Make sure that the trajectory is clear of bone fragments, so that no bending of the
307 needle/capillary is observed.

308

NOTE: The presented coordinates refer to an injection of the reprogramming factors into the striatum of NG2-Cre mice. Coordinates corresponding to other brain regions may be used. Always test the coordinates for injection first using a dye (e.g., Trypan Blue), colored bead injection or a reporter viral vector prior to injection of reprogramming factors into the brain of a new mouse strain. If injecting Trypan Blue, the animals need to be sacrificed immediately after surgery and the brain dissected out. Fresh brains can be cut using a microtome, while frozen, and the site of injection determined by visualization of the dye position in the brain. If testing coordinates using colored beads, it is possible to determine the site of injection on perfused and cut brains a week after injection. Alternatively, an injection with a viral vector carrying a reporter gene can be used, and the site of injection determined in perfused cut brains.

==Highlight-start==
2.3.4   Inject 1 µL of the viral solution at a rate of 0.4 µL/min. When the whole volume is injected, allow for a diffusion period of 2 min before syringe withdrawal.
==Highlight-end==

==Highlight-start==
2.3.5   After diffusion, slowly retract the syringe until the tip of the capillary is completely out of the brain.
==Highlight-end==

2.3.6   Place a piece of cotton gauze over the wound and flush the syringe with saline solution.

2.3.7   Move the M/L-D/V arm of the stereotaxic frame out of the working area.

2.4   Wound closing and post-operative procedures

2.4.1   Carefully suture the incision using suture thread.

NOTE: All suture threads used in injected animals should be disposed in a cup/vial containing anti-viral detergent solution. The same solution is used to clean all surfaces surrounding the surgery area that have been in contact with surgical materials. All surgical tools are thoroughly washed and autoclaved at the end of each surgery day.

2.4.2   Remove the animal from the stereotaxic frame and place it in a post-operative station, which includes a clean, heated cage, access to food and water, and where the animal stays until fully awake. During this period, monitor the animal closely until consciousness is regained.

2.4.3   Do not house operated animals with not operated animals until the former have fully recovered from surgery.

2.4.4   Supervise operated animals daily. Depending on the sutures used, make sure they are removed if necessary.

350　NOTE: If operating using the same syringe in two consecutive days, flush it with water, followed
351　by ethanol 70% and water again. Leave the syringe filled with water over-night, to allow
352　dissolution of any possible residues.
353
354　**3　　Electrophysiological recordings**
355
356　**3.1　　Tissue slice preparation for electrophysiology**
357
358　CAUTION: A well-executed tissue preparation is necessary in order to achieve good
359　electrophysiological recordings. Prepare the room carefully and place tools for perfusion and
360　dissection on ice.
361
362　3.1.1　Prepare ice-cold and oxygenized (95% O_2 and 5% CO_2) Krebs–Henseleit solution for
363　perfusion, dissection and cutting (prepare this on the same day from the 10x stock by diluting
364　the stock solution in ultrapure water and adding $NaHCO_3$ and glucose). The components in the
365　Krebs–Henseleit solution in mM (after dilution to 1x) are: 126 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4 \cdot H_2O$,
366　1.3 $MgCl_2 \cdot 6H_2O$, and 2.4 $CaCl_2 \cdot 6H_2O$, 22 $NaHCO_3$, 10 glucose. Adjust the pH of the solution to
367　7.4.
368
369　3.1.2　Perform a calibration (Vibracheck) for the vibratome with a new razor blade.
370
371　3.1.3　Start the cooling block of the vibratome (or fill the surrounding chamber with ice), fill
372　the cutting chamber with Krebs–Henseleit solution, and gas it with 95 % O_2 and 5 % CO_2 at least
373　30 min before use.
374
375　3.1.4　Put a Petri-dish on ice and fill it with oxygenized Krebs–Henseleit solution. Place the
376　blade, scissors and mounting plate on ice.
377
378　CAUTION: Bring the cage containing the mouse to the room at least 1 h before starting the
379　procedure for acclimatization. Stress has a negative effect on the condition of the brain tissue
380　sections.
381
382　3.1.5　Anesthetize the mouse and transcardially perfuse the animal with ice-cold Krebs–
383　Henseleit solution for 2-3 min (at a rate of 10-20 mL/min).
384
385　3.1.6　Rapidly, but carefully, dissect out the brain and put it upside down in the Petri-dish that
386　is placed on ice (containing Krebs–Henseleit solution).
387
388　NOTE: In order to compare the functional maturation of the reprogrammed neurons, sacrifice
389　the animals for recordings at different time-points post-viral injection. Assess the firing
390　properties of distinct subtypes of neurons based on existing literature[15] .
391
392　3.1.7　Make a coronal cut along the mid cerebellum and glue this side onto the mounting plate
393　(also ice-cold) for vibratome cutting.

394
395 3.1.8   Dip the glued brain carefully into the buffer chamber in the vibratome.

396
397 CAUTION: Be careful not to touch the razor blade for your own safety, and also for not moving
398 the blade that has been calibrated.

399
400 3.1.9   Cut from the most rostral part of the brain down to the striatal level at a high speed.
401 Then cut the striatum coronally at 275 μm at slow speed (0.10 mm/s).

402
403 3.1.10 After each cut, carefully remove the non-injected striatal side (e.g., with a bended
404 needle) and transfer the other side into another vial in a water bath, containing a bottom net in
405 oxygenized Krebs–Henseleit at RT. Keep this at RT until all sections are cut.

406
407 3.1.11 Slowly increase the temperature of the water bath to 37 °C and leave it for 30 min. Then
408 turn off the heater and let cool down until RT.

409
410 NOTE: At this point, you can pause until you start recording. The sections last for 4-6 h.

411
412 **3.2   Whole-cell patch-clamp recordings**

413
414 3.2.1   Transfer the first tissue section to the recording chamber submerged in a continuous
415 flow of Krebs–Henseleit solution. Mount the section using light weights and submerge the
416 objective.

417
418 3.2.2   Identify the striatal region in the microscope and search for GFP-positive
419 (reprogrammed) neurons. Select a neuron that is extensive in morphology and not covered by
420 fiber bundles or blood vessels.

421
422 3.2.3   Prepare borosilicate glass pipettes (3–7 MΩ) for patching and fill with the following
423 intracellular solution (in mM): 122.5 potassium gluconate, 12.5 KCl, 0.2 EGTA, 10 HEPES, 2
424 MgATP, 0.3 Na$_3$GTP and 8 NaCl, adjusted to pH 7.3 with KOH.

425
426 3.2.4   For biocytin filling, add 1 mg of biocytin salt to 1 mL of internal solution and vortex.

427
428 CAUTION: Make sure to filter the internal solution with biocytin as this can otherwise clog the
429 electrode.

430
431 3.2.5   Attach the glass pipette to the recording electrode and dip into the solution. Double-
432 check the resistance of the electrode. Then slowly approach the cell with the pipette, keeping a
433 slight positive pressure in the electrode to avoid plugging the tip.

434
435 CAUTION: Be careful to keep track of your cell while descending the electrode and not to bleach
436 the fluorescence in the cell (i.e., turn off the fluorescence lamp when you do not need it).

437

438   3.2.6   Rinse the surrounding tissue carefully using positive pressure of the electrode and
439   approach the cell with the electrode. Locate the electrode right on top of the cell and descend
440   until the membrane is reached. Make a Giga-Ω seal between the electrode and cell membrane,
441   and with negative pressure pulses, rupture the membrane to create a whole-cell patch.

442

443   CAUTION: Reprogrammed neurons are sensitive. Be careful when patching and do not to put
444   too much negative pressure when reaching a Giga-Ω seal or opening the cell membrane. Also,
445   patching on animals that are not juvenile requires practice and patience as their connective
446   tissue is thicker and it is harder to visualize the neurons.

447

448   3.2.7   Check the resting membrane potentials immediately after breaking-in in current-clamp
449   mode and write down for analysis.

450

451   3.2.8   In current clamp, maintain the cell between -60 mV to -80 mV and inject 500 ms
452   currents from -20 pA to +90 pA, with 10 pA increments to induce action potentials.

453

454   3.2.9   Transfer into voltage-clamp and measure the inward sodium and delayed rectifying
455   potassium currents at depolarizing steps of 10 mV.

456

457   NOTE: Voltage-clamp recordings are better assessed using a different internal solution,
458   composed of chemicals that more efficiently clamp the membrane. However, for
459   reprogrammed neurons, the number of cells that you can record from is few and the number of
460   tissue sections with these neurons is limited. Hence, it is an advantage to record both in
461   current-clamp and voltage-clamp from the same cell.

462

463   3.2.10 In voltage-clamp mode, record spontaneous postsynaptic activity at -70 mV. Distinguish
464   inhibitory postsynaptic events at a membrane potential of 0 mV from excitatory events at -70
465   mV.

466

467   3.2.11 After reaching a stable baseline, add Picrotoxin, the GABA$_A$ receptor antagonist, to the
468   Krebs–Henseleit solution that flows into the recording chamber, to extract excitatory events
469   (add a stock solution of Picrotoxin to a final concentration of 50 mM in separate buffer syringe
470   that is connected to the chamber perfusion. Connect the outflow of buffer from the chamber to
471   a vacuum bag for safe disposal).

472

473   3.2.12 After 20 min, add CNQX (20 mM), an AMPA antagonist, to the Krebs–Henseleit solution
474   that flows into the recording chamber, to block the inhibitory events. Leave in for another 20
475   min and then wash out with Krebs–Henseleit solution. Remove the tissue section from the
476   chamber.

477

478   3.2.13 After finishing the recordings, do an offline analysis of spontaneous excitatory post-
479   synaptic currents (EPSC) and inhibitory post-synaptic currents (IPSC) using a threshold-event
480   detection (>5 pA) in the analysis program.

481

482 3.2.14 During the whole recording, the cell will be slowly filled with the biocytin-containing
483 internal solution. Keep the patch for at least 20 min before slowly removing the electrode in
484 order to achieve complete filling of the cell.
485
486 CAUTION: Be careful not to have any positive pressure in the electrode that could destroy the
487 morphological analysis of the cells afterwards.
488
489 3.2.15 For biocytin-visualization, put the tissue section in 4 % paraformaldehyde (PFA)
490 overnight at 4 °C. Rinse the section in 0.02 M potassium phosphate buffer (KPBS) with 0.1 %
491 Triton. Then, stain for streptavidin- Cy3 (1: 600 in KPBS-T, 2 h), for identification of the biocytin-
492 filled cell and morphological visualization of the reprogrammed neuron.
493
494 NOTE: Biocytin filling can be used for morphological analysis or illustration of the neurons.
495
496 **4   Immunohistochemistry, stereology and quantification**
497
498 NOTE: Dedicate a specific group of mice for immunohistochemistry, as tissue sections used for
499 electrophysiology are not optimal for immunohistochemistry.
500
501 4.1.   Anaesthetize mice with an i.p. injection of an overdose of pentobarbital and mount the
502 animal for perfusion.
503
504 4.2.   Transcardially perfuse the mice, first with a saline solution to remove blood, and then
505 with ice-cold 4% PFA.
506
507 4.3.   Dissect the brains and post-fix for at least 12 h in 4% PFA.
508
509 4.4.   Put the brains in 25% sucrose solution (for cryoprotection) for approximately 12 h.
510
511 NOTE: The brains are ready to be cut when they sink in the 25% sucrose solution, indicating
512 that the sucrose has penetrated the entire mouse brain.
513
514 4.5.   Make a coronal cut across the cerebellum, use the flat, most caudal part of the brain to
515 place the brain on a microtome and fix it in place using optimal cutting temperature compound
516 (OCT).
517
518 4.6.   Cut the brain into coronal slices with a thickness of 35 μm and divide the brain into
519 consecutive series placed in vials or wells containing 0.02 M KPBS.
520
521 4.7.   Process the sections for immunohistochemistry using antibodies against GFP and
522 interneuron markers such as GAD65/67, PV, Chat (choline acetyltransferase) and NPY
523 (Neuropeptide Y), according to standard protocols[16, 17.]
524
525 NOTE: Brain slices can be kept at 4 °C or -20 °C in anti-freeze solution for long periods.

526

527 4.8.   After staining is complete, mount the sections on a glass slide and cover with a glass
528 coverslip, using a mounting solution to fix it in place. Leave the coverslipped slides to dry over-
529 night.

530

531 NOTE: For our studies, whole brains are cut in 1:8 series (i.e., each vial containing sections will
532 hold one eighth of the mouse brain[11]).

533

534 4.9.   Analyze the sections using an inverted fluorescence and/or confocal microscope.

535

536 CAUTION: Fluorescent microscopy is useful for a general overview of results and capturing
537 images for calculating reprogramming efficiency. For a more detailed analysis of phenotypic
538 identity by observation of double-positive cells, confocal imaging should be used.

539

540 4.10.   For quantification of different markers expressed in GFP$^+$ neurons in the striatum, count
541 the number of double-positive cells in relation to the total number of GFP$^+$ cells (i.e., the
542 reprogrammed neurons), in at least two fields of each striatal section.

543

544 **4.11**   For determining the approximate extrapolated total number of reprogrammed neurons
545 per brain, count the GFP$^+$ neurons present in the striatum of one of the brain series cut earlier,
546 and then multiply by the total number of series.

547

548 NOTE: Different methods of quantification can be used to express reprogramming efficiency,
549 number of reprogrammed neurons per animal and percentage of neurons that express certain
550 phenotypic markers. For more information, see previous publication[11].

551

552 **4.12**   Analyze the differences between conditions using Graph Pad Prism or similar.

553

554 NOTE: For efficiency calculation, we injected *NG2*-Cre mice with the CRE-dependent ALN
555 conversion vectors and the GFP reporter. To estimate the conversion efficiency, we also
556 injected animals with a Cre-dependent GFP under the ubiquitous cba promoter, rendering all
557 targeted cells GFP$^+$. We found that the vectors efficiently targeted NG2 glia, and estimated that
558 66.81% ± 38.38% of targeted cells converted into neurons. For validation of expression of each
559 one of the genes, complementary double immunofluorescent-staining can be performed for
560 GFP/Ascl1, GFP/Lmx1a and GFP/Nurr1. Additionally, genome sequencing such as single cell RNA
561 sequencing can reveal the presence of each one of the genes in the reprogrammed cells.

562

563 **REPRESENTATIVE RESULTS:**
564 The injection of AAV vectors is used to successfully reprogram resident NG2 glia cells into
565 neurons in the NG2-Cre mouse striatum (**Figure 1A**). To specifically target NG2 glia, FLEX
566 vectors with reprogramming/reporter genes, are inserted in an antisense direction and flanked
567 by two pairs of antiparallel, heterotypic loxP sites (**Figure 1B**). Each of the three reprogramming
568 genes (Ascl1, Lmx1a and Nurr1) is placed under the control of the ubiquitous cba promoter on
569 individual vectors. In order to make sure GFP expression is restricted to reprogrammed neurons

570 originating from a Cre-expressing cell, GFP is placed under the control of the neuron-specific
571 synapsin promoter (also in a FLEX vector).

572

573 The use of the combination of reprogramming and reporter constructs allows for the
574 generation of GFP-positive neurons in the mouse striatum (**Figure 1C,C'**). The use of the
575 reporter construct without the presence of reprogramming genes does not yield GFP-positive
576 neurons (**Figure 1D**).

577

578 The reprogrammed neurons that are biocytin-filled are visible after post-mortem immuno-
579 staining (**Figure 2A**). If conversion is successful, there should be extensive neuronal
580 morphology. The electrophysiological recordings of reprogrammed neurons show presence of
581 postsynaptic functional connections with spontaneous activity measures (**Figure 2B,C**). This can
582 be blocked with either ionotropic GABAergic or glutamatergic blocker (Picrotoxin or CNQX),
583 suggesting both excitatory and inhibitory synaptic input to the reprogrammed neurons. The
584 occurrence of spontaneous activity increases with time post viral injection (**Figure 2D**),
585 indicating a gradual maturation.

586

587 Current-induced action potentials are present in functional neurons. The action potentials
588 increase in number over time after conversion (**Figure 2E**). This further indicates maturation in
589 neuronal function. In an immature neuron, current will induce either none or very few action
590 potentials (**Figure 2F**).

591

592 The firing patterns observed in recorded cells are specific for different types of neurons
593 depending on factors such as the morphology of the cells and channel expression[15]. The
594 recorded patterns in the in vivo reprogrammed neurons can be distinguished into groups and
595 compared to that of endogenous neuronal subtypes (e.g., cells similar to fast-spiking
596 interneurons (Cell Type B, **Figure 3B**) or other cell types (**Figure 3A,C,D**)). The observed
597 electrophysiological differences amongst in vivo reprogrammed neurons were confirmed by the
598 presence of specific subtype markers and co-expression with GFP (**Figure 3E-H**). Altogether, this
599 data indicates that the reprogrammed neurons present in the striatum have properties of
600 different types of interneurons, such as Parvalbumin-, ChAt- and NPY- expressing interneurons,
601 as well as striatal medium spiny neuron identity (DARPP32+) (**Figure 3E-H**).

602

603 **FIGURE AND TABLE LEGENDS:**
604 **Figure 1: In vivo reprogramming of resident NG2 glia into neurons.** (**A**) Schematic
605 representation of AAV virus-mediated in vivo reprogramming of striatal NG2 glia. (**B**) Schematic
606 representation of AAV5 FLEX constructs used for in vivo reprogramming, in which gene
607 expression is regulated by Cre expression in the targeted cells. (**C** and **C'**) In vivo reprogrammed
608 neurons, resulting from Syn-GFP + ALN injection into the Striatum. (**D**) Absence of
609 reprogrammed neurons when no reprogramming factors are added into the viral cocktail, and
610 only reporter construct is injected in vivo. Scale bars: (C) 100 μm, (C') 25 μm, (D) 25 μm.

611

612 **Figure 2: In vivo reprogrammed neurons are functional and show maturation over time. (A)**
613 Post recorded biocytin-filled reprogrammed neuron, shows mature neuronal morphology,

614 including dendritic spines. Traces shows (**B**) inhibitory (GABAergic) activity that is blocked with
615 picrotoxin, a $GABA_A$ receptor antagonist and (**C**) excitatory activity that is blocked with CNQX,
616 an AMPA receptor antagonist. (**D**) Patched neurons show repetitive firing already at 5 weeks
617 post-injection (w.p.i.) and continue to show that at 8 and 12 w.p.i. (**E**) The number of neurons
618 with postsynaptic activity increases over time. (**F**) Current-induced action potential and
619 postsynaptic activity of an immature neuron, showing few synaptic events and few action
620 potentials compared to B and D. Scale bar: 25 μm
621
622 **Figure 3: In vivo reprogrammed neurons show immunohistochemical and electrophysiological**
623 **properties of striatal interneurons.** (**A-D**) The firing patterns of patched in vivo reprogrammed
624 neurons can be of distinct types: (**A**) Type A is similar to endogenous medium spiny neuron
625 (DARPP32+); (**B**) similar to fast-spiking (PV+) interneurons; (**C**) similar to low-threshold spiking
626 neurons with prominent sag (NPY+); (**D**) neurons firing with large after-hyperpolarization
627 (Chat+). (**E-H**) Confocal images showing co-localization of GFP and the interneuron markers PV
628 (**E**), ChAT (**F**), NPY (**G**), and projection neuron marker DARPP32 (**H**). All scale bars: 50 μm.
629
630 **DISCUSSION:**
631 In vivo direct reprogramming can be achieved using AAV FLEX vectors in Cre-expressing mouse
632 strains. However, it is important to note that differences between mouse strains with regards
633 to reprogramming efficacy have been observed. For in vivo reprogramming in the striatum, the
634 NG2-Cre mouse line has proved to be the more efficient when compared with other strains.
635 Prior to starting to use a new animal strain, it is important to check the mouse provider
636 guidelines regarding Cre expression over time, as the age of animals often impacts the
637 specificity of this protein expression. In our studies, animals older than 12 weeks were not used
638 for in vivo reprogramming as there was a risk for Cre expression in cells other than NG2 glia.
639 The constant presence and monitoring of control animals that have been injected only with
640 Synapsin-FLEX-GFP construct is advised. This allows monitoring of the animals for GFP-positive
641 cells that should not be present if no reprogramming genes (ALN) are used.
642
643 To identify the newly reprogrammed neurons, a neuron-specific identification method such as
644 the one described in this protocol, using synapsin-driven GFP vector, is of utmost importance as
645 this allows for a proper identification and distinction of the reprogrammed neurons from the
646 endogenous surrounding cells. This is of particular importance when the reprogramming into
647 neurons occurs in a homotopic region.
648
649 It is also important to target the correct structure for viral injection. Therefore, the stereotaxic
650 surgery for viral injection is important and needs to be approached with accuracy, especially
651 when targeting smaller structures of the brain.
652
653 We have previously shown[11] that it is not reliable to predict the outcome of in vivo
654 reprogramming based on in vitro reprogramming experiments using the same reprogramming
655 factors. All factors of interest therefore need to be tested in vivo. In our hands, many different
656 factor combinations give the same subtype of neurons (i.e., interneurons[11] in vivo) despite the
657 fact that these genes have been implicated in the development of other neurons.

658

659 Whole-cell patch clamp for reprogrammed neurons is a delicate technique and tissue
660 processing is very important for a good outcome. Perfusion with ice-cold Krebs–Henseleit
661 solution improves tissue quality. Also, patched neurons need to be treated carefully. Even if
662 maturation and phenotypic identity of reprogrammed neurons can be somewhat assessed
663 using whole-cell patch clamp, these cells are not fully comparable to their endogenous
664 counterparts, a factor that is important to remember when further assessing these cells for
665 therapeutic use. Additional types of analysis, such as genome sequencing (e.g., RNA
666 sequencing) should be used to further confirm reprogrammed cell identity.

667

668 The technique described herein is currently considered of relevance for the development of
669 future therapies where neuronal replacement is needed in the brain. Although in vivo
670 reprogramming is still at its early stages and the translation into humans is not yet foreseen,
671 this technique could provide a method to assess exogenous gene function in the brain and
672 study cell maturation in vivo.

673

679

680 **DISCLOSURES:**
681 The authors have nothing to disclose.

682

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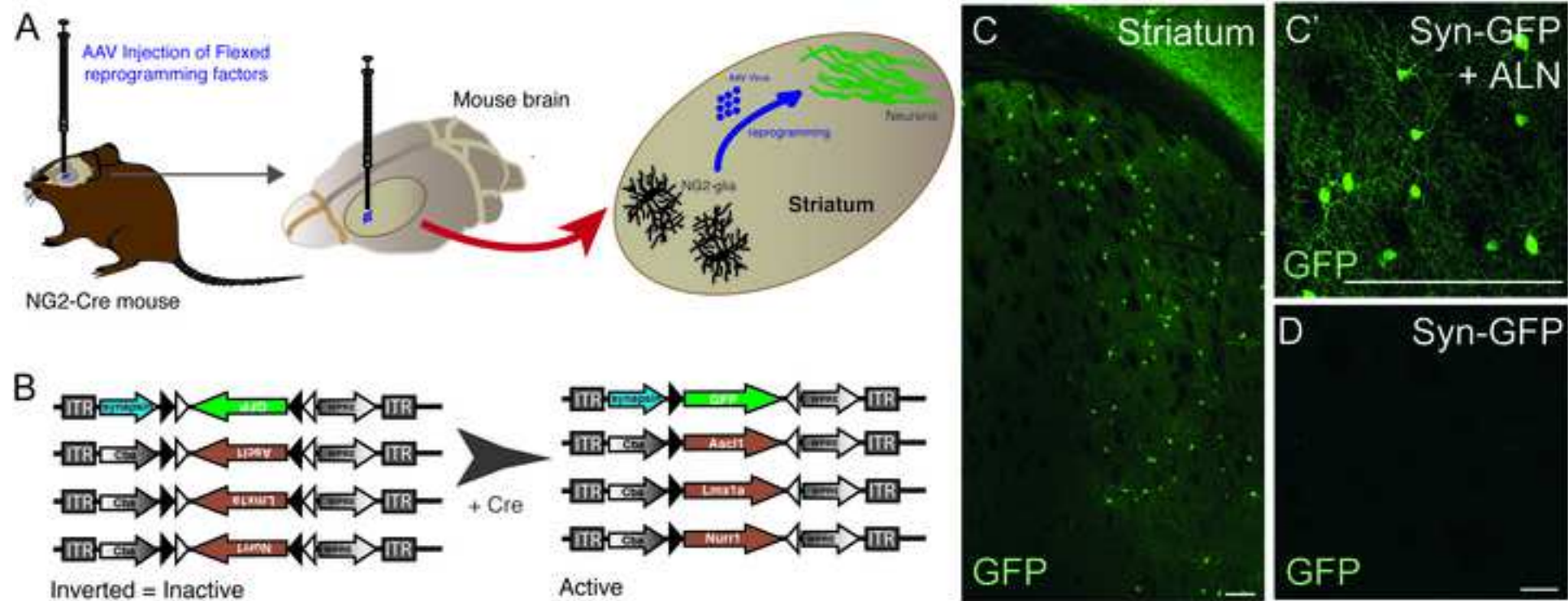717   Therapy Methods & Clinial Development.* **10** 1-7, (2018).

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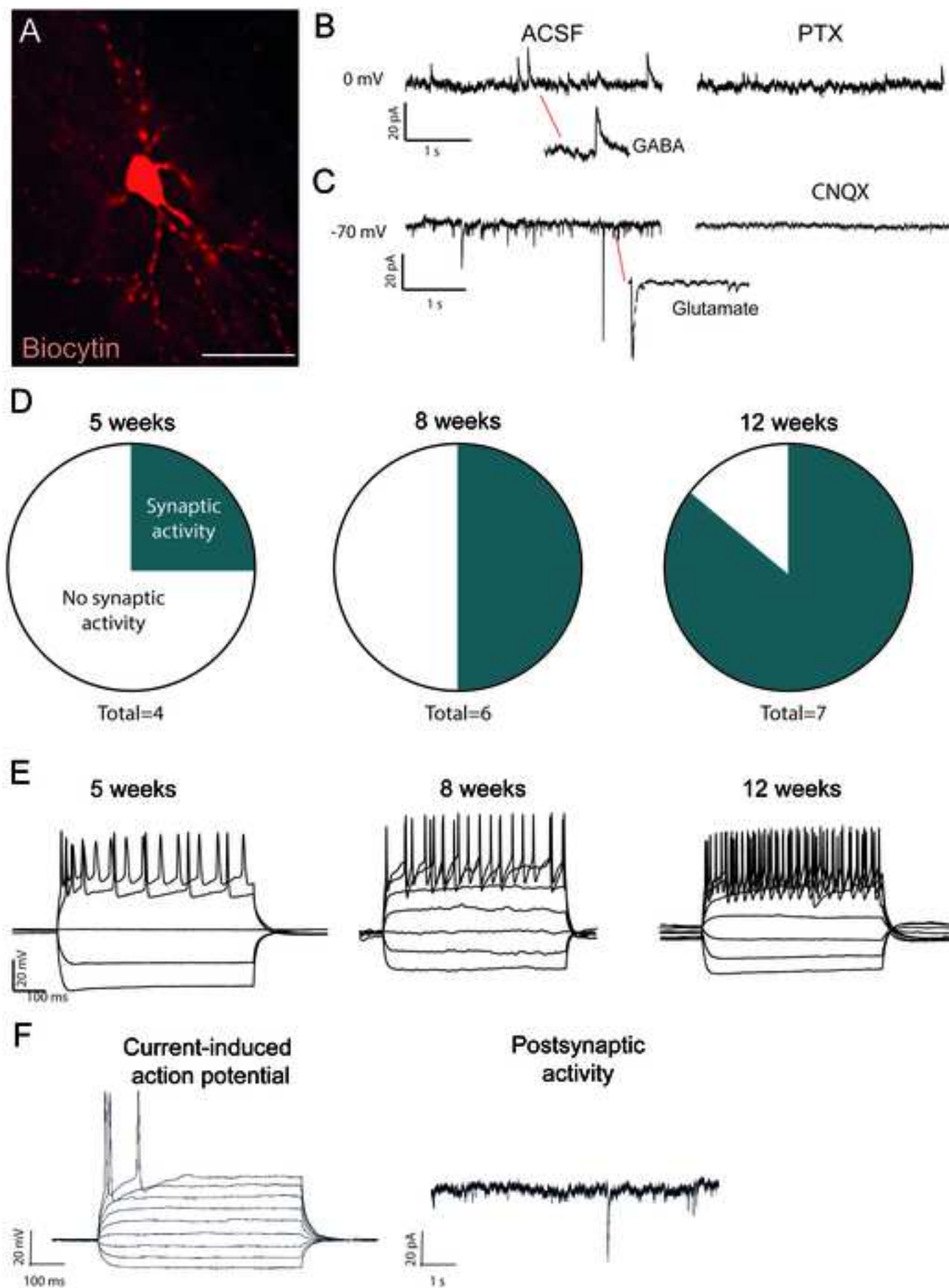720   (17), 7038-7043, (2013).

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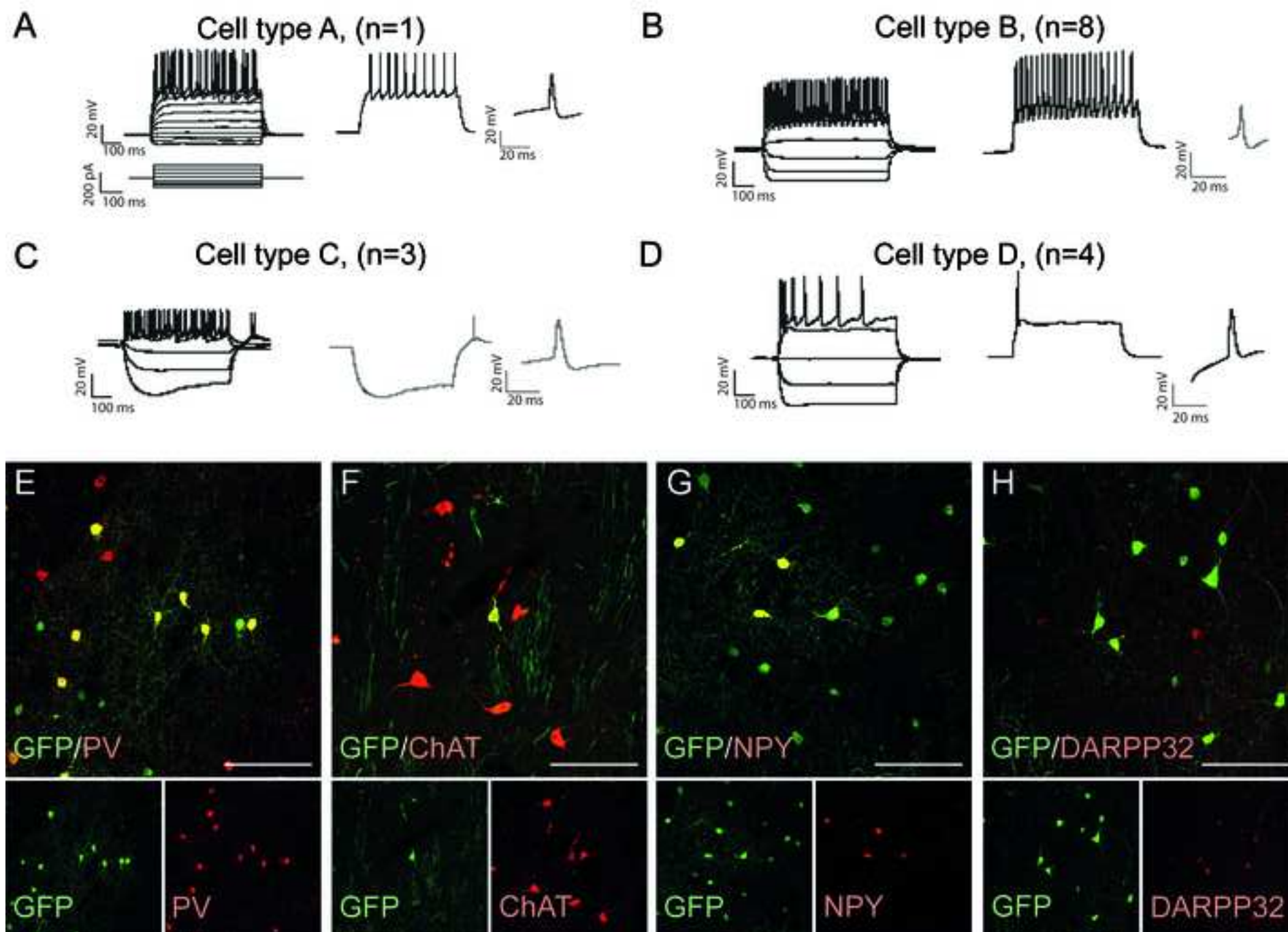
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731

Figure 2

Click here to access/download;Figure;Figure 2.tif ⬇

A — Biocytin

B — ACSF / PTX
0 mV
20 pA / 1 s
GABA

C — CNQX
-70 mV
20 pA / 1 s
Glutamate

D
5 weeks — Synaptic activity / No synaptic activity — Total=4
8 weeks — Total=6
12 weeks — Total=7

E
5 weeks
8 weeks
12 weeks
20 mV / 100 ms

F
Current-induced action potential
Postsynaptic activity
20 mV / 100 ms
20 pA / 1 s

Figure 3

A Cell type A, (n=1)

B Cell type B, (n=8)

C Cell type C, (n=3)

D Cell type D, (n=4)

E GFP/PV

F GFP/ChAT

G GFP/NPY

H GFP/DARPP32

GFP | PV

GFP | ChAT

GFP | NPY

GFP | DARPP32

Name of Material/ Equipment

REAGENTS FOR AAV5 CLONING AND VIRAL VECTOR PREPARATION

pAAV-CA-FLEX

Ascl1

Lmx1a

Nurr1

GFP-syn

LoxP (FLEX) sequence 1

LoxP (FLEX) sequences 2

pDP5

pDP6

Phosphate-Buffered Saline (PBS)

FBS (Fetal bovine serum)

Penicillin streptomycin

DMEM (Dulbecco's Modified Eagle Medium)+ Glutamax

DPBS (Dulbecco's Phosphate Buffer Saline)

HEK293 cells

Flasks

Tris H-CL

EDTA

Ultrapure water

$CaCl_2$

Dulbecco´s phosphate-buffered saline (DPBS)

NaCl

$MgCl_2$

Ultracentrifuge sealing tubes

OptiPrep Density Gradient Medium

10-mL syringe-18G needle

Laboratory glass bottles

Anion exchange filter

Centrifugal filter unit

Endotoxin-Free Plasmid DNA Isolation Kits

Na_2PO_4

HEPES

Falcon tube

Falcon tube

Glass Vials

Forward Primer for Inverted Terminal Repeat (ITR) sequence

Reverse Primer for Inverted Terminal Repeat (ITR) sequence

5´FAM / 3´BHQ1 probe

0.22 mm filter

EQUIPMENT AAV5 VIRAL VECTOR PREPARATION

Freezer -20 °C

Freezer -80 °C

Fridge +4 °C

AAV viral room

Ultrapure Water system

Vortex mixer

Ultracentrifuge

Autoclave

Polymerase Chain Reaction (PCR)

Quantitative PCR (qPCR)

Centrifuge

Water Bath

ANIMAL MODEL

NG2-Cre mice

REAGENTS FOR INJECTION OF REPROGRAMMING FACTORS INTO THE BRAIN

Water

Saline

Ethanol

Isolfurane

Virkon

Pentobarbital

Sucrose

Trypan Blu

Retrobeads

Buprenorphine

EQUIPMENT FOR INJECTION OF REPROGRAMMING FACTORS INTO THE BRAIN

Scissors

Tweezers

Forcep

Scalpel holder

Scalpel

Stereotaxic frame

Mouse ear bars

Syringe with Removable needle

Glass capilaries

Glass capillary puller

Dental drill

Shaver

Isoflurane Chamber and pump

Syringes

Needles

Mouse and neonatal rat adaptor for stereotaxic fram

Heating pad

Cotton gauze

Rubber tube

Cotton swabs

REAGENTS FOR WHOLE-CELL PATCH CLAMP RECORDINGS

NaCl

KCl

NaH_2PO_4-H_2O

$MgCl_2$-6 H_2O

$CaCl_2$-6 H_2O

MgSO4-7 H_2O

$NaHCO_3$

Glucose

Ultrapure Water

KOH

K-D-gluconate

KCl

KOH-EGTA (Etilene glycol-bis-N-tetracetic acid)

KOH- Hepes acid (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

NaCl

Mg2ATP

Na3GTP

Biocytin

Picrotoxin

CNQX

Ice

EQUIPMENT FOR WHOLE-CELL PATCH CLAMP RECORDINGS

Borosilicate glass pipette

Glass capillary puller

Vibratome

WaterBath

Clampfit software

Multiclamp software

REAGENTS FOR IMMUNOHISTOCHEMISTRY

Paraformaldehyde (PFA)

Triton X-100

Serum

4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI)

streptavidin- Cy3

Anti-GAD65/67

Anti-PV

Anti-Chat

Anti-NPY

K_2HPO_4

NaCl

KH_2PO_4

Anti-GFP

Mounting solution
Glycerol
etylengligerol
NaHPO$_4$
Distilled water
Anti-freeze solution

EQUIPMENT FOR IMMUNOHISTOCHEMISTRY
Inverted fluorescence microscope
Confocal microscope
Prism
Microtome

Company	Catalog Number
AddGene	38042
AddGene	67291
AddGene	33013
AddGene	35000
AddGene	30456
Plasmid Factory	PF435
Plasmid Factory	PF436
Thermo Fisher Scientific	10010023
Thermo Fisher Scientific	10500064
Thermo Fisher Scientific	15140122
Thermo Fisher Scientific	61965026
Thermo Fisher Scientific	14190094
Thermo Fisher Scientific	85120602-1VL
BD Falcon	10078780
Sigma Aldrich	10812846001
EDTA: Invitrogen	EDTA: AM9260G
SigmaAldrich	C5080
Thermo Fisher Scientific	14190136
Sigma-Aldrich	S3014
Sigma-Aldrich	M8266-100G
Beckman Coulter	Quick-Seal Polypropylene Tube
Sigma Aldrich	D1556-250ML
BD	305064
VWR	
PALL laboratory	MSTG25Q6
Merck	Z740210-24EA
Thermo Fisher Scientific	A33073
Sigma-Aldrich	S7907
Sigma-Aldrich	H7523
Thermo Fisher Scientific	Corning 352196
Thermo Fisher Scientific	Corning 352070
Novatech	30209-1232
Merck	SLGV004SL

Merck	Milli-Q IQ 7000
VWR	444-0004
Beckman Coulter	Beckman Optima LE-80K Ultracentrifuge
Tuttnauer	2540 EL
BioRad	C1000 Touch Thermal Cycler
Roche	LightCycler 480 System
Thermo Fisher Scientific	Sorvall ST16
Thermo Fisher Scientific	TSGP02
Jackson	NG2-CrexB6129, Stock #008533
Apoteket AB	
Solveco	
Apoteket AB	
Viroderm	7511
Apoteket AB	P0500000
Merck	S0389-500G
Thermo Fisher Scientific	15250061
Lumafluor	R170
Apoteket AB	
VWR	233-1552
VWR	232-0007
VWR	232-0120
VWR	RSGA106.621
VWR	RSGA106.200
Stoelting Europe	51500D
Stoelting Europe	51648
Hamilton Company	65
Stoelting	50811
Sutter company	P-1000
Agnthos AB	1464
Agnthos AB	GT420
Agnthos AB	8323101
Merck	Z118400-30EA
Merck	Z192414 Aldrich
Stoelting	51625
Braintree scientific, inc	53800M
Fisher Scientific	22-037-902
Elfa Distrelec	HFT-A-9.5/4.8 PO-X BK 150

Fisher Scientific	18-366-473
Sigma-Aldrich	S3014
Sigma-Aldrich	P9333
Sigma-Aldrich	S9638
Sigma-Aldrich	M2670
Sigma-Aldrich	C8106
Sigma-Aldrich	230391
Sigma-Aldrich	S5761
Sigma-Aldrich	G7021
Sigma-Aldrich	P5958-500G
Sigma-Aldrich	G4500 Sigma
Sigma-Aldrich	P9541
Sigma-Aldrich	E3889 Sigma
Sigma-Aldrich	H7523
Sigma-Aldrich	S3014
Sigma-Aldrich	A9187
Sigma-Aldrich	G8877
Sigma-Aldrich	B4261
Merck	P1675 Sigma
Merck	C239 Sigma
Sutter Company	B150-86-10
Sutter company	P-1000
Leica	Leica VT1000 S
Thermo Fisher Scientific	TSGP02
Molecular Devices	
Molecular Devices	
Merck Millipore	1040051000
Fisher Scientific	10254640
Merck Millipore	S30-100ML
Sigma Aldrich	D9542
Thermo Fisher Scientific	434315
Abcam	
Sigma	P3088
Merk	AB143
Immunostar	22940
Sigma-Aldrich	P3786
Sigma-Aldrich	S3014
Sigma-Aldrich	NIST200B

Abcam	ab13970
Merck	10981
Sigma-Aldrich	G9012-100ML
Tissue Pro Technology	AFS05-1000N, 1000 mL/ea.
Leica	DMI6000 B
Leica	TCS SP8 laser scanning confocal microscope.
GraphPad	
Leica	SM2010R

Comments/Description

NM_008553.4
NM_0033652.5
NM_013613.2

GATCTccataacttcgtataaagtatcctatacgaagttatatcaaaataggaagaccaatgcttcaccatcgacccgaattgccaagcatcaccatc
tactagtataacttcgtataggatactttatacgaagttatcattgggattcttcctattttgatccaagcatcaccatcgaccctctagtccacatctcacc

175 cm^2
For TE buffer use 10 mM, pH 8.0; for lysis buffer use 50mM, pH 8.5; for IE buffer use 20 mM, pH 8.0; for
For TE buffer use 1 mM EDTA
see Ultrapure water system
2.5 M

FOR HBS use 140 mM; for Lysis Buffer use 150 mM; for IE buffer use 15 mM; for elution buffer use 250 m
For lysis Buffer: 1 mM

Acrodisc unit with Mustang Q membrane
Amicon Ultra-4 device

15 mL

CGGCCTCAGFGAGCGA
GGAACCCCTAGTGATGGAGTT
CACTCCCTCTCTGCGCGCTCG

Millex-GV filter

Refer to local biosafety guidelines when handling adeno-associated virus (AAV).

70%

Dilute to 1% solution with warm water.

From RSG Solingen.

From Biochem.

Number 20.

5 µl
0.75 inner diameter and 1.5 outer diameter.

2 mL
25G

From Covidien 2187.

see Ultrapure Water system

Prepare 1 or 2M.

Use at a concentration of 4%. CAUTION: PFA is a potent fixative. Avoid ingestion and contact with skin.

Use at a concentration of 0.1%.

Donkey.Use at a concentration of 1 : 400.

Reconstitute the powder in Milli-Q water to 1 mg/mL. Aliquot and store at -20°C, light sensitive. Use at a 1: 600 in KPBS-T.

1:5000, rabbit.

1:2000, mouse.

1:200, goat.

1:5000, rabbit.

1:1000, chicken.

Polyvinyl alcohol mounting medium with DABCO[®], antifading

gacccataacttcgtataatgtatgctatacgaagttatactagtcccgggaaggcgaagacgcggaagaggctctaga
catcgacccataacttcgtatagcatacattatacgaagttatgtccctcgaagaggttcgaattcgtttaaacGGTACCCTCGAC

elution buffer use 20 mM, pH 8.0

nM

concentration of 1 : 500.

jove
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Title of Article:	In vivo direct reprogramming of resident glial cells into interneurons by intracerebral injection of viral vectors
Author(s):	Maria Pereira, Marcella Birtele and Daniella Rylander Ottosson

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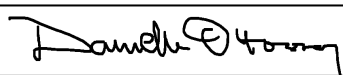
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Editorial comments:

The manuscript has been modified and the updated manuscript, **59465_R2.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

We thank the editor for this valuable input to our protocol. We have made corrections on all the following points and hope that the protocol has now improved to a level that is required for JoVe publication.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have had an English native speaker to read though the manuscript.

2. Please number all figures in the order of their appearance in the manuscript. For example, figure 2 is mentioned first in the manuscript, so it should be numbered as figure 1.

We would like to keep the numbering order of the figures, if that is ok with the Editor. However, we do agree that figure number should reflect their order of appearance in the text. Therefore, we have removed the text where Figure 2 is referred for the first time (this will not change the information regarding Figure 2 present in the manuscript since it is referred again in the results' section).

3. Please do not abbreviate journal titles for all references.

We apologize for this and have now written out the full name of the journals.

4. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Jackson, etc.

The mentioned commercial language has been removed from the text and sufficient information provided in the Materials Table.

5. Step 1.2.1: Please specify the composition of the media.

We have now specified the composition of the media with: DMEM Glutamax + 10% FBS + penicillin 100 U/ml + streptomycin (100 ug/ml) and added this in the Table of Materials

6. 1.2.2: Please split this long step into more sub-steps.

We have now devided into sub-steps.

7. 1.2.3: How much transfection mix is added?

We have now added: Transfection mix volume 1.8 ml.

8. 2.1.18: Please write this step in the imperative tense.

We have re-written the sentence to:

2.1.18   At the end of the drilling, check that the dura mater remain intact and exposed for injection.

9. 1.2: Please write this step in the imperative tense.

We are not sure of which point the Editor refers to here. 1.2 is a title: AAV5 viral vector production. We have anyhow read though and checked that all steps are written in imperative tense.

10. 3.2.6: Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have re-written the point to :

3.2.2   Rinse the surrounding tissue carefully using positive pressure and approach the cell with the electrode. Locate the electrode right on top of the cell and descend until the membrane is reached. Make a Giga-Ω seal with the electrode and cell membrane, and with negative pressure pulses, rupture the membrane to create a whole-cell patch.

11. 4.9: Please write this step in the imperative tense.

4.1.   We have re-written to: Analyze the sections using an inverted fluorescence and/or confocal microscope.