Supplementary Materials for

In vivo Chemical Reprogramming of Astrocytes into Functional Neurons

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Materials and Methods

Mice

Homozygous TauEGFP knock-in mice (*14*), *mGfap-Cre* transgenic mice (*15*) and Rosa-CAG-LSL-tdTomato mice (*16*) were purchased from the Jackson Laboratory. TauEGFP heterozygous mice were generated by crossing the homozygous TauEGFP knock-in mice with wild-type ICR mice. *mGfap-Cre*/*Rosa26*-tdTomato mice were generated by crossing the *mGfap-Cre* transgenic mouse strain, which expresses Cre recombinase under the control of the glial fibrillary acidic protein (GFAP) promoter, with the *Rosa26*-tdTomato mouse strain, in which the *Rosa26* locus was inserted using a CAG promoter and was followed by a LoxP-Stop-LoxP cassette-controlled fluorescent tdTomato locus. All animal experiments had received prior approval by the Animal Protection Guidelines of Peking University, China.

Cell Culture

Primary mouse astrocytes were isolated from postnatal day 0 to day 3 mice. After disinfection, the mice were decapitated. Their cerebra were transferred into pre-cooled HBSS (Gibco). Remove the meningeal and cut up the cerebra into small pieces. The debris of the tissue were digested with 5 mL 0.25 % trypsin (Gibco) and 1 % DNaseI (Roche) for 15 min at 37 °C, then the digestion was stopped with 5 mL astrocyte culture medium (Dulbecco's Modified Eagle Medium/F12 (DMEM) medium (Gibco) containing 10 % fetal bovine serum (Gibco) and 1 % penicillin-streptomycin (Invitrogen)). The cells were centrifuged for 5 min at 1500 rpm and were suspended in culture medium, a total of 10×10^6 cells were plated onto a 10 cm culture dish for differential adhesion. Thirty minutes later, the supernatant was transferred into a 75 cm^2 culture flask. The flask was incubated at 37 °C in a 5 % CO_2 / 95 % air. Refresh the medium every 3 days. Upon reaching 100 % confluency, the cultures were shaken at 260 rpm for 18 h to remove contaminating cells.

Detailed Protocol for Chemically Inducing Neurons from astrocytes

Culture Medium Preparation

Neuronal induction medium: Neurobasal (Invitrogen) with 0.5 % N2 (Invitrogen), 1 % B-27 (Invitrogen), 1 % GlutaMAXTM-I (Invitrogen), 1 % penicillin-streptomycin (Invitrogen), bFGF (100 ng/mL; Origene). The small molecules were as follows: dbcAMP, 100 μM; Forskolin, 10 μM; ISX9, 40 μM; CHIR99021, 20 μM; I-BET151, 2μM and Y-27632, 10 μM.

Neuronal maturation medium: Neurobasal (Invitrogen) with 0.5 % N2 (Invitrogen), 1 % B-27 (Invitrogen), 1 % GlutaMAXTM-I (Invitrogen), 1 % penicillin-streptomycin (Invitrogen), 10 ng/mL bFGF, 20 ng/mL BDNF, 20 ng/mL GDNF. The small molecules were as follows: 10 μM Forskolin, 3 μM CHIR.

Small molecules used in this process are listed in Table S4.

Chemical Induction of neurons

1. Thaw matrigel (growth factors reduced; BD Biosciences) on ice according to the manufacturer's instruction and dilute it in pre-cold PBS with a ratio of 1: 30.
2. Add diluted matrigel to 6-well plates to cover the entire growth surface of the plates and keep the plates in 37 °C for 2 h to be ready to use.
3. Dissociate primary TauEGFP astrocytes or *mGfap-Cre*/*Rosa26*-tdTomato/TauEGFP astrocytes with 0.25 % trypsin and neutralized with astrocyte culture medium.
4. Resuspend the cells with astrocyte culture medium and seed them into the matrigel-coated plates at a density of 100,000 cells per well of a 6-well plate. Cells are grown in DMEM/F-12 (Invitrogen) medium supplied with 10 % fetal bovine serum for 5-7 days until reaching 100 % confluent and the medium was refreshed every 4 days.
5. Change the medium into neuronal induction medium (day 0) and the medium was refreshed every 4 days during the chemical induction period.

Maturation of CiNs

After chemical treatment for about 16 days (the induced cells become TauEGFP-positive), the induced cells were progressed for further maturation: Replate and co-culture directly with primary wildtype mouse astrocytes.

1. Wildtype astrocytes, isolated from the C57BL/6 mice were plated at a density of 100,000 cells per well of a 6-well plate and cultured to 100 % confluency before co-cultured with induced cells.
2. Dissociate the induced cells gently by using 0.25 % trypsin for 3 min at 37 °C and neutralize with astrocyte culture medium.
3. Centrifuge for 3 min at 1000 rpm at room temperature.
4. Carefully discard the supernatant, gently resuspend the cells with maturation medium and replate them at a density of 200,000 cells per well of a 6-well plate to co-culture with pre-existing primary wild-type astrocytes.
5. Replated cells were co-cultured for about 14 days to become functional mature. Maturation medium was changed every 4 days.

Chemical infusion into the mouse brain

Small molecules were resolved in the neuronal induction medium with 300 ng/mL bFGF as follows: Forskolin, 300 μM; CHIR, 60 μM; ISX9, 120 μM; I-BET151, 6 μM; Y27632, 30 μM. For chemical infusion, a total volume of 100 μL neuronal induction medium were loaded into the Alzet osmotic minipumps to obtain a constant release at the rate of 0.25 μL/hr for 14 days (Alzet 1002; Cupertino, CA). In the vehicle group, same amount of neuronal induction medium without small molecules were infused into the mouse brains. Stereotaxic surgeries were performed on two-month old mice for chemical infusion. Mice were anesthetized with 0.8 % Pelltobarbitalum Natricum and heads were shaved and swabbed with 70 % ethanol and iodophor. Erythromycin eye ointment was applied to prevent corneal drying and a heat pad (RWD, Shenzhen, China) was used to hold body temperature at 37 °C. A small midline incision was made to expose the skull. Remove the periosteal connective tissue which adheres to the skull. Keep the skull dry, which permits good adhesion of the dental cement which is later used to secure the cannula. Insert the L-shaped cannula into the striatum (coordinates: AP 0.5 mm, ML -2 mm, DV -3 mm) or the cortex (coordinates: AP 0.5 mm, ML -2 mm, DV -0.5 mm) and the pump subcutaneously on the back. The cannula was secured to the skull with instant adhesive (Tonsan 1454, China) and dental cement. The incision was closed by suture thread. Animals were then placed in a clean warm cage on a heating pad until mobile.

Immunostaining

Mice were anesthetized and perfused with 0.9 % saline solution followed by 4 % ice-cold phosphate-buffered paraformaldehyde (PFA). Brains were removed and post-fixed in 4 % PFA overnight, then sequentially into 20 % and 30 % sucrose to cryoprotect the tissues. After embedding with OCT solution (Tissue-Tek), coronal sections were cut on a freezing microtome at a thickness of 15 μm and stored at -20 °C.

Immunofluorescence on cells or brain slices were all carried out as previously described (*10*). Primary antibodies included those specific to TUJ1 (Covance, rabbit anti, 1:500), MAP2 (Sigma-Aldrich, mouse anti, 1:500), NF-H (Abcam, rabbit anti, 1:400), SYN1(Cell Signaling Technology, rabbit anti, 1:500), NEUN (Millipore, mouse anti, 1:500), NEUN (Abcam, rabbit anti, 1:500), vGLUT2 (Invitrogen, rabbit anti, 1:400), GAD67 (Millipore, mouse anti, 1:500). GFAP (Agilent, rabbit anti, 1:500), NEUROD1 (Abcam, rabbit anti, 1:500), NEUROG2 (Abcam, rabbit anti, 1:500), DCX (Abcam, rabbit anti, 1:500), S100B (Sigma-Aldrich, mouse anti, 1:1000), IBA1 (Abcam, goat anti, 1:500), O4 (Millipore, mouse anti, 1:500) and MBP (Abcam, rabbit anti, 1:200). Secondary antibodies used in this study including: 488-conjugated secondary antibodies, Cy3-conjugated secondary antibodies and 647-conjugated secondary antibodies (Jackson ImmunoResearch).

Electrophysiology

For whole-cell patch-clamp recordings, the chamber was perfused with the artificial cerebrospinal fluid (ACSF), containing (all in mM) 141 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 1.25 NaH_2PO_4, 10 glucose and 10 HEPES, and the pH was adjusted to 7.4 with NaOH. Patch pipettes were pulled (6 MΩ tip resistance) with a P97 micropipette puller (Sutter Instruments) filled with internal pipette solution contained (in mM) 140 potassium gluconate, 1 $CaCl_2$, 10 EGTA, 2 $MgCl_2$, 5 Na_2ATP, and 10 HEPES, the pH was adjusted to 7.4 with KOH. Patch-clamp recordings were taken using an EPC-10 amplifier (HEKA) with PatchMaster. For current-clamp recordings, a hyperpolarized current was injected into the $EGFP^+$ cells after co-cultured with mouse primary astrocytes to membrane potentials around -70 mV. Action potentials were elicited by step-depolarized currents with sweep intervals of 2 s. The cells were held at 0 pA to record the resting membrane potentials. All recordings were conducted at room temperature.

For brain slice recording, the lineage tracing mice brains were dissected and placed in ice-cold oxygenated artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$-6H_2O, 1.25 NaH_2PO_4-2H_2O, 25 $NaHCO_3$ and 10 glucose, at pH 7.4. Coronary slices of 300 mm thick were made with a vibratome (VT-1200S; Leica) and were maintained in an incubation chamber with oxygenated (95 % O_2 / 5 % CO_2) aCSF at 37 °C for 15 min and then transferred to room temperature for 30 min before being transferred to the recording chamber.

Virus injection

To trace the astrocytes, *mGfap-Cre* mice were used, and a total volume of 0.5μl mixed with rAAV-EF1α-DIO-His-EGFP-2a-TVA-WPRE-pA (2×10^{12} vg/mL) and rAAV-EF1α-DIO-RVG-WPRE-pA (2×10^{12} vg/mL) at the ratio of 1:1 was injected into the cortex (coordinates: AP 0.5 mm; ML -2 mm; DV -0.5 mm). All three virus used in this paper were purchased from BrainVTA Wuhan, China. For the virus injection, the micropipette was held in place before the injection for 5 min. Viruses were then injected into the cortex at a constant rate of 0.1μL per minute. To prevent any backflow, the pipette was held for another 5 min before the slowly withdrawing. One week later, similar procedures as described above for chemical treatment were adopted, which was within the cortex and the same coordinates of the viral injection. To label the induced neurons, 0.3 μL of RV-ENVA-ΔG-DsRed (2.5×10^8 IFU/mL) was injected into the same site one week prior to perfusion. Brain slices were made as 30 μm thick.

Cell counting

To systemically quantify CiNs distribution, slice with the largest cannula track were considered as injection core. 5 brain slices at 120-μm intervals per mouse were analyzed on either side throughout the rostral-caudal extent. For dosage optimization, single small molecule omission and time scale analysis, 3 representative slices were analyzed. The cell counting area on brain slices was selected surrounding the injection site: upper edge was extended from the needle hole to the corpus callosum (Cc); the bottom, left and right edges were the outward extensive zone of 100 μm from the astrocytes hypertrophy area.

For the PRV tracing mice, the numbers of $EGFP^+/DsRed^+$ cells in every slice along the injection area was counted. Only cell bodies in which the nucleus was clearly visible were counted.

For induction efficiency analysis in vitro and lineage tracing analysis, Images were randomly selected from independent batches of cell cultures for quantification.

Figure S1. A chemical cocktail efficiently converts astrocytes into functional neurons *in vitro*.

(A) Scheme of chemically induced astrocyte-to-neuron reprogramming.

(B) Immunofluorescence analyses of initiating astrocytes at 0 dpi for astrocyte marker GFAP, chemical induced TauEGFP$^+$ cells at 16 dpi for pan-neuronal markers (TUJ1, MAP2, SYN1 and NF-H) and at 30 dpi for mature neuronal markers (NEUN, GAD67, vGLUT2) after co-cultured with primary astrocytes for 2 weeks.

(C) Quantification of TUJ1$^+$ cells and TUJ1$^+$ cells / TauEGFP$^+$ cells at 16 dpi (n = 6).

(D) Analyses of the influence of each small molecule on the induction efficiency of NF-H$^+$ CiNs at 16 dpi (n = 2).

(E) Quantification of vGLUT2$^+$, GAD67$^+$ and NEUN$^+$ cells at 30 dpi after co-cultured with primary astrocytes for 2 weeks (n = 3).

(F) Electrophysiological analyses of the induced TauEGFP$^+$ CiNs. Action potentials and inactivating inward currents were recorded at 30 dpi after co-cultured with primary astrocytes for two weeks.

Scale bars: 50 μm (GFAP, GAD67), 250 μm (TauEGFP, TUJ1, MAP2, SYN1 and NF-H), 25 μm (NEUN, vGLUT2).

Error bars represent s. e. m.

Figure S2. Neuronal conversion of astrocytes derived from *mGfap-Cre/Rosa26*-tdTomato mice.

(A) Scheme of lineage-tracing constructs.

(B) Immunofluorescence analyses showed that GFAP-tdTOMATO cells co-expressed astrocytes markers GFAP and S100B, but not oligodendrocytes marker O4, microglia marker IBA1, neural progenitor marker DCX or neuronal marker NEUN.

(C) Quantification of cultured GFAP-tdTOMATO cells. Images were randomly selected from each of the independent batches of cell cultures for quantification (n = 3).

(D) Quantification, in percentage, of tdTOMATO$^+$/TUJ1$^+$ cells induced from astrocytes carrying GFAP-tdTomato at 16 dpi.

Scale bar: 100 μm.

Error bars represent s. e. m.

Figure S3. In vivo chemical cocktail optimization.

(A) Dosage optimization of the FICBY cocktail in adult mouse striatum. Quantification of $NEUN^+$/$tdTOMATO^+$ cells in representative images of chemically treated mice at 1, 3, and 10 times of dosage in vitro (n = 2).

(B) Single small molecule omission in adult mouse striatum based on the FICB cocktail. Quantification of $NEUN^+$/$tdTOMATO^+$ cells in single small molecule omission analysis at 8 wpi (n = 2).

Error bars represent s. e. m.

Figure S4. Scheme of monosynaptic tracing methodology based on PRV.

Two Cre-dependent FLEX-AAV vectors encoding TVA, histone-EGFP and RVG were injected into the cortex of *mGfap-Cre* mice, resulting in their expression restricted to astrocytes. After EGFP labeled astrocytes were converted into neurons, inject PRV-carrying DsRed into the same site to label the CiNs and to trace endogenous host neurons that made synaptic contact with CiNs.

Figure S5. Validation of the specificity of PRV tracing system.

Immunohistochemistry analyses of the $EGFP^+$ cells after injection of helper AAVs, rAAV-EF1α-DIO-His-EGFP-2a-TVA-WPRE-pA and rAAV-EF1α-DIO-RVG-WPRE-pA. Arrowheads highlight the co-expression of EGFP and S100B.Co-expression of EGFP and NEUN was not detected.

Scale bars: 25 μm.

Table S1. Electrophysiological properties of induced neurons after co-culture, related to figure S1F.

Induction	Rm(MΩ)	Cm(pF)	RMP (mV)	AP threshold(mV)	AP amp(mV)	In-max(pA)	N
CiNs	555.025± 83.854	21.980± 2.638	-70.094± 2.077	-32.503± 1.543	77.213± 4.996	-1249.945± 215.113	12/12
Cell1	1000.0	16.06	-84.23	-29.79	106.66	-639.1	1/12
Cell2	116.4	22.52	-69.37	-39.70	85.42	-2539.7	1/12
Cell3	537.5	34.06	-69.82	-34.64	72.66	-870.4	1/12
Cell4	525.3	26.77	-68.18	-34.21	88.17	-1866.5	1/12
Cell5	838.9	16.13	-70.07	-28.84	90.85	-1036.4	1/12
Cell6	261.0	13.94	-70.53	-32.59	79.93	-1299.4	1/12
Cell7	479.8	21.18	-71.62	-32.81	94.88	-2357.8	1/12
Cell8	951.0	10.35	-72.05	-19.74	78.24	-373.2	1/12
Cell9	326.5	14.80	-72.11	-38.47	50.78		1/12
Cell10	590.3	34.34	-71.78	-35.55	59.63	-595.7	1/12
Cell11	791.3	37.49	-70.04	-35.40	66.35	-1125.5	1/12
Cell12	242.3	16.11	-51.33	-28.29	52.98	-1045.7	1/12

Rm: input resistance;
Cm: membrane capacitance;
RMP: resting membrane potential;
APthreshold: action potential threshold;
APamp: action potential amplitude;
In-max: inward maximum currents;
All the data are mean+/- SEM.

Table S2. Electrophysiological properties of CiNs at different induced time points, related to Figure 3.

4 wpi

Induction	Rm(MΩ)	Cm(pF)	N
CiNs	260.825±65.484	1.928±0.570	4/4
Cell1	235.5	2.38	1/4
Cell2	196.3	1.30	1/4
Cell3	260.9	2.44	1/4
Cell4	350.6	1.59	1/4

6 wpi

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	AP threshold(mV)	AP amp(mV)	N
CiNs	293.020±45.551	33.980±2.619	-73.706±2.573	-40.030±7.615	115.828±8.664	5/9
Cell1	262.7	25.46	-66.22	-46.11	104.80	1/9
Cell2	371.0	36.28	-80.81	-9.98	149.81	1/9
Cell3	426.8	36.52	-76.45	-49.29	111.02	1/9
Cell4	210.7	40.62	-69.73	-43.78	102.45	1/9
Cell5	193.9	31.02	-75.32	-50.99	111.06	1/9

8 wpi

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	AP threshold(mV)	AP amp(mV)	N
CiNs	298.433±62.955	25.190±3.508	-74.482±2.627	-41.680±5.293	102.181±3.534	9/23
Cell1	131.7	15.59	-74.49	-54.38	111.82	1/23
Cell2	211.9	51.30	-71.14	-51.06	111.63	1/23
Cell3	154.6	18.13	-63.94	-35.98	86.12	1/23
Cell4	144.0	26.24	-89.20	-51.97	109.63	1/23
Cell5	153.0	22.84	-84.87	-58.07	115.91	1/23
Cell6	476.1	18.01	-72.14	-15.38	93.66	1/23
Cell7	603.5	23.91	-68.45	-16.51	100.49	1/23
Cell8	541.2	26.44	-71.62	-45.75	99.76	1/23
Cell9	269.9	24.25	-71.01	-46.02	90.61	1/23

10 wpi

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	AP threshold(mV)	AP amp(mV)	N
CiNs	204.371±17.473	22.434±4.908	-70.477±2.954	-46.666±2.052	91.899±5.304	7/7
Cell1	193.3	36.47	-57.74	-36.25	81.76	1/7
Cell2	160.3	41.87	-68.36	-43.98	82.89	1/7
Cell3	200.3	22.76	-79.16	-52.12	111.21	1/7
Cell4	289.8	6.13	-79.68	-50.67	108.76	1/7
Cell5	187.7	12.93	-74.40	-45.19	98.91	1/7
Cell6	239.1	23.37	-67.32	-49.13	80.99	1/7
Cell7	160.1	13.51	-66.68	-49.32	78.77	1/7

12 wpi

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	APthreshold(mV)	APamp(mV)	N
CiNs	267.875±53.828	45.185±9.341	-73.296±1.352	-43.014±3.292	107.584±1.718	4/4
Cell1	240.1	72.58	-70.43	-35.95	104.80	1/4
Cell2	426.8	36.52	-76.45	-49.29	111.02	1/4
Cell3	210.7	40.62	-69.73	-43.78	102.45	1/4
Cell4	193.9	31.02	-75.32	-50.99	111.06	1/4

15 wpi

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	AP threshold(mV)	AP amp(mV)	N
CiNs	224.700±16.493	31.580±3.387	-65.529±2.090	-42.334±3.792	90.225±2.617	18/19
Cell1	309.5	24.64	-48.61	-36.61	82.79	1/19
Cell2	175.4	24.72	-56.24	-48.55	75.45	1/19
Cell3	168.6	41.39	-76.90	-47.94	97.41	1/19
Cell4	164.4	48.21	-71.58	-49.19	91.74	1/19
Cell5	315.4	35.57	-66.01	-47.21	95.89	1/19
Cell6	318.3	42.81	-75.71	-47.55	96.65	1/19
Cell7	284.8	27.50	-75.84	-44.62	100.98	1/19
Cell8	231.9	28.17	-61.51	-47.55	91.67	1/19
Cell9	347.5	27.38	-68.48	-44.13	81.12	1/19

Cell10	218.4	23.06	-69.49	-46.92	96.22	1/19
Cell11	226.8	22.90	-70.96	-47.19	104.14	1/19
Cell12	108.2	9.78	-61.80	-45.53	90.18	1/19
Cell13	197.4	9.03	-45.38	-38.70	64.79	1/19
Cell14	291.0	21.37	-64.85	20.72	81.82	1/19
Cell15	183.6	44.51	-68.88	-47.03	95.64	1/19
Cell16	175.9	62.20	-58.35	-48.52	82.24	1/19
Cell17	184.3	52.97	-70.92	-47.70	99/79	1/19
Cell18	143.2	24.03	-68.02	-47.79	105.10	1/19
Cell15	237.7	97.52	-69.49	-48.83	105.07	1/18
Cell16	321.6	67.46	-70.19	-48.31	91.03	1/18

8 wpi

Induction	**RMP(mV)**	**AP threshold(mV)**	**AP amp(mV)**	**N**
CiNs	-72.070±2.072	-48.684±1.977	99.950±5.411	5/8
Cell1	-71.50	-50.17	82.92	1/8
Cell2	-79.62	-53.10	112.95	1/8
Cell3	-72.54	-41.84	109.82	1/8
Cell4	-68.88	-51.30	99.49	1/8
Cell5	-67.81	-47.01	94.57	1/8

Rm: input resisitance;
Cm: membrane capacitance;
RMP: resting membrane potential;
APthreshold: action potential threshold;
APamp: action potential amplitude;
All the data are mean+/- SEM.

Table S3. Electrophysiological properties of endogenous neurons, related to Figure 3.

Induction	Rm(MΩ)	Cm(pF)	RMP(mV)	AP threshold(mV)	AP amp(mV)	N
Neurons	145.000±9.430	61.844±7.987	-72.584±0.353	-48.108±3.439	92.328±5.508	5/5
Cell1	153.2	63.27	-71.78	-59.78	95.58	1/5
Cell2	122.1	61.08	-71.87	-46.63	99.18	1/5
Cell3	154.3	51.89	-73.67	-47.12	97.11	1/5
Cell4	124.4	90.27	-72.97	-48.74	99.30	1/5
Cell5	171.0	42.71	-72.63	-38.27	70.47	1/5

Rm: input resisitance;
Cm: membrane capacitance;
RMP: resting membrane potential;
APthreshold: action potential threshold;
APamp: action potential amplitude;
All the data are mean+/- SEM.

Table S4. Small molecules used in reprogramming, related to Figure 2 and S1.

FULL NAME	ABBREVIATION	SOURCE	MOLECULAR WEIGHT
Dibutyryl-cAMP	D	Santa Cruz, cat.no. sc-201567	491.37
Forskolin	F	Enzo, cat. no.BML-CN100-0100	410.50
ISX9 (Isoxazole 9)	I	Tocris, cat. no.4439	234.28
CHIR99021	C	WUXI APPTEC	465.34
I-BET151	B	WUXI APPTEC	415.44
Y-27632	Y	Tocris, cat. no.1254	247.34