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Supplemental Information

Region-Restrict Astrocytes Exhibit Heterogeneous Susceptibility to Neuronal Reprogramming

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Supplemental Figures and Legends

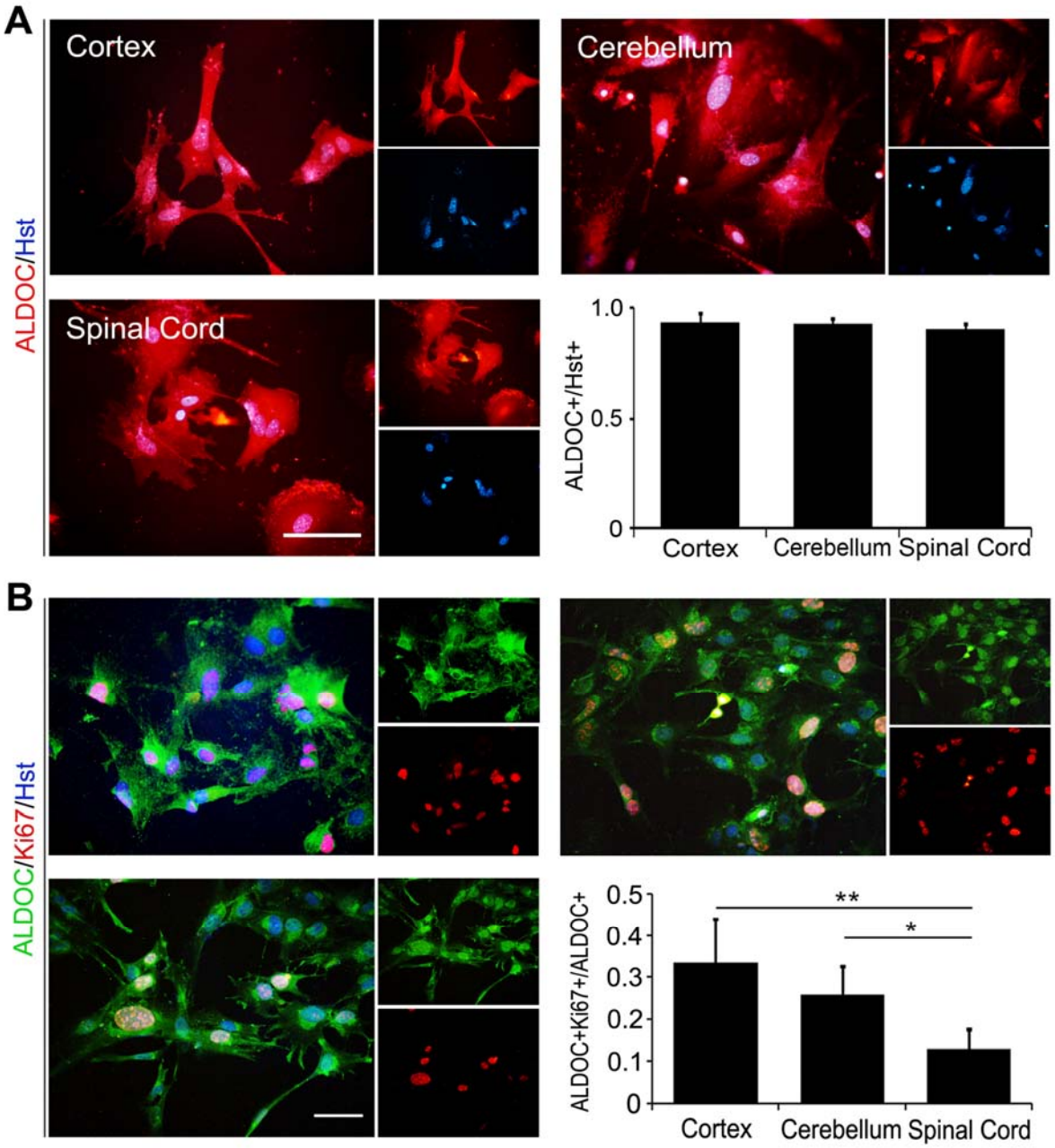

Figure S1 Establishment and characterization of primary astrocyte culture from adult mouse. (A) The purity of adult mouse astrocytes was determined by staining with anti-Aldoc and Hoechst (n = 3; cells = 1.000-1,500 for each condition). **(B)** Analysis of the proliferation of cultured adult mouse astrocytes (n = 3; cells = 1.000-1,500 for each condition). **P*<0.05, ***P*<0.01 by ANOVA Tukey's post hoc test. Scale bar = 50 μm.

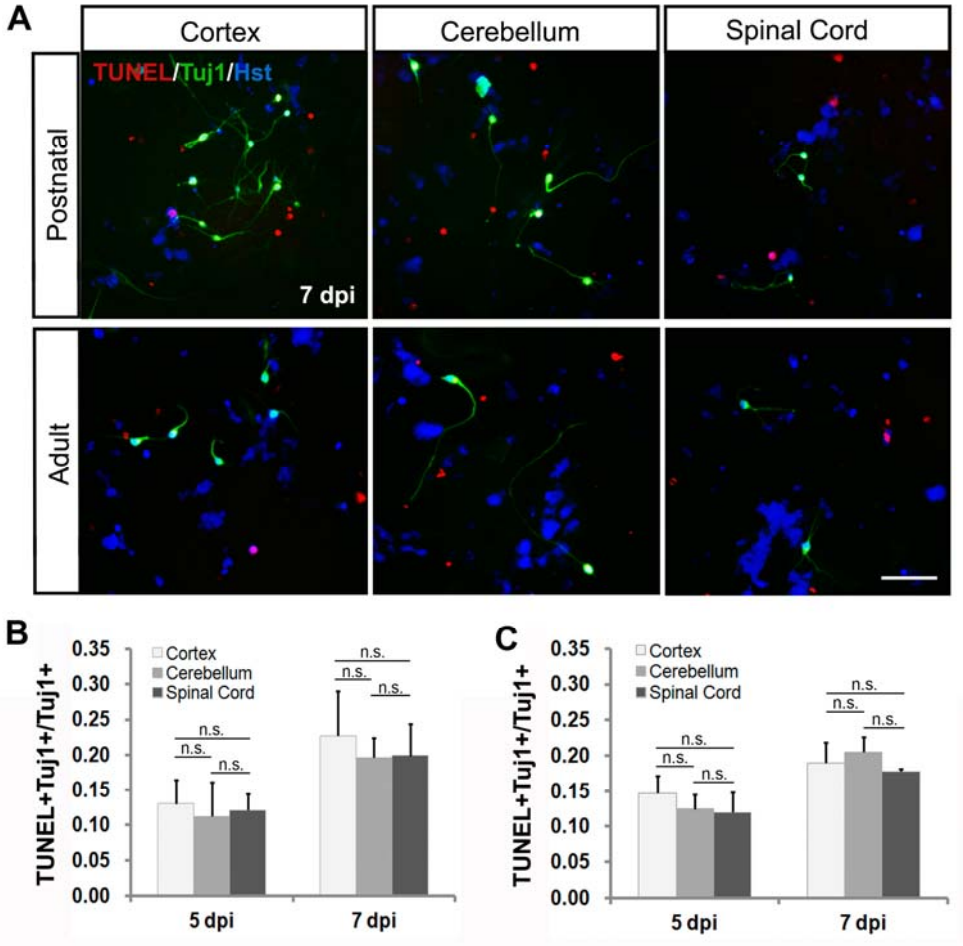

Figure S2 Neuronal apoptosis was quantitatively analyzed by TUNEL staining. (A) Representive micrographs of TUNEL (Cat: 12156792910, Roche) staining in NGN2-induced neurons from postnatal and adult astrocytes at 7 dpi. **(B and C)** Histograms showing the percentage of NGN2-induced neurons from postnatal (B) and adult (C) astrocytes as indicated at 5 and 7 dpi (n = 3; cells = 200-600 for each condition). $P \geq 0.05$, no statistically significant difference (n.s.); ANOVA Tukey's post hoc test. Scale bar = 100 μm.

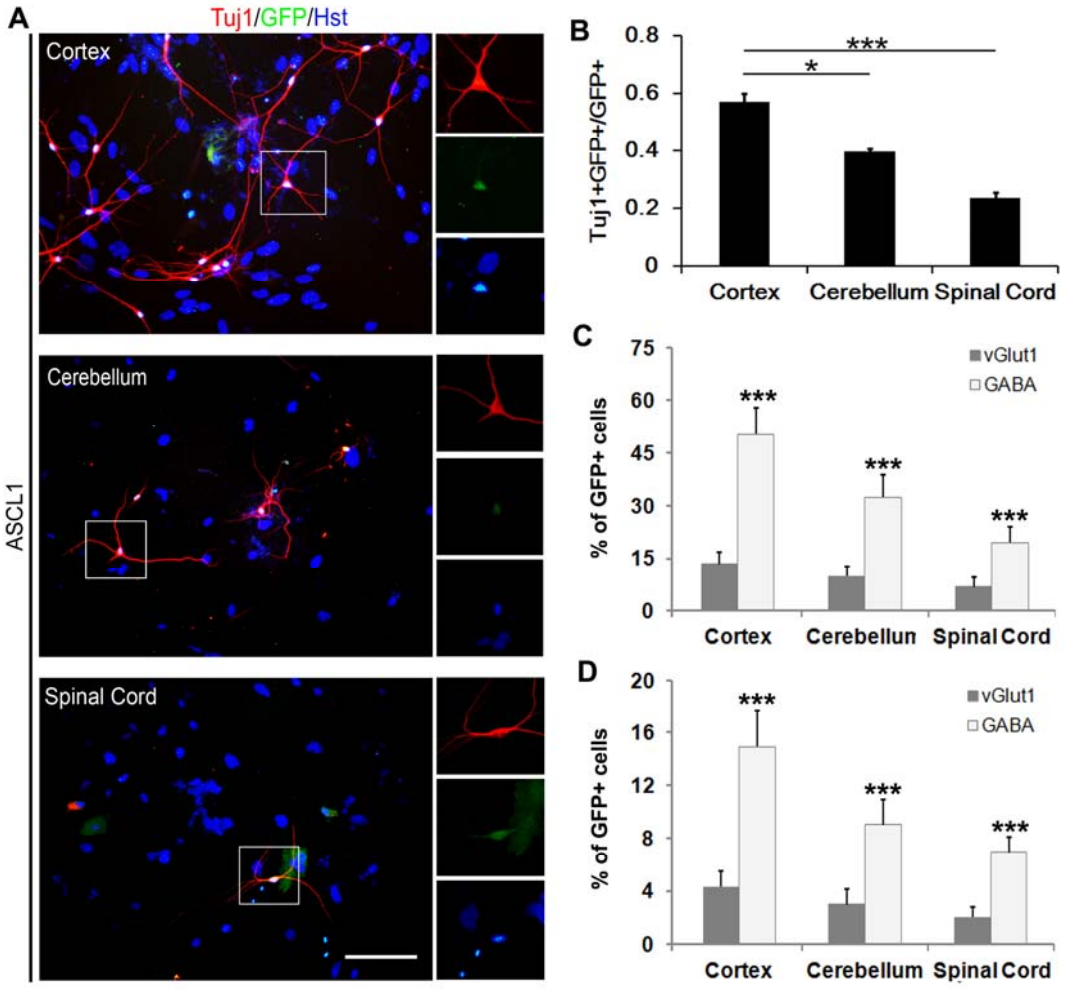

Figure S3 The ASCL1-induced neuronal reprogramming of postnatal astrocytes. (A) Representative pictures of ASCL1-induced neurons from postnatal astrocytes by staining with Tuj1 at 12dpi. **(B)** ASCL1-induced neuronal reprogramming efficiency of postnatal astrocytes (n = 3; cells = 900-1,500 for each condition). **P*<0.05, ****P*<0.001 by ANOVA Tukey's post hoc test. **(C and D)** ASCL1-induced neurons mainly matured into GABAergic neurons. Quantification of GABAergic and glutamatergic neurons reprogrammed from different region-derived postnatal (C) or adult (D) astrocytes at 18 dpi (n = 3; cells = 300-600 for each condition). ****P*<0.001 by Student's t test. Scale bar = 100 μm.

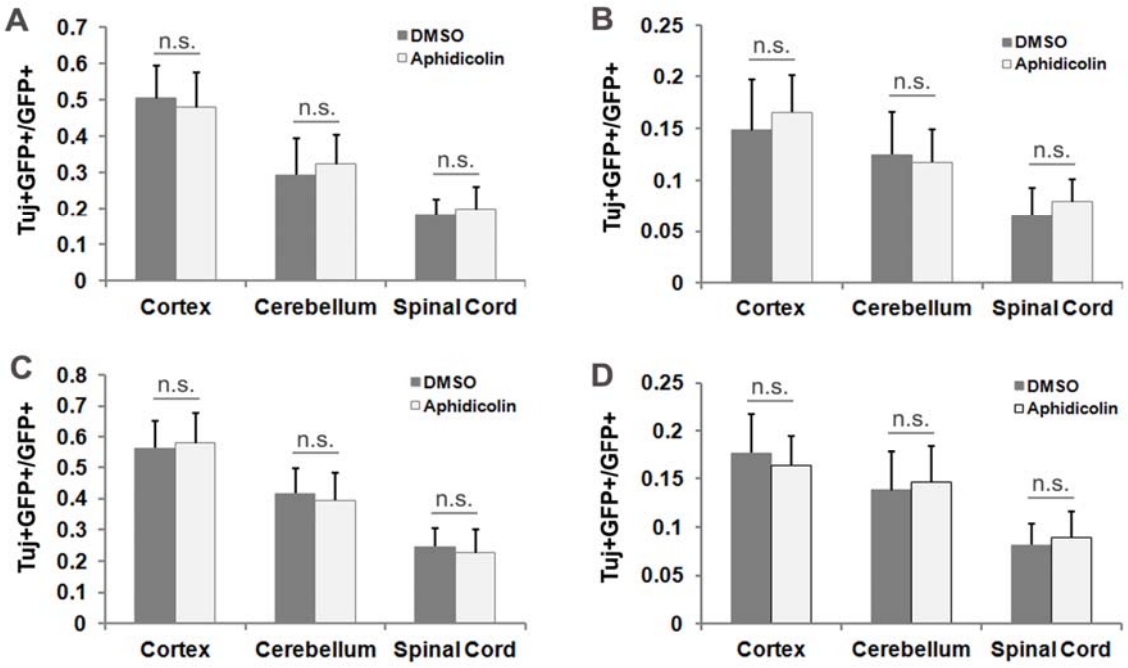

Figure S4 Effect of astrocyte proliferation on the neuronal reprogramming efficiency of region-restrict astrocytes. (A and B) The cultured postnatal (A) and adult (B) astrocytes were treated with aphidicolin (Cat: ab142400, Abcam; 5 μg/mL, dissolved in DMSO) to block cell proliferation. DMSO treatment was used as control. At 12 dpi, the efficiency of NGN2-induced neuronal reprogramming was analyzed in aphidicolin group compared with DMSO group (n = 3; cells = 700-1,400 for each condition). **(C and D)** After treatment of cultured postnatal (C) and adult (D) astrocytes with aphidicolin, the efficiency of ASCL1-induced neuronal reprogramming was analyzed between aphidicolin and DMSO groups (n = 3; cells = 700-1,500 for each condition) at 12 dpi. $P \geq 0.05$, no statistically significant difference (n.s.); Student's t test.

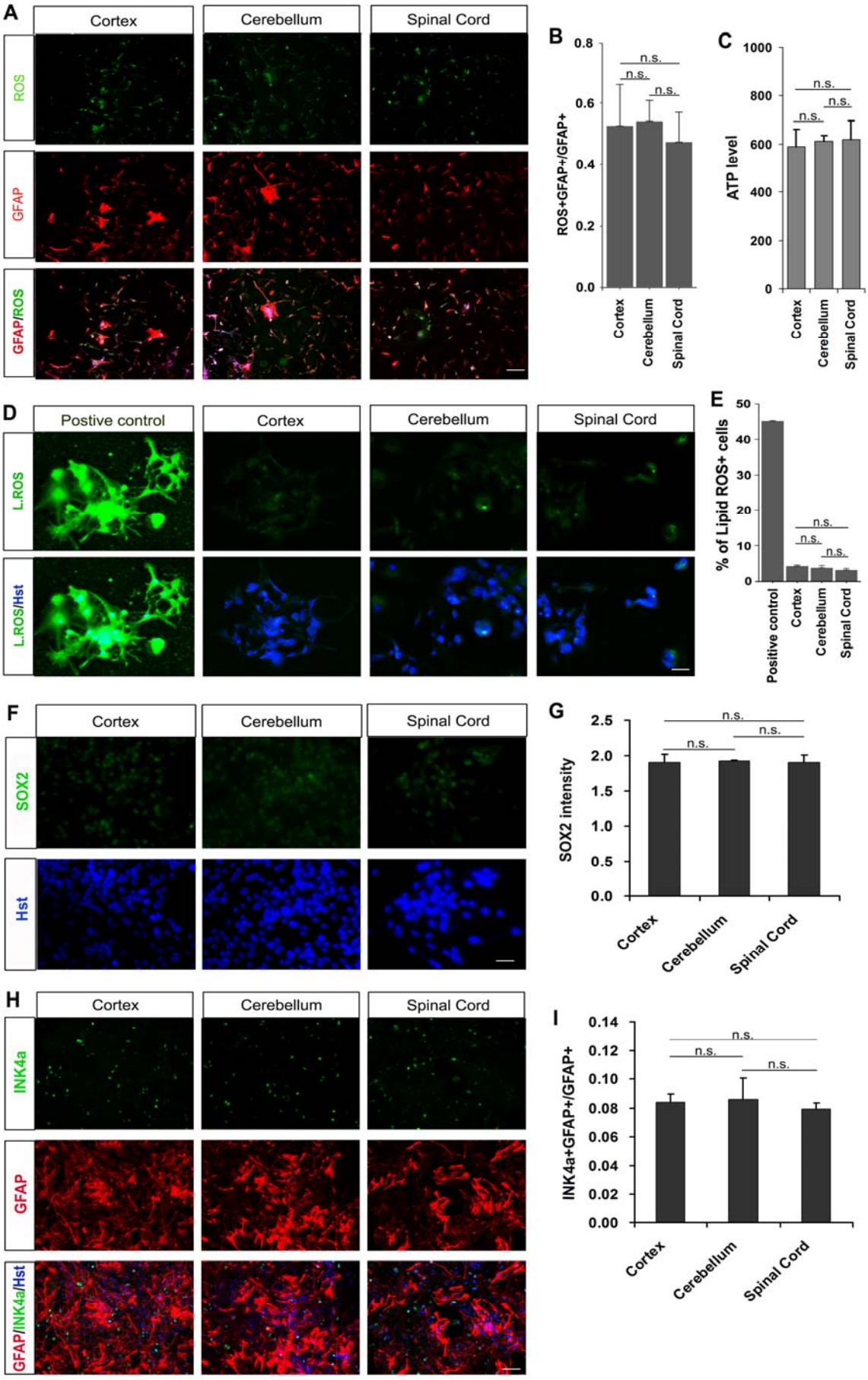
A
Cortex
Cerebellum
Spinal Cord
ROS
GFAP
GFAP/ROS
B
ROS+GFAP+/GFAP+
0.8
0.6
0.4
0.2
0.0
n.s.
n.s.
n.s.
Cortex
Cerebellum
Spinal Cord
C
ATP level
1000
800
600
400
200
0
n.s.
n.s.
n.s.
Cortex
Cerebellum
Spinal Cord
D
Postive control
Cortex
Cerebellum
Spinal Cord
L.ROS
L.ROS/Hst
E
% of Lipid ROS+ cells
50
40
30
20
10
0
n.s.
n.s.
n.s.
Positive control
Cortex
Cerebellum
Spinal Cord
F
Cortex
Cerebellum
Spinal Cord
SOX2
Hst
G
SOX2 intensity
2.5
2.0
1.5
1.0
0.5
0.0
n.s.
n.s.
n.s.
Cortex
Cerebellum
Spinal Cord
H
Cortex
Cerebellum
Spinal Cord
INK4a
GFAP
GFAP/INK4a/Hst
I
INK4a+GFAP+/GFAP+
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
n.s.
n.s.
n.s.
Cortex
Cerebellum
Spinal Cord

Figure S5 Analysis of the metabolic status and molecular expression of astrocytes. (A and B) The general ROS in postnatal astrocytes was detected with CellROX Oxidative Stress Reagent (Cat: C10448, Molecular probes) (n = 3; cells = 300-500 for each condition). **(C)** ATP levels of postnatal astrocytes were measured with ATP Assay Kit (Cat: S0026, Beyotime). **(D and E)** Analysis of lipid peroxidation in postnatal astrocytes with The Click-iT Lipid peroxidation Kit based on linoleamide alkyne (LAA) reagent (Cat: C10446, Life technologies) (n = 3; cells = 200-500 for each condition). Treatment of astrocytes with 100 μM cumene hydroperoxide was used as positive control. **(F-I)** Analysis of the expression of SOX2 and INK4a in astrocytes. Immunocytochemical analysis of SOX2 (Cat: AB5603A4, Milipore; F and G) and INK4a (Cat: Ab211542, Abcam; H and I) was performed in cultured postnatal astrocytes from cortex, cerebellum and spinal cord (G and I, n = 3, cells = 500-800 for each condition). $P \geq 0.05$, no statistically significant difference (n.s.); ANOVA Tukey's post hoc test. Scale bar: 100 μm (A, H), 50 μm (D, F).

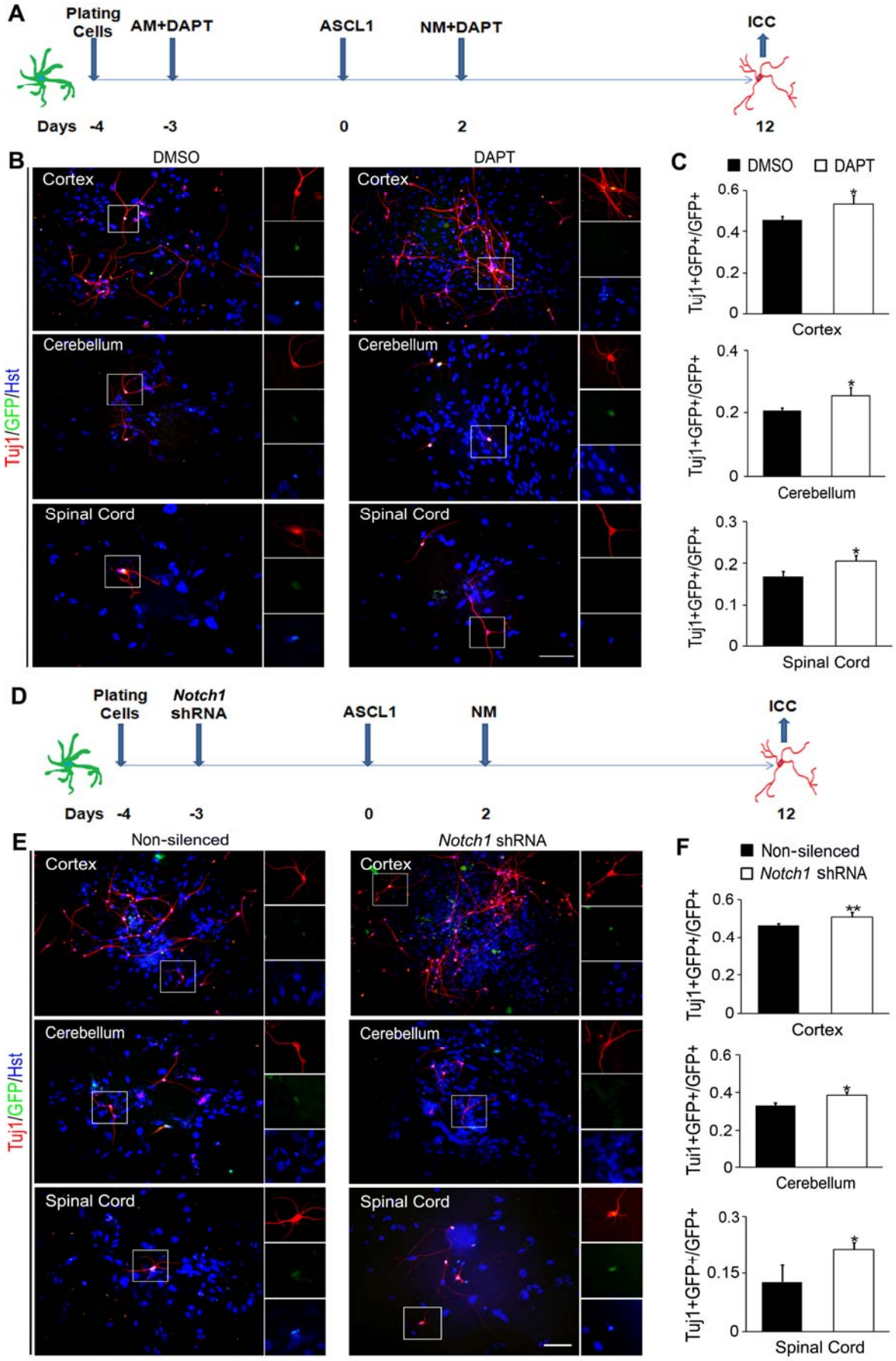
A
Plating Cells
AM+DAPT
ASCL1
NM+DAPT
ICC
Days -4
-3
0
2
12
B
DMSO
DAPT
Cortex
Cerebellum
Spinal Cord
Tuj1/GFP/Hst
C
DMSO
DAPT
Tuj1+GFP+/GFP+
Cortex
Cerebellum
Spinal Cord
D
Plating Cells
Notch1 shRNA
ASCL1
NM
ICC
Days -4
-3
0
2
12
E
Non-silenced
Notch1 shRNA
Cortex
Cerebellum
Spinal Cord
Tuj1/GFP/Hst
F
Non-silenced
Notch1 shRNA
Tuj1+GFP+/GFP+
Cortex
Cerebellum
Spinal Cord

Figure S6 Effects of blocking NOTCH1 signaling on ASCL1-mediated neuronal reprogramming. (A-C) DAPT treatment increased the efficiency of ASCL1-induced neuronal reprogramming from postnatal astrocytes. (A) Experimental scheme of blocking NOTCH1 signaling in astrocytes by DAPT during neuronal reprogramming. (B) Representative pictures of ASCL1-induced neurons from postnatal astrocytes through staining with Tuj1 at 12dpi. (C) The efficiency of ASCL1-induced neuronal reprogramming in DAPT group compared with control group (n = 3; cells = 900-1,500 for each condition). *P<0.05 by ANOVA Tukey's post hoc test. **(D-F)** Interference of Notch1 signaling in astrocytes increased ASCL1-induced neuronal reprogramming efficiency. (D) Experimental scheme of *Notch1* knock-down in astrocytes by lentivirus-mediated shRNA during neuronal reprogramming. (E) Representative pictures of ASCL1-induced neurons from postnatal astrocytes by staining with Tuj1 at 12dpi. (F) The efficiency of ASCL1-induced neuronal reprogramming in *Notch1* shRNA group compared with control group (n = 3; cells = 900-1,600 for each condition). *P<0.05, **P<0.01 by ANOVA Tukey's post hoc test. Scale bar = 100 μm.

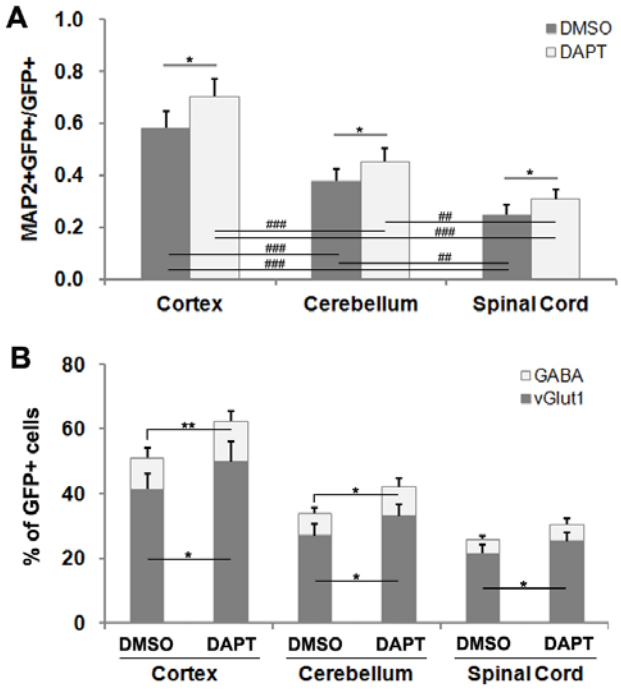

Figure S7 DAPT treatment did not affect the maturation and cell subtype of NGN2-induced neurons from postnatal astrocytes. (A) After treated with DMSO or DAPT, quantitative analysis of mature neurons induced from different region-derived postnatal astrocytes was performed at 18 dpi (n = 3; cells = 800-1,200 for each condition). Although DAPT treatment resulted in an increase in the induced MAP2-positive mature neurons, just as the distinct reprogramming efficiency observed in astrocytes cultured from cortex, cerebellum and spinal cord, the ratio of MAP2-positive neurons was still highest in cortex group and lowest in spinal cord group, suggesting that the maturation of induced neurons was not affected by DAPT treatment. **(B)** Neuronal subtype was analyzed at 18 dpi (n = 3; cells = 300-600 for each condition). DAPT treatment resulted in an increase in both vGlut1^{+} and GABA^{+} neurons, while astrocytes derived from cortex, cerebellum and spinal cord were all still mainly reprogrammed into vGlut1^{+} neurons, suggesting that the specification of induced neurons was not affected by DAPT treatment. **P*<0.05, ***P*<0.01 by Student's t test; $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001 by ANOVA Tukey's post hoc test.

Table S1 Gene primer sequences for quantitative real-time PCR

Gene	Primer sequence (5'-3')
Gfap	sense: GAAACCAACCTGAGGCTGGA antisense: CCACATCCATCTCCACGTGG
Aldoc	sense: CGGAGACCATGACCTCAAAC antisense: GGTCACTCAGGGCCTTGTAT
Aldh1l1	sense: CCAGCCTCCCAGTTCTTCAA antisense: GGACATTGGGCAGAATTCGC
CD44	sense: TCTGCCAGGCTTTCAACAGT antisense: CTGCACAGATAGCGTTGGGA
Glast	sense: CTCACGGTCACTGCTGTCAT antisense: TCCTCATGAGAAGCTCCCCA
Glt-1	sense: ATCACTGCTCTGGGAACTGC antisense: ACGAATCTGGTCACACGCTT
Vimentin	sense: CCAGAGAGAGGAAGCCGAAA antisense: GGTCAAGACGTGCCAGAGAA
P53	sense: TCCAGCCTAGAGCCTTCCAA antisense: TCAGGCCCCACTTTCTTGAC
Shh	sense: AGACCCAACTCCGATGTGTTC antisense: ATATAACCTTGCCTGCCGCTG
Bmp4	sense: CCCGGAAGCTAGGTGAGTTC antisense: AATCCCATCAGGGACGGAGA
Wnt3a	sense: CTCGCATGGCATAGATGGGT antisense: ACGTAGCAGCACCAATGGAA
Nrpb1	sense: ATTGGTGCAGTCAGCATGGA antisense: CCTACAGCTACGACCGCTTT
Etv5	sense: GAGTGGCCGCTCAGGAGTA antisense: AACCCATCCATGGTGCTTCC
Notch1	sense: TGTGGCTTCCTTCTACTGCG antisense: CTTTGCCGTTGACAGGGTTG
Numb	sense: CAACACTGCTCCATCCCCAT antisense: AATCCCCGGAAAGAGCCTTG
Hes1	sense: CAGCCAGTGTCAACACGACAC antisense: TCGTTCATGCACTCGCTGAG
Nrarp	sense: CCTCGCACTTAGGAAGGGAAGGGGACGC antisense: GGACAGCCAGTGACGCTCCAGCACCTC
Gapdh	sense: AAATGGTGAAGGTCGGTGTG antisense: AGGTCAATGAAGGGGTCGTT

Supplemental Experimental Procedures

ROS level detection

The CellROX Oxidative Stress Reagent (Cat: C10448, Molecular probes) were used to measure the ROS levels of region-restrict astrocytes, in which the green fluorescence and nuclear localization were acquired via DNA binding upon oxidation (Gascon et al. 2016). Postnatal astrocytes were cultured in 24-well plates with coverslips. The CellROX Reagent was added into the cultured medium at a final concentration of 5 μM. The green signal was analyzed within 24 hours.

Detection of lipid peroxidation

The Click-iT Lipid peroxidation Kit based on linoleamide alkyne (LAA) reagent (Cat: C10446, Life technologies) was used to detect the lipid peroxidation of region-restrict astrocytes (Gascon et al. 2016). The cells were labeled according to the specifications described in the product data sheet. After fixed with 4% PFA, the direct fluorescence of the reporters was detected with an Olympus fluorescence microscope.

ATP level measurement

ATP Assay Kit (Cat: S0026, Beyotime) was used to measure the ATP levels of region-restrict astrocytes (Wang et al. 2007). Briefly, astrocytes cultured in 12-well plates were lysed with 100 μl Lysis buffer for each well, and the lysate were then centrifuged by 12000g for 5min, finally producing the supernatant liquor for next investigation. To prepare for the measurement of ATP standard curve, the standard solution in the kit was diluted into several reference samples with different concentration in the following: (0, 0,03 μM, 0.1 μM, 0.3 μM, 1 μM, 3 μM). 100 μl ATP test reagent was added into every 1.5 ml-tube, waiting for 5min RT to consume the ATP in background, and then the cell lysate or reference samples was added into each tube. After full mixing, the RLU value of each sample was measured using the luminometer (GLOMAX E5311) and plot the standard curve for calculating the ATP value.

Supplemental References

Gascon S, Murenu E, Masserdotti G, Ortega F, Russo GL, Petrik D, Deshpande A, Heinrich C, Karow M, Robertson SP and others. 2016. Identification and Successful Negotiation of a Metabolic Checkpoint in Direct Neuronal Reprogramming. Cell Stem Cell 18(3):396-409.

Wang YM, Pu P, Le WD. 2007. ATP depletion is the major cause of MPP+ induced dopamine neuronal death and worm lethality in alpha-synuclein transgenic C. elegans. Neurosci Bull 23(6):329-35.