Research Article

Lineage tracing of direct astrocyte-to-neuron conversion in the mouse cortex

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Abstract

Regenerating functional new neurons in the adult mammalian central nervous system has been proven to be very challenging due to the inability of neurons to divide and repopulate themselves after neuronal loss. Glial cells, on the other hand, can divide and repopulate themselves under injury or diseased conditions. We have previously reported that ectopic expression of NeuroD1 in dividing glial cells can directly convert them into neurons. Here, using astrocytic lineage-tracing reporter mice (Aldh1l1-CreER^{T2} mice crossing with Ai14 mice), we demonstrate that lineage-traced astrocytes can be successfully converted into NeuN-positive neurons after expressing NeuroD1 through adeno-associated viruses. Retroviral expression of NeuroD1 further confirms that dividing glial cells can be converted into neurons. Importantly, we demonstrate that for *in vivo* cell conversion study, using a safe level of adeno-associated virus dosage (10^{10}–10^{12} gc/mL, 1 μL) in the rodent brain is critical to avoid artifacts caused by toxic dosage, such as that used in a recent bioRxiv study (2×10^{13} gc/mL, 1 μL, mouse cortex). For therapeutic purpose under injury or diseased conditions, or for non-human primate studies, adeno-associated virus dosage needs to be optimized through a series of dose-finding experiments. Moreover, for future *in vivo* glia-to-neuron conversion studies, we recommend that the adeno-associated virus results are further verified with retroviruses that mainly express transgenes in dividing glial cells in order to draw solid conclusions. The study was approved by the Laboratory Animal Ethics Committee of Jinan University, China (approval No. IACUC-20180330-06) on March 30, 2018.

Key Words: adeno-associated viruses; astrocyte; dosage; glia-to-neuron conversion; *in vivo* reprogramming; lineage tracing; neuron; retrovirus

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Introduction

The idea of directly converting one type of cells into another cell type for tissue regeneration has fascinated biologists for decades (Zhou and Melton, 2008). Transcription factor MyoD has been first reported to convert dermal fibroblasts, chondroblasts and retinal pigmented epithelial cells into contracting muscle cells (Choi et al., 1990; Murry et al., 1996; Lattanzi et al., 1998). Similarly, transcription factor C/EBP reprograms B lymphocytes into macrophages (Xie et al., 2004), while transcription factor Math1 transforms non-sensory cells into hair cells in the ear (Zheng and Gao, 2000; Izumikawa et al., 2005). Neural transcription factor NeuroD has been reported to convert embryonic ectoderm cells into neurons in *Xenopus* (Lee et al., 1995). The cell transdifferentiation field entered into a new era after the success of Shinya Yamanaka and his colleagues show reprogramming of fibroblast cells into induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Okita et al., 2007; Takahashi et al., 2007). In particular, using combinations of transcription factors and small molecules, many labs around the globe have been able to directly convert different types of cells into neurons both *in vitro* and *in vivo*. For example, Vierbuchen et al. (2010) converted skin fibroblast cells into neurons using transcription factors Ascl1, Brn2, and Myt1l. Shortly after that, many somatic cells such as fibroblasts, hepatocytes, pericytes, astrocytes, and peripheral T cells in cell culture have been successfully trans-differentiated into various subtypes of induced neurons including but not limited to glutamatergic, GABAergic, dopaminergic, motor neurons, and retinal neurons *in vitro* (Berninger et al., 2007; Addis et al., 2011; Caiazzo et al., 2011; Marro et al., 2011; Pfisterer et al., 2011; Encinas et al., 2011; Yoo et al., 2011; Giorgetti et al., 2012; Karow et al., 2012; Ladewig et al., 2012; Liu et al., 2013; Xue et al., 2013; Li et al., 2015; Zhang et al., 2015; Tanabe et al., 2018; Heinrich et al., 2010; Victor et al., 2014; Colasante et al., 2015). As for *in*

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vivo reprogramming, our group has previously reported that a single neural transcription factor NeuroD1 can convert reactive glial cells into fully functional neurons in mouse brains with injury or Alzheimer's disease (Guo et al., 2014). More recently, we demonstrated that NeuroD1 adeno-associated viruses (AAV)-based gene therapy can regenerate and protect a large number of functional neurons to restore brain functions after ischemic injury in adult mice (Chen et al., 2020). We also reported that AAV-mediated expression of NeuroD1 and Dlx2 can reprogram striatal astrocytes into GABAergic medium spiny neurons and hence improve the motor functions and extend the life span in Huntington's disease mouse models (Wu et al., 2020). In another attempt to reprogram glial cells into neurons, researchers converted astrocytes into neuroblasts with transcription factor Sox2 and then further differentiated them into neurons in mouse brain and spinal cord (Niu et al., 2013, 2015; Su et al., 2014; Islam et al., 2015; Wang et al., 2016). Many other groups have also successfully transdifferentiated glial cells into neurons *in vivo* through ectopic expression of Ascl1 (Liu et al., 2015) or combinations of transcription factors such as Ascl1 + Lmx1a + Nurr1 (Torper et al., 2015; Pereira et al., 2017), or Ascl1 + Sox2 (Heinrich et al., 2014), or Neurogenin-2 + Bcl-2 (Gascón et al., 2016), or Neurogenin-2 plus growth factors fibroblast growth factor 2 and epidermal growth factor (Grande et al., 2013). A mixture of NeuroD1, Ascl1, Lmx1a, and microRNA 218 transformed mouse astrocytes into dopaminergic neurons (Rivetti di Val Cervo et al., 2017). In addition, overexpression of Ascl1 in mouse retina also converted Müller glia into inner retinal neurons in both young and adult mice with N-methyl-d-aspartate damage (Ueki et al., 2015; Jorstad et al., 2017), and application of Otx2, Crx and Nrl after β-catenin expression could reprogram Müller glia into rod photoreceptors which restored lost vision in adult mice (Yao et al., 2018). Different from overexpression of transcription factors, Qian et al. (2020) recently reported that knockdown of the RNA-binding protein Ptbp1 in the substantia nigra can convert midbrain astrocytes into dopaminergic neurons and restore motor functions in Parkinson's disease mouse model. Surprisingly, Zhou et al. (2020) reported that striatal astrocytes can also be converted into dopaminergic neurons by CRISPR-mediated Ptbp1 knockdown, which has been disputed by Qian et al. (2020). Taken together, direct glia-to-neuron conversion has been successfully achieved both *in vitro* and *in vivo*, through overexpression of neural transcription factors or knockdown of RNA-binding protein Ptbp1. Given such broad success both *in vitro* and *in vivo*, it is rather surprising that Wang et al. (2020) challenged the entire field of glia-to-neuron conversion based on one set of experiments using a high dose of AAV (2×10^{13} gc/mL, 1 μL) that produced apparent artifacts in the mouse cortex. The current study aims at clarifying the confusion about the leakage *versus* conversion caused by highly toxic level of AAV used by Wang et al. (2020). We demonstrate here that lineage traced-astrocytes can be successfully converted into neurons by NeuroD1 in Aldh1l1-CreER^{T2} mice crossed with Ai14 mice. We further employ retrovirus to express NeuroD1 in dividing glial cells to unambiguously demonstrate *in vivo* glia-to-neuron conversion. Together, our astrocytic lineage-tracing study provides strong support that brain internal glial cells can be directly converted into neurons *in situ*.

Materials and Methods

Animals

Fifteen male and 15 female mice aged 8–10 weeks were used in this experiment. The wild-type C57BL/6J mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), Aldh1l1-CreER^{T2} transgenic mice (031008) and Ai14 knock in mice (#007914) were from Jackson Laboratory. All animals were housed in a 12 hours light/dark cycle and supplied with sufficient food and water. All the experiments were approved by the Laboratory Animal Ethics Committee of Jinan University, China (approval No. IACUC-20180330-06) on March 30, 2018.

Virus information

Single strand adenovirus-associated viral (ssAAV, AAV for short) vector hGFAP::Cre and FLEX-CAG::mCherry were constructed as previously described (Chen et al., 2020), and used for Cre experiment. A short version of hGFAP promoter (681 bp) was also used in this study (Lee et al., 2008) for the lineage tracing experiment. AAV serotype 9 (AAV9) and 5 (AAV5) were produced by PackGene® Biotech, LLC, purified through iodixanol gradient ultracentrifuge and subsequent concentration. Purified AAV viruses were tittered using a quantitative PCR-based method. All AAV used in this study was prepared in 0.001% Pluronic F-68 solution (Poloxamer 188 Solution, PFL01-100ML, Caisson Laboratories, Smithfield, UT, USA). Retroviral vector CAG::NeuroD1-IRES-GFP were constructed, packaged and concentrated as previously described (Chen et al., 2020) for the retrovirus experiment.

Mouse model of ischemic injury and virus injection

Endothelin-1 was injected into motor cortex of the adult WT C57BL/6J mice to create a focal ischemic injury as described (Chen et al., 2020), for the Cre experiment. Briefly, the mice were anesthetized with 20 mg/kg 1.25% Avertin (a mixture of 12.5 mg/mL of 2,2,2-tribromoethanol and 25 μL/mL 2-methyl-2-butanol; Sigma, St. Louis, MO, USA) through intraperitoneal injection and then placed in a prone position in the stereotaxic frame. 1 μL of endothelin-1 (1 μg/μL dissolved in phosphate buffer saline (PBS)) was injected at the following coordinate: + 0.2 mm anterior-posterior, ± 1.5 mm medial-lateral, 1.2 mm dorsal-lateral at the speed of 100 nL/min. After injection, the pipette was kept in place for about 10 minutes and then slowly withdrawn. Seven days later, 1 μL of virus mixture AAV9 hGFAP::Cre (1×10^{10} gc/mL) and FLEX-CAG::mCherry (1×10^{12} gc/mL) was injected at the same coordinates.

For intact mouse cortex, 1 μL of retroviruses CAG::NeuroD1-IRES-GFP (1×10^{7} TU/mL) or 1 μL of AAV5 GFAP::NeuroD1 (1×10^{12} gc/mL) were injected at the similar coordinates described above.

Immunofluorescence

The mice were anesthetized with 1.25% Avertin and then sequentially perfused intracardially first with saline solution (0.9% NaCl) and then with 4% paraformaldehyde. The brains were collected and post-fixed in 4% paraformaldehyde overnight and sequentially placed in 20% and 30% sucrose at 4°C until the tissue sank. The dehydrated brains were embeded in Optimal Cutting Temperature (Tissue-Tek® O.C.T. Compound, Sakura® Finetek, Torrance, CA, USA), and then serially sectioned at the coronal plane on the cryostat (Thermo Scientific, Shanghai, China) at 30 μm thickness. For immunofluorescence, free floating brain sections were first washed with PBS and blocked for 1 hour at room temperature in 5% normal donkey serum, 3% bovine serum albumin and 0.3% TritonX-100 prepared in PBS, and then incubated overnight at 4°C with primary antibodies diluted in blocking solution. After additional washing with 0.2% PBST (0.2% Tween-20 in PBS), the samples were incubated with 4′,6-diamidino-2-phenylindole (DAPI; F. Hoffmann-La Roche, Natley, NJ, USA) and appropriate donkey anti-mouse/rabbit/rat/chicken secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (1:1000, Life technologies, Carlsbad, CA, USA) for 2 hours at room temperature, followed by extensive washing with PBS. Samples were finally mounted with VECTASHIELD® mounting medium (VECTOR Laboratories, Burlingame, CA, USA) and sealed with nail polish. Representative Images were taken with confocal microscope (LSM880, Zeiss, Jena, Germany).

Primary antibodies used were listed as follows: rabbit anti-

GFAP (a marker for astrocytes, 1:1000, Cat# Z0334, DAKO), rabbit anti-NeuN (a marker for neurons 1:1000, Cat# ab177487, Abcam, Cambridge, Massachusetts, USA), rabbit anti-S100β (a marker for astrocytes, 1:500, Cat# ab52642, Abcam), mouse anti-Cre recombinase (1:500, Cat# MAB3120, Millipore), chicken anti-mCherry (1:1000, Cat# ab205402, Abcam), rabbit anti-Sox9 (1:500, Cat# AB5535, Millipore).

Results

Direct astrocyte-to-neuron conversion revealed through astrocytic lineage tracing using Aldh1l1-CreER^{T2} mice

Our group has previously published a series of articles on NeuroD1-mediated direct glia-to-neuron conversion both *in vitro* and *in vivo* using retroviruses or AAV system (Guo et al., 2014; Zhang et al., 2018; Ge et al., 2019; Puls et al., 2019; Chen et al., 2020; Liu et al., 2020; Wu et al., 2020). Recently, we have further demonstrated that overexpression of NeuroD1, or Neurogenin2, or Ascl1 can directly convert glioma cells into neurons (Wang et al., 2020). However, a recent study raised some doubt on *in vivo* glia-to-neuron conversion after observing some artifacts with the use of highly toxic level of AAV (Wang et al., 2020). Here, to clarify the confusion surrounding *in vivo* glia-to-neuron conversion, we performed astrocytic lineage tracing experiments to provide unambiguous evidence on the neuronal conversion from lineage-traced astrocytes. A transgenic mouse line Aldh1l1-CreER^{T2} has been reported before to be an excellent mouse line for the purpose of astrocytic lineage tracing with minimal leakage into neurons (Srinivasan et al., 2016). We crossed Aldh1l1-CreER^{T2} mice with Ai14 mice and administered tamoxifen to induce Cre-mediated recombination so that some of the astrocytes are lineage-traced as tdTomato-positive cells (**Figure 1A**, schematic illustration of experimental design). AAV5 GFAP::NeuroD1 was injected into one side of the brain to induce astrocyte conversion, and the contralateral side without viral injection served as the control. As expected, the tdTomato-labeled cells in non-viral infected cortex (contralateral side) were immunopositive for astrocyte markers Sox9 (**Figure 1B**) and GFAP/S100β (**Figure 1F**). In contrast, in AAV5 GFAP::NeuroD1-infected cortex, some of the tdTomato-labeled cells lost astrocytic markers such as Sox9 (**Figure 1D**) or GFAP/S100β signal (**Figure 1H**), and displayed typical neuronal morphology. Quantitative analysis revealed that in the contralateral control side, tdTomato-labeled cells were 88% Sox9$^+$ (**Figure 1C**) and 98% GFAP/S100β$^+$ (**Figure 1G**), indicating that they are essentially all astrocytes determined by the Aldh1l1-lineage tracing model. In contrast, in NeuroD1-injected side, the percentage of Sox9$^+$ cells among the tdTomato-labeled cells dropped to 42% (**Figure 1C**) and GFAP/S100β$^+$ cells dropped to 35% (**Figure 1G**), suggesting that the number of astrocytes decreased after NeuroD1 infection (**Figure 1E** and **I**). Where did the tdTomato-labeled astrocytes go? Did they disappear or did they convert into neurons?

To answer this question, we performed immunostaining with neuronal marker NeuN to investigate potential neuronal identity of some of the tdTomato-labeled cells in NeuroD1-infected cortex (**Figure 2A–C**). We first demonstrated that NeuroD1 expression was clearly detected in tdTomato-labeled astrocytes at 7 days post viral injection (**Figure 2B**), confirming that our AAV5 GFAP::NeuroD1 viruses did infect astrocytes as expected. Interestingly, by 135 days post NeuroD1 viral infection, many NeuroD1-infected tdTomato-labeled cells were NeuN$^+$ and showed clear neuronal morphology with long neuronal processes (**Figure 2C2**), whereas in the contralateral control side the tdTomato-labeled cells were still in astrocytic morphology with numerous short processes and rarely colocalized with NeuN (**Figure 2C1**). Quantitative analysis found that about half of the tdTomato-labeled astrocytes in the NeuroD1 group had converted into NeuN$^+$ neurons (**Figure 2D**, control group, < 1% NeuN$^+$; NeuroD1 group, 48% NeuN$^+$). Accordingly, the total number of tdTomato-labeled neuronal cells increased significantly compared to the contralateral side (**Figure 2E**). Together, these astrocytic lineage tracing experiments demonstrate unambiguously that astrocytes can be directly converted into neurons by NeuroD1 *in situ*, consistent with our series of publications in recent years (Guo et al., 2014; Zhang et al., 2018; Ge et al., 2019; Puls et al., 2019; Chen et al., 2020; Liu et al., 2020; Wu et al., 2020).

Neuronal conversion induced by retrovirus overexpressing NeuroD1 in dividing glial cells

While AAV has the advantage of low immunogenicity and relatively safe as a gene therapy vector for the treatment of neurological disorders, its capability to infect both neurons and glial cells may cause confusion if AAV dosing and promoter are not used properly. Therefore, if one's main research purpose is not to generate as many neurons as possible to treat certain neurological disorders, retroviruses that mainly target dividing glial cells may be a better choice to study basic molecular mechanisms of glia-to-neuron conversion. This is because neurons cannot divide and retroviruses cannot enter neuronal nuclei, only dividing glial cells can allow retroviruses enter glial nuclei to express transgene. Therefore, to avoid direct viral infection of neurons, retroviruses should be used if any confusion arises regarding AAV or lentivirus results. We have previously reported that retroviruses expressing NeuroD1 can convert dividing glial cells into neurons (Guo et al., 2014; Chen et al., 2020). Here, we provide further evidence using retrovirus, instead of AAV, to ectopically express NeuroD1 and convert glial cells into neurons (**Figure 3**). Specifically, at 6 days post retroviral injection, many cells infected by CAG::NeuroD1-GFP retroviruses showed typical astrocytic morphology with numerous short processes and GFAP signal (**Figure 3A** and **B**). By 14 days post viral injection, while some NeuroD1-GFP infected glial cells still showed glial morphology with GFAP signal (**Figure 3C**), other NeuroD1-GFP infected cells displayed clear neuronal morphology with dendritic tree pattern (**Figure 3D**). Note that many NeuroD1-GFP infected cells were also NeuN$^+$, although their signals were relatively weak compared to the non-converted preexisting neurons (**Figure 3A–D**), suggesting that these are newly converted immature neuronal cells. Since retroviruses cannot enter neuronal nuclei, these experiments further demonstrate that NeuroD1 can directly convert dividing glial cells into neurons.

AAV GFAP::Cre should express Cre in astrocytes not in neurons

It is perhaps not too difficult to understand that injecting too much viruses into the brain will cause toxic effects. However, it appears that not everyone knows the importance of viral dosing. A perfect example is the recent bioRxiv paper posted by Wang et al. (2020), where 2×10^{13} gc/mL × 1 μL AAV particles were injected into the mouse cortex, producing artifacts that led the authors to challenge the entire field of *in vivo* reprogramming. In fact, it has previously been reported that high dosing AAV can produce harmful effects on both neurons and glial cells in mammalian brains (Ortinski et al., 2010; Mingozzi and High, 2011; Rogers et al., 2011; Hinderer et al., 2014; Lavin et al., 2019; Xiong et al., 2019). In particular, Ortinski et al. (2010) has reported that high titre AAV will cause astrocytic gliosis and impair synaptic transmission. Xiong et al. (2019) has also reported AAV toxicity in the retina when using much lower dose than that used by Wang et al. (2020). Unfortunately, Wang et al. (2020) appeared to be unaware of these very important works in the field and conducted all their experiments based on a single toxic dosing of AAV (2×10^{13} gc/mL × 1 μL) in the mouse cortex. Wang et al. (2020) reported that when they injected AAV GFAP::Cre into the mouse brain, which should express Cre in GFAP$^+$ astrocytes under the control of astrocytic promoter GFAP, they instead observed Cre expression predominantly in neurons (see their

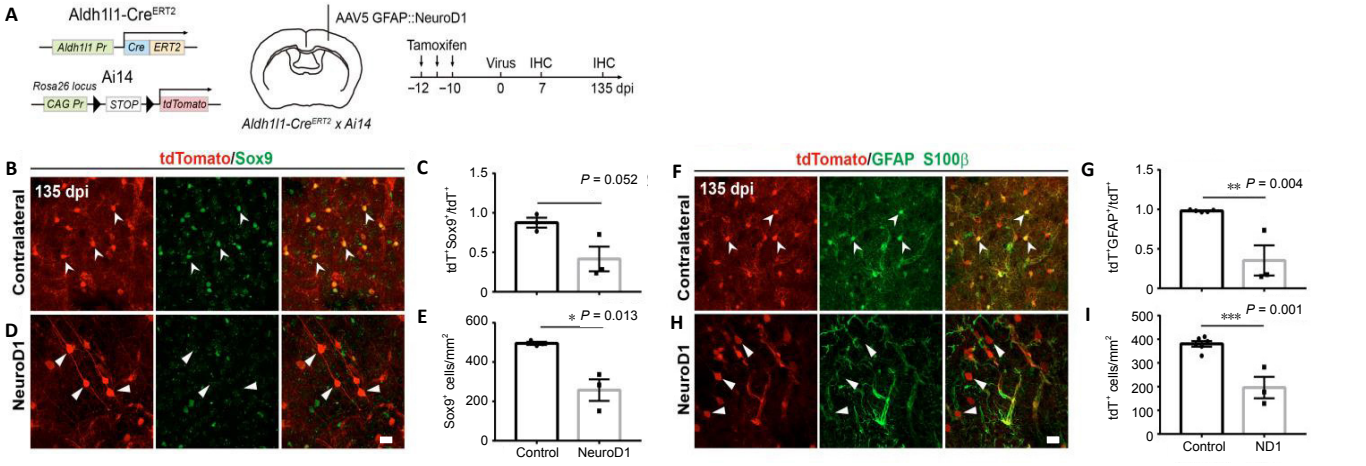
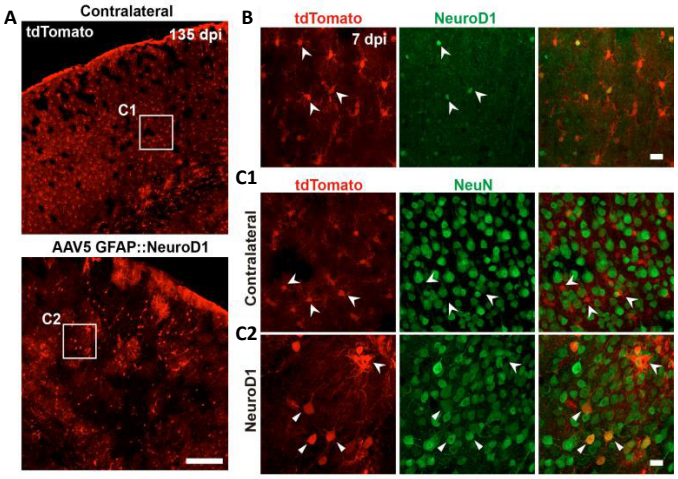


Figure 1 | **Lineage-traced astrocytes in Aldh1l1-CreER^{T2} mice crossed with Ai14 mice.**
(A) Schematic diagram illustrating the experimental design in this study. (B–E) Representative images illustrating astrocytic marker Sox9 among tdTomato (tdT)-labeled cells in non-infected contralateral cortex (B) and NeuroD1-infected side (D). Quantified data are shown in bar graphs (C, ratio of Sox9$^+$ cells among tdT$^+$ cells; E, total Sox9$^+$ cells in local NeuroD1-converted areas or contralateral side). n = 3 for both the control group and the NeuroD1-infected group. *P < 0.05, Student's t-test. (F–I) Representative images illustrating astrocytic marker GFAP/S100b among tdT-labeled cells in non-infected contralateral cortex (F) and NeuroD1-infected side (H). Quantified data are shown in bar graphs (G, ratio of GFAP$^+$/S100b$^+$ cells among tdTomato$^+$ cells; I, total tdT$^+$ cells in local NeuroD1-converted areas or contralateral side). Scale bar: 20 μm. n = 5 for the control group, n = 3 for the NeuroD1-infected group. **P < 0.01, ***P < 0.001, Student's t-test. dpi: Day post injection; GFAP: glial fibrillary acidic protein; IHC: immunohistochemistry.



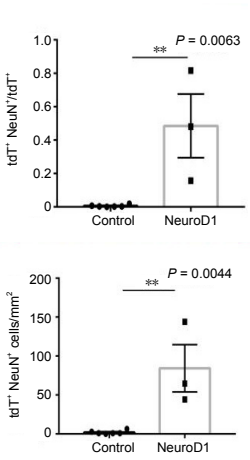
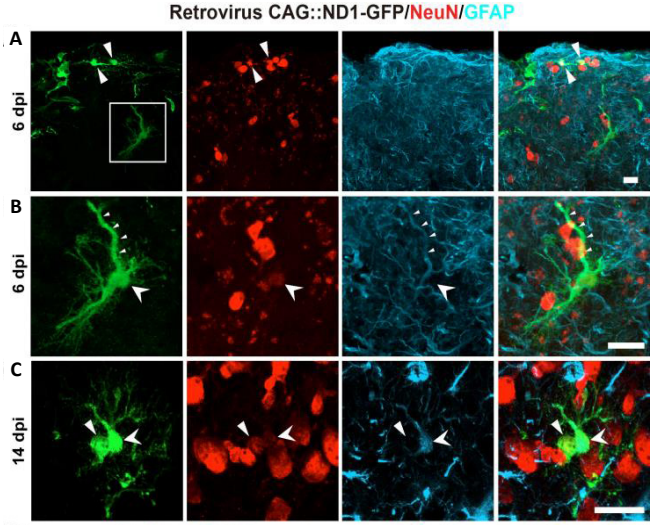


Figure 2 | **NeuroD1 converts lineage-traced astrocytes into neuronal nuclei (NeuN)$^+$ neurons.**
(A) tdTomato(tdT)-labeled cells in non-infected contralateral cortex (top panel) and AAV5 NeuroD1-infected cortex (bottom panel) in Aldh1l1-CreER^{T2} x Ai14 mice. Scale bar: 200 μm. (B) NeuroD1 expression detected in tdTomato$^+$ cells in Aldh1l1-CreERT2 X Ai14 mice at early days of AAV infection (7 dpi, arrowheads). Scale bar: 20 μm. (C1) In contralateral cortex without viral injection (Box C1 in panel A), tdT-labeled cells were NeuN negative, as expected. (C2) In NeuroD1-infected cortex (Box C2 in panel A), some tdT-labeled cells were NeuN positive and showed clear neuronal morphology. Scale bar for C1 and C2: 20 μm. (D, E) Quantitative analysis revealed that NeuN and tdT double-positive cells increased significantly in the NeuroD1-infected group, compared to the contralateral control side. n = 5 for the control group, n = 3 for the NeuroD1-infected group. **P < 0.01, Student's t-test. dpi: Day post injection.



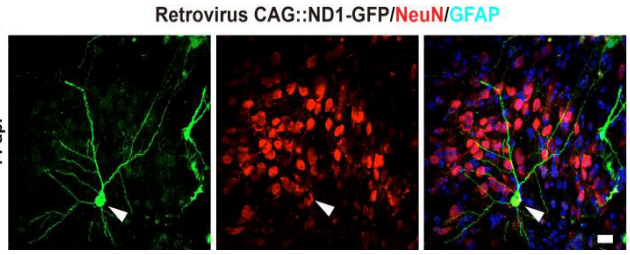


Figure 3 | **Retroviral expression of NeuroD1 in dividing glial cells further demonstrates *in vivo* glia-to-neuron conversion.**
(A–B) Representative images illustrating retrovirus-infected cells at early stage (6 days post viral injection, 6 dpi) showing typical astrocytic morphology with numerous fine processes and immunostained with glial fibrillary acidic protein (GFAP) signal. A few NeuroD1-GFP infected cells also showed weak neuronal nuclei (NeuN) signal (arrowhead). Scale bars: 20 μm. (C, D) By 14 dpi, some of the NeuroD1-GFP infected cells were still going through a transitional stage with co-localized signal of GFAP and NeuN together (C), whereas other NeuroD1-GFP infected cells had converted into NeuN$^+$ neurons with typical neuronal morphology (D). Scale bar: 20 mm. Retroviruses were CAG::NeuroD1-GFP (1 × 10^7 TU/mL, 1 μL).

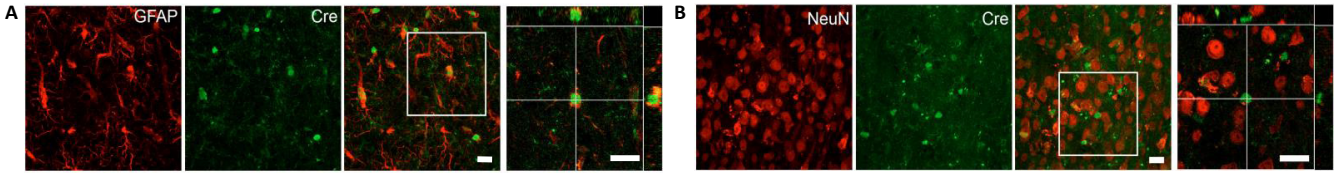


Figure 4 | **Cre recombinase expression in glial fibrillary acidic protein (GFAP)$^+$ astrocytes but not neuronal nuclei (NeuN)$^+$ neurons after infected by adeno-associated virus serotype-9 (AAV9) GFAP::Cre in the mouse cortex.**
(A) Representative high-resolution confocal images showing colocalization of Cre recombinase with GFAP signals at 14 days post viral injection (14 dpi). AAV9 hGFAP::Cre at 1×10^{10} gc/mL + FLEX-CAG::mCherry at 1×10^{12} gc/mL, 1 μL in mouse cortex. (B) Representative high-resolution confocal images showing that Cre recombinase was not colocalized with NeuN signals (14 dpi) . Scale bars: 20 μm.

Figure 3, 14 days post viral injection). Typically, when one sees such abnormal result, one would immediately lower the AAV dosing and repeat the experiments until find the right dosing so that GFAP::Cre is properly expressed in GFAP$^+$ astrocytes. However, it is surprising that the authors continued their experiments with such high level of AAV which is toxic to the central nervous system as reported before (Ortinski et al., 2010; Rogers et al., 2011; Mingozzi and High, 2011; Hinderer et al., 2014; Lavin et al., 2019; Xiong et al., 2019). Therefore, it is not surprising that Wang et al. (2020) would observe their Cre expression and other transgenes in neurons, an artifact that is directly linked to the toxic level of viruses they used.

Such artifacts caused by high dosing of AAV reported by Wang et al. (2020) can be easily avoided through the use of lower AAV dosage. We have performed many GFAP::Cre experiments and never observed such high expression level of Cre transgene in neurons, because we usually use much lower dosing AAV to express Cre in astrocytes (10^{10}–10^{11} gc/mL, 1 μL, in rodents). **Figure 4** illustrates a typical example of Cre expression in astrocytes (GFAP/S100b$^+$), but not in neurons (NeuN$^+$) at 14 days post AAV injection (AAV9 hGFAP::Cre at 1×10^{10} gc/mL + FLEX-CAG::mCherry at 1×10^{12} gc/mL, 1 μL). The Cre recombinase was typically detected in the nucleus of astrocytes, and surrounded by astrocytic marker protein GFAP, which is a filamentous protein in the cytoplasm of astrocytes (**Figure 4**). Moreover, Cre signal was clearly separated from NeuN in our experiments (**Figure 4**), which should be the right pattern because Cre expression is controlled by GFAP promoter. Together, it is critical to design experiments properly using a right dosage of AAV before starting any experiments.

Discussion

In recent years, many groups have used AAV-mediated ectopic expression of transcription factors or knockdown of PTBP1 to convert resident glial cells into neurons. However, Wang et al. (2020) used a rather high dosage of AAV (10–1000 folds higher than that used in our lab or other labs) to challenge the field of *in vivo* reprogramming. In this work, we provide unambiguous data through lineage tracing experiments that NeuroD1 can convert brain internal astrocytes into neurons *in situ*. Moreover, the AAV results are further substantiated by retroviruses that only express transgenes in dividing glial cells.

Given the artifacts arising from a prominent lab, we feel that it is important to suggest a few rules to follow regarding how to conduct and evaluate *in vivo* glia-to-neuron conversion work:

1) For any factor(s) that is claimed to be capable of converting glial cells into neurons, we recommend conducting both *in vitro* and *in vivo* studies and use both retrovirus and AAV (or lentivirus) to unambiguously demonstrate the glia-to-neuron conversion. Note that, AAV is great for *in vivo* work but not for *in vitro* cultured astrocytes (low infection efficiency). For cultured astrocytes, retroviruses work much more efficiently.

2) One must test different doses of the delivery vehicles (viral or non-viral) to find optimal dosing for certain experiments. In particular, the toxic effects of high dosing should be tested because it is obvious that our brain cannot tolerate a huge amount of viral particles (or non-viral particles).

3) One must take a wholistic view on the entire *in vivo* glia-to-neuron conversion field before focusing on one single experiment, which can be an artifact produced by a specific person.

NeuroD1-induced astrocyte-to-neuron conversion through lineage tracing

Wang et al. (2020) reported their lineage tracing experiments by crossing tamoxifen-inducible Aldh1l1-CreER^{T2} transgenic mice with a reporter line (R26R-YFP or Ai14) to trace astrocytes labeled by YFP. We have conducted almost the same experiments using Aldh1l1-CreER^{T2} mice crossed with a different reporter line Ai14. Surprisingly, while we report here clear astrocyte-to-neuron conversion through astrocyte lineage-tracing experiments, Wang et al. (2020) reached opposite conclusion of not detecting NeuN$^+$ neurons. Comparison between these two studies immediately identified the difference in terms of the timing when results were reported after NeuroD1 AAV injection: we found clear evidence of neuronal conversion from tdTomato-traced astrocytes at 135 days post AAV NeuroD1 injection (experiment delayed by COVID-19); while Wang et al. (2020) stopped short of their experiments at 28 days post AAV NeuroD1 injection. We have already informed the senior author of Wang et al. (2020) about our lineage tracing results, and look forward to seeing their updated data at longer time points after NeuroD1 expression. In fact, even in the data of Wang et al. (2020), the morphology of the NeuroD1-infected YFP-traced astrocytes was obviously different from that of the control group. In their NeuroD1 group (Wang et al. 2020; Figures 5F and 6F), the NeuroD1-infected YFP-traced astrocytes displayed clear morphological changes toward neuronal like structures with many fine processes retracted in comparison to their control group. It is rather astonishing that the authors of Wang et al. (2020) would ignore such evident morphological changes and abruptly ended their experiments at 28 days post NeuroD1 AAV infection. We sincerely hope that Wang et al. (2020) will soon provide data at 60–100 days after NeuroD1 infection to clarify whether those morphologically changed YFP-traced astrocytes will eventually become NeuN$^+$ neurons.

AAV *versus* retrovirus

AAV has been approved by FDA for various clinical trials due to its relatively low immunogenicity, and some gene therapy products based on AAV have been marketed for therapeutic use. However, since AAV particles can be produced in very high titre, as high as 10^{13}–10^{14} gc/mL, extra caution should be taken in carrying out the AAV infection experiments. In the gene therapy field, it is well-known that AAV dosing is critical when considering how much AAV should be administered into the body. For the brain, because AAV can infect both neurons and glial cells, it is even more important to use minimal effective dosing of AAV to avoid any brain damage. The artifacts produced by Wang et al. (2020) due to the use of high titre of AAV send an alarming signal to the entire field

of *in vivo* reprogramming that AAV dosage should be carefully controlled and a series of different doses must be tested to avoid similar artifacts arising again.

Because AAV can infect both dividing and non-dividing cells, another safeguard is to use retroviruses that only express transgenes in dividing cells, which are glial cells in the central nervous system. Any strange results obtained from AAV infection should be double checked using retroviruses in order to prevent wrong conclusions being reached. In general, if someone has any doubt on whether certain transcription factor(s) can convert glial cells into neurons or not, then using retroviruses to express the specific transgene(s) only in dividing glial cells should be a safe way to unambiguously test glia-to-neuron conversion without the complication of AAV.

Virus dosing is critical to avoid artifacts

Injecting high dosage of AAV into the brain may result in some artifacts that are counter intuitive. Wang et al. (2020) injected 1 μL of 2×10^{13} gc/mL AAV5 GFAP::Cre into the mouse cortex and found that the Cre signal was predominantly detected in neurons instead of astrocytes. It is certainly difficult to understand why their Cre expression was found in neurons under the control of astrocytic promoter GFAP. In their discussion part, they attributed this phenomenon to exosomes or tunneling nanotubes induced by some uncertain genetic manipulations (Wang et al., 2020). While this is one possibility, a more straightforward explanation is the toxic effects to neurons caused by high dosage of virus administration. In our previous studies, we proved that $10^{10–11}$ gc/mL at 1 μL of AAV GFAP::Cre (only 1/100 to 1/1000 of the dosage used by Wang et al. (2020) was sufficient to trigger Cre-mediated recombination in the mouse cortex and striatum (Chen et al., 2020; Wu et al., 2020). More importantly, as repeated here in this study, Cre expression was restricted to astrocytes in low dose as it should be. It is incomprehensive why Wang et al. (2020) applied 1000-fold higher dosage of GFAP::Cre without questioning their own data of Cre expression in neurons. One should have investigated immediately why injecting GFAP::Cre would result in the expression of Cre in neurons. If lowering AAV dosage, one would have found the right answer quickly without falsefully challenging the field of *in vivo* reprogramming based on one set of improperly designed experiments.

How to interpret the BrdU data in a right way?

Quiescent or resting astrocytes are more resistant to cell conversion compared to reactive astrocytes, which explains why previous studies targeted more on reactive astrocytes for *in vivo* reprogramming (Grande et al., 2013; Guo et al., 2014; Gascón et al., 2016; Wang et al., 2016; Brulet et al., 2017; Rivetti di Val Cervo et al., 2017; Ge et al., 2019; Chen et al., 2020; Qian et al., 2020; Wu et al., 2020; Zhou et al., 2020). However, Wang et al. (2020) used BrdU-incorporation experiment to declare that converted neurons were not derived from $BrdU^+$ reactive astrocytes, largely due to their poorly designed experiments for BrdU-labeling. The major flaw of their BrdU experiment is that they have administered BrdU for such a long-time span of weeks after AAV NeuroD1 injection, leading to a large number of $BrdU^+$ astrocytes that have never had a chance to be infected by AAV NeuroD1. Therefore, they of course could not detect many $BrdU^+$ neurons. The right experiment should be to inject AAV NeuroD1 at the end of their BrdU labeling in order to convert many BrdU-labeled astrocytes into neurons. BrdU labeling should be stopped immediately after AAV injection to prevent wrong conclusions.

How to understand the puzzle of neuronal density not changed after conversion?

Wang et al. (2020) were puzzled by the fact that after astrocyte-to-neuron conversion, there was no significant increase of neuronal density. We have essentially observed the same phenomenon in non-injured non-diseased mouse brains. However, in injured brains with substantial neuronal loss, we always detect a significant increase of neuronal density across the entire injury/diseased areas. In fact, from the data presented by Wang et al. (2020), the tissue repair is so obvious in their Figure 2C and E and their Figure 6F, as shown by significantly reduced cortical tissue loss in the NeuroD1 group compared to their control group, which is also consistent with our reported findings (Lavin et al., 2019). Wang et al. (2020) ignored the apparent tissue repair in the center of lesion core in the NeuroD1 group, and asked why neuronal density did not increase significantly in the less injured surrounding areas. This is actually similar to our findings in the mouse striatum of Huntington's disease model where the neuronal density did not change much after conversion but the overall striatal atrophy was alleviated (Wu et al., 2020). Wang et al. (2020) assumed that the neuronal density should increase after conversion, but they probably did not realize that their highly toxic AAV dosage already damaged many neurons, eventually leading to a balance between the newly converted neurons and the loss of preexisting neurons. We hypothesize that there should be some kind of homeostatic control to keep the neuron density in certain brain regions relatively constant to maintain normal functions, which surely warrants further studies.

Besides neuronal density, there is also some concern in the field that astrocyte-to-neuron conversion might lead to the depletion of astrocytes in the converted areas. Fortunately, we have never observed depletion/absence of astrocytes in NeuroD1-converted areas in mouse, rat, and monkey brains. In fact, the results from Wang et al. (2020) confirmed our observations that astrocytes were not depleted in NeuroD1-expressed areas, consistent with the notion that astrocytes are dividing cells with proliferative capability (Barnabé-Heider et al., 2010; Burda and Sofroniew, 2014). Our recent study detected more proliferative astrocytes ($Ki67^+$) in the converted areas, indicating that astrocytes can repopulate themselves after some of the astrocytes being converted into neurons (Lavin et al., 2019; Wu et al., 2020).

Recommendation for future research

Given the fact that C-L Zhang's lab was among the early pioneers who reported *in vivo* glia-to-neuron conversion, the impact of the pre-print article of Wang et al. (2020) would pose grave danger to the field of *in vivo* reprogramming if their flawed design and wrong interpretations were not corrected immediately. While it is up to every single scientist to make his or her own judgement, we do want to reiterate the importance of using different types of viral vectors, different dose, and perform both *in vitro* and *in vivo* experiments to prove or disprove any hypothesis regarding glia-to-neuron conversion. We do have every reason to believe that Wang et al. (2020) might have good intention to raise a potential problem to the field, but such hasty deposit of improperly designed experiments based solely on one single high dosing of AAV without verification by retrovirus and *in vitro* studies, should be highly discouraged in future studies.

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