

Limited astrocyte-to-neuron conversion in the mouse brain using NeuroD1 overexpression

The central nervous system (CNS) is largely unable to generate new neurons to compensate for the loss of neurons caused by disease or injury. One potentially promising approach to replace CNS neurons is to convert resident astrocytes into neurons *in situ* by gene therapy. Conversion of astrocytes to neurons *in vitro* by overexpression of the transcription factor *Pax6* was first reported nearly 20 years ago.¹ Since then, multiple groups have reported astrocyte-to-neuron conversion *in vitro* and *in vivo* following various genetic manipulations.^{2–11} Recently, overexpression of NeuroD1^{12–17} and knockdown of the RNA splicing factor *Ptbp1*^{18,19} were assessed for their ability to convert astrocytes to neurons *in vivo* in multiple CNS areas, with therapeutic effects in mouse models of disease, ischemia, and injury. However, key findings from this work were not readily reproduced,²⁰ prompting responses from the original authors^{21,22} and generating controversy around astrocyte-to-neuron conversion. Here we report limited, brain-area-specific astrocyte-to-neuron conversion in mice and compare our findings with recent reports.

We initially set out to test astrocyte-to-neuron conversion across the mouse CNS using systemic delivery of the blood-brain-barrier-penetrating AAV-PHP.eB capsid²³ and a shortened human *GFAP* promoter (hGFAP)²⁴ to restrict expression to astrocytes. We achieved improved broad expression in astrocytes with self-complementary AAV genomes²⁵ (Figures 1A–1C; Figure S1B) and confirmed that miR-124 targeting sites (miR-124-TSs)²⁶ in the 3' untranslated region (UTR) helped restrict expression to astrocytes (Figures S1A and S1B). However, the expression level was generally low, and we noted trace levels of off-target expression in neurons (Figures 1C and S1B) as well as scattered expression in the liver (data not shown). These results underscore the difficulty of targeting astrocytes specifically by systemic AAV injection.

We instead decided to permanently label astrocytes with tdTomato (tdTom) in *Aldh111*^{CreERT2}; *Rosa26*^{LSL-tdTom} mice²⁷ and deliver AAV1 vectors expressing conversion factors directly into the brain parenchyma. Tamoxifen administration in these mice led to tdTom expression in the vast majority of astrocytes, with only rare expression in neurons (data not shown). To test NeuroD1-mediated conversion, we designed expression cassettes with hGFAP promoters driving NeuroD1.P2A.EGFP or EGFP alone. Both cassettes included the 3'UTR miR-124-TS. We also designed an artificial microRNA²⁸ targeting *Ptbp1* (miPtbp1) and confirmed its activity *in vitro* (Figure S1C). We then cloned miPtbp1 or a control microRNA²⁹ (miControl) into the 3'UTR of a hGFAP-EGFP transgene. Four weeks after tamoxifen administration, we infused AAV1 vectors with each of these four transgenes unilaterally into the striatum, hippocampus, and cerebellum at 1×10^9 vector genomes (vg) per injection site (Figure 1D). We perfused the mice after 4 weeks and collected their brains for histology.

We next performed immunofluorescence for the neuronal marker NeuN to identify fate-mapped tdTom-positive cells that had converted to neuron-like cells. In mice treated with NeuroD1, we observed extensive tdTom/NeuN overlap in the hippocampus and cerebellar cortex but not in the striatum (Figures 1E and 1F). In the hippocampus, viral expression was largely confined to the molecular layers of the dentate gyrus (DG) and cornu ammonis 1 (CA1), and apparent conversion was entirely confined to these regions (Figure S1D). Concerningly, conversion in the cerebellar cortex was localized to regions with a damaged granule cell layer (Figure S1D). In the hippocampus and cerebellar cortex, conversion was always within a few hundred micrometers of the needle track. However, despite the proximity to the needle track, NeuN staining in the far-red channel in converted cells was not due to autofluorescence, which was punctate and faint (data not shown). In addition, the hGFAP promoter remained active in induced NeuN-positive cells, unlike in mature neurons, because we noted

continued EGFP expression (Figures 1E and 1F). In contrast to NeuroD1, we observed no evidence of astrocyte-to-neuron conversion in mice treated with the EGFP control virus (Figure S1E), miPtbp1 virus (Figure 1G; Figure S1D), or miControl virus (data not shown).

Although the NeuroD1 results were encouraging, apparent astrocyte-to-neuron conversion occurred in a narrow region close to the injection site. It has been proposed that reactive astrocytes may be more readily converted into neurons than nonreactive astrocytes.³⁰ Therefore, we decided to test astrocyte-to-neuron conversion in *Tpp1*^{-/-} mice,³¹ a model of late infantile neuronal ceroid lipofuscinosis. These mice develop progressive astrogliosis beginning by 9 weeks of age and some neurodegeneration by the end stage at 21 weeks.³² As expected, we observed elevated levels of the reactive astrocyte marker *Gfap* by 11 weeks of age that increased by 14 weeks (Figure S1F). We next infused the cerebral cortex, striatum, hippocampus, and cerebellum of 10-week-old *Tpp1*^{-/-} mice with 1×10^9 vg of the same viruses as above (Figure 1H). To identify converted neurons, we performed NeuN immunofluorescence and identified transduced astrocytes by EGFP expression. In NeuroD1-treated *Tpp1*^{-/-} mice, we detected no EGFP/NeuN overlap in the cerebral cortex (Figure 1I). In brain areas tested previously in *Aldh111*^{CreERT2}; *Rosa26*^{LSL-tdTom} mice, results were comparable in *Tpp1*^{-/-} mice; we observed no NeuN/EGFP overlap in the striatum but significant overlap in the molecular layers of the hippocampus and in the cerebellar cortex (Figure 1I). As above, cerebellar regions with apparent conversion were marked by a damaged granule cell layer, and we noted particularly strong *Gfap* expression in these areas (Figures 1I). We again saw no evidence of astrocyte-to-neuron conversion in *Tpp1*^{-/-} mice injected with EGFP control (Figure S1G) or miPtbp1 viruses (data not shown). Therefore, the astrogliosis in *Tpp1*^{-/-} mice did not enhance astrocyte-to-neuron conversion compared with wild-type mice.

In summary, we observed evidence of NeuroD1-mediated astrocyte-to-neuron conversion in the mouse hippocampus and cerebellar cortex but not in the cerebral cortex

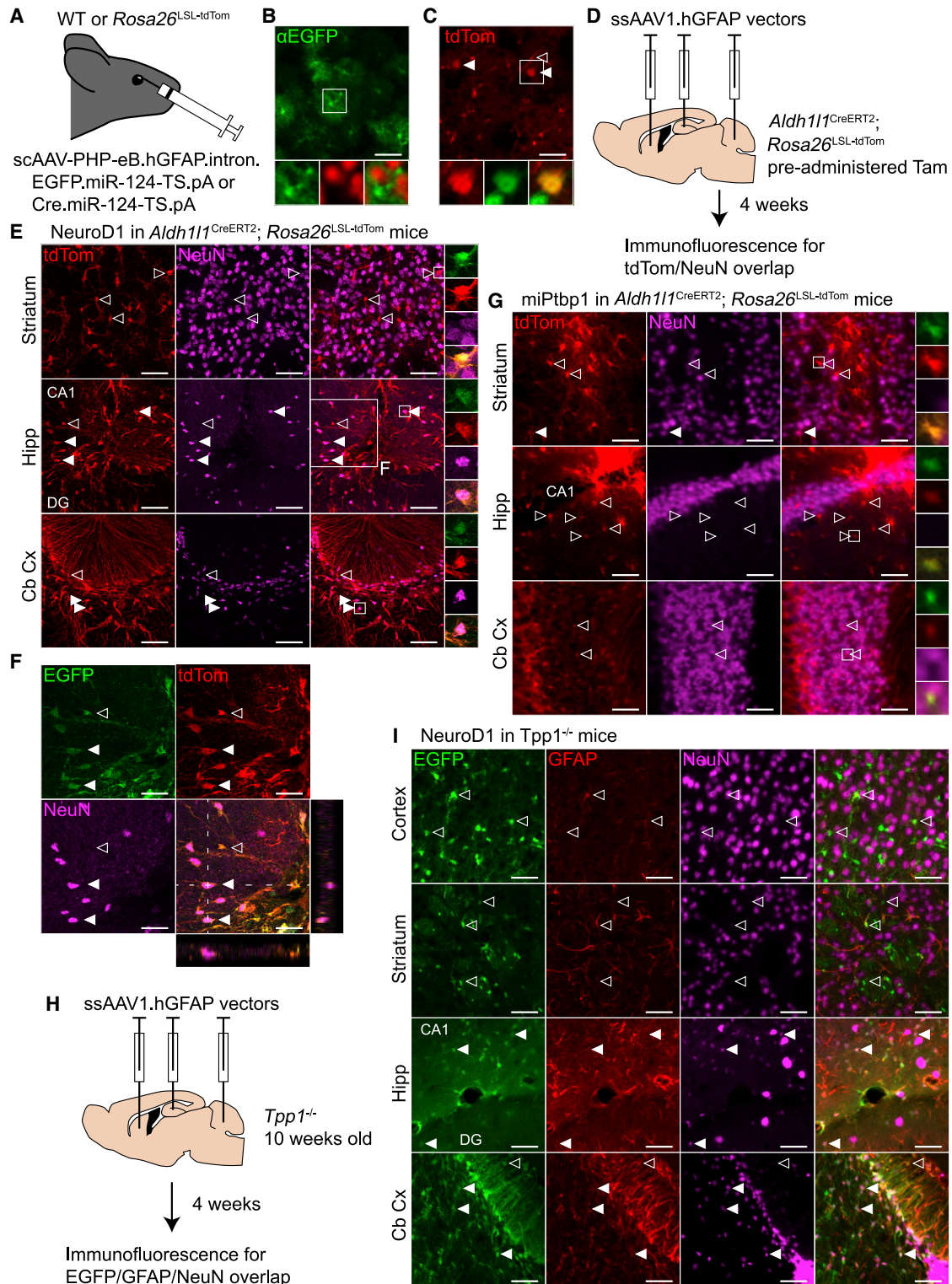


Figure 1. Overexpression of NeuroD1 induces NeuN expression in astrocytes in the mouse hippocampus and cerebellar cortex

(A) Optimized AAVs expressing EGFP (3.16×10^{11} vg) or Cre (1.00×10^{11} vg) from a hGFAP promoter were injected retro-orbitally into wild-type (WT) or *Rosa26*^{LSL-tdTom} mice, respectively. (B) Widespread but faint EGFP immunofluorescence (green, main and bottom left) did not co-localize with NeuN immunofluorescence (red, bottom center)

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or striatum. Where conversion was observed, it was limited spatially to within a few hundred micrometers of the injection site. This may be due to a localized dose effect or damage and inflammation along the needle track. In addition, converted cells were likely not fully mature neurons by 4 weeks after injection because the hGFAP promoter remained highly active in these cells. We did not follow converted cells for a longer time period because of the limited scope of conversion. In contrast to NeuroD1 overexpression, delivery of an artificial microRNA targeting *Ptbp1* did not lead to astrocyte-to-neuron conversion in any brain area, although we did not quantify *Ptbp1* knockdown *in vivo*. One caveat regarding our results is the small scale of this pilot study. However, the results were consistent across multiple animals in two mouse lines, including *Aldh111^{CreERT2}* mice, which enable robust fate mapping of astrocytes. We also note that *Aldh111* and *Gfap*, the genes on which our Cre and AAV reporter systems are based, are expressed in neural stem cells in the subgranular zone (SGZ), which normally generate granule cells in the DG granule cell layer. Thus, it is possible that neural stem cells, rather than astrocytes, gave rise to the apparent converted neurons in the hippocampus. This possibility is made less likely by the distance between the SGZ and the converted neurons, the farthest of which were across the hippocampal fissure in CA1, and the relatively short time frame of the experiments.

Published results on astrocyte-to-neuron conversion using NeuroD1 overexpression vary widely.^{12–14,16,17,30} In a recent publication, Wang et al.²⁰ propose that the *Neurod1*

DNA sequence upregulates GFAP promoter activity in neurons and that this can lead to spurious reports of astrocyte-to-neuron conversion. When they instead fate mapped astrocytes in *Aldh111^{CreERT2}; Rosa26^{LSL-tdTom}* mice, as we did above, they reported no NeuroD1-mediated conversion in the cerebral cortex and striatum. This is consistent with our results showing a lack of conversion in these brain areas but some conversion in the hippocampus and cerebellar cortex. However, Xian et al.²² do report conversion in the cerebral cortex and striatum in these mice. We cannot rule out the possibility of efficient conversion in the cerebral cortex and striatum with alternative viral vectors, a higher dose, or a longer time period after injection. Similar to NeuroD1-mediated conversion, widely varying efficiencies of astrocyte-to-neuron conversion by *Ptbp1* knockdown have been reported.^{18–20,33} The degree of *in vivo* knockdown of *Ptbp1* is difficult to compare between these studies and may be critical to achieve efficient conversion. Overall, we are concerned by how difficult it is to reproduce efficient astrocyte-to-neuron conversion. Additional work is clearly needed to verify the results of *in vivo* conversion experiments and establish the precise conditions that permit conversion.

Although astrocyte-to-neuron conversion may be possible, important questions remain unanswered regarding its therapeutic application. For us, NeuN expression was only induced in astrocytes very close to the injection site. Conversion on this scale is unlikely to have a therapeutic effect in the mouse brain and even less likely in the much larger human brain. Therefore, the route of administration and dose will have to be carefully

considered to optimize conversion while minimizing toxicity. Systemic delivery of AAVs may be an option for broader conversion, but this would likely require a high dose of AAVs and lead to unintended expression in the periphery, as noted above for AAV-PHP.eB in mice. If more widespread conversion is achievable, long-term studies using rigorous methods will be needed to determine whether induced NeuN-positive cells develop into mature neurons that can integrate into neural circuits and rescue disease phenotypes. Although these are major challenges, it may be premature to abandon astrocyte-to-neuron conversion without further investigation.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.ymthe.2022.01.028>.

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AUTHOR CONTRIBUTIONS

D.L. designed the research, performed experiments, and analyzed data. D.L. and A.M.M. designed the artificial microRNA targeting *Ptbp1*. Y.H.C. and A.M.M. designed the ITR block sequence. B.L.D. designed and supervised the research. D.L. and B.L.D. wrote the manuscript with input from all authors.

in the primary visual cortex after AAV administration in WT mice (n = 2). Scale bar, 50 μ m. (C) tdTom fluorescence (red, main and bottom left) and NeuN immunofluorescence (green, bottom center) in primary visual cortex following AAV administration in *Rosa26^{LSL-tdTom}* mice (n = 2). Solid arrowheads, tdTom- and NeuN-positive neurons; open arrowhead, tdTom-positive endothelial cell. Scale bar, 50 μ m. (D) ssAAV1 vectors with conversion factors or controls were infused into the striatum, hippocampus, and cerebellum in *Aldh111^{CreERT2}; Rosa26^{LSL-tdTom}* mice at a dose of 1×10^9 vg per site. Mice were perfused after 4 weeks for histology. (E) tdTom and NeuN immunofluorescence following NeuroD1 overexpression in the striatum, the molecular layers of the DG and CA1 in the hippocampus, and the cerebellar cortex (n = 2). All cells marked with arrowheads are tdTom+ and EGFP+. Closed arrowheads represent transduced, fate-mapped astrocytes that express NeuN. Scale bars, 50 μ m. (F) Magnification of the hippocampus in (E), showing co-localization of EGFP, dTom, and NeuN, with orthogonal views through a NeuN+ cell. Scale bars, 100 μ m. (G) Fluorescence images following miPtbp1 expression with the same channels and brain areas as in (E). Only a low, background level of tdTom/NeuN co-localization was observed (n = 1). Scale bars, 50 μ m. (H) 10-week-old *Tpp1^{-/-}* mice were infused with the same viruses as the *Aldh111^{CreERT2}; Rosa26^{LSL-tdTom}* mice, with an added infusion in the cerebral cortex above the hippocampus. Mice were perfused after 4 weeks for histology. (I) Immunofluorescence images from *Tpp1^{-/-}* mice (n = 3) treated with NeuroD1. Shown are EGFP (green, left), Gfap immunofluorescence, and NeuN immunofluorescence in the cerebral cortex, striatum, hippocampus, and cerebellar cortex. Scale bars, 50 μ m. In all panels, transduced cells that do and do not colocalize with NeuN are labeled with solid and open arrowheads, respectively. Tam, tamoxifen; Hipp, hippocampus; Cb Cx, cerebellar cortex; DG, dentate gyrus. See also Figure S1.

DECLARATION OF INTERESTS

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Reply to *In vivo* confusion over *in vivo* conversion

We strongly disagree with Dr. Gong Chen's criticisms of our recently published *Cell* paper¹ in his recent letter to the editor.²

In our publication,¹ we demonstrated that stringent lineage tracing is essential for *in vivo* studies on astrocyte-to-neuron conversion. As described in Dr. Chen's prior publications,^{3,4} we observed the same phenomenon: the adeno-associated virus (AAV)-expressed fluorescence reporter was detected predominantly in astrocytes in the control group and in neurons in the NEUROD1 group. On the basis of this phenomenon, Dr. Chen concluded that resident astrocytes were converted into neurons with perfect identity and connectivity by forced expression of NEUROD1. In contrast, our experiments using lineage mapping and retrograde tracing unambiguously showed that those viral reporter-labeled perfect neurons were in fact native neurons and were not derived from resident astrocytes.¹ We further showed that NEUROD1 mutants lacking neurogenic activity still induced neuronal expression of the viral reporter, ruling out the possibility of astrocyte-to-neuron conversion by forced expression of NEUROD1 in the adult mouse brain. Mechanistically, we showed that cell-type specificity of the promoter in AAVs could be altered by the downstream genes such as NEUROD1.

Regarding the issue of viral titers, Dr. Chen did not provide direct evidence that the viral reporter-labeled neurons in his publications originated from astrocytes rather than native neurons. Second, we used the same amount of virus for both the control and NEUROD1 groups; however, only the latter group showed robust leaky neuronal expression of the viral reporter, excluding a role for viral titers in such a phenomenon. This conclusion is further supported by our results following injections of a mixture of the NEUROD1 and control viruses, showing that the reporter from the NEUROD1 virus but not from the control virus was easily detected in native neurons. Third, NEUROD1-induced neuronal expression of the viral reporter was also detected in areas distant from the injection core and where the virus concentration is very low. Finally, "different groups use different methods to titer, and even for a given stock large differences can be reported, as was well illustrated in a trial where multiple groups measured the titer of the same stock, with differences of up to 100-fold reported."⁵ As such, the viral titers used by Dr. Chen, us, and other groups cannot be directly compared.

Similarly, Dr. Chen's assertion that "the Cre-loxP recombination...may have created a higher barrier for cell conversion" is not supported by any available evidence. Cre-loxP-mediated lineage tracing methods have been extensively used in biological research for decades. They were also suc-

cessfully applied by us and others to study cell conversions in the adult mouse brain and spinal cord.^{6–13} In Dr. Chen's study using the lineage tracing method,¹⁴ there were concerns about experimental design. As they did not use a reporter-expressing NEUROD1 virus as in all their other studies,^{3,4} there was no evidence to show that those genetically labeled neurons actually were derived from NEUROD1-transduced cells. We repeated Dr. Chen's experiment by using double reporters, one for the NEUROD1 virus and the other for the lineage-traced astrocytes.¹ Despite many viral reporter-positive neurons, none showed evidence of being derived from genetically lineage-traced astrocytes.¹ Additionally, we detected occasional leakage of the genetic reporter in neurons in the mouse line that was used by Dr. Chen.¹⁴ Such mice with neuronal leakage might explain Dr. Chen's observation of some genetically labeled neurons, but these mice should have been excluded from their study. Dr. Chen's claim of Cre toxicity in lineage tracing is self-contradictory, as his group has frequently used AAV-expressed Cre, which should be even more toxic because of higher copy numbers and expression.

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