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***In vivo* direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model**

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Summary

Loss of neurons after brain injury and in neurodegenerative disease is often accompanied by reactive gliosis and scarring, which are difficult to reverse with existing treatment approaches. Here, we show that reactive glial cells in the cortex of stab-injured or Alzheimer's disease (AD) model mice can be directly reprogrammed into functional neurons *in vivo* using retroviral expression of a single neural transcription factor, NeuroD1. Following expression of NeuroD1, astrocytes were reprogrammed into glutamatergic neurons, while NG2 cells were reprogrammed into glutamatergic and GABAergic neurons. Cortical slice recordings revealed both spontaneous and evoked synaptic responses in NeuroD1-converted neurons, suggesting that they integrated into local neural circuits. NeuroD1 expression was also able to reprogram cultured human cortical astrocytes into functional neurons. Our studies therefore suggest that direct reprogramming of reactive glial cells into functional neurons *in vivo* could provide an alternative approach for repair of injured or diseased brain.

Keywords

NeuroD1; reactive astrocytes; NG2 cells; reprogram; *in vivo*; Alzheimer's disease; brain injury; cortex; neurons

Introduction

Gliosis is a common pathological process after brain injury, involving the activation of glial cells to proliferate and become hypertrophic to occupy the injured brain areas (Pekny and Nilsson, 2005; Robel et al., 2011; Sofroniew and Vinters, 2010). Glial cells, including astrocytes, NG2 cells, and microglia, undergo reactive response to injury in order to form a defense system against the invasion of micro-organisms and cytotoxins into surrounding

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AUTHOR CONTRIBUTIONS

Ziyuan Guo performed the major part of the experiments and data analysis. Lei Zhang contributed significantly on brain injury experiments. Zheng Wu performed cortical slice recordings. Yuchen Chen made the GFAP promoter constructs and contributed to immunostaining. Fan Wang made viruses and contributed to immunostaining. Gong Chen conceived and supervised the entire project, analyzed the data and wrote the manuscript.

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tissue (Pekny and Nilsson, 2005; Robel et al., 2011; Sofroniew and Vinters, 2010). However, once activated, many reactive glial cells will stay in the injury sites and secrete neuroinhibitory factors to prevent neuronal growth, eventually forming glial scar inside the brain (Sofroniew and Vinters, 2010). Reactive glial cells have also been widely reported after stroke, spinal cord injury, glioma, and neurodegenerative disorders such as Alzheimer's disease (Gwak et al., 2012; Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010; Verkhratsky et al., 2010; Verkhratsky et al., 2012). However, despite substantial progress in understanding the molecular pathways of reactive gliosis (Robel et al., 2011), there has been little success in efforts to reverse glial scarring after its formation.

Reprogramming adult skin fibroblasts into pluripotent stem cells has opened a new field for potential stem cell therapy (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). Many studies have since demonstrated trans-differentiation across different cell lineages, including reprogramming mouse or human fibroblasts directly into neurons (Ambasudhan et al., 2011; Caiazzo et al., 2011; Kim et al., 2011; Ladewig et al., 2012; Liu et al., 2013; Liu et al., 2011; Meng et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Qiang et al., 2011; Son et al., 2011; Torper et al., 2013; Vierbuchen et al., 2010; Yoo et al., 2011) or oligodendroglial cells (Najm et al., 2013; Yang et al., 2013). It has also been demonstrated that astroglial cells can be trans-differentiated into neurons (Heinrich et al., 2010; Torper et al., 2013) or reprogrammed into neuroblast cells (Niu et al., 2013). However, it is unclear whether such trans-differentiation studies can be applied to brain repair after brain injury or neurodegeneration.

We demonstrate here that after brain injury, reactive glial cells including both astrocytes and NG2 cells can be reprogrammed into functional neurons in the adult mouse cortex when infected with retrovirus encoding a single transcription factor NeuroD1. Electrophysiological recordings revealed both spontaneous and evoked synaptic responses in NeuroD1-converted neurons. Interestingly, astrocytes were mainly reprogrammed into glutamatergic neurons whereas NG2 cells were reprogrammed into both glutamatergic and GABAergic neurons after NeuroD1 expression. We also demonstrated that forced expression of NeuroD1 in a mouse model for Alzheimer's disease was capable of reprogramming reactive glial cells into functional neurons. Furthermore, NeuroD1 was capable of reprogramming cultured human astrocytes into functional neurons efficiently. Thus, *in vivo* regeneration of functional neurons from reactive glial cells may provide a potential therapeutic approach to restore lost neuronal function in injured or diseased brain.

Results

***In vivo* reprogramming of reactive glial cells into functional neurons after brain injury**

A signature of brain injury is the loss of functional neurons and activation of glial cells. In the adult mouse cortex, astrocytes are usually quiescent and not proliferative unless activated by injury or diseases (Ge et al., 2012; Robel et al., 2011; Tsai et al., 2012). Besides astrocytes, NG2 cells and microglia can also be activated and proliferate rapidly in the injury sites or in diseased brain (Aguzzi et al., 2013; Hines et al., 2009; Kang et al., 2013). To test whether reactive glial cells can be reprogrammed into functional neurons for brain repair, we decided to inject retroviruses encoding neural transcription factors into adult mouse cortex *in vivo*. We chose retroviral delivery for *in vivo* injection because, unlike lentiviruses or adeno-associated viruses, retroviruses only infect dividing cells such as progenitor cells or reactive glial cells, and do not infect non-dividing cells such as neurons (Zhao et al., 2006). As a control, we first injected retroviruses expressing GFP alone under the control of CAG promoter (pCAG-GFP-IRES-GFP) (Zhao et al., 2006) into mouse cortex to examine what type of cells will be infected by the retrovirus after stab injury. As expected, many GFP-labeled cells were immunopositive for astrocytic marker GFAP (Fig. 1A; $52.1 \pm 4.3\%$ were

GFAP+, n = 3 animals). We did not observe any neuronal cells infected by control retrovirus expressing GFP alone (Suppl. Fig. 1).

Our strategy for reprogramming reactive glial cells into neurons involved construction of a retrovirus encoding NeuroD1, a bHLH proneural transcription factor that plays an important role during embryonic brain development and adult neurogenesis (Cho and Tsai, 2004; Gao et al., 2009; Kuwabara et al., 2009). We first tested the effect of NeuroD1 in a brain injury model, where reactive glial cells were induced by stab injury during stereotaxic injection of retroviruses into mouse somatosensory cortex. We limited our injection to cortical areas without penetrating the hippocampus or subventricular zone, where adult neural stem cells are known to reside. Interestingly, 3 days post injection (DPI) of the retrovirus encoding NeuroD1 (pCAG-NeuroD1-IRES-GFP) into mouse cortex, many NeuroD1-GFP infected cells showed bipolar morphology and were immunopositive for doublecortin (DCX), an immature neuronal marker (Fig. 1B). One week after viral injection, NeuroD1-infected cells started to show staining for neuronal nuclei (NeuN), a typical neuronal marker (Fig. 1C). Three weeks after viral injection, NeuroD1-infected cells showed extensive neurites and the NeuN signal reached the level of non-infected mature neurons in the same vicinity (Fig. 1D). Quantitatively, we detected a large number of NeuroD1-GFP labeled newborn neurons (DCX) at 3 DPI (19.3 ± 3.7 per 0.1 mm^2 , n = 5 animals), and the number of converted neurons gradually declined during the maturation process (Fig. 1E). Nevertheless, at any given time point after NeuroD1 retroviral infection, the majority of NeuroD1-infected cells were DCX or NeuN-positive neurons, whereas control GFP viral infection resulted in no neurons at all (Fig. 1E; Suppl. Fig. 1).

We found that NeuroD1-converted neurons were usually located in the deep cortical layer, with some exceptions in the cingulate cortex or superficial layer of the cortex, as illustrated with a general cortical neuron marker Tbr1 (Fig. 1F). To further test the neuronal properties of NeuroD1-converted neurons, we used the deep layer cortical neuron marker Ctip2 and found that NeuroD1-converted neurons were indeed immunopositive for Ctip2 (Fig. 1G). No GFP-labeled neurons were detected in the dentate gyrus or the subventricular zone, because our viral injection was restricted to the cortical layers.

Interestingly, we found that NeuroD1-converted neurons at 3 DPI were typically localized within $100 \mu\text{m}$ from the injection site. However, 1–2 weeks after injection, NeuroD1-infected cells were found in more broad areas, ranging from $100 - 500 \mu\text{m}$ away from the injection site (Suppl. Fig. 2). It is possible that this gradual spread is a reflection of distant cells being exposed to a lower level of viral infection and thus having later conversion, or that the newly-converted neurons migrate away from the injury core. Further investigation will be needed to distinguish between these possibilities.

To test whether NeuroD1-converted neurons have functional activity, we performed cortical slice recordings on NeuroD1-GFP infected cells ~1 month after retroviral injection. The NeuroD1-converted neurons showed large sodium currents ($3840 \pm 302 \text{ pA}$, n = 5) and potassium currents ($4672 \pm 602 \text{ pA}$, n = 5) (Fig. 1H), and were capable of firing repetitive action potentials (Fig. 1I, n = 4). Importantly, we recorded robust spontaneous synaptic events in NeuroD1-converted neurons in cortical slice recordings (Fig. 1J; frequency, $1.96 \pm 0.43 \text{ Hz}$; amplitude, $23.7 \pm 2.0 \text{ pA}$; n = 8; 25–31 DPI), suggesting that these NeuroD1-converted neurons formed functional synapses with other neurons. Moreover, we placed a stimulating electrode nearby ($50 \mu\text{m}$) to stimulate axon fibers and recorded evoked synaptic responses in the NeuroD1-converted neurons (Fig. 1K; n = 3 animals), suggesting an integration of converted neurons into local neural circuits. The NeuroD1-converted neurons can survive for a long time in mouse brain *in vivo*, at least 2 months after the retroviral injection, and they showed clear dendritic spines and large spontaneous synaptic events

(Suppl. Fig. 3, n = 3 animals). Thus, NeuroD1 can reprogram brain injury-induced reactive glial cells into functional neurons in mouse brain *in vivo*.

NeuroD1 reprograms astrocytes into glutamatergic neurons

After a brain injury, many glial cells are activated and become proliferative. To examine whether reactive astrocytes, a major subtype of reactive glial cells in gliosis, can be reprogrammed into neurons by NeuroD1, we generated a retrovirus expressing NeuroD1 under the control of human GFAP promoter. After injecting GFAP::NeuroD1-IRES-GFP retrovirus into the mouse cortex, we found that NeuroD1-infected cells were mostly immunopositive for the neuronal markers NeuN (Fig. 2A; $92.8 \pm 2\%$ were NeuN positive, 8 DPI; n = 4 animals) and Tuj1 (Fig. 2B; n = 4 animals). Therefore, reactive astrocytes induced by brain injury can be reprogrammed into neurons *in vivo* after overexpressing a single transcription factor NeuroD1.

To further characterize the properties of astrocyte-converted neurons, we infected cultured mouse cortical astrocytes with GFAP::NeuroD1-IRES-GFP retrovirus. Our mouse astrocyte cultures were enriched with GFAP-positive astrocytes, with little contamination from microglia or NG2 cells (Suppl. Fig. 4A). Similar to our *in vivo* reprogramming, cultured mouse astrocytes can be efficiently reprogrammed into NeuN-positive neurons after GFAP::NeuroD1 infection (Fig. 2C). Quantitatively, we found that the NeuroD1-induced reprogramming efficiency dramatically increased in the first 3 days after GFAP::NeuroD1 infection and reached >90% after 7 DPI (Fig. 2D). To examine whether GFAP::NeuroD1-converted neurons are glutamatergic or GABAergic, we performed immunostaining with VGluT1 and GAD67-specific antibodies, and found that the majority of neurons were positive for VGluT1 (Fig. 2E–F) but virtually none stained for GAD67 (Fig. 2F; Suppl. Fig. 4B). Thus, NeuroD1 reprograms astrocytes into glutamatergic neurons. We also used superficial and deep layer cortical neuronal markers to characterize the NeuroD1-converted neurons. The GFAP::NeuroD1-converted neurons were positive for the deep layer markers Ctip2 and Otx1, as well as Tbr1 (Fig. 2G–H), but negative for the superficial layer markers Cux1 and Lhx2 (Fig. 2H; Suppl. Fig. 4C). This finding may explain why we observed more NeuroD1-converted neurons in deep cortical layer in mouse brain *in vivo*.

We also functionally characterized the cultured mouse astrocyte-converted neurons after NeuroD1 infection and detected large GABA (100 μ M), glutamate (100 μ M) and NMDA (100 μ M) currents (Fig. 2I). The mouse astrocyte-converted neurons also showed repetitive action potentials (Fig. 2J, n=18) and large Na⁺ and K⁺ currents (Fig. 2K; I_{Na}, 2979 \pm 626 pA, n = 11; I_K, 5136 \pm 1181 pA, n=11, 14 DPI). More importantly, we recorded robust synaptic events in astrocyte-converted neurons, which were blocked by glutamate receptor antagonist CNQX (10 μ M) but not by GABA_A receptor antagonist bicuculline (BIC, 20 μ M) (Fig. 2L; frequency, 1.15 \pm 0.71 Hz; amplitude, 21.5 \pm 0.71 pA, n = 13; 14 DPI), confirming that astrocyte-converted neurons are glutamatergic neurons.

NeuroD1 reprograms NG2 cells into glutamatergic and GABAergic neurons

In addition to reactive astrocytes, NG2 cells also proliferate significantly in response to brain injury and accounted for about 19.0 \pm 3.9 % of control retrovirus-infected cells in our stab injury model, whereas only 6% infected cells were microglia. To investigate whether NG2 cells can be reprogrammed into neurons by NeuroD1, we generated a retrovirus expressing NeuroD1 under the control of the human NG2 promoter (NG2::NeuroD1-IRES-GFP). Interestingly, we discovered that injecting NG2::NeuroD1 retrovirus into mouse cortex also reprogrammed NG2 cells into NeuN and Tuj1 positive neurons (Fig. 3A–B; 42.5 \pm 6.6% GFP-labeled cells were NeuN positive, 8 DPI, n = 3 animals). We then characterized NG2-converted neurons using cultured NG2 cells dissociated from mouse cortex. In our

mouse NG2 cultures, the majority of cells were NG2-positive ($79.2 \pm 3.2\%$, $n = 3$ repeats of cultures) (Suppl. Fig. 4D). Consistent with our *in vivo* study, we found that NG2::NeuroD1 also efficiently reprogrammed cultured mouse NG2 cells into neurons (Fig. 3C–D; 7 DPI, $98.2 \pm 1.8\%$, $n = 484$, 4 repeats). While the majority of NG2::NeuroD1-converted neurons were also glutamatergic (VGluT1 positive), about 10% NG2-converted neurons were immunopositive for GAD67 and presynaptic GABAergic terminals (GAD65) were found on neuronal dendrites (Fig. 3E–G). Therefore, it appears that NG2 cells can be reprogrammed into both glutamatergic and GABAergic neurons after expressing NeuroD1. Immunostaining with cortical layer markers revealed that NG2-converted neurons also stained mainly for the deep layer markers Ctip2 and Otx1, but rarely for Cux1 and Lhx2 (Fig. 3H–I; Suppl. Fig. 4E–F). Patch-clamp recordings demonstrated that NG2-converted neurons generated after NeuroD1 infection were able to fire repetitive action potentials (Fig. 3J), showed large Na^+ and K^+ currents (Fig. 3K), and large glutamate- and GABA-evoked receptor currents (Fig. 3L–M; $I_{\text{Glu}} = 438 \pm 78$ pA, $n = 7$; $I_{\text{GABA}} = 496 \pm 32$ pA, $n = 7$). Moreover, we detected both glutamatergic and GABAergic events in NG2-converted neurons (Fig. 3N), confirming that NG2 cells can be reprogrammed into both glutamatergic and GABAergic neurons. Therefore, a single transcription factor NeuroD1 not only reprograms astrocytes into glutamatergic neurons but also reprograms NG2 cells into glutamatergic and GABAergic neurons.

Reactive glia-neuron conversion in Alzheimer's disease mouse model

Besides activation by mechanical injury, reactive astrocytes have been widely reported in the cortex of Alzheimer's disease patients or animal models (Rodriguez et al., 2009; Steele and Robinson, 2010). We employed a transgenic mouse model with AD (5xFAD) (Oakley et al., 2006) to test whether reactive astrocytes in the AD brain can be reprogrammed into functional neurons. We first confirmed that there were indeed many reactive astrocytes in the cortex of 5xFAD mice compared to WT (Fig. 4A). Next, we injected NeuroD1-GFP retrovirus (CAG promoter) into the cortex of 5xFAD mice and observed NeuN-positive neuron-like cells (Fig. 4B, 14 DPI). To further confirm that the reactive astrocytes in the AD model mouse brain can be reprogrammed into neurons, we injected GFAP::NeuroD1-GFP retrovirus to infect cortical astrocytes specifically, and indeed observed NeuroD1-converted neurons labeled by NeuN (Fig. 4C). Immunostaining with VGluT1 and GAD65 revealed glutamatergic and GABAergic terminals innervating NeuroD1-converted neurons in the 5xFAD mouse brain (Fig. 4D). As AD is a progressive neurodegenerative disorder, we wondered whether *in vivo* reprogramming could occur in very old animals. Remarkably, when we injected NeuroD1-GFP retrovirus into 14-month old 5xFAD animals, we observed many NeuroD1-GFP infected cells immunopositive for NeuN (Fig. 4E). Interestingly, we found that the number of NeuroD1-converted neurons was higher in 5xFAD than in WT mouse brain, and even higher in 14-month old animals than in 7-month old animals (Fig. 4F). This increase might occur because there are more reactive glial cells in older diseased animals that can be reprogrammed into neurons. Using cortical slice recordings, we further demonstrated that the NeuroD1-converted neurons in 5xFAD mouse brain were functional, with the peak amplitude of I_{Na} at 2270 ± 282 pA ($n = 5$) and I_{K} at 5498 ± 706 pA ($n = 5$) (Fig. 4G). More importantly, we recorded robust synaptic events in NeuroD1-converted neurons in cortical slice recordings (Fig. 4H–I; frequency, 2.80 ± 0.95 Hz; amplitude, 20.5 ± 2.7 pA; $n = 7$), suggesting that these newly reprogrammed neurons are functionally connected with surrounding neurons in 5xFAD mouse brain. Therefore, overexpression of NeuroD1 in reactive glial cells has the potential to regenerate functional neurons in an AD model brain.

Reprogramming cultured human astrocytes into functional neurons

We next investigated whether NeuroD1 can reprogram human astrocytes into functional neurons using a human cortical astrocyte cell line (ScienCell, San Diego, California). The majority of our cultured human astrocytes were immunopositive for GFAP and S100 β (Fig. 5A; Suppl. Fig. 5), but with very low level of neuroprogenitor marker Sox2 or Musashi (Suppl. Fig. 5A–B). We infected human astrocytes with GFAP::NeuroD1-IRES-GFP retrovirus and found that the majority of NeuroD1-infected cells were immunopositive for NeuN (Fig. 5B), suggesting that human astrocytes can also be efficiently reprogrammed into neurons by expressing NeuroD1. We then examined the time course of NeuroD1-induced astrocyte-neuron conversion using a series of neuronal markers including DCX, NeuN, and MAP2. We found that the conversion efficiency increased dramatically between 3 to 5 DPI, with 90% of NeuroD1-infected human astrocytes becoming neurons by 5 DPI (Fig. 5C–F). Infection by NeuroD1 significantly changed the cell morphology from astrocytes to neurons, as shown by the phase contrast images in Fig. 5G. To investigate whether NeuroD1-induced reprogramming involved a transient neuroprogenitor stage, we monitored the trans-differentiation process from 24 hours till 5 days after NeuroD1 infection of human astrocytes (Suppl. Fig. 5C–D). No transient increase in the expression level of the neural stem cell markers Sox2 (Suppl. Fig. 5C) or Musashi (Suppl. Fig. 5D) occurred during the early conversion period. In fact, after only 3 days of infection by NeuroD1, some astrocytes already became neuron-like cells with clearly extended neurites (Suppl. Fig. 5C–D). Therefore, NeuroD1 appears to reprogram astrocytes directly into neurons without transition through neuroprogenitor stage.

Next, we investigated what types of neurons were reprogrammed from human astrocytes. Immunostaining with VGluT1 and GAD67 revealed that human astrocytes infected by NeuroD1 were mainly reprogrammed into glutamatergic neurons as shown by immunopositive for VGluT1 (Fig. 5H), but not GAD67 (Suppl. Fig. 6B), consistent with our observations for mouse astrocyte conversion. Using cortical layer markers, we found that, as for mouse astrocytes, human astrocyte-converted neurons also stained positive for the cortical neuron marker Tbr1 and the deep layer markers Ctip2 and Otx1 (Fig. 5I–L), but much less for the superficial layer markers Cux1 and Lhx2 (Fig. 5L; Suppl. Fig. 6C–D). To investigate whether human microglia can be reprogrammed into neurons, we cultured human microglia and infected them with NeuroD1-GFP retrovirus, but did not detect any DCX-positive neurons (Suppl. Fig. 6E–H; 0 DCX+ neurons out of 33 NeuroD1-GFP infected microglial cells). However, this apparently different result might be influenced by the low infection efficiency of microglia by retrovirus ($9.9 \pm 0.8\%$, $n = 3$ batches of culture), compared to the high infection efficiency of astrocytes ($51.6 \pm 2.9\%$, $n = 3$ batches) or NG2 cells ($57.8 \pm 5\%$, $n = 3$ batches).

To examine whether NeuroD1-converted human neurons are functionally connected, we performed immunostaining with the synaptic marker SV2 and the glutamatergic synapse marker VGluT1 (Fig. 6A–B). After NeuroD1-induced conversion, we observed numerous SV2 puncta on MAP2-labeled neuronal dendrites (Fig. 6A, 45 DPI). Some neurons even showed mushroom-like mature spines, which were co-localized with VGluT1 puncta (Fig. 6B). Next, we employed patch clamp recordings to test the function of reprogrammed human neurons. Human astrocyte-converted neurons started to show detectable NMDA receptor currents at 20 DPI, but very small GABA or glutamate receptor currents at this stage (Fig. 6C). However, after 30 – 40 DPI, we detected large glutamate receptor currents (548 ± 138 pA, $n = 7$; 31–35 DPI), GABA_A receptor currents (599 ± 114 pA, $n = 8$; 31–35 DPI), and NMDA receptor currents (966 ± 101 pA, $n = 8$; 40 DPI) (Fig. 6C–D). At 20 DPI, we also detected clear sodium (I_{Na}) and potassium currents (I_K) (Fig. 6E), which increased dramatically by 40 DPI (Fig. 6E–F). Accordingly, we recorded repetitive action potential firing in human astrocyte-converted neurons (Fig. 6G, $n = 15$). Furthermore, we detected

functional synaptic events in NeuroD1-converted human neurons (frequency, 1.6 ± 0.3 Hz; amplitude, 23.2 ± 0.8 pA; $n = 13$), which were blocked by CNQX ($10 \mu\text{M}$) but not by BIC ($20 \mu\text{M}$) (Fig. 6H). Thus, it appears that NeuroD1 can reprogram human astrocytes into functional glutamatergic neurons.

DISCUSSION

We show here that reactive glial cells generated after brain injury or in Alzheimer's disease model can be directly reprogrammed into functional neurons by a single transcription factor NeuroD1 in mouse brain *in vivo*. Interestingly, after expressing the same transcription factor NeuroD1, astrocytes are mainly reprogrammed into glutamatergic neurons, whereas NG2 cells can be reprogrammed into both glutamatergic and GABAergic neurons. Such different cell fate after reprogramming by the same transcription factor may provide important clues regarding the lineage relationship between neurons and glial cells. We also show that human astrocytes in culture can be reprogrammed into functional neurons after expressing NeuroD1. The *in vivo* reprogramming of reactive glial cells into functional neurons after brain injury or in diseased mouse brain could potentially provide a therapeutic approach for treating reactive gliosis, which is widely associated with nerve injury and neurodegenerative disorders.

Direct reprogramming of reactive astrocytes into functional neurons

During mammalian brain development, neurogenesis typically occurs in embryonic stage before birth, while gliogenesis usually occurs in postnatal stage (Miller and Gauthier, 2007). In the central nervous system, neurons, astrocytes, oligodendrocytes, and NG2 cells (oligodendrocyte precursor cells) are all generated from neural stem cells, whereas microglial cells are generated from hematopoietic stem cells. Astrocytes maintain proliferative properties after differentiation from neural stem cells, and the majority of astrocytes in the cortex are generated locally by astrocytes themselves in the postnatal stage (Ge et al., 2012). Astrocyte proliferation largely stops after one month of age in rodents (Ge et al., 2012; Tsai et al., 2012). However, after brain injury or neurodegenerative disorders, astrocytes become activated and start to proliferate again (Robel et al., 2011; Sofroniew and Vinters, 2010). Previous studies reported that reactive astrocytes after injury may have certain stem cell properties, since isolating and culturing reactive astrocytes *in vitro* can generate neurospheres (Buffo et al., 2008; Lang et al., 2004; Shimada et al., 2012; Sirko et al., 2013; Sirko et al., 2009). However, a number of studies have pointed out that the reactive astrocytes cannot generate neurons *in vivo*, although they may be able to generate glial cells (Buffo et al., 2008; Shimada et al., 2012). Therefore, injury itself can activate astrocytes to proliferate and even dedifferentiate to acquire certain properties of progenitor cells, but reactive astrocytes genetically remain within glial lineages. Our forced expression of NeuroD1 in reactive astrocytes may help them to overcome trans-lineage barriers that cannot be surmounted by injury alone. We selected NeuroD1 for *in vivo* astrocyte-neuron conversion because NeuroD1 has been reported to be essential for adult neurogenesis (Gao et al., 2009; Kuwabara et al., 2009). NeuroD1 has also been shown to induce terminal neuronal differentiation (Boutin et al., 2010) and help reprogramming human fibroblast cells into induced neurons when combined with Brn2, Ascl1, and Myt1l (Pang et al., 2011). Other transcription factors such as neurogenin-2 and Dlx2 have been shown to reprogram cultured mouse astrocytes into neurons (Heinrich et al., 2010). Therefore, it only takes a single neural transcription factor to change glial fate into neuronal fate both *in vitro* and *in vivo*.

Reactive astrocytes activated under different pathological conditions seem to have different proliferation rates. In particular, stab injury and ischemic stroke-induced reactive astrocytes can be highly proliferative, whereas reactive astrocytes in APPPS1 or CK/p25 mice have

lower rate of proliferation (Sirko et al., 2013). Sonic hedgehog (SHH) also plays a critical role in regulating the proliferative rate (Sirko et al., 2013). Although stab injury-induced reactive astrocytes were found to express nestin, they were not reported to express Sox2 or Musashi. Therefore, it is possible that SHH alone can promote the proliferation of reactive astrocytes, but may not be sufficient to reverse reactive astrocytes into genuine neuroprogenitor cells. Interestingly, forced expression of Sox2 has been shown to de-differentiate astrocytes into neuroblast cells (Niu et al., 2013). Sox2 is a critical marker for neural stem cells, and expression of Sox2 in fibroblast cells has been shown to induce neural stem cells (Ring et al., 2012). It is important to note that after Sox2-induced astrocyte-neuroblast conversion, addition of BDNF and noggin is required to further induce differentiation of neuroblasts into neurons (Niu et al., 2013). In contrast, our NeuroD1 expression reprograms reactive astrocytes directly into functional neurons *in vivo* without going through a neuroprogenitor stage. Thus, our NeuroD1 reprogramming strategy can produce functional neurons rapidly after injury. Furthermore, our application of retroviral vectors targets proliferative glial cells that are typically activated by injury or diseases in the adult brain, without affecting quiescent glial cells. Thus, NeuroD1-induced reprogramming may be particularly well suited for therapeutic intervention and not interfere significantly with normal astrocyte functions. Moreover, we demonstrate that NeuroD1-induced reactive glia-neuron conversion can occur in very old animals and even old animals with a model of Alzheimer's disease. Therefore, NeuroD1-induced *in vivo* reactive astrocyte-neuron conversion could potentially be useful for regeneration of new neurons in the aging brain. In addition, the reprogramming of human astrocytes into functional neurons suggests that such reactive glia-neuron conversion approach is potentially applicable to human patients.

NeuroD1 reprograms NG2 cells into glutamatergic and GABAergic neurons

NG2 cells are the major proliferative glial cells in the adult brain under normal physiological conditions (Buffo et al., 2008; Kang et al., 2010). NG2 cells can receive synaptic inputs from neurons although the function of such neuron-glia synapses is not well understood (Bergles et al., 2010). In our stab injury model, we found that our CAG-GFP retrovirus infected cells are mainly GFAP-positive cells, and NG2 cells only account for about 20% of total infected cells. This bias might be due to the preferential infection of astrocytes by the retrovirus we used, or due to the higher proliferation rate of reactive astrocytes than NG2 cells in our stab injury model. An unexpected finding in our study is that NeuroD1 not only reprograms astrocytes into functional neurons, but also reprograms NG2 cells into functional neurons. More interestingly, NeuroD1 reprograms astrocytes into glutamatergic neurons but reprograms NG2 cells into both glutamatergic and GABAergic neurons, suggesting that different glial cells may be associated with different neuronal fate in terms of lineage differentiation. Since glutamatergic and GABAergic neurons are the two major subtypes of neurons in the cortex, our finding that NeuroD1 can reprogram astrocytes and NG2 cells into glutamatergic and GABAergic neurons may have important functional implications. The simultaneous generation of both excitatory and inhibitory neurons by NeuroD1 alone could potentially make it possible to balance excitation and inhibition in the cortex after reprogramming.

Conclusion

Our findings suggest that *in situ* reprogramming of reactive astrocytes and NG2 cells into functional neurons may offer a new approach to use internally reprogrammed neurons for brain repair. One outstanding question is whether it is possible to use virus-free or small molecule strategy to effect the reprogramming *in vivo* (Bayart and Cohen-Haguenaer, 2013; Chambers et al., 2012; Hou et al., 2013; Kaji et al., 2009; Li et al., 2013; Shi et al., 2008). An equally challenging question is whether the *in vivo* reprogramming can ultimately

rescue behavioral deficits, such as cognitive impairment in a diseased brain. Nevertheless, our *in situ* reprogramming of reactive glial cells into functional neurons suggests that it may be possible to replace neurons lost after nerve injury or diseases by direct reprogramming as a first step toward brain repair.

Experimental procedures

Animals and *in vivo* assays

In vivo experiments were conducted on wildtype C57/BL6 and AD transgenic mice (5xFAD). AD transgenic mice were purchased from The Jackson Laboratory (B6SJL-Tg (APP^SwF1L^{on}, PSEN1*^{M146L}*^{L286V}) 6799Vas/Mmjax) (Oakley et al., 2006) and mated with C57/BL6 mice. Mice were housed in a 12 hr light/dark cycle and supplied with enough food and water. Experimental protocols were approved by The Pennsylvania State University IACUC and in accordance with guidelines of the National Institutes of Health.

Stereotaxic viral injection

Surgeries were performed on 1–14 months old WT and AD mice for virus injection. The mice were anesthetized by injecting 20 ml/kg 2.5% Avertin (a mixture of 25 mg/ml of Tribromoethylethanol and 25 μ l/ml T-amyl-alcohol) into the peritoneum and then placed in a stereotaxic setup. Artificial eye ointment was applied to cover and protect the eye. The animals were operated with a midline scalp incision and a drilling hole on the skulls above somatosensory cortex. Each mouse received injection (position: AP 1.25 mm, ML 1.4 mm, DV –1.5 mm) of virus with a 5 μ l syringe and a 34 gauge needle. The injection volume and flow rate were controlled as 3 μ l at 0.2 μ l/min, and the needle was moved up during the injection at speed of 0.1 mm/min. After injection, the needle was kept for at least 5 additional minutes and then slowly withdrawn. The needle injection itself was used as stab injury model.

Mouse cortical astrocyte and NG2 culture

For astrocyte culture, postnatal (P3–P5) mouse cortical tissue was dissociated and plated onto 25 cm² flasks (Wu et al., 2012). Cells were cultured for 5–6 days, and flasks were rigorously shaken daily to remove neurons and non-astrocytic cells. After reaching confluence, astrocytes were centrifuged for 5 min at 1,000 rpm, re-suspended, and plated on poly-D-lysine (Sigma) coated coverslips (12 mm). Astrocyte culture medium contained DMEM/F12 (Gibco), 10% fetal bovine serum (Gibco), penicillin/ streptomycin (Gibco), 3.5 mM glucose (Sigma), and supplemented with B27 (Gibco), 10 ng/mL epidermal growth factor (EGF, Invitrogen), and 10 ng/mL fibroblast growth factor 2 (FGF2, Invitrogen). For mouse NG2 culture, the cortical tissue of postnatal mice (P3–P5) was dissociated and plated in 25 cm² flasks coated with poly-D-lysine (Sigma). The cells were maintained in DMEM/F12 (Gibco) with 10% fetal bovine serum (Gibco) for 9 days, with a medium change every 3 days. On the 9th day, the flasks were shaken rigorously and the supernatant was collected and centrifuged to harvest NG2 cells with a small number of neurons and microglia cells. After centrifuge, cells were resuspended and seeded on poly-D-lysine (Sigma) coated coverslips (12 mm). The cells were cultured in serum-free DMEM medium (Gibco) with N2 supplements (STEMCELL™) and 10 ng/ml platelet-derived growth factor (PDGF, Invitrogen), 10 ng/mL epidermal growth factor (EGF, Invitrogen), and 10 ng/mL fibroblast growth factor 2 (FGF2, Invitrogen) for 3 days. Cells were maintained at 37°C in humidified air with 5% CO₂.

Human cortical astrocyte and microglia culture

Human cortical astrocytes (HA1800) were purchased from ScienCell (California). Cells were subcultured when they were over 90% confluent. For subculture, cells were trypsinized by TrypLE™ Select (Invitrogen), centrifuged for 5 min at 1,000 rpm, re-suspended, and plated in a medium consisting of DMEM/F12 (Gibco), 10% fetal bovine serum (Gibco), penicillin/ streptomycin (Gibco), 3.5 mM glucose (Sigma), and supplemented with B27 (Gibco), 10 ng/mL epidermal growth factor (EGF, Invitrogen), and 10 ng/mL fibroblast growth factor 2 (FGF2, Invitrogen). The astrocytes were cultured on poly-D-lysine (Sigma) coated coverslips (12 mm) at a density of 50,000 cells per coverslip in 24-well plates (BD Biosciences). Human primary microglial cells were obtained from Clonexpress, Inc. (Maryland). The cells were cultured in DMEM/F-12 (Gibco) supplemented with 5% FBS, 10 ng/ml of macrophage colony-stimulating factor (M-CSF, Invitrogen), 10 ng/mL epidermal growth factor (EGF, Invitrogen), and 10 ng/mL fibroblast growth factor 2 (FGF2, Invitrogen). Cells were maintained at 37 °C in humidified air with 5% CO₂.

Retrovirus production

The mouse NeuroD1 gene was subcloned from the pAd NeuroD-I-nGFP (Zhou et al., 2008) (Addgene) and inserted into a pCAG-GFP-IRES-GFP retroviral vector (Zhao et al., 2006) (gift of Dr. Fred Gage) to generate pCAG-NeuroD1-IRES-GFP. The human GFAP promoter gene was subcloned from hGFAP Promoter-Cre-MP-1 (Addgene) and replaced CAG promoter to generate pGFAP-NeuroD1-IRES-GFP or pGFAP-GFP-IRES-GFP retroviral vector. The human NG2 promoter gene was subcloned from hNG2 Promoter-GLuc (GeneCopoeia) and replaced CAG promoter to generate pNG2-NeuroD1-IRES-GFP or pNG2-GFP-IRES-GFP retroviral vector. Viral particles were packaged in gpg helperfree HEK (human embryonic kidney) cells to generate VSV-G (vesicular stomatitis virus glycoprotein)-pseudotyped retroviruses encoding neurogenic factors in CellMax™ hollow fiber cell culture system (Spectrum Laboratories). The titer of viral particles was about 10⁸ particles/μl, determined after transduction of HEK cells.

Trans-differentiation of glial cells into neurons

Twenty-four hours after infection of astrocytes, NG2 cells, or microglia with GFP or NeuroD1 retrovirus, the culture medium was completely replaced by a differentiation medium including DMEM/F12 (Gibco), 0.5% FBS (Gibco), 3.5 mM glucose (Sigma), penicillin/streptomycin (Gibco), and N2 supplement (Gibco). Brain-derived neurotrophic factor (BDNF, 20 ng/mL, Invitrogen) was added to the cultures every four days during the differentiation to promote synaptic maturation (Song et al., 2002). Due to the morphological change from astrocytes or NG2 cells to neurons during conversion, we filled the empty space with additional human or mouse astrocytes to support the functional development of converted neurons.

Immunocytochemistry

For brain section staining, the mice were anesthetized with 2.5% Avertin and then sequentially perfused, first with saline solution (0.9% NaCl) to wash the blood off and then with 4% paraformaldehyde (PFA) to fix the brain. The brains were removed and post fixed in 4% PFA overnight at 4 °C, and then cut at 45 μm sections by a vibratome (Leica). Coronal brain sections were first pretreated in 0.3% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 1 hour, followed by incubation in 3% normal goat serum, 2% normal donkey serum and 0.1% Triton X-100 in PBS for 1 hour.

For cell culture staining, the cultures were fixed in 4% PFA in PBS for 15 min at room temperature. Cells were first washed three times by PBS and then pretreated in 0.1% Triton

X-100 in PBS for 30 min, followed by incubation in 3% normal goat serum, 2% normal donkey serum and 0.1% Triton X-100 in PBS for 1 hour. Primary antibodies were incubated with either brain slices or cultures overnight at 4°C in 3% normal goat serum, 2% normal donkey serum and 0.1% Triton X-100 in PBS. After additional washing in PBS, the samples were incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488, Alexa 546, Alexa 647 (1:300, Molecular Probes), or Dylight (1:500, Jackson ImmunoResearch) for 1 h at room temperature, followed by extensive washing in PBS. Coverslips were finally mounted onto a glass slide with an anti-fading mounting solution with DAPI (Invitrogen). Slides were first examined with an epifluorescent microscope (Nikon TE-2000-S) and further analyzed with a confocal microscope (Olympus FV1000). Z-stacks of digital images, which can either release single confocal images or collapse as one resulting picture, were acquired and analyzed using FV10-ASW 3.0 Viewer software (Olympus). For detailed antibodies list, please see supplementary material.

Patch-clamp recordings in cell cultures

For glial cell-converted neurons, whole-cell recordings were performed using Multiclamp 700A patch-clamp amplifier (Molecular Devices, Palo Alto, CA) as described before (Deng et al., 2007), and the chamber was constantly perfused with a bath solution consisting of 128 mM NaCl, 30 mM glucose, 25 mM HEPES, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂. The pH of bath solution was adjusted to 7.3 with NaOH, and osmolarity at 315–325 mOsm/L. Patch pipettes were pulled from borosilicate glass (3–5 M Ω) and filled with a pipette solution consisting of 135 mM KCl, 5 mM Na-phosphocreatine, 10 mM HEPES, 2 mM EGTA, 4 mM MgATP, and 0.5 mM Na₂GTP, pH 7.3 adjusted with KOH. The series resistance was typically 10–30 M Ω . For voltage-clamp experiments, the membrane potential was typically held at –70 or –80 mV. Drugs were applied through a gravity-driven drug delivery system (VC-6, Warner Hamden, CT). NMDA currents were recorded in Mg²⁺ free bath solution (128 mM NaCl, 30 mM D-glucose, 25 mM HEPES, 5 mM KCl, and 2 mM CaCl₂, pH 7.3, adjusted with NaOH) plus 10 μ M glycine, 0.5 μ M TTX, and 20 μ M BIC. Data were acquired using pClamp 9 software (Molecular Devices, Palo Alto, CA), sampled at 10 kHz and filtered at 1 kHz. Na⁺ and K⁺ currents and action potentials were analyzed using pClamp 9 Clampfit software. Spontaneous synaptic events were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA). All experiments were conducted at room temperature.

Brain slice recordings

Cortical slices were prepared typically ~1 month after virus injection and cut at 300 μ m thick coronal slices with a Leica vibratome in ice cold cutting solution (containing 75 mM sucrose, 87 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl₂, 7 mM MgCl₂, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄ and 20 mM glucose). Slices were maintained in artificial cerebral spinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 1.3 mM MgCl₂, 2.5 mM CaCl₂ and 10 mM glucose. Slices were incubated in ACSF, continuously bubbled with 95% O₂ and 5% CO₂, first at 34 °C for 30 minutes, and then at room temperature. Whole-cell recordings were performed using a pipette solution containing 135 mM K-Gluconate, 10 mM KCl, 5 mM Na-phosphocreatine, 10 mM HEPES, 2 mM EGTA, 4 mM MgATP, and 0.5 mM Na₂GTP (pH 7.3 adjusted with KOH, 290 mOsm/L). Pipette resistance was 3–5 M Ω , and series resistance was typically 20–40 M Ω . The holding potential for voltage-clamp experiments was –70 mV. Data were collected using pClamp 9 software (Molecular Devices, Palo Alto, CA), sampled at 10 kHz and filtered at 1 kHz, analyzed with Clampfit and Synaptosoft softwares.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- NeuroD1 converts reactive glial cells into functional neurons *in vivo* after injury
- NeuroD1 induces glia-neuron conversion in Alzheimer's disease mouse model
- NeuroD1 reprograms cultured human astrocytes into glutamatergic neurons
- NeuroD1 reprograms NG2 cells into glutamatergic and GABAergic neurons

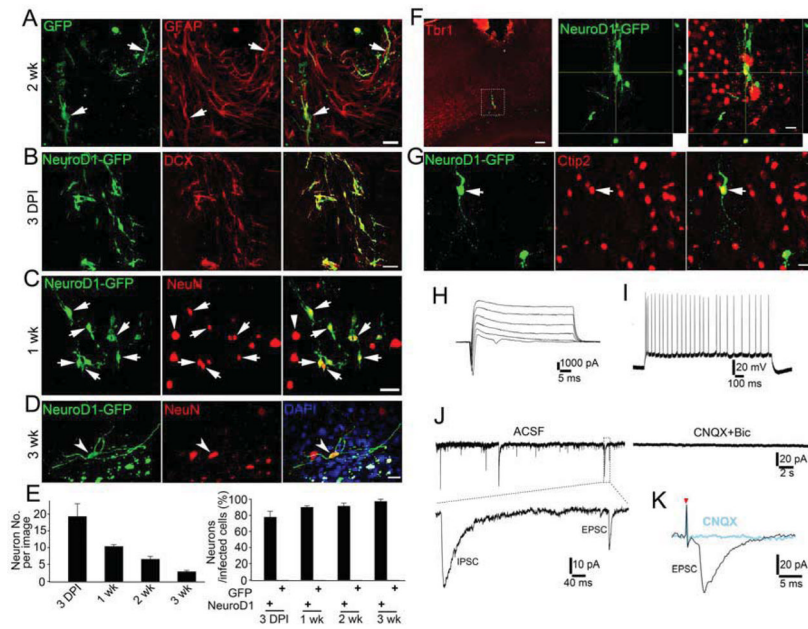


Figure 1. *In vivo* conversion of reactive glial cells into functional neurons after brain injury (A) Injecting control retrovirus expressing GFP (green) into mouse cortex revealed GFAP-positive reactive astrocytes (red) in the injury site (14 days post injection, DPI). (B–C) NeuroD1-IRES-GFP infected cells (green) were immunopositive for neuronal markers DCX (B, 3 DPI) and NeuN (C, 7 DPI). Note a significant number of NeuN-positive neurons in the injury site after NeuroD1 infection. (D) After 21 DPI, NeuroD1-converted neurons (NeuN-positive, arrowhead) showed extensive neurites. Scale bar, 20 μm for (A) and (D); 40 μm for (B) and (C). (E) Quantified data showing the number of converted neurons per imaged area (40x, 0.1 mm^2) and conversion efficiency after NeuroD1 infection. (F–G) NeuroD1-converted neurons were immunopositive for cortical neuron marker Tbr1 (F) and deep layer marker Ctip2 (G, 12 DPI). Scale bars: 100 μm for low power image, 40 μm for high power image. (H–I) Representative traces from cortical slice recordings showing Na^+ and K^+ currents (H) and repetitive action potentials (I) in NeuroD1-converted neurons (30 DPI). (J) Representative traces showing spontaneous synaptic events in a NeuroD1-converted neuron (26 DPI) in cortical slice recording (CNQX, 10 μM ; Bic, 20 μM). (K) Evoked synaptic events recorded from a converted neuron. See also Suppl. Fig. 1–3.

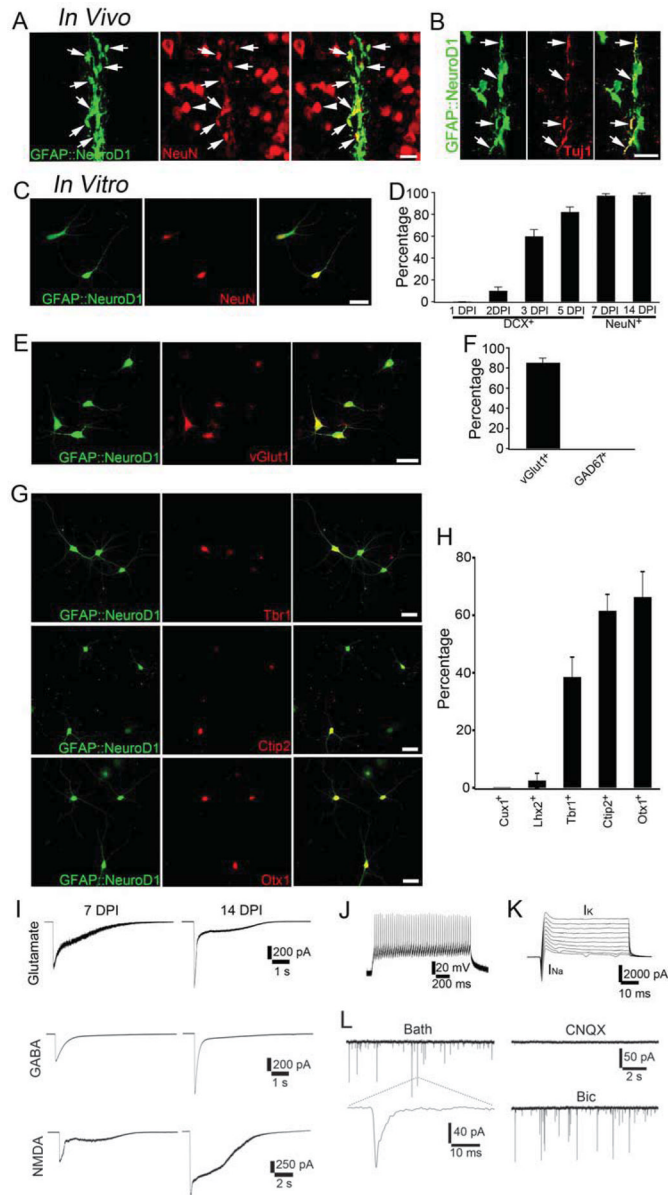


Figure 2. NeuroD1 converts astrocytes into glutamatergic neurons

(A–B) *In vivo* injection of GFAP promoter-driven NeuroD1-IRES-GFP (green) retrovirus revealed astrocyte-converted neurons immunopositive for NeuN (A) and Tuj1 (B). (C) Cultured mouse cortical astrocytes were converted into NeuN-positive neurons. (D) Time course of GFAP::NeuroD1 conversion efficiency after infecting cultured mouse astrocytes. (E–F) Astrocyte-converted neurons were positive for VGluT1 (E) but negative for GAD67 (F). (G–H) Immunostaining with cortical layer neuronal markers showed deep layer neuronal properties (Ctip2 and Otx1) after NeuroD1-induced conversion. Scale bars: 20 μm for (A–C) and (E), and 40 μm for (G). (I) Mouse astrocyte-converted neurons showed large glutamate, GABA, and NMDA receptor currents within 2 weeks after NeuroD1 infection. Average GABA current, 7 DPI, 405 ± 97 pA, $n=8$; 14 DPI, 861 ± 55 pA, $n=13$. Average glutamate current, 7 DPI, 517 ± 145 pA, $n=7$; 14 DPI, 1060 ± 159 pA, $n=9$. Average NMDA current, 7 DPI, 676 ± 118 pA, $n=7$; 14 DPI, 1315 ± 95 , $n=7$. (J–K) Mouse astrocyte-converted neurons showed repetitive action potentials (J) and large I_{Na} and I_{K} (K). (L)

Spontaneous synaptic events recorded from mouse astrocyte-converted neurons. All events were blocked by CNQX but not Bic, suggesting that they were glutamatergic events. Also see Suppl. Fig. 4.

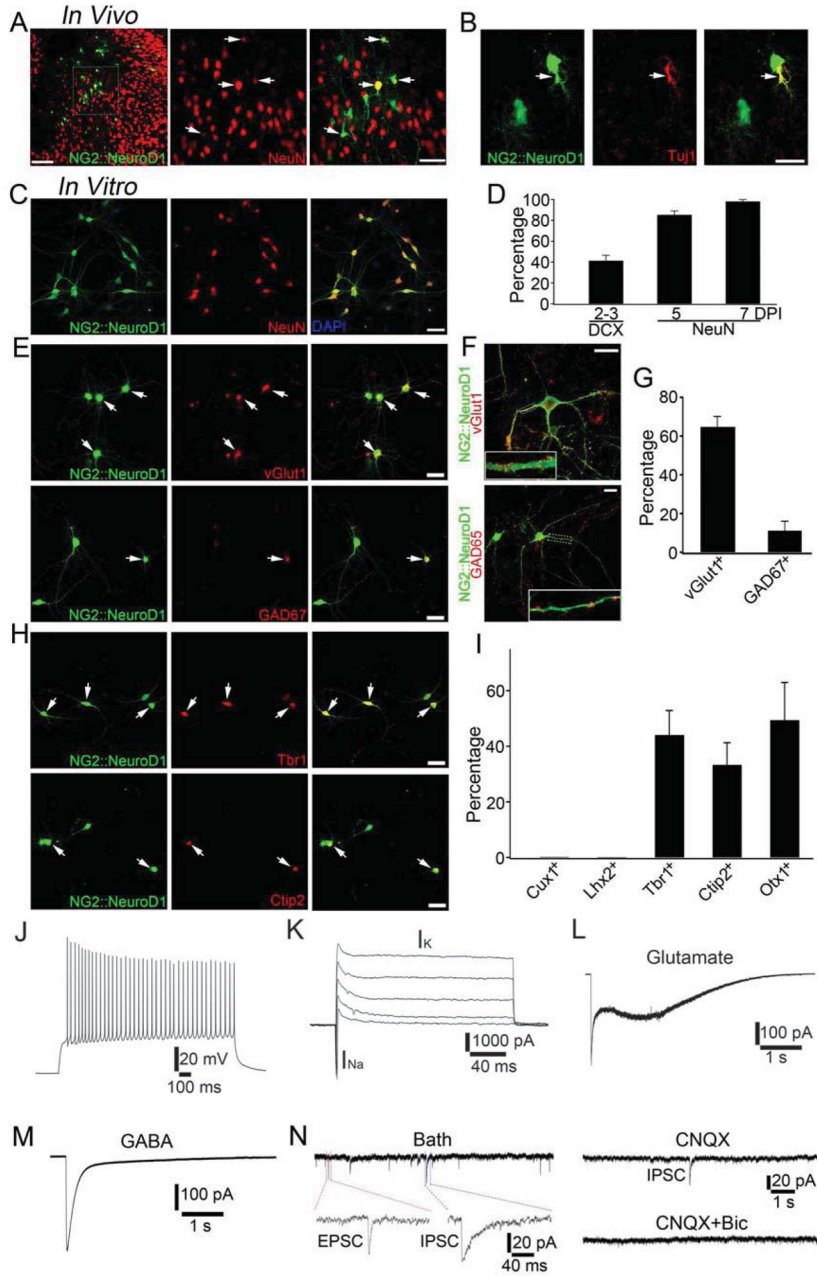


Figure 3. NeuroD1 converts NG2 cells into glutamatergic and GABAergic neurons
 (A–B) *In vivo* injection of NG2::NeuroD1-GFP retrovirus revealed the conversion of NG2 cells into neuronal cells positive for NeuN (A) or Tuj1 (B) (8 DPI). (C–D) Cultured NG2 cells were converted into NeuN-positive neurons within one week after infection by NG2::NeuroD1. (E–G) NG2 cell-converted neurons after NeuroD1-infection were immunopositive for both vGluT1 (>60%) and GAD67 (10%). vGluT1 and GAD65 immunostaining also showed glutamatergic and GABAergic puncta on converted neural dendrites (F). (H–I) Cortical layer neuronal marker immunostaining showed deep layer neuronal properties (Ctip2 and Otx1) after NeuroD1-induced conversion of NG2 cells. Scale bars: 40 μ m for (A), (C), (E), and (H); 20 μ m for (B) and (F). (J–K) NG2-converted neurons showed repetitive action potentials (J; n = 9) and large sodium and potassium currents (K; n

= 10). (L–M) NG2-converted neurons showed large glutamate-evoked current (L; n = 7) and GABA-evoked current (M; n = 7). (N) Spontaneous synaptic events recorded from NG2-converted neurons showed both glutamatergic and GABAergic events, confirming that NeuroD1 can convert NG2 cells into both excitatory and inhibitory neurons.

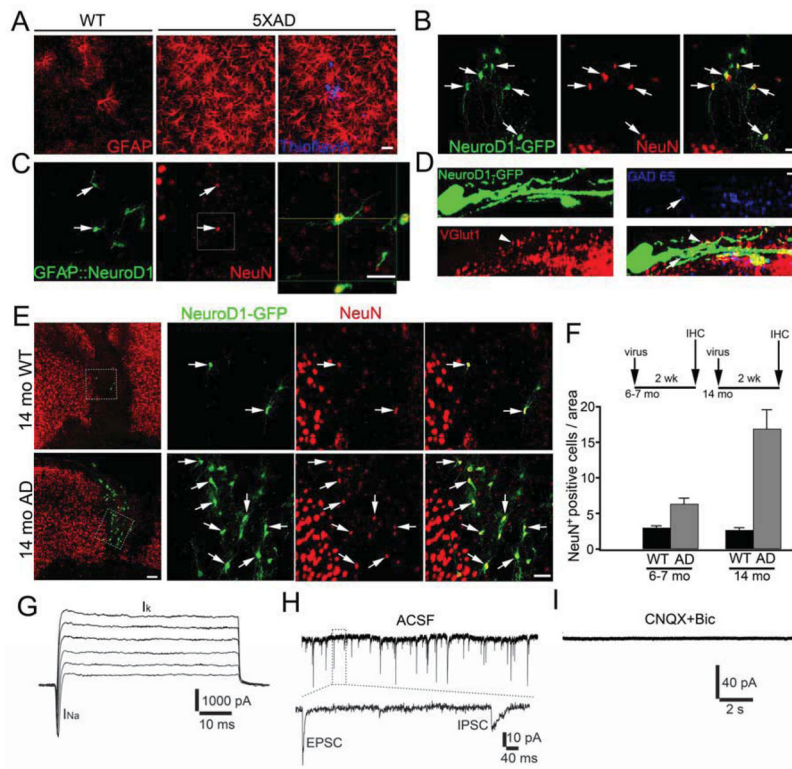


Figure 4. NeuroD1 converts reactive glial cells into functional neurons in AD mouse brain *in vivo* (A) Reactive astrocytes (labeled by GFAP, red) in 5xFAD mouse cortex (5-month old) were significantly increased compared to that in WT cortex. A β plaques were labeled by thioflavin-S (blue). (B) NeuroD1-infected cells (16 DPI) in AD mouse cortex (7-month old) showed clear neuron-like morphology (green) and NeuN staining (red). (C) Injecting GFAP::NeuroD1 retrovirus into AD cortex also converted astrocytes into NeuN-positive neurons (7 DPI). (D) NeuroD1-converted neurons in the AD brain were innervated by glutamatergic (VGlut1, red) and GABAergic terminals (GAD65, blue). Scale bars: 20 μ m for (A) and (C); 40 μ m for (B); 5 μ m for (D). (E) Efficient induction of many new neurons in 14-month old AD animals after NeuroD1-GFP retroviral infection. Scale bar: 100 μ m for low power image, 40 μ m for high power image. (F) Quantified data showing enhanced neural conversion in AD animals compared to WT animals, likely due to more reactive glial cells in old AD brain. (G) Representative traces of sodium and potassium currents recorded from NeuroD1-infected cells in AD cortical slices. (H) Spontaneous synaptic events recorded from NeuroD1-converted neurons (28 DPI) in AD cortical slices. (I) All synaptic events were blocked by CNQX (10 μ M) and BIC (20 μ M).

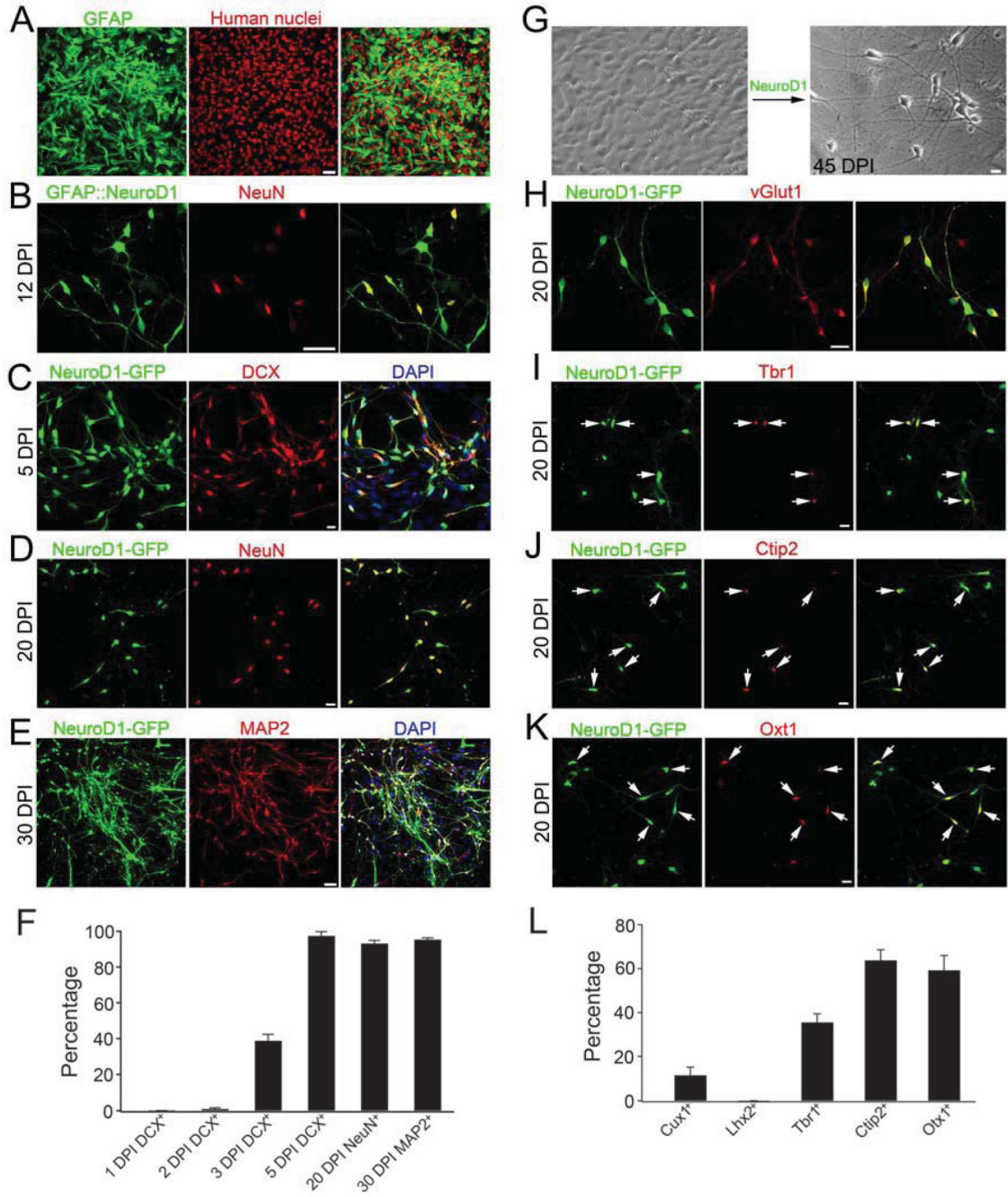


Figure 5. Conversion of cultured human astrocytes into functional neurons
 (A) The majority of cultured human astrocytes were labeled by GFAP (green). (B) Infection by GFAP::NeuroD1 retrovirus converted human astrocytes into NeuN-positive neurons. (C–E) NeuroD1-induced conversion of human astrocytes into neurons as shown by a series of neuronal markers DCX (C), NeuN (D), and MAP2 (E). (F) Quantified data showing a significant increase of conversion efficiency during 3 – 5 DPI. (G) Phase contrast images showing NeuroD1-induced morphological change from astrocytes (left) to neurons (right, 45 DPI). (H) Human astrocyte-converted neurons were immunopositive for vGluT1. (I–K) Cortical layer neuronal markers revealed that human astrocyte-converted neurons were immunopositive for Tbr1 (I), Ctip2 (J), and Otx1 (K). (L) Quantitative analysis of human

astrocyte-converted neurons labeled by superficial (Cux1 and Lhx2) or deep layer (Ctip2 and Otx1) neuronal markers. Scale bars: 50 μm for (A) and (E); 20 μm for panels (C–D) and (G–K); 40 μm for panel (B). See also Suppl. Fig. 5–6.

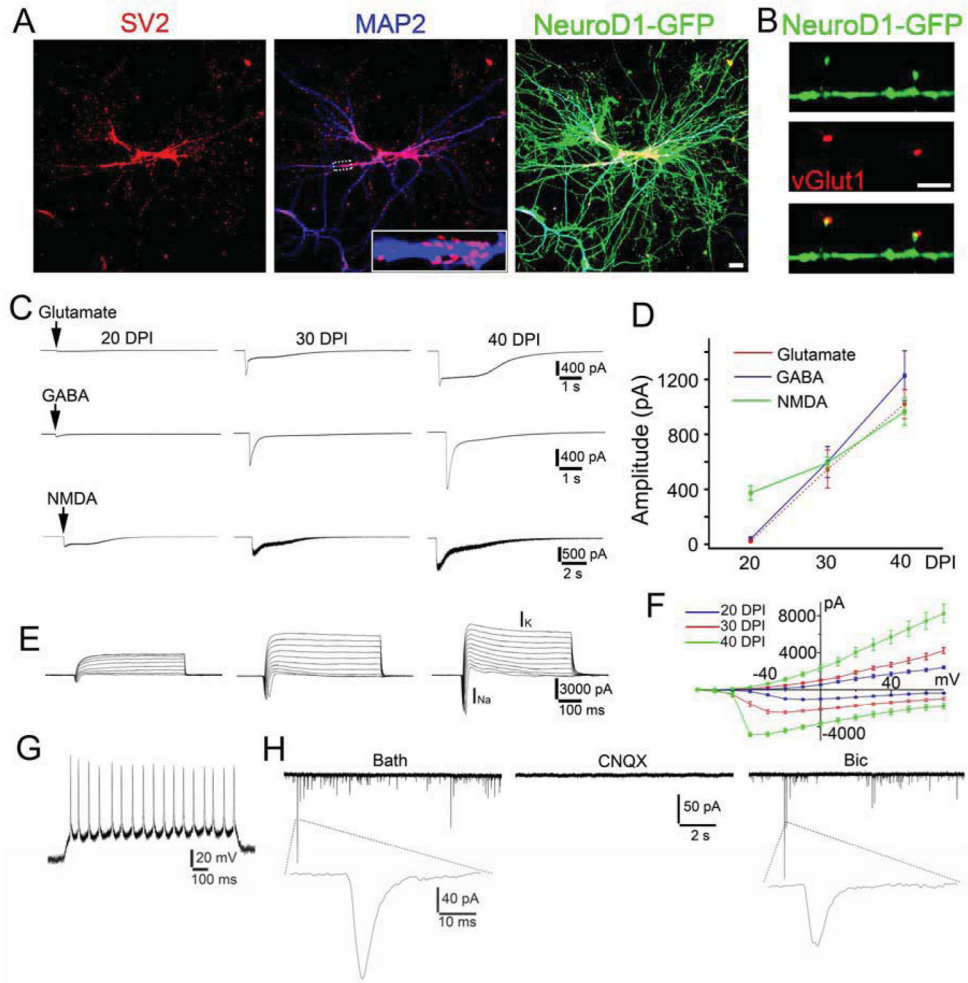


Figure 6. Functional characterization of human astrocyte-converted neurons

(A) Synaptic puncta (SV2, red) on the dendrites (MAP2, blue) of human astrocyte-converted neurons (green, 45 DPI) after NeuroD1 infection. (B) High power image showing vGlut1 puncta (red) co-localized with dendritic spines on NeuroD1-converted neurons. Scale bars: 20 μm for panel (A); 10 μm for panel (B). (C–D) Representative traces (C) and quantitative analysis (D) of the receptor currents induced by bath application of glutamate (100 μM), GABA (100 μM), and NMDA (100 μM). (E–F) Representative traces of Na^+ and K^+ currents (E) and their I–V curve (F) recorded from NeuroD1-converted neurons. (G) Representative trace of repetitive action potentials in NeuroD1-converted neurons (20 DPI). (H) Representative traces of spontaneous synaptic events in NeuroD1-converted human neurons (40 DPI). Note that all synaptic events were blocked by CNQX (10 μM) but not by Bic (20 μM), suggesting that human astrocyte-converted neurons induced by NeuroD1 expression were glutamatergic neurons.