CHEMICAL REPROGRAMMING OF ASTROCYTES INTO FUNCTIONAL NEURONS FOR CNS REPAIR

A Dissertation in

Biology

by

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The mammalian central nervous system (CNS) possesses very limited self-repair capability: very few newborn neurons are generated during adulthood. Regeneration of neurons in the CNS when injured or under pathological conditions remains a major challenge for functional recovery.

Current efforts largely focus on cell replacement therapy with exogenous cells derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) to generate neurons (Sahni and Kessler, 2010; Takahashi et al., 2007; Takahashi and Yamanaka, 2006). In spite of the great promise, cell transplantation approaches face significant hurdles, such as poor survival rate, immunorejection, tumorigenesis and differentiation uncertainty (Lee et al., 2013; Lukovic et al., 2014).

Glial cells represent a large reservoir for generating neurons locally. In response to CNS injury, glial cells (e.g., astrocytes, NG2 cells and microglia) are activated to proliferate and become hypertrophic to occupy the injured CNS area, thus limiting the spreading of injury in the acute stage (Pekny and Nilsson, 2005; Robel et al., 2011; Sofroniew and Vinters, 2010). On the other hand, long-term occupancy of the injury sites by reactive glial cells will result in the secretion of neuroinhibitory factors that prevent neuronal growth, eventually forming glial scars inside the CNS (Sofroniew and Vinters, 2010). Reactive glial cells have been widely reported after brain injury, spinal cord injury and neurodegenerative disorders, such as Alzheimer’s disease (AD) (Burda and Sofroniew, 2014; Gwak et al., 2012; Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010; Verkhratsky et al., 2012).

Recent studies, including our own, have demonstrated that astroglial cells can be directly converted into functional neurons in vitro (Guo et al., 2014; Heinrich et al., 2010; Zhang et al., 2015) and in vivo (Grande et al., 2013; Guo et al., 2014; Heinrich et al., 2010; Liu et al., 2013; Torper et al., 2013) by ectopic overexpression of neural transcription factors (TFs). So far, conversion of glial cells into neurons has been largely...
achieved using viral-based expression of TFs, but clinical applications might be hampered due to complex brain surgery and genetic alteration.

Here we report a novel technology, chemical reprogramming that uses defined small molecules to reprogram cultured human astrocytes into functional neurons with high efficiency. Chemically converted human neurons can survive in long-term culture and in the mouse brain. Moreover, they form elaborate neuronal networks in culture and can integrate into mouse neural circuits.

We further examined the mechanisms underlying small molecule-induced glia-to-neuron conversion. Our results suggest that human neurons can be directly generated by conversion of human astrocytes without a transient stem cell stage. Intriguingly, epigenetic silencing of glial genes and transcriptional activation of neural TFs, such as NEUROD1 and NGN2, are involved in chemical reprogramming. In addition, we evaluated the functional role of each individual small molecule included in the cocktail and identified the core molecules that are essential for highly efficient glia-to-neuron reprogramming.

Towards future therapeutic applications, we also tested the effect of a small-molecule cocktail on neurogenesis in vivo. Interestingly, the small molecules injected into mouse brains induced stem cell properties in cortical and striatal astrocytes. Moreover, adult neurogenesis was significantly enhanced by local injection of small molecules in adult mouse hippocampus.

In conclusion, our study opens a new avenue using chemical compounds to reprogram reactive glial cells into functional neurons for CNS repair. Our chemical reprogramming method may potentially be translated into clinical therapy to help patients suffering from CNS disorders.
Graphic Abstract
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ALKs</td>
<td>activin receptor-like kinases</td>
</tr>
<tr>
<td>Ascl1</td>
<td>achaete-scute homolog 1</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>Brn2a</td>
<td>POU domain, class 3, transcription factor 2</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
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<tr>
<td>BS-seq</td>
<td>bisulfite sequencing</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ChABC</td>
<td>chondroitinase ABC</td>
</tr>
<tr>
<td>CNS</td>
<td>central nerve system</td>
</tr>
<tr>
<td>CHIP-qPCR</td>
<td>chromatin immunoprecipitation - quantitative real-time PCR</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-responsive element-binding protein</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>DAPT</td>
<td>N-[N-(3,5-difluorophenacetyl)-L-alanyl]- S-phenylglycine t-butylerster</td>
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<td>DCX</td>
<td>doublecortin</td>
</tr>
<tr>
<td>Dlx2</td>
<td>distal-less homeobox 2</td>
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<tr>
<td>DMNT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DPI</td>
<td>days post cell injection</td>
</tr>
<tr>
<td>EAAT2 or GLT1</td>
<td>excitatory amino acid transporter 2</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EPSCs</td>
<td>excitatory postsynaptic currents</td>
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<td>ESC</td>
<td>embryonic stem cell</td>
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<tr>
<td>FGF2</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Glt1</td>
<td>glial glutamate transporter 1</td>
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<tr>
<td>GM</td>
<td>glial medium</td>
</tr>
<tr>
<td>Gsx2</td>
<td>genetic-screened homeobox 2</td>
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<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HA</td>
<td>human astrocyte</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HMTase</td>
<td>histone methyltransferase</td>
</tr>
<tr>
<td>HP</td>
<td>holding potential</td>
</tr>
<tr>
<td>hSC</td>
<td>human stem cell</td>
</tr>
<tr>
<td>HuNu</td>
<td>human nuclei</td>
</tr>
<tr>
<td>H3Ac</td>
<td>acetylation of histone 3</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>trimethylation of histone 3 at lysine 4</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>trimethylation of histone 3 at lysine 27</td>
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<tr>
<td>IGF</td>
<td>insulin-like factor</td>
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### Glossary

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>IPSCs</td>
<td>inhibitory postsynaptic currents</td>
</tr>
<tr>
<td>LCN2</td>
<td>lipocalin 2</td>
</tr>
<tr>
<td>Lmx1a</td>
<td>LIM homeobox transcription factor 1, α</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MeCP2</td>
<td>methyl DNA binding protein</td>
</tr>
<tr>
<td>MeDIP-seq</td>
<td>methylated DNA immunoprecipitation followed by sequencing</td>
</tr>
<tr>
<td>Myt1l</td>
<td>myelin transcription factor 1-like</td>
</tr>
<tr>
<td>NDM</td>
<td>neuronal differentiation medium</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
</tr>
<tr>
<td>NG2</td>
<td>neural/glial antigen 2</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>Ngn2</td>
<td>neurogenin 2</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NPC</td>
<td>neural progenitor</td>
</tr>
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<td>NSC</td>
<td>neural stem cell</td>
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<tr>
<td>NT3</td>
<td>neurotrophin-3</td>
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<td>Nurr1</td>
<td>nuclear receptor related 1 protein</td>
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<tr>
<td>Olig2</td>
<td>oligodendrocyte transcription factor</td>
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<td>Pax6</td>
<td>paired box protein 6</td>
</tr>
<tr>
<td>Purmo</td>
<td>purmorphamine</td>
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<tr>
<td>RA</td>
<td>retinoid acid</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<tr>
<td>RE1</td>
<td>repressor element 1</td>
</tr>
<tr>
<td>REST</td>
<td>RE1 silencing transcription factor</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>SAG</td>
<td>smoothened agonist</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>Sox2</td>
<td>sex determining region Y-box 2</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
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<tr>
<td>TACE</td>
<td>tumor necrosis factor-α-converting enzyme</td>
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<td>TGFβ</td>
<td>transforming growth factor β</td>
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<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
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<tr>
<td>TLE1-4/Grg</td>
<td>Transducin-like E/Groucho-related gene</td>
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<tr>
<td>TSS</td>
<td>transcription start site</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<tr>
<td>Tuj1</td>
<td>β-tubulin 3</td>
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<tr>
<td>t-PA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>VACHT</td>
<td>vesicular acetylcholine transporter</td>
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<tr>
<td>VGluT1</td>
<td>vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid</td>
</tr>
</tbody>
</table>
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Chapter 1
Introduction

1.1 CNS injury

Central nerve system (CNS) disorders, such as injury and neurodegenerative diseases, cause enormous suffering to individuals and put a heavy burden on society. Although the etiology and pathology of CNS injury have been intensively studied, very few therapeutic approaches have proved to be effective for CNS repair and functional recovery. This is partially due to the complex nature of the CNS response to insult and the limited capability of the CNS for self-repair. Thus, understanding of the cellular and molecular mechanisms underlying CNS disorders will facilitate the development of therapeutic strategies.

1.1.1 Neuronal loss leads to CNS functional deficits

CNS trauma and diseases often result in permanent damage to functional neurons. The neuronal death process begins immediately (minutes to hours) in response to varying degrees of insult (e.g., mechanical force, ischemia and toxin) and mostly occurs as a result of acute disruption of the plasma membrane (Farkas et al., 2006; Geddes et al., 2003). As a consequence of primary neuronal damage, a cascade of biochemical, metabolic and molecular events is triggered that, in turn, contributes to the spread of the damage. These events include the excessive release of excitatory neurotransmitters, free radicals, tissue ions, biogenic amines and lipid-dependent enzymes; alteration of energy states; and activation of neural inflammation (Hovda et al., 1991; Yoles et al., 1991; Yoshino et al., 1991). All of these changes disrupt cellular homeostasis for hours to days,
leading to even greater neuronal loss than that might have been expected from the severity of primary injury (Kipnis et al., 2001).

The neuronal loss inevitably leads to dysfunction of affected CNS regions. For instance, the loss of motor neuron in the case of spinal cord injury (SCI) might cause deficits in locomotor function (e.g., paralysis) (Ebrahim et al., 2008). Damage to hippocampal neurons is strongly associated with learning and memory decline (Padurariu et al., 2012). On the other hand, neuroprotective innervations had beneficial effects on functional recovery after stroke, indicating the importance of CNS neurons (Majid, 2014).

Notably, even for neurons that survive the primary and secondary injuries, their axons may be damaged. The Axon degeneration breaks down the communication inside the nervous system, thus contributing to the dysfunction of neural network (Conforti et al., 2014). As a simple example, paralysis in lower limbs, in the case of injury to the cervical spinal cord, is essentially mediated by the degeneration of the afferent and efferent axon tracts, rather than direct damage to the motor neurons in control (Kerschensteiner et al., 2005).

Collectively, an effective treatment approach for CNS repair should consider multiple processes, including protecting and regenerating injured neurons, as well as bridging the axons to their original targets.

1.1.2 Limited regenerative capability of adult CNS

The mammalian CNS possesses limited capability for self-repair, as indicated by the small number of newborn neurons produced in response to injury, as well as the impaired potential for axon regrowth.

Neurogenesis is largely completed before birth in mammalian animals. In the adult brain, only a small number of new neurons are generated in two major neurogenic regions: the subventricular zone (SVZ) lining the lateral ventricles, and the subgranular zone (SGZ) in the hippocampus. More recently, new sites inside the brain have been discovered with
limited neurogenic capability, such as the hypothalamus and the striatum (Sousa-Ferreira et al., 2013; Ernst et al., 2014). Several lines of evidence have suggested that ischemic stroke is able to enhance neurogenesis in SVZ, from where newly generate neurons migrate to the injured striatum for cell replacement (Arvidsson et al., 2002; Guo et al., 2012; Hou et al., 2008; Li et al., 2010; Wang et al., 2007; Yamashita et al., 2006; Zhang et al., 2014). However, the newborn neurons are few and limited to certain brain regions. Outside the neurogenic niches in other brain areas and the entire spinal cord, neurogenesis is tightly repressed under physiological conditions. Although recent studies reported that astroglial cells residing in the striatum regain neurogenic potential and contribute to neurogenesis in post-stroked striatum (Bi et al., 2011; Duan et al., 2015; Magnusson et al., 2014), these astrocyte-derived new neurons are limited in number with immature properties. Their long-term survival and functions need to be evaluated. In addition, astrocytes in other CNS regions, such as the cortex and spinal cord, which are physically vulnerable to injury, almost completely lack neurogenic capability (Arvidsson et al., 2002; Sirko et al., 2013). SVZ-derived precursor cells give rise to glial cells, but not neurons in the cortex (Faiz et al., 2015). Thus, the evidence supports the restricted capability of CNS in neuroregeneration after damage.

1.1.3 Reactive glial cells and CNS repair

Glial cells are the most abundant cell types in the mammalian brain. They provide fundamental support to the nervous system and participate in physiological and pathological processes (Barres, 2008). Three major glial types include astrocytes, oligodendrocytes and microglia. Astrocytes exhibit star-like morphology with many major processes and send thousands of fine end feet to contact neurons and blood vessels to regulate brain functions (Nave, 2010; Sofroniew and Vinters, 2010).

In pathological conditions, the crucial role of astrocytes has been well recognized. As part of the early wound-healing process, astrocytes respond to injury immediately and become activated, as indicated by the increased proliferative rate, the hypertrophic
morphology, and the upregulation of protein markers, such as glial fibrillary acidic protein (GFAP), nestin and vimentin (Bardehle et al., 2013; Bush et al., 1999; Rolls et al., 2009).

In the acute phase after injury (hours to days), these reactive astrocytes occupy the injured area, forming a boundary to separate the lesion area from the healthy neural tissues (Fitch and Silver, 1997; Reier and Houle, 1988). As a result, the toxic factors induced by primary insults are isolated and restricted within the lesion site (Chen et al., 2001; Cui et al., 2001). In support of this notion, experimental ablation of the scar-forming astrocytes after brain stab injury, results in the enhanced leukocyte infiltration, impaired blood-brain barrier (BBB) and substantial neuronal degeneration, suggesting an essential role of reactive astrocytes in the early wound healing (Bush et al., 1999; Stichel and Muller, 1998). In addition to sealing the site of injury, astrocytes also exhibit neuroprotective effect by reducing nitric oxide toxicity (Chen et al., 2001) and neural inflammation (Ousman and Kubes, 2012).

In the sub-acute phase (weeks after injury) and the chronic phase (months to years), long-term stay of reactive astrocytes forms a scar-like tissue, which is generally considered as a physical barrier against axon penetration. In addition, the inhibitory molecules secreted by glial scar, such as chondroitin sulfate proteoglycans (CSPGs), negatively regulate axon regeneration (Rolls et al., 2009). A growing body of evidence has demonstrated the CSPG effect on neurite retraction and growth cone collapse (McKeon et al., 1991). On the other hand, removal of CSPG by specific enzymes or antibodies leads to a dramatic increase in axonal regrowth (Caggiano et al., 2005; Faissner et al., 1994; Smith-Thomas et al., 1995). Moreover, once activated, astrocytes may lose neuroprotective functions and even gain neurotoxic properties by secreting neurotoxic factors. For example, astrocytes can uptake the extracellular neurotransmitter glutamate through excitatory amino acid transporter 2 (EAAT2 or GLT1); in reactive astrocytes, GLT1 deficiency contributes to excessive glutamate, which often leads to excitatory neuronal damage (Bi et al., 2013; Tanaka et al., 1997). In addition, inducible factor lipocalin 2 (LCN2) is secreted by reactive astrocytes and shows selective toxic effect on neurons (Bi et al., 2013; Lee et al., 2012; Zamanian et al., 2012). Collectively, despite an initial positive effect, long-term effect of reactive astrocytes is more negative in the neural repair process.
after CNS injury.

The timing of scar formation and removal should be taken into account in evaluating its effects. Emerging evidence suggests that reactive astrocytes are required in the acute phase after injury for sealing the lesion and cleaning the injury. On the other hand, the long-term existence of the scar tissue results in the profound axon inhibition, which significantly impairs neural repair. In agreement with this time-dependent view, the removal of glial scar on day 0 or day 7 after spinal cord injury led to detrimental or beneficial effects, respectively (Rolls et al., 2008).

Notably, complete ablation of astrocytes may cause excessive neural inflammation and impair neuroregeneration (Bush et al., 1999; Anderson et al., 2016). In a recent paper in *Nature*, Anderson et al. utilized genetic loss-of-function methods to prevent or completely remove reactive astrocytes from injured mouse spinal cord (Anderson et al., 2016). Consequently, the transected axon failed to regrow. Therefore, they concluded that astrocyte scar formation aids rather than prevents CNS axon regeneration. However, complete depletion of astrocytes is an abnormal process that severely disrupts neuron-glia interactions in the CNS. More importantly, without showing the evidence that more severe astroglial scars can achieve better axonal regeneration, it is not convincing to claim that the glial scar benefits neural repair. In addition, this finding contradicts many reports, including one published earlier by the same research group. In a *Neuron* paper (1999) published by the same group, the researchers used a toxin to deplete GFAP-positive astrocytes and observed a pronounced increase in local neurite outgrowth (Bush et al., 1999). Despite the controversy, complete ablation of glial scar by genetic targeting may create an unnatural environment and disrupt the delicate homeostasis needed for CNS repair. Therefore, a different method is needed to reduce the reactivity of glial scar without killing the endogenous cells.

1.2 Current therapeutic options

Currently, the therapeutic strategies to treat CNS injury are limited to lesion
restriction, rehabilitation, and prevention of complications. Unfortunately, all of these have limitations. Some must be applied in an extremely narrow time window. For example, recombinant tissue plasminogen activator (t-PA) (1995) reduces stroke infarction size, but only if administrated within 3 hours of the onset of ischemia, which limits its wide clinical application. The curative effects of other interventions are still under debate. For instance, with its anti-inflammatory and antioxidant properties, methylprednisolone used to be employed to treat acute spinal cord injury. However, contradictory results from several clinical trials (Baron, 2006; Bracken et al., 1990; Roberts et al., 2004) have put its therapeutic effects in doubt. In addition, the side effects of high doses of glucocorticoid, such as gastric bleeding, also restrict its application (Kwon et al., 2004; Short et al., 2000). Nevertheless, attempts to find therapies continue. In the past several decades, research has extended our understanding of CNS injury at the molecular and cellular levels, and several approaches have been developed for pre-clinical or clinical trials.

1.2.1 Molecular therapeutic interventions

A wide variety of strategies at the molecular level has been developed to facilitate the neural tissue repair: 1) to protect the neurons from secondary injury, 2) to improve the microenvironment for axon re-growth, 3) to promote the endogenous neurogenesis for replacement of the damaged neurons.

1.2.1.1 Neuroprotective molecules for CNS repair

Growth factors, such as brain-derived neurotrophic factor (BDNF), insulin-like factor (IGF), nerve growth factor (NGF) and glial cell line–derived neurotrophic factor, have been well studied for their neuroprotective effects in animal experiments. Perfusion of growth factors into the lesion site facilitates functional recovery in spinal cord injury and stroke (Bradbury et al., 1999; Oudega and Hagg, 1996; Sharma et al., 2005). However, the
beneficial effect in lab animals may not be translated in clinic: clinical trials have failed, which shows either a lack of efficacy or significant side effects (Apfel, 2002). Based on the observation that excessive and prolonged neuroinflammation leads to neuronal death, anti-inflammatory drugs, such as methylprednisolone, Minocycline or IL-10, have been tested in animal models of CNS injury and have shown certain neuroprotective effects (Brewer et al., 1999; Casha et al., 2012; Popovich and Longbrake, 2008). However, as mentioned before, the ambiguous curative effect and severe secondary effects have invited broad debate for their clinical application.

1.2.1.2 Molecules promoting axon regeneration for CNS repair

Primary damage in the CNS triggers a series of events leading to an axon-inhibitory environment. Various therapies have been developed to target and overcome those inhibitors of axon regeneration. Several laboratories tried to use antibodies for neutralization of Nogo-A, an axon-growth inhibitor secreted by myelin debris in damaged brain or spinal cord (Bregman et al., 1995; Schnell and Schwab, 1990; Thallmair et al., 1998). Promising results from animal models showed improved axon regrowth and functional recovery. Currently, Phase I clinical trials are in progress to test the effect of anti-Nogo-A therapy (M. Schwab with Novartis). The results related to the efficacy have not yet been disclosed (Nagoshi and Fehlings, 2015).

As mentioned above, reactive glial cells impair the axon regrowth by secreting axon-inhibitory molecules, such as CSPGs (Thuret et al., 2006). Bacterial enzyme chondroitinase ABC (ChABC) was developed to degrade CSPG. Delivery of ChABC in the lesion site has shown beneficial effects on axon regeneration and recovery of locomotor function in SCI rodent models (Bradbury et al., 2002; Garcia-Alias et al., 2008), which also led to the Phase II clinical trials in testing ChABC for herniated lumbar discs (Seikagaku Corporation). Despite promising results, the bacterial origin of ChABC, as well as the digestion products, raise concerns about potential immune responses, which may hinder its clinical translation (Pires and Pego, 2015).
Thus, although a number of therapeutic approaches targeting axon regrowth have been proposed and showed a promise when applied in mouse models of CNS injury, it is unclear whether they can be translated into clinical applications. Take SCI for example, even though the afferent and efferent axon tracts are regenerated, if the damaged motor neurons cannot be protected or regenerated, the functional recovery might be rather limited.

1.2.1.3 Enhancement of endogenous neurogenesis

Since neural stem cells (NSCs) with neurogenic potential have been characterized in the adult CNS, the possibility to harness such cells for CNS repair has been intensively explored. Ideally, if the endogenously generated new neurons could replace the damaged or dead ones, the neural network will be rewired, and CNS functions will be rebuilt. In fact, enhanced neurogenesis has been demonstrated in mouse SVZ in response to ischemic stroke, as indicated by achaete-scute homolog 1 (Ascl1)-positive neuroblasts and doublecortin (DCX)-positive newborn neurons migrate out of SVZ towards the lesion site (Arvidsson et al., 2002; Parent et al., 2002). However, due to the limited self-repair capability, those newly generated neurons occur in only small numbers, and most of them exhibit the maturation deficit (Arvidsson et al., 2002; Magnusson et al., 2014). To utilize endogenous neurogenesis for CNS repair, a number of molecules have been tested in animal models to determine if they can expand the stem cell pool or promote neural differentiation. Interestingly, basic fibroblast growth factor (FGF2) facilitates cortical cell replacement and functional recovery after cortical injury in adult rats (Leker et al., 2007). Similarly, delivery of epidermal growth factor (EGF), FGF2, to the injured spinal cord results in a limited number of new neurons that express DCX (Ohori et al., 2006). Furthermore, genetically engineered neural progenitors have shown neurogenic capabilities in injured brain areas (Buffo et al., 2005).

In spite of these promising findings, cell replacement from endogenous NSCs has limitations. The potential side effects must be carefully investigated. For example, high
doses of FGF2 yielded inhibitory effect on the oligodendrocyte differentiation and myelin production (Assanah et al., 2006; Butt and Dinsdale, 2005). Moreover, excessive expansion of stem cells may lead to tumorigenesis. In support of this view, glioma, the most aggressive brain tumor, may be derived from endogenous stem cells (Bao et al., 2008; Singh et al., 2004).

1.2.2 Cell-based therapeutic interventions

Given the relatively low level of endogenous neurogenesis, transplantation of exogenous cells or tissues might be another solution for cell replacement. To date, various types of cells have been grafted to injured CNS in rodent models, and many stem cell–based clinical trials are in progress.

1.2.2.1 Pluripotent stem cells

Embryonic stem cells (ESCs) can self-renew and possess the ability to differentiate into almost any type of somatic cells including neurons. Thus, they are considered to be an ideal cell source for cell replacement therapy. In many laboratories, ESCs have been transplanted into adult rodent spinal cords or brains after CNS injury, and exhibited modest efficacy in functional improvement (Harper et al., 2004; McDonald et al., 1999). However, based on the results from animal experiments, several concerns hinder clinical applications: 1) The most common fate of transplanted cells is cell death (Bakshi et al., 2005). Having large amounts of dead cells may cause the microenvironment in lesion site to deteriorate. 2) To facilitate the survival of allogeneic cells, the immune system needs to be purposely suppressed, which may lead to uncontrollable side effects, such as bacterial or fungal infection. 3) The infinite proliferative nature of pluripotent stem cells raises the risk of tumorigenesis. 4) The neuronal differentiation of ESCs is relatively poor without specific modulation. 5) Although ESCs retain the capacity to generate the desired cell
types after transplantation, uncontrollable and inappropriate differentiation may result in undesired cell types in CNS. 6) The maturation and integration of differentiated neurons is less clear. 7) Even upon the successful differentiation and survival, the newly generated neurons may still be restricted in transplantation site by glial scar.

To address these concerns, modifications have been made to ESCs. The discovery of induced pluripotent stem cells (iPSCs) from skin fibroblasts (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) opened a brand new avenue for cell replacement therapy, as the autologous pluripotent stem cells are accessible and can be transplanted back to the injured CNS for replacement therapy (Medvedev et al., 2010). In the case of autologous transplantation, immunorejection is no longer a concern. However, the other limitations remain unsolved.

1.2.2.2 Neuronal lineage-restricted progenitors

To avoid teratoma formation, pluripotent stem cells have been pre-differentiated towards neuronal lineage before transplantation. A variety of differentiation protocol have been developed for a high yield of NSCs from ESCs or iPSCs (Chambers et al., 2009; Chambers et al., 2012; Kim et al., 2002; Liour and Yu, 2003; Lowell et al., 2006; Zhang et al., 2001). In addition, specific subtypes of neurons or neural progenitors (NPCs) have been induced for treatment of diseases, such as dopaminergic neurons for treating Parkinson’s disease (Kim et al., 2002; Mendez et al., 2008; Morizane et al., 2006). Promisingly, several groups have reported the functional improvement after neuroprogenitor transplantation in injured or diseased CNS (Cummings et al., 2005; Iwanami et al., 2005; Mitsui et al., 2005; Ogawa et al., 2002; Thuret et al., 2006).

However, the challenges remained in cell survival, differentiation, maturation and integration as mentioned above. Notably, limited neuronal differentiation has been shown in most studies, and glial differentiation dominates cell fates after transplantation (Horky et al., 2006). Thus, a clear correlation between newly generated neurons and behavioral recovery still needs to be demonstrated.
1.2.2.3 Translation from bench to clinic therapies

With considerable optimism from animal experiments, stem cell therapies have been expected to bring substantial benefit to patients with a variety of CNS diseases and injuries. Currently, a large number of cell transplantation therapies in clinical trials utilize a wide range of cell types, including ESC-derived NSCs, adult NSCs, fetal-derived progenitors and bone marrow–derived progenitors (Ratcliffe et al., 2013; Trounson and DeWitt, 2016). While many clinical trials are in progress, it is still too early to make predictions about their clinical efficacy. Based on the clinical data available, although severe side effects such as tumorigenesis are rarely reported, there is little evidence showing functional benefit of stem cell therapy for patients suffering with a CNS injury, such as SCI and stroke (Doeppner and Hermann, 2014; Savitz et al., 2014; Savitz et al., 2011). In addition, many cell types, especially bone marrow progenitors, exhibit trophic influences only within a transient time window after transplantation. The long-term effect of stem cells on cell replacement and functional restoration needs to be clarified.

1.2.3 Developing an optimal therapy for neuroregeneration

During the past several decades, tremendous efforts have been made in research into CNS repair, and encouraging results are accumulating from laboratory animal experiments. However, no fully restorative therapies have been discovered to date.

Exogenous cell transplantation holds the promise for replacement of the damaged cells. However, several hurdles need to be overcome before clinical application, as mentioned above. Of note, even upon the successful survival and differentiation of new neurons, it is difficult for the axons to grow and make connections to the targeting neurons in the presence of glial scar.

On the other hand, molecular therapies targeting axon-inhibitory factors have shown certain benefit to the regeneration of damaged axons. However, only if the glial scar is attenuated can the restriction to axon regrowth be largely lifted. In addition, the
functional deficits caused by the damaged or dead neurons will not be restored without cell replacement.

A successful therapy aimed at neuronal regeneration should provide solutions to at least two basic questions: 1) To restore the neural function, how can we replace the dead/malfunctioning neurons with functional ones? 2) To rebuild the neuronal network, how can we improve the microenvironment from axon-inhibitory to permissive by breaking down the mechanical and biochemical barrier such as glial scar? In this context, a revolutionary strategy is required to achieve an optimal therapeutic efficacy.

1.3 Reprogramming of glial cells into neurons for CNS repair

Neurogenesis outside the neurogenic regions is extremely restricted in mammalian brain. However, non-neurogenic cells, such as glial cells and pericytes, may be amenable to exogenous reprogramming cues, allowing them to generate neurons in vivo (Grande et al., 2013; Guo et al., 2014; Heinrich et al., 2014; Niu et al., 2013; Su et al., 2014; Torper et al., 2013). The in vivo reprogramming, which regenerates neurons at the expense of reactive glial cells is emerging as one of the most promising approaches for patient-specific cell therapies.

Different from conventional stem-cell-based cell replacement, in vivo reprogramming enables fast generation of autologous neurons by circumvention of time-consuming cell production and sophisticated cell transplantation surgery. In addition, the transformation of inhibitory glial scar into neural tissue may improve the environment to be more conducive to neurons. Therefore, it could be an attractive option for regenerative therapy.
Figure 1-1. Illustration of CNS injury and astrocyte-to-neuron reprogramming

CNS injury causes substantial neuronal loss and neuroinflammation. Astrocytes undergo activation process in acute injury stage and form a fence to separate the lesion area from the spared neural tissues. However, in the sub-acute and chronic phases, long-term stayed reactive astrocytes form glial scar to inhibit the neural regeneration by serving as the physical barrier and secreting axon-inhibitory molecules. Reprogramming of reactive astrocytes into neurons may regenerate new neurons and reduce the glial scar, and thus facilitate the CNS repair.

1.3.1 Direct conversion of glial cells into proliferative neuroblasts

The observation that adult NSCs share many features with resident astrocytes invited great interest to explore the possibility of utilizing astrocytes for neuron production. Recently, several lines of evidence demonstrated the neurogenic potential of astrocytes. In a study on ischemic stroke, genetic inhibition of Notch signaling in striatum enhanced the transition from astrocytes to neuroblasts (Magnusson et al., 2014). Another study led by
Dr. Nakafuku demonstrates the ectopic expression of TF genetic-screened homeobox 2 (Gsx2) and Ascl1 in mature astrocytes could induce many features of NSCs, including substantial proliferation and neurogenesis in vivo (Andersen et al., 2014; Lopez-Juarez et al., 2013). Employing a similar approach, a research group led by Dr. Zhang at UT Southwestern found that by genetically overexpressing TF sex determining region Y-box 2 (Sox2) in reactive astrocytes, the infected cells obtained neuroblasts properties, as indicated by the increased proliferative rate and upregulated expression of the neuroblast markers, such as DCX (Niu et al., 2013; Su et al., 2014). Immunostaining of Ki67 and BrdU-incorporation, which are indicators of cell proliferation, confirmed the presence of persistently dividing cells. In addition, by adding neurotrophic factors BDNF and Noggin, those reprogrammed neuroblasts could differentiate into functional neurons and survive in adult brain for 2 months. By using this technology, they reprogrammed resident astrocytes into neuroblasts in both stab injured mouse brain and traumatically injured spinal cord (Niu et al., 2013; Su et al., 2014). It will be interesting to investigate the efficacy on functional restoration. In addition to TFs, microRNAs reprogram in situ striatal astrocytes into neuroblasts with the help of valproic acid (VPA). However, the neuronal function and integration remain to be investigated (Ghasemi-Kasman et al., 2015). In spite of the encouraging therapeutic potential, the possible side effect should not be overlooked. As mentioned above, expansion of dividing progenitors in brain may raise the risk for tumorigenesis. How to precisely control the cell proliferation versus neural differentiation remains to be solved.

1.3.2 Direct conversion of glial cells into functional neurons

Starting in early 2000, studies have reported that astrocyte cell fate can be switched to neurons in vitro by ectopic expression of TFs associated with neural development, such as paired box protein 6 (Pax6), oligodendrocyte TF (Olig2), Ascl1 and neurogenin 2 (Ngn2) (Berninger et al., 2007a; Berninger et al., 2007b; Blum et al., 2011; Buffo et al., 2005; Heinrich et al., 2010; Heins et al., 2002). These proof-of-principle studies showed the
neural plasticity of cultured astrocytes. Interestingly, neurons derived from cultured rodent astrocytes are mostly glutamatergic. Later, Heinrich et al. reported that, with forced expression of distal-less homeobox 2 (Dlx2), a TF pivotal in GABAergic neuron development, the cultured postnatal cortical astrocytes can be converted into GABAergic neurons (Heinrich et al., 2010). In addition, other glial types, such as neural/glial antigen 2 (NG2) glia and pericytes, can be reprogrammed into neurons in the culture dish with Ngn2 transduction. Those in vitro studies laid the groundwork for the recent explorations on translating the neural reprogramming method into in vivo.

Torper and colleagues transduced the Ascl1, Brn2a and myelin TF 1-like (Mytl1) into human astrocytes in vitro, and activated the transgenes via tamoxifen after transplanting the cells into mouse brain. Six weeks after, human neurons expressing neuronal marker hNCAM were observed, indicating the successful reprogramming (Torper et al., 2013). Using the same three factors, Torper et al. (2013) showed that glial cells in vivo might also be converted into neuron-like cells, but no detailed analysis was carried out. In a separate study, Grande et al. (2013) observed newborn neurons generated from resident glial cells in the stab-injured neocortex and striatum after Ngn2 transduction, and perfusion of growth factors further enhanced neurogenesis (Grande et al., 2013). However, the Ngn2-induced neurogenic efficiency is relatively low, which significantly limits its therapeutic applications.

In contrast, our group demonstrated that highly efficient glia-to-neuron conversion can be achieved by forced expression of a single TF, NeuroD1, in mouse brain (Guo et al., 2014). After brain stab injury, most of the reactive astrocytes transduced with NeuroD1-retrovirus changed their cell fate into neuronal cells expressing the neuronal markers DCX, β-tubulin 3 (Tuj1) and NeuN. Those NeuroD1-converted neurons were capable of firing repetitive action potentials and formed synaptic connections with surrounding neurons, indicating they were fully functional and have integrated to the host neural network. The lack of stem cell marker expression suggested the neurons were directly converted from astrocytes without going through a transient progenitor stage. Interestingly, majority of the newly generated neurons obtained deep cortical layer properties and were mostly glutamatergic (Guo et al., 2014). We also applied the same technology in mouse brain of
Alzheimer’s disease models and demonstrated the potential of NeuroD1-induced glia-to-neuron conversion in treating neurodegenerative disorders. Our data suggested that the \textit{in vivo} reprogramming of reactive astrocytes into functional neurons is possible and could be utilized for brain repair (Guo et al., 2014).

In addition to astrocytes, NG2 glia can be converted to neurons. Our own experiments demonstrated that ectopic expression of NeuroD1 in NG2 glia induced a mixture of glutamatergic and GABAergic neurons (Guo et al., 2014). After brain injury, Buffo et al. infected reactive glial cells with retroviruses encoding Sox2 and observed the generation of substantial number of DCX-expressing new neurons. Lineage tracing results revealed the majority of DCX-positive cells arise from NG2 glia (Heinrich et al., 2014). Plus, Ascl1 could further enhance the neuronal conversion induced by Sox2. More recently, Torper et al. reported that forced expression of Ascl1, LIM homeobox transcription factor 1, \(\alpha\) (Lmx1a), and nuclear receptor related 1 protein (Nurr1) were able to reprogram NG2 glia into mostly GABAergic neurons in mouse striatum (Torper et al., 2015).

To explore the feasibility for therapeutic application, our lab and others asked if human glia could be reprogrammed to functional neurons by the similar approach. Intriguingly, by use of the same TF, NeuroD1, human astrocytes have been efficiently converted into functional glutamatergic neurons (Guo et al., 2014). Likewise, Karow and colleagues demonstrated that two TFs, Sox2 and Ascl1, converted cultured human pericytes into neurons, which acquired molecular markers and electrophysiological properties (Karow et al., 2012).

### 1.3.3 Challenges for clinical translation

Emerging evidence supports the principle that glial cells can be utilized for neuronal regeneration \textit{in vivo}. Moreover, animal studies suggested a promising potential for the \textit{in vivo} reprogramming approach on brain and spinal cord repair. However, there are many challenges ahead before this novel technology can be translated into the clinic.

Firstly, the \textit{in vivo} reprogramming of glial cells into glutamatergic and GABAergic
neurons has been achieved by many independent laboratories. However, so far, dopaminergic and cholinergic neuronal fates have not been obtained from direct glia-to-neuron conversion. In addition, more effort needs to be paid to achieve high yields of the desired subtype of neurons with high purity. After all, the therapeutic significance of \textit{in vivo} reprogramming relies upon the highly efficient induction of the cell type desired for treating specific CNS disorders.

Secondly, long-term assessment of the functional consequences after \textit{in vivo} reprogramming is necessary. It will be important to understand whether the newly generated neurons fully integrate into neural circuits and how they contribute to the functional recovery of brain or spinal cord.

Furthermore, the current glia-to-neuron reprogramming largely depends on virus-transduction-mediated gene delivery. For future clinical use, a more efficient and safer method needs to be developed (e.g., using virus-free methods for gene delivery (Yilmazer et al., 2013) or defined small-molecule compounds).

1.4 Advantages of chemical reprogramming for CNS repair

Small molecules are chemical compounds with low molecular mass (< 900 daltons). They modulate a broad spectrum of targets, including signaling pathways and transcriptional and epigenetic mechanisms, and thus are powerful tools for cell reprogramming. By targeting the signaling pathways related to neurogenesis, small molecules might direct the glia-to-neuron conversion. Compared to virus-based genetic manipulation, small molecules have several advantages: 1) they are more cost-effective and easier to synthesize, 2) they are more convenient to control temporally due to their rapid and reversible effects, and 3) they provide a higher degree of spatial control as their effects can be confined to different cell types (Li et al., 2013). Notably, the optimal effect can be determined by varying concentrations and combinations. With these advantages, small-molecule–based cell reprogramming strategies could have more chances to be translated into therapeutic applications.
A chemical approach has been widely applied in regenerative medicine and stem cell research. A number of studies used small molecules to induce neurons from a variety of cell types. Small molecules targeting the neural developmental pathways have been broadly utilized in research labs and industry to induce neural differentiation from stem cells (Chambers et al., 2012; Ding et al., 2012; Li et al., 2013). Dual inhibitors of SMAD signaling, including transforming growth factor β (TGFβ) inhibitor SB431542 and bone morphogenetic protein (BMP) inhibitor LDN193189, are highly efficient inducers for neural stem cell production from ESCs or iPSCs (Chambers et al., 2012; Ladewig et al., 2012). Sonic hedgehog (Shh) pathway activators smoothened agonist (SAG), purmorphamine (Purmo), together with retinoid acid (RA), have been commonly used to induce motor neurons or dopaminergic neurons from pluripotent stem cells (Hester et al., 2011; Lee et al., 2000; Xi et al., 2012). N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenylglycinet-butyl ester (DAPT), a gamma secretase inhibitor, could efficiently differentiate neuroprogenitors into neurons by indirectly blocking notch signaling (Borghese et al., 2010; Crawford and Roelink, 2007). Glycogen synthase kinase 3 (GSK3) inhibitor lithium significantly enhances the neural differentiation from adult neural stem cells (Vazey and Connor, 2010).

Small molecules can also facilitate cell reprogramming induced by TFs (Ladewig et al., 2012; Li et al., 2014; Liu et al., 2013). Huangfu et al. reported that, by adding the histone deacetylase (HDAC) inhibitor VPA to the Yamanaka factors (Sox2, Oct4, Klf4, c-Myc), iPSCs reprogramming efficiency is greatly increased by over 100-fold (Huangfu et al., 2008). Similarly, Rho-associated kinase (ROCK) inhibitor Y-23672 remarkably improved the iPSC reprogramming efficiency from human fibroblasts (Lai et al., 2010). In addition, SMAD signaling inhibitors SB431542 and noggin facilitate the neuronal conversion from fibroblasts induced by Ascl1 and Ngn2 (Ladewig et al., 2012). Forskolin, a cyclic adenosine monophosphate (cAMP) promoter, together with BMP inhibitor dorsomorphin, enabled Ngn2 to directly convert human fibroblasts into cholinergic neurons (Liu et al., 2013).

To improve the reprogramming efficiency and avoid the possible safety concerns brought by the transgenic method, a large effort has been applied to develop small molecules to replace TFs in cell reprogramming. Importantly, a pure chemical reprogramming approach induces pluripotent stem cells (Hou et al., 2013) or neuroprogenitor cells (Cheng et al., 2014)
from mouse fibroblasts, without the use of exogenous factors. More recently, pure chemical protocols have been developed to directly convert skin fibroblasts into neurons without bypassing the stem cell stage (Hu et al., 2015; Li et al., 2015). However, a pure small-molecule approach to convert glial cells into neurons has not yet been described.

1.5 Hypothesis and brief introduction

In my dissertation, I tested a novel hypothesis that human astrocytes may be directly reprogrammed into functional neurons using a defined combination of small molecules. To test this hypothesis, I have screened a variety of small molecules that target signaling pathways important for neurogenesis and gliogenesis. After numerous trial-and-error, I identified a group of nine small molecules that can successfully reprogram human astrocytes into functional neurons. By treating the cultured human astrocytes with three groups of small molecules in a sequential manner, we observed the majority of astrocytes gradually changed from flat, sheet-like shape to elongated neuronal morphology and extended long thin processes reaching out to neighboring neuronal cells. These chemically generated neurons are comparable to normal brain neurons: they were immunopositive for neuronal markers and functional by electrophysiology experiments. This small-molecule-induced conversion is likely mediated through epigenetic regulation, and involves the silencing of glial genes and the activation of endogenous neural TFs, such as NGN2 and NEUROD1. The neurons converted from human astrocytes survived for longer than 5 months in culture and formed functional synaptic networks. After transplanted into the neonatal mouse brain in vivo, the small-molecule-converted human neurons can survive for more than 1 month and integrate into the host circuits. Our study opens a new avenue using chemical compounds to reprogram reactive glial cells into functional neurons for brain repair.
Chapter 2
Reprogramming of human astrocytes into functional neurons by defined small molecule cocktail

In this study, Jiuchao Yin, Hana Yeh, Grace Lee and Ningxin Ma participated in the primary screening of small-molecule compounds and the characterization of glia-to-neuron conversion. Dr. Gangyi Wu performed an electrophysiological analysis on human astrocyte characterization, calcium images, and time-lapse imaging. This work was published in Cell Stem Cell (Zhang et al, 2015).

2.1 Identification of small-molecule candidate pool for glia-to-neurons conversion

To identify the small molecules for astrocyte-to-neuron reprogramming, we searched the literature carefully and selected 20 small molecules as our starting candidate pool. We based our selections on two major criteria: one was to inhibit glial signaling pathways, and the other was to activate neuronal signaling pathways. Some molecules were included because they modulate DNA or histone structure to increase reprogramming efficiency. Most of these molecules were documented as neural inducers from stem cells or they could facilitate the translineage reprogramming. The 20 small molecules selected for our initial screening were SB431542, RepSox, LDN193189, dorsomorphin, DAPT, BMS299897, CHIR99021, TWS119, thiazovivin, Y23652, SAG, purmorphamine, TTNPB, RA, VPA, forskolin, BIX 01294, RG-108, ISX9, and statta. We hypothesized that, to achieve glia-to-neuron conversion, glial genes should be inhibited, and neuronal genes should be activated. In this context, we arranged the 20 molecules into different categories, according to their documented functions (Table 2-1).
For instance, TWS119, CHIR 99021, SAG, purmorphamine, TTNPB, RA, and ISX9 were considered as neuron-inducers since they promote proneural gene expression; LDN193189, SB431542, DAPT, RepSox, dorsomorphin and stattic functioned as glial pathway inhibitors.

**Table 2-1. Small-molecule candidates for primary screening**

<table>
<thead>
<tr>
<th>Category</th>
<th>Molecule Name</th>
<th>Function/Pathway</th>
<th>Conc.</th>
<th>References</th>
</tr>
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<tr>
<td>Neuron inducers</td>
<td>CHIR 99021</td>
<td>Potent and highly selective inhibitor of GSK-3 (IC50 values are 6.7 and 10 nM for GSK-3β and GSK-3α respectively). Exhibits &gt;500-fold selectivity for GSK-3 over closely related kinases; also displays &gt;800-fold selectivity against 45 additional enzymes and receptors. In combination with tranylcypromine, enables reprogramming of mouse embryonic fibroblasts, transduced by Oct4 and Klf4 only, into iPSCs. Enhances mouse and human ESC self-renewal when used in combination with PD 0325901.</td>
<td>1.5 - 20 μM</td>
<td>(Hou et al., 2013; Hu et al., 2015; Li et al., 2009b; Li et al., 2015)</td>
</tr>
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<td></td>
<td>TWS 119</td>
<td>Inhibitor of GSK3β with IC50 of 30 nM, capable of inducing neuronal differentiation from stem cells.</td>
<td>1 μM</td>
<td>(Ding and Schultz, 2004; Ding et al., 2003)</td>
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<td></td>
<td>Retinoid acid</td>
<td>Endogenous agonist for retinoic acid receptors (RAR, IC50 = 14 nM for RARα, RARβ and RARγ receptors). Also promotes differentiation of mouse ESCs into adipocytes, neurons, and glia in vitro.</td>
<td>1 nM - 1μM</td>
<td>(Okada et al., 2004; Tonge and Andrews, 2010)</td>
</tr>
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<td></td>
<td>TTNPB</td>
<td>Extremely potent analog of retinoic acid, selective for the RAR subtype. Enhances reprogramming efficiency in chemically induced pluripotent stem cells (CiPSCs).</td>
<td>1 μM</td>
<td>(Hou et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>ISX9</td>
<td>Promote neurogenesis by increasing expression of NeuroD1 transcription factor. Induces neuronal</td>
<td>20μM</td>
<td>(Li et al., 2015; Schneider et al., 2016)</td>
</tr>
<tr>
<td>Glial inhibitors</td>
<td>Description</td>
<td>IC50</td>
<td>References</td>
<td></td>
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<tr>
<td>Purmorphamine</td>
<td>Directly binds to and activates the 7-transmembrane Smo receptor of the Hedgehog signaling pathway</td>
<td>1 µM</td>
<td>(Amoroso et al., 2013)</td>
<td></td>
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<tr>
<td>SAG</td>
<td>A chlorobenzothiophene-containing compound which acts as an SMO agonist but inhibits hedgehog signaling at high concentrations (&gt;1 µM). It directly binds to SMO and can block SMO inhibition by cyclopamine. Binding of SAG alters the conformation of SMO, leading to its accumulation in cilia and activation of gene transcription. SAG improves the conversion of human iPSCs to the neural lineage.</td>
<td>1 µM</td>
<td>(Carney and Ingham, 2013; Hu et al., 2015)</td>
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<tr>
<td>Forskolin</td>
<td>Cell-permeable activator of adenylyl cyclase. Hypotensive and vasodilatory agent. Induces neuronal differentiation in stem cells and in several neuroblastomas.</td>
<td>10-50uM</td>
<td>(Hou et al., 2013; Li et al., 2015)</td>
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<tr>
<td>LDN 193189</td>
<td>Inhibits SMAD 1/5/8 phosphorylation by BMP type I receptors, which are known as activin receptor-like kinases (ALKs), with an IC50 value of 4.9nM. Induces neuronal differentiation from stem cells</td>
<td>100 nM</td>
<td>(Chambers et al., 2012; Sanvitale et al., 2013; Surmacz et al., 2012)</td>
<td></td>
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<tr>
<td>Dorsomorphin</td>
<td>Potent and selective inhibitor of AMP-activated protein kinase (AMPK). Also, inhibits BMP type I receptors (ALK 2,3,6). Promotes neuronal differentiation from stem cells.</td>
<td>5 µM</td>
<td>(Mak et al., 2012; Rodriguez-Martinez et al., 2012)</td>
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<tr>
<td>SB 431542</td>
<td>SB431542 inhibits the TGF-β-mediated activation of SMAD proteins. Potent and selective inhibitor of TGFβ superfamily type I ALK receptors.</td>
<td>10uM</td>
<td>(Chambers et al., 2009; Chambers et al., 2012; Li et al., 2015)</td>
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<td>RepSox</td>
<td>Potent and selective inhibitor of the TGF-β type I receptor/ALK5 (IC50 values are 4 and 23 nM for TGF-β type I receptor autophosphorylation and</td>
<td>1 µM</td>
<td>(Hu et al., 2015; Li et al., 2009a)</td>
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<tr>
<td>Epigenetic modulators</td>
<td>Valproic Acid (VPA)</td>
<td>An inhibitor of HDACs, inhibiting Class I HDACs. VPA also inhibits GSK3 and depletes cellular inositol-1,4,5-trisphosphate (1,4,5-IP3). VPA at 1 mM also has pronounced effects on stem cell differentiation and self-renewal.</td>
<td>1 mM</td>
<td>(Hsieh et al., 2004; Hu et al., 2015)</td>
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<td></td>
<td>BIX 01294</td>
<td>BIX 01294 is a selective histone methyltransferase (HMTase) inhibitor. It selectively impairs G9a HMTase and the generation of H3K9me2 in vitro. Bix-01294 may replace the requirement for ectopic OCT4 (POU5F1) in iPSC recipes.</td>
<td>1 μM</td>
<td>(Hou et al., 2013)</td>
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<td></td>
<td>RG 108</td>
<td>DNA methyltransferase (DMNT) inhibitor. It activates tumor suppressor genes in tumor cells by DNA demethylation.</td>
<td>20–40 μM</td>
<td>(Hou et al., 2013)</td>
</tr>
<tr>
<td>Cell death protectors</td>
<td>Thiazovivin</td>
<td>ROCK inhibitor. Greatly enhances the efficiency of fibroblast reprogramming to generate iPSCs when used in combination with SB 431542 and PD 0325901.</td>
<td>2 μM</td>
<td>(Narsinh et al., 2011; Zhang et al., 2013)</td>
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<tr>
<td></td>
<td>BMS-299897</td>
<td>Orally active potent Y-secretase inhibitor with IC50 of 12 nM. Induces neuronal differentiation from stem cells.</td>
<td>1 μM</td>
<td>(Weissmiller et al., 2015)</td>
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<td></td>
<td>DAPT</td>
<td>NOTCH signaling pathway plays a crucial role in gliogenesis. DAPT inhibits Y-secretase and thus indirectly blocks Notch pathway. DAPT induces neuronal differentiation from stem cells.</td>
<td>2.5–10uM</td>
<td>(Borghese et al., 2010; Crawford and Roelink, 2007)</td>
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<td></td>
<td>Stattic</td>
<td>STAT3 is crucial for astrocyte development and astrocytes reactivity. Stattic inhibits binding of tyrosine-phosphorylated peptide motifs to STAT SH2 domain and inhibits STAT3 activation, dimerization, and nuclear translocation.</td>
<td>2.5 μM</td>
<td>(Herrmann et al., 2008; Schust et al., 2006)</td>
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<tr>
<th>Epigenetic modulators</th>
<th>Valproic Acid (VPA)</th>
<th>An inhibitor of HDACs, inhibiting Class I HDACs. VPA also inhibits GSK3 and depletes cellular inositol-1,4,5-trisphosphate (1,4,5-IP3). VPA at 1 mM also has pronounced effects on stem cell differentiation and self-renewal.</th>
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<td>2 μM</td>
<td>(Narsinh et al., 2011; Zhang et al., 2013)</td>
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We combined the glial inhibitory molecules with neural inducing molecules for screening. The small-molecule concentration that exhibited the best effect with no obvious cell toxicity was selected, based on pilot tests. The small-molecule-treated cells were examined under the light microscope every 2 days to trace their morphological transition. If neuron-like cells with elongated cell body and long thin processes were observed, the cells were fixed and immunostained for newborn-neuron-markers, such as doublecortin (DCX) and βIII tubulin (Tuj1). To evaluate the drug effect on neurogenesis, the DCX- or Tuj1-positive cells were counted, and the conversion efficiency was measured by Tuj1-positive cell number/total cell number indicated by DAPI × 100%. Small-molecule combinations yielding higher than 10% conversion efficiency were highlighted. Further tests were performed by regrouping the small molecules, refining the concentrations and optimizing the timing for treatment. In the control group, according volume of DMSO was added to evaluate the solvent-mediated effect.

2.2 Characterization of human astrocyte culture

We mainly used primary human astrocyte cultures (HA1800, ScienCell, San Diego, CA, USA) isolated from fetal cortex for chemical reprogramming, aiming at future clinical applications for human brain repair. Human astrocytes were isolated and subcultured in culture medium with 10% fetal bovine serum (FBS) to reduce possible contamination of progenitor cells because FBS stimulates glial differentiation of progenitors.

Before reprogramming, we carefully characterized the properties of cultured human cells. The results (Fig. 2-1 A, C) confirmed the dominant astrocytic identity as

<table>
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<th>Compound</th>
<th>Selective ROCK inhibitor. Increases survival rate of human ESCs undergoing cryopreservation and dissociation.</th>
<th>5-10 μM</th>
<th>(Hu et al., 2015; Ichikawa et al., 2011)</th>
</tr>
</thead>
</table>
indicated by 79.3 ± 4.9% cells were immunopositive for GFAP, which is an intermediate filament highly expressed in astrocytes. In agreement, 82.5 ± 4.3% cells exhibited immunoreactivity for Glt1, an astrocyte-specific glutamate transporter. No cells were positive for the neuronal markers DCX, microtubule-associated protein 2 (MAP2) or Neuronal nuclei (NeuN) (n = 3 batches) (Fig. 2-1 B, C).

We further examined the astrocyte functions by performing patch-clamp recordings and demonstrated that our cultured human astrocytes were with large K⁺ and glutamate transporter currents, but no Na⁺ currents (Fig. 2-1 D-F), and gap junctions were identified among the cultured astrocytes (Fig. 2-1 F-H).
Figure 2-1. Characterization of cultured human cortical astrocytes

(A) Representative images showing the cultured human astrocytes were immunopositive for astrocytic markers GFAP, astrocytic glutamate transporter GLT-1, and S100β.

(B) No signals were detected for neuronal markers doublecortin (DCX) and microtubule-associated protein 2 (MAP2).

(C) Quantitative analysis of the human astrocyte cultures (HA1800, ScienCell). The majority of cells in our human astrocyte cultures were immunopositive for astrocytic
marker GFAP (79.3 ± 4.9%), Glt-1 (82.5 ± 4.3%), and to a lesser degree S100β (39.3 ± 1.8%). No cells were immunopositive for neuronal markers NeuN, MAP2 or DCX. HuNu, human nuclei, a marker for human cells. N = 3 batches.

(D-F) Functional analyses of cultured human astrocytes (HA1800). Electrophysiological recordings revealed large K⁺ current but no Na⁺ current (D), gap junctional coupling between neighboring astrocytes (E), and glutamate (500 μM) transporter current (F) (G) Representative image showing dye-coupling among local astrocyte domain after recording.

(H) Immunostaining revealed the presence of gap junction protein connexin 43 (Cxn 43) in human astrocyte culture.

Data are represented as mean ± SEM. Scale bars = 20 μm.

To make sure the induced neurons were converted from astrocytes but not differentiated from contaminating neural stem cells, we carefully characterized our astrocytes culture and excluded the latter possibility. Firstly, when compared to cultured human NPCs, we found that our astrocyte cultures exhibited low expression levels of neural stem cell markers, such as Sox2, Musashi, and Nestin, and a significantly lower cell proliferative capability as indicated by Ki67 immunostaining (Fig. 2-2 A, B). Furthermore, we purposely treated human astrocytes in neural differentiation medium supplemented with growth factors (BDNF, NT3, and NGF) for 1 month. The neural stem cells, if present, would be forced to differentiate into neurons. However, our data suggest the majority of cells after a 1-month culture were astrocytes, immunopositive for S100 β, GFAP, glutamine synthetase (GS) and Glt1, and not neurons or NG2 cells (Fig. 2-2 C, D).
Figure 2-2. Exclusion of progenitor contamination in human astrocyte culture

(A) Human cortical astrocytes (HA1800, ScienCell) cultured in glial medium (GM, with 10% FBS) or N2 medium (for reprogramming, without FBS) and immunostained with neural stem cell markers Musashi, Nestin, and Sox2. Note that in both culture media, there were no neuroprogenitor cells.

(B) Quantitative analyses of neural stem cell markers revealed low expression levels of Musashi, Nestin and Sox2 in cultured human astrocytes, compared to human neuroprogenitors (NPCs). *** P < 0.0001, one-way ANOVA followed by Dunnett’s test. N = 3 batches.

(C-D) Human astrocytes (HA1800, ScienCell) were cultured for 1 month in neuronal differentiation medium (NDM) supplemented with BDNF, NT3, and NGF to ensure neural differentiation if there were any neural stem cells in the astrocyte cultures. Quantitative analyses revealed that the majority of cells were immunopositive for
astrocytic markers S100β (74.7 ± 1.5%), GFAP (83.6 ± 1.2%), glutamate synthetase (GS) (94.3 ± 0.7%) and Glt-1 (91.4 ± 1.5%). A few cells were positive for DCX (5.18 ± 0.67) and Tuj1 (8.98 ± 0.75%), but no cells were positive for NeuN nor NG2. N = 4 batches.

Data are represented as mean ± SEM. Scale bars = 20 μm.

2.3 Development of small-molecule protocol for efficient astrocyte-to-neuron conversion

By trying hundreds of combinations and concentrations, eventually, we selected nine small molecules giving the most efficient neuronal induction: SB431542, LDN193189, TTNPB, CHIR99201, DAPT, VPA, SAG, purmorphamine and thiazovivin. These nine small molecules are hereafter referred to as the master conversion molecules (MCMs). Initially, we applied the MCMs into human astrocyte (HA) cultures. However, massive cell death was observed after 2 days of drug treatment. Thus, we decided to apply the compounds stepwise as shown in Fig. 2-3 A.

When human astrocytes reached 90% confluences in the 24-well plate, we primed the cells by reducing the serum content to half for overnight (D0) (Fig. 2-3 B). The next day (D1), we withdrew serum and changed to N2 medium supplemented with SMAD signaling dual inhibitors SB431542 (5 μM) and LDN193189 (0.5 μM), retinoid acid receptor (RAR) agonist TTNPB (0.5 μM) and ROCK inhibitor thiazovivin (5 μM). During the 48-hour incubation with the first group of compounds, the cells started to change morphology with smaller soma size and thinner processes (Fig. 2-3 B). At Day 3, the first group of compounds was replaced by GSK3 signaling inhibitor CHIR99021 (1.5 μM), Notch inhibitor DAPT (5 μM), HDAC inhibitor VPA (0.5 mM) and ROCK inhibitor thiazovivin (5 μM). Small-molecule-treated cells were elongated and started to form three-dimensional clusters. Although cell number indicated by DAPI showed no significant decrease, around 30% open space was observed in the culture dish due to cell clustering (Fig. 2-3 B). After 48-hour culture (D5), VPA was withdrawn from the second group to minimize cell death.
Around 70% cells started to show neuronal morphology: soma size was further reduced and processes became even thinner and longer (Fig. 2-3 B). At Day 7, sonic hedgehog agonist SAG (0.5 µM) and purmorphamine (0.5 µM) were applied for 2 more days, at which time around 70% cells showed clear neuronal morphology. In the meantime, cells with astrocytic morphology proliferated to cover the coverslip, leading to the increased total cell number (Fig. 2-3 B). To protect massive cell death, ROCK inhibitor thiazovivin (5 µM) was applied throughout the chemical reprogramming processes. Immunostaining results confirmed the neuronal identity of converted cells, as they were immunopositive for immature neuronal markers DCX and TuJ1 (Fig. 2-3 C). A conversion efficiency of 67.1 ± 0.8% (n = 4 batches) was achieved at D8 after small-molecule treatment. In contrast, few neurons were observed in the control group without small molecules (3.3 ± 0.5%, n = 4 batches, student t-test, P < 0.0001 ***) (Fig. 2-3 C, D), in which group cells were treated with N2 medium supplemented with 1% DMSO. At D10, the medium was completely replaced by neuronal differentiation medium (NDM) supplemented with neurotrophic factors (BDNF, NT3, and IGF-1) to promote neuronal maturation after astrocyte-to-neuron conversion. The successful reprogramming strategy is illustrated in Fig. 2-3 A.
Figure 2-3. Sequential exposure to a defined group of small molecules converts human astroglial cells into neuronal cells

(A) Schematic illustration of our strategy of using a cocktail of small molecules to convert cultured human astrocytes into neurons. Note that different subsets of small molecules were used at different reprogramming stages.
(B) Representative images showing the morphological changes from astrocytes to neurons during and after small-molecule treatment.

(C) At the end of small-molecule treatment, a large number of cells immunopositive for neuronal markers DCX and β-III tubulin (Tuj1) were observed. In contrast, very few neurons were detected in human astrocytes without small-molecule treatment. MCM stands for master conversion molecules, including the nine small molecules for reprogramming together.

(D) Quantitative analysis revealed small-molecule treatment achieved high conversion efficiency after 8-day exposure to MCM (67.1 ± 0.8%, Tuj1+ neurons/total cells labeled by DAPI, n = 4 batches, *** P < 0.0001, student t-test).

After 14 days with small-molecule treatment, the human astrocyte–converted neurons polarized to grow MAP2-positive dendrites (Fig. 2-4 B). The neurons gradually developed to become mature neurons expressing NeuN and extending complex dendrites at D30 (Fig. 2-4 C). Notably, astrocyte-derived human-neurons survived for more than 4 months in culture and developed robust axons demonstrated by SMI312, as well as elaborate dendrites labeled by MAP2 (Fig. 2-4 D). Moreover, the presence of chloride and potassium cotransporter KCC2 in cell membrane indicates the maturation of chemically converted neurons (Fig. 2-4 E). In support of this notion, synapses were identified by synaptophysin staining on synaptic puncta at D60 (Fig. 2-4 F), suggesting the established neural networks among converted neurons.
Figure 2-4. Small-molecule-induced neurons survive well and become mature in culture

(A) Control human astrocyte cultures without small-molecule treatment had very few cells immunopositive for neuronal markers DCX (green), Tuj1 (red) or MAP2 (cyan).

(B) Sequential exposure of human astrocytes to small molecules resulted in a massive number of neuronal cells, which were immunopositive for DCX (green), Tuj1 (red) and MAP2 (cyan). Analyzed at 14 days after initial small-molecule treatment.

(C) At 30 days after initial small-molecule treatment, human astrocyte-converted neurons developed extensive dendrites (MAP2, green) and were immunopositive for mature neuronal marker NeuN (red).

(D) Small-molecule-converted human neurons survived for 4 months in culture and showed robust dendritic trees (MAP2, green), as well as extensive axons (SMI312, red).

(E) Small-molecule-induced neurons express chloride transporter KCC2 (red).

(F) Immunostaining revealed presynaptic vesicles labeled by synaptophysin (Synt, red) distributing along the dendrites (MAP2, green), indicating the synapse formation.

N = 3 batches. Scale bars = 20 µm.
2.4 Conversion of multiple human astrocyte lines by master conversion molecules

To exclude the possibility that the MCM cocktail–induced astrocyte-to-neuron conversion was cell-line specific artifact, we obtained human cortical astrocytes HA Gibco from different sources (GIBCO, Invitrogen, Carlsbad, CA). HA Gibco cells were converted into Tuj1-positive neurons at D14 with an efficiency of 36.9 ± 4.1% by using the same protocol (n = 3 batches) (Fig. 2-5 B, C). In contrast, no neurons were observed in DMSO control group (Fig. 2-5 A). In addition, HA Gibco-converted neurons survived for 2 months and formed synaptic connections indicated by synaptophysin puncta (Fig. 2-5 D).

Figure 2-5. Chemical conversion of human cortical astrocytes from different sources into neurons
(A–C) Human brain astrocytes from a different source (Gibco) were also successfully reprogrammed into neurons using the same small-molecule protocol with an efficiency of 41.1 ± 3.6%. N = 4 batches. *** P < 0.0001, Student t test.

(D) Immunostaining showing synaptophysin (red) and MAP2 (green) signal in 2-month old neurons converted from Gibco human astrocytes. Thus, different sources of human brain astrocytes could be successfully converted into neurons using the same small molecule strategy.

Scale bar for panel D is 10 µm, and the rest are 20 µm.

To test whether the same small-molecule cocktail also converts astrocytes isolated from different CNS regions, we further obtained human midbrain astrocytes and human spinal cord astrocytes from ScienCell. Both samples were carefully characterized and excluded the contamination from neuronal cells as shown in Fig. 2-6 A. Interestingly, human midbrain astrocytes were efficiently reprogrammed into neurons using our stepwise 9-small-molecule strategy (Fig. 2-6 B-E), whereas human spinal cord astrocytes could not be reprogrammed into neurons by the same protocol (data not shown). This piece of result suggests that our chemical reprogramming protocol is more suitable for astrocytes with human brain origin.
Figure 2-6. Chemical conversion of human midbrain astrocytes into neurons

(A) Characterization of human midbrain astrocytes (HA midbrain, ScienCell), which were immunopositive for astrocytic markers GFAP, S100β, glutamine synthetase (GS) and Glt1, but showed low expression level of neural stem cell markers Sox2 and Nestin.

(B) Sequential exposure of human midbrain astrocytes to small molecules resulted in a large number of neuronal cells, which were immunopositive for DCX (green) and Tuj1 (red). In contrast, no cells immunopositive for neuronal markers were detected in control human midbrain astrocytes. Analyzed at 16 days after initial small-molecule treatment.

(C) At 1-month after small-molecule treatment, induced neurons were immunopositive for mature neuronal markers NeuN (red) and MAP2 (green).
(D) Immunostaining of axonal marker SMI312 (green) at the 1-month-old culture of human midbrain astrocytes without or with small-molecule treatment.

(E) Long-term survival of human neurons converted from human midbrain astrocytes. A large number of synaptic puncta (SV2, red) were distributed along dendrites (MAP2, green) in NeuN positive (cyan) neurons.

N = 3 batches. Scale bar for panel A is 10 µm, and the rest are 20 µm.

2.5 Visualization of astrocyte-to-neuron conversion by time-lapse imaging

To visualize the reprogramming process from astrocytes to neurons, we labeled human astrocytes with low-tittered retroviruses encoding EGFP so that only a small portion of EGFP-positive astrocytes were observed in each coverslip (Fig. 2-7). We monitored the morphological changes of human astrocytes in response to small-molecule treatment by performing time-lapse imaging. In the control group without small molecules, the human astrocytes displayed consistent morphology from day 0 to day 8 (Fig. 2-7 A). GFP-positive cells were immunopositive for glial marker GFAP, but not for the neuronal marker NeuN (Fig 2-7 C). In contrast, we observed a clear transition from flat astroglial morphology to neuronal morphology with the extension of long neurites from day 8 to day 10 (Fig. 2-7 B). Immunostaining results confirmed the neuronal identity of the neuron-like GFP+ cells (Fig. 2-7 D).
Figure 2-7. Time-lapse imaging of the astrocyte-to-neuron conversion process during small-molecule treatment

(A) Control human astrocytes infected by CAG::GFP retrovirus (green) remained astrocytic morphology during 8-day treatment with 1% DMSO. Imaged daily with a Nikon 2000 epifluorescent microscope.

(B) Two GFP-labeled human astrocytes were traced from 1 day before (D-1) to 10 days after (D10) small-molecule treatment. There was a clear transition of cell morphology from astrocytes at D0 to neuron-like cells at D9.

(C) Representative image showing GFP+ cells in the control group were immunopositive for astrocytic marker GFAP (red) but not for neuronal marker NeuN (red) after 21-day culture.
(D) After time-lapse imaging, cells were fixed and immunostained with neuronal markers DCX, Tuj1, and NeuN. The GFP-labeled cells (green, arrowhead) after small-molecule treatment were immunopositive for DCX (red), NeuN (red) and Tuj1 (cyan). Scale bars = 20 µm.

To prove the principle that the neurons came from astrocytes, we further constructed the GFP under astrocyte-specific promoter GFAP. We infected the human astrocytes with GFAP::GFP retrovirus. Around 91 ± 6.7% GFP-labeled cells were immunopositive for GFAP (Fig. 2-8 A). At 18 days after small-molecule treatment, GFP-labeled astrocytes were efficiently reprogrammed into NeuN+ neurons (68.7 ± 4.2%, Fig. 2-8 B, n = 5 batches). In contrast, in the absence of small molecules, no neurons were detected in the control group (Fig. 2-8 C, D). LCN2 encoded by the LCN2 gene was reported to be expressed in reactive glial cells. We used LCN2::GFP retrovirus to trace astrocyte-to-neuron conversion and found 88.5 ± 3% of GFP+ cells were astrocytes expressing GFAP (Fig. 2-8 E). At 18 days after small-molecule treatment, 54.4 ± 5.3% of LCN2::GFP-labeled astrocytes became NeuN+ neurons (n = 4 batches) (Fig. 2-8 G). The conversion efficiency obtained with astrocyte-specific lineage tracing experiments was consistent with the overall conversion efficiency induced by small-molecule treatment (MCM, 67.1 ± 0.8% Tuj1+, n = 4 batches).
Figure 2-8. Lineage tracing experiments revealed that neurons are directly converted from astrocytes

(A) Human astrocytes were infected with GFAP::GFP retroviruses for lineage tracing. GFP-labeled cells were all GFAP⁺ (red).

(B) Immunostaining at 18 days after small-molecule treatment revealed most GFP-labeled astrocytes (arrowhead) were converted into neuronal cells immunopositive for NeuN (red).

(C and D) Without small-molecule treatment, GFP⁺ cells were still GFAP⁺ astrocytes (C, red), with no neurons detected (D) after 18 days of culture. N = 3 batches. Scale bars = 10 µm.
(E to G) Human astrocytes infected with LCN2::GFP retroviruses were immunopositive for GFAP (E, red) and LCN2 (E, cyan). Without small-molecule treatment, GFP+ cells retained the astrocyte morphology and remained immunopositive for GFAP (F, red) for 18 days of culture. In contrast, after small-molecule treatment, GFP+ cells became immunopositive for NeuN (G, red) indicating their neuronal identity. N = 3 batches. Scale bars = 10 µm.

2.6 Chemically converted human neurons are fully functional

Next, we investigated whether the chemically converted neurons are fully functional. Patch-clamp recordings revealed significant inward sodium and outward potassium currents in astrocyte-converted neurons, which gradually increased during neuronal maturation (Fig. 2-9 A-D; 2-months: $I_{\text{Na}}$ peak = 1889 ± 197 pA, n = 10; $I_{\text{K}}$ peak = 2722 ± 263 pA, n = 10). In addition, the converted neurons exhibited the robust response to locally puffed glutamate (Fig. 2-9 E), indicating the development of glutamate receptors on cell membrane. These human neurons developed from the immature stage at 1 month, when a single action potential can be fired (Fig. 2-9 F), to the mature stage at 2 months, when neurons could fire repetitive action potentials (Fig. 2-9 G,H). More importantly, small-molecule-converted neurons showed robust spontaneous synaptic events. High-frequency excitatory postsynaptic currents were detected (EPSCs; frequency = 0.66 ± 0.14 Hz; amplitude = 24.8 ± 8.2 pA, n = 15) that were blocked by TTX and the AMPA receptor antagonist DNQX (Fig. 2-9 I-J). In contrast, only a few inhibitory postsynaptic currents (IPSCs; frequency = 0.48 ± 0.21 Hz; amplitude = 23.3 ± 6.3 pA, n = 2) were detected that could be blocked by the GABA A receptor antagonist bicuculline (Fig. 2-9 K), suggesting the presence of small numbers of GABAergic neurons.
Figure 2-9. Functional analyses of human astrocyte-converted neurons induced by small molecule treatment

(A - C) Representative traces showing Na\(^+\) and K\(^+\) currents recorded from 1- (A) and 2-month (B) -old human neurons induced by small molecules. Panel C shows the blockade of Na\(^+\) currents by tetrodotoxin (TTX) (2 μM).

(D) Quantitative analyses of peak Na\(^+\) and K\(^+\) currents in 2–3-month-old neurons converted from human astrocytes by small molecules.

(E) Representative traces showing the current response to glutamate.

(F–H) Representative traces showing action potentials recorded from 1- (F) and 3-months (G) -old human neurons induced by small molecules. Panel H shows the blockade of
action potentials by TTX (2 µM).

(I and J) Representative traces showing spontaneous synaptic events in 2-months-old converted human neurons. Note the synaptic events were blocked by AMPA receptor antagonist DNQX (10 µM). Holding potential (HP) = -70 mV. (J) Expanded trace from (I).

(K) Inhibitory GABAergic events detected in human astrocyte–converted neurons held at a holding potential of 0 mV (2 months old). The events were blocked by GABA_A receptor antagonist bicuculline (BIC, 10 µM).

N = 3 batches. Data were presented as mean ± SEM.

Notably, when converted neurons were older than 3 months in culture, they established elaborate synaptic networks, indicated by substantial synaptic puncta labeled by SV2 (Fig. 2-10 A red). In support of this notion, patch-clamp recordings revealed large periodic burst activities in converted neurons (Fig. 2-10 B, C), which can be abolished by TTX or DNQX (Fig. 2-10 D), indicating that these neurons formed functional networks and started to fire synchronously. Dual whole-cell recordings confirmed that two adjacent neurons showed synchronous burst activities (Fig. 2-10 E). Furthermore, Fura-2 Ca^{2+} ratio imaging revealed synchronized Ca^{2+} spikes in chemically reprogrammed neurons (Fig. 2-10 F), indicating that these neurons have been functionally networked together. Therefore, human astrocytes can be chemically reprogrammed into fully functional neurons with defined small molecules.
Figure 2-10. Small-molecule-converted neurons form synaptic networks

(A) Long-term survival of small molecule–induced human neurons (5 months in culture) and a massive number of synaptic puncta (SV2, red) along the dendrites (MAP2, green). Scale bar: 20 μm.

(B–C) Representative traces showing spontaneous burst activities in 3-month-old small molecule–induced human neurons. HP = −70 mV. (C) An expanded view of a burst in (B).

(D) The burst activities were blocked by TTX (2 μM). The majority of synaptic events at −70 mV were blocked by glutamate receptor antagonist DNQX (10 μM), suggesting that they were glutamatergic events.
(E) Dual whole-cell recordings illustrating that the small molecule–converted human neurons formed robust synaptic networks and fired synchronously.
(F) The Ca$^{2+}$ ratio imaging further illustrating that the small molecule–converted human neurons were highly connected and showed synchronous activities.

2.7 Small molecules reprogram human astrocytes into forebrain glutamatergic neurons

We further examined neuronal properties of human astrocyte–converted neurons. We found the majority of chemically converted neurons were immunopositive for forebrain marker FoxG1 (97.1 ± 1.1%, Fig. 2-11 A, n = 3 batches), but negative for spinal cord and hindbrain markers HOX B4 and HOX C9 (Fig. 2-11 B-C, n = 3 batches). Next, we performed a series of immunostaining with a variety of cortical neuron markers, such as Tbr1, Cux1, Ctip2, and Otx1. Interestingly, a majority of MAP2-labeled neurons were immunopositive for pan-cortical marker Tbr1 (86.4 ± 3.4%, n = 3 batches) (Fig. 2-11 G, I) as well as deep layer markers Ctip2 (71.4 ± 3%, n = 5 batches) (Fig. 2-11 E, I) and Otx1 (87.4 ± 3.2%, n = 3 batches) (Fig. 2-11 F, I). In contrast, only a few MAP2-labeled neurons were immunopositive for superficial layer marker Cux1 (3.1 ± 1.9%, n = 4 batches) (Fig. 2-11 D, I). In addition, hippocampal neuronal marker Prox1 was detected in the majority of converted neurons (73.4 ± 4.4%, n = 4 batches) (Fig. 2-11 H, I). Therefore, our chemically reprogrammed neurons were mainly forebrain deep layer...
Figure 2-11. Small molecule-converted neurons obtain forebrain properties

(A–C) Immunostaining with anterior-posterior neuronal markers revealed that the small molecule-converted human neurons were positive for forebrain marker FoxG1 (A) but negative for hindbrain and spinal cord markers HOX B4 (B) and HOX C9 (C).

(D–F) Immunostaining with cortical neuron markers revealed that small molecule-induced human neurons were negative for superficial layer marker Cux1 (D), but positive for deep layer markers Ctip2 (E) and Otx1 (F).

(G–H) The small molecule-converted human neurons were also immunopositive for general cortical neuron marker Tbr1 (G) and hippocampal neuron marker Prox1 (H).
(I) Quantitative analyses of small molecule–induced human neurons (FoxG1, 97.1 ± 1.1%, n = 3 batches; Cux1, 3.1 ± 1.9%, n = 4 batches; Ctip2, 71.4 ± 3%, n = 4 batches; Otx1, 87.4 ± 3.2%, n = 3 batches; Tbr1, 86.4 ± 3.4%, n = 3 batches; Prox1, 73.4 ± 4.4%, n = 4 batches). Scale bars: 20 μm.

We further investigated neuronal subtypes based on the neurotransmitters they contained and transported. We found that the vast majority of human astrocyte–converted neurons were immunopositive for glutamate transporter VGlut1 (88.3 ± 4%, n = 4 batches) (Fig. 2-12 A, F); only a small portion of converted neurons were immunopositive for the GABA synthetic enzyme GAD67 (8.2 ± 1.5%, n = 4 batches) (Fig. 2-12 B, F). This result was consistent with the electrophysiological data, as robust ESPCs, yet stagnant IPSCs were detected by patch-clamp recording. On the other hand, the astrocyte-converted neurons were largely immuno-negative for cholinergic transporter VACHT (Fig. 2-12 C, F), spinal motor neuron marker Isl1 (Fig. 2-12 D, F), or L-DOPA synthetic enzyme TH (Fig. 2-12 D, F), suggesting no cholinergic, dopaminergic or motor neurons were generated. Taken together, the glutamatergic neurons are the major subtype using our small-molecule reprogramming protocol.
Figure 2-12. Small molecules reprogram human astrocytes into glutamatergic neurons

(A) MCM-converted human neurons were immunopositive for vesicular glutamate transporter 1 (VGlut1).
(B) A small portion of MCM-converted human neurons was glutamate decarboxylase (GAD) 67-positive.
(C–E) MCM-converted neurons were immuno-negative for cholinergic neuronal marker vesicular acetylcholine transporter (VACht) (C), spinal motor neuron marker Isl1 (D) or dopaminergic neuronal marker tyrosine hydroxylase (TH) (E).
(F) Quantitative analyses of small molecule–converted human neurons (VGlut1, 88.3 ± 4%, n = 4 batches; GAD67, 8.2 ± 1.5%, n = 4 batches). Scale bars: 20 μm.
2.8 Summary

Together, by use of defined small-molecule compounds, we converted cultured human brain astrocytes into neurons with high efficiency. Lineage tracing experiments confirmed the neurons were converted from astrocytes. Notably, the astrocyte-derived neurons survived in culture for up to 5 months and became fully functional. Interestingly, the human neurons were predominantly glutamatergic and obtained properties of deep layer cortical neurons.
Chapter 3

Mechanisms underlying small molecule-induced chemical reprogramming from human astrocytes to functional neurons

To understand the mechanisms underlying small molecule-mediated glia-to-neuron conversion, we designed a number of experiments looking into the molecular and epigenetic events during the reprogramming process.

In this study, we collaborated with Dr. Yanming Wang in the Department of Biochemistry and Molecular Biology at Pennsylvania State University on histone modifications studies, and with Dr. Peng Jin at Emory University on DNA methylation study. Hana Yeh analyzed the stem cell marker expression during chemical conversion. Grace Lee participated in PCR array and real-time PCR experiments. Jiuchao Yin and Grace Lee studied the functional role of each individual molecule on glia-to-neuron conversion. Yi Hu performed western blot experiments. This part of work was published in Cell Stem Cell (Zhang et al, 2015).

3.1 Small molecules directly convert human astrocytes into neurons

We hypothesized our chemical conversion was directly from astrocytes into neurons without de-differentiation of human astrocytes into neuroprogenitor cells. To test this hypothesis, we monitored the expression of stem cell markers, such as Sox2, Nestin, and Pax6, during the chemical reprogramming process from day 0 to day 10, and compared to iPSC-derived NPCs (Fig. 3-1 A-C, E-G, n = 3 batches). While Sox2 showed a slight increase during reprogramming, it never reached the level of NPCs (Fig. 3-1. A, E, one-way ANOVA with Dunnett’s test, n = 3 batches). Nestin and Pax6 did not show much increase during small-molecule treatment (Fig. 3-1 B-C, F-G, one-way ANOVA with Dunnett’s test, n = 3 batches), suggesting that there were no progenitor cells that can expand and give rise to neurons.
Neurons are post-mitotic, whereas NPCs are highly proliferative. If NPCs occur in culture, we will be able to observe cell expansion during reprogramming. However, our results suggest a decreased number of proliferating cells immunopositive for Ki67 after small-molecule treatment (Fig. 3-1 D, H, one-way ANOVA with Dunnett’s test, n=3 batches). In support of this notion, time-lapse imaging revealed that there was no obvious cell expansion during conversion (Fig. 2-7 B), suggesting the direct conversion from astrocytes to neurons.
**Figure 3-1. Small molecules convert astrocytes to neurons without a stem cell stage**

(A–C) Representative images showing low expression level of neural stem cell markers Sox2 (A, red), Nestin (B, green) and Pax6 (green) at different days of small-molecule treatment, compared to human NPC culture.

(D) Representative images showing no significant cell expansion during reprogramming, as indicated by cell proliferation marker Ki67 (red).

(E–G) Quantitative analyses of the fluorescence intensity of Sox2 (E), Nestin (F) and Pax6 (G) during chemical reprogramming normalized to the intensity at D0. Compared to D0, Sox2 expression level was slightly increased at D4-D10 but much lower than NPCs (E). Nestin expression level was very low compared to NPCs (F). Similarly, the Pax6+ cell number was also very low when compared to NPCs (G).

(H) Quantitative analyses of Ki67+ cell number to assess the cell proliferation rate during chemical reprogramming. Compared to D0, cell proliferation was significantly reduced at D2–D6 after small-molecule treatment. The overall proliferation rate of human astrocytes was significantly lower than NPCs. The reduced proliferation rate in the presence of small molecules suggested that no cell expansion occurred during chemical reprogramming.

N = 3 batches. Scale bars = 20 μm. ** P < 0.001; *** P < 0.0001; one-way ANOVA followed by Dunnett’s multiple comparison test. Data are represented as mean ± SEM.

To trace the birthday of newly regenerated neurons, we utilized 5-bromo-2'-deoxyuridine (BrdU) labeling assay. BrdU is an analog of thymidine, so it can be taken up by proliferating cells and passed to progeny. When we labeled human astrocytes with BrdU before chemical treatment (Fig. 3-2 A), many converted neurons were BrdU-positive (Fig. 3-2 B, E, n = 3 batches). However, when we labeled cell cultures with BrdU at day 10 after small-molecule treatment (Fig. 3-2 C), essentially all converted neurons were negative for BrdU (Fig. 3-2 D, E, n = 3 batches), suggesting that all glia-to-neuron conversion occurred during the presence of small molecules.

In addition, if we applied BrdU from D1 to D8 when cells were under small-molecule treatment (Fig. 3-2 F), the majority of converted neurons (95.7 ± 2.3 %, n = 3
batches) showed no BrdU (Fig. 3-2 G) labeling, indicating those neurons bypassed cell cycle before they adopted the neuronal cell fate. In other words, the converted neurons were directly converted from astrocytes.

Figure 3-2. BrdU birth dating reveals that small molecule-induced neurons are directly converted from astrocytes

(A–B) When BrdU was applied in culture medium at 1 day before small-molecule treatment (A), the majority of BrdU-labeled cells were immunopositive for neuronal marker NeuN at day 30 after small-molecule treatment (B). Arrows in (B) point to cells
with co-localization of BrdU and NeuN. Therefore, neurons were born during or after small-molecule treatment.

(C–D) When BrdU was applied in culture medium at the end of small-molecule treatment (D), very few BrdU-labeled cells were immunopositive for neuronal marker NeuN (D), indicating neurons were born during small-molecule treatment but not after.

(E) Quantitative analyses of BrdU-labeled neurons in B and D. A large number of cells showed co-localization of BrdU and NeuN (77.3 ± 3.8%) when BrdU was added before small-molecule treatment. Very few NeuN+ cells were co-localized with BrdU if BrdU was added after small-molecule treatment (D10 to D30, 1.75 ± 0.73%), indicating that neurons were mostly converted in the presence of small molecules.

(F–G) When BrdU was applied during small-molecule treatment from D1 to D8, most of the NeuN-positive neurons were absent of BrdU labeling (95.7 ± 2.3 %, n = 3 bathes), suggesting those neurons were directly converted from astrocyte without a cell expansion.

### 3.2 Transcriptional activations of endogenous neural transcription factors during chemical reprogramming

To understand the molecular mechanisms underlying chemical reprogramming from astrocytes to neurons, we first employed a PCR Array (Qiagen) to investigate gene profile changes. We observed a significant increase in the transcriptional levels of neural TFs, such as ASCL1, NGN1/2 and NEUROD1, at day 4 with small-molecule treatment (Fig. 3-3 A, n = 3 batches). Note that NGN2 expression was increased by up to 300-fold. At day 8, the neuronal genes were turned on, for instance, DCX showed a 2000-fold increase in the transcriptional level (Fig. 3-3 B), suggesting a large number of small molecule–treated cells are immature neurons. Interestingly, the neural TFs were switched down at D8. In cultured astrocytes without small-molecule treatment, there was no significant change in gene expression (Fig. 3-3 C, D).
We further investigated the time course of transcriptional change of neural TFs NGN2 and NEUROD1, as well as astroglial genes GFAP and ALDH1L1, during chemical reprogramming process (Fig. 3-3 E-H, n = 3 batches). Interestingly, we found that NGN2 transcription increased from day 2 and peaked at day 4 (Fig. 3-3 E), while NEUROD1 peaked at day 6 during small-molecule treatment (Fig. 3-3 F), consistent with their sequential expression during early brain development. In contrast, transcriptional level of glial gene GFAP was dramatically reduced as early as day 2 and reached the peak at 200-fold at D4 (Fig. 3-3 G), coinciding with the activation of neural TFs (Fig. 3-3 E-F). Similarly, the transcriptional level of another astrocytic gene ALDH1L1 was also downregulated (Fig. 3-3 H). In contrast, control experiments without small-molecule treatment showed little transcriptional changes (Fig. 3-3 I-L). Therefore, our small-molecule treatment activates neural transcriptional factors and, in the meantime, inhibits astrocytic genes.
Figure 3-3. Transcriptional regulation in chemical reprogramming

(A–B) PCR array revealed substantial transcriptional activation of neural TFs (NGN1/2, NEUROD1, and ASCL1) and immature neuronal gene DCX at day 4 (A) or day 8 (B) after small-molecule treatment. Note that DCX increased > 2000-fold at D8 compared to the control. The genes showing a significant change in the PCR array assay were presented (P < 0.05, Mann-Whitney t test).

(C–D) PCR array revealed no significant change of gene expression in control human astrocyte cultures at day 4 (C) or day 8 (D) without small-molecule treatment, but with 1% DMSO as vehicle control.
(E–H) The time course of transcriptional changes revealed by quantitative real-time PCR analyses. Neural transcriptional factors NGN2 (E) and NEUROD1 (F) showed a peak transcription at D4 and D6, respectively, whereas astroglial genes GFAP (G) and ALDH1L1 (H) were significantly downregulated.

(I–L) In control human astrocyte cultures (1% DMSO), quantitative real-time PCR also revealed little transcriptional changes in neural transcriptional factors NGN2 (I) and NEUROD1 (J), or glial genes GFAP (K) and ALDH1L1 (L). N = 3 batches.

In summary, the transcription data suggest the neural induction from astrocytes was initiated at early days and was accomplished at the end of chemical treatment.

3.3 Epigenetic regulation during chemical reprogramming

Given the dramatic changes in transcription level, we hypothesized that epigenetic regulation also play a role in chemical reprogramming. We first investigated DNA methylation in astroglial gene GFAP and neural gene NEFM. DNA methylation of the gene promoter affects the accessibility of transcriptional factor binding and hence impairs the gene transcription. DNA methylation was considered the rate-limiting factor in reprogramming of pluripotent stem cells (Papp and Plath, 2013; Yao and Jin, 2014). To examine the methylation levels of the genes of interest, before and after small-molecule treatment, we performed methylated DNA immunoprecipitation, followed by sequencing (MeDIP-seq).

As expected, the promoter region of GFAP gene was initially absent of methylation signals in human astrocytes before small-molecule treatment (D0), but a clear peak signal was detected after 8 days of small-molecule treatment, indicating increased DNA methylation (Fig. 3-4 A). This increased methylation peak was further confirmed by targeted bisulfite sequencing (BS-seq) (Fig. 3-4 B), which gives higher resolution into single nucleotides. Notably, this GFAP promoter region contains the TF binding sites for
STAT3 and AP1, which play critical roles in the activation of GFAP gene (Cheng et al., 2011; Condorelli et al., 1994; Fan et al., 2005). BS-seq data revealed that the flanking regions of STAT3 and AP1 binding locus were hypermethylated (Fig. 3-4 B) in response to small-molecule treatment. As a result, TF binding to these sites could be impaired, thus leading to the significant down-regulation of GFAP transcription (Fig. 3-3 G) (Xu et al., 2015). In addition, an increase in DNA methylation at the GFAP transcription start site (TSS) after small-molecule treatment was revealed by MeDIP-seq and was confirmed by BS-seq (Fig. 3-4 C).

In contrast to glial gene GFAP, proneural TF NEUROD1 exhibited reduced DNA methylation at the promoter region in response to small-molecular treatment (Fig. 3-4 D), which is consistent with the transcriptional upregulation of NEUROD1. Similarly, another neuronal gene NEFM, encoding a midsized neurofilament specific to neurons, showed a significant decrease of methylation signal at the promoter region after small-molecule treatment (Fig. 3-4 E), suggesting the activation of neuronal genes.
Figure 3-4. Epigenetic regulation in chemical reprogramming

(A–C) Epigenetic regulation of GFAP promoter and TSS during chemical reprogramming. MeDIP-seq revealed a significant increase of methylation in the GFAP promoter region (A, box region) after 8 days of small-molecule treatment, which was confirmed by subsequent BS-seq (B). Note that the hypermethylated sites were located in the flanking regions of two important TF-binding sites, STAT3 and AP1, which significantly inhibited GFAP transcription. BS-seq also showed a significant increase of methylation level at GFAP TSS and 5’ UTR regulatory region (C), further suggesting an inhibition of GFAP transcription.
through DNA methylation.

(D) MeDIP-seq results revealed the reduction of methylation signal at the promoter region of proneural TF NEUROD1.

(E–F) MeDIP-seq and BS-seq revealed a significant decrease of methylation at the promoter region of neuronal gene NEFM (neurofilament-M), suggesting transcriptional activation of neuronal genes during chemical reprogramming of human astrocytes into neurons.

We also investigated epigenetic regulation of TF NGN2, an important gene involved in neuronal differentiation. MeDIP-seq analyses suggested the methylation level of the NGN2 promoter region was quite low before and after small-molecule treatment (Fig. 3-5 A), consistent with previous reports (Covic et al., 2010). In addition to DNA methylation, histone modifications can also regulate gene expression by modulating the chromatin configuration. Generally, acetylation of histone 3 (H3Ac) and trimethylation of histone 3 at lysine 4 (H3K4me3) promote the euchromatin configuration and thus function as the transcription activating factors. In contrast, trimethylation of histone H3 at lysine 27 (H3K27me3) usually leads to the heterochromatin, so that are considered as transcription inhibitory factors. We targeted NGN2 promoter region, as well as TSS, and investigated histone modifications as illustrated in Fig. 3-5 B. Consistent with the application of histone deacetylation (HDAC) inhibitor VPA during chemical reprogramming, chromatin immunoprecipitation (CHIP) coupled to detection by quantitative real-time PCR (qPCR) revealed a significant increase of histone acetylation at D8 (Fig. 3-5 C). Interestingly, the H3K4me3 level was significantly increased at promoter region (Fig. 3-5 D), whereas H3K27me3 level was significantly reduced at TSS after small-molecule treatment (Fig. 3-5 E), consistent with transcriptional activation of NGN2 induced by small-molecule treatment.
Figure 3-5. Chromatin modifications during chemical reprogramming

(A) MeDIP-seq analyses revealed low methylation levels at the Neurogenin 2 (NGN2) promoter region.

(B) Cartoon illustration showing two sites we examined for chromatin configuration at NGN2 promoter and TSS region.

(C) CHIP-qPCR revealed a significant increase of histone acetylation in NGN2 promoter region after small-molecule treatment, likely caused by the HDAC inhibitor VPA.

(D–E) The methylation level of H3K4 was increased significantly at NGN2 promoter region (D), whereas H3K27 methylation at TSS showed a significant decrease (E), indicating epigenetic activation of NGN2 through histone modification.
In addition, we observed a gradual reduction in the immuno-reactivity of transcriptional repressor REST (RE1 silencing transcription factor) during chemical reprogramming (Fig. 3-6 A-B). Since REST is a key regulator of many neuronal genes, this observation is consistent with the increased expression of neuronal genes, as we showed with our PCR array data. In parallel, the expression of methyl-DNA binding protein (MeCP2) was gradually increased in converted neurons (Fig. 3-6 C-D). As a key regulator of many non-neuronal genes, MeCP2 is highly expressed in neurons. These data are consistent with the reduced gene expression of glial genes.
Figure 3-6. Expression of MeCP2 and REST during chemical reprogramming

(A–B) Immunostaining and quantitative analysis revealed a significant increase in MeCP2 expression (A, arrow, red) during small-molecule treatment.
(C–D) Immunostaining and quantitative analysis revealed a significant reduction in REST expression (C, arrow, red) during small-molecule treatment.

Our results suggest that epigenetic regulation is involved in chemical reprogramming and may influence the conversion by mediating global gene expression.

3.4 Translational increase of endogenous neural transcription factors during chemical reprogramming

To corroborate our transcriptional and epigenetic results, we used immunostaining to examine the protein expression changes during the chemical reprogramming process (Fig. 3-7, one-way ANOVA with Dunnett's test, n = 3 batches). After a 2-day treatment with LDN193189, SB431542, and TTNPB, the Ascl1 expression level was significantly increased (Fig. 3-7 A and G). Ngn2 expression peaked at D4 after CHIR99021, DAPT, and VPA treatment (Fig. 3-7 B and H). Compared to Ascl1 and Ngn2, the expression of NeuroD1 appeared to be delayed, with a peak level at D6 after small-molecule treatment (Fig. 3-7 C and I), consistent with our transcriptional studies (Fig. 3-3 E-F). In addition, neuronal markers, such as DCX, were detected at D4–D6 and gradually increased until the end of small-molecule treatment (Fig. 3-7 D). Neurons positive for the mature neuronal marker NeuN appeared at D8–D10 (Fig. 3-7 E and J). The neuronal markers were generally expressed after the neural TFs.

In the meantime, while neuronal markers showed increased expression, levels of the astrocytic protein GFAP were significantly lower after small-molecule treatment (Fig. 3-7 F and K), consistent with epigenetic silencing and transcriptional downregulation of GFAP
gene (Fig. 3-3 G).

![Image]

**Figure 3-7. The increase of the protein expression level of neural transcription factors during chemical reprogramming**

(A–C) Representative images illustrating the gradual activation of endogenous neural TFs Ascl1 (A), Ngn2 (B), and NeuroD1 (C) at different days of small-molecule treatment.

(D–E) Representative images showing the gradual increase of neuronal markers DCX (D) and NeuN (E) during the conversion process from D0 to D10.

(F) Representative images showing the decrease of astrocytic marker GFAP from D0 to D10. Scale bars: 20 μm
(G–I) Quantitative analyses of the protein expression level of Ascl1 (G), Ngn2 (H), and NeuroD1 (I). Note that Ascl1 significantly increased at day 2 by threefold, while Ngn2 peaked at day 4 and NeuroD1 peaked at day 6. N = 3 batches.

(J) Quantified data showing a significant increase of NeuN from day 6 to day 10. N = 3 batches.

(K) Quantified data showing a significant decrease of GFAP from D0 to D10. N = 3 batches. *** P < 0.0001, one-way ANOVA with Dunnett’s test. Data are presented by mean ± SEM.

Control astrocytes in the absence of small-molecule treatment were largely immuno-negative for neuronal markers and consistently showed high expression of astrocytic protein GFAP (Fig. 3-8).
Figure 3-8. The absence of neuronal transcription factor expression in human astrocytes without small-molecule treatment

(A–C) Immunostaining revealed very low protein expression of the endogenous neural TFs Ascl1 (A), Ngn2 (B) and NeuroD1 (C) in control human astrocytes (1% DMSO). (D) NeuN staining showed few neurons in the control condition. (E) Representative images showing constant expression of GFAP (red) in the control condition. (F–H) Quantitative analyses of the fluorescence intensity of Ascl1 (F), Ngn2 (G), and NeuroD1 (H) during D2-D10 cultures, normalized to the intensity at D0.
(I) Quantitative analysis showing few NeuN-positive cells in control human astrocyte cultures from D0 to D10.

(J) Quantitative analysis showing GFAP expression remained high from D0 to D10 in control human astrocyte cultures. N = 3 batches. Scale bars = 20 µm. Data are represented as mean ± SEM.

To summarize, these results suggest that small molecules successfully activated endogenous neural TFs, which may play an important role in reprogramming astrocytes into neurons.
3. 5 Functional roles of each individual molecule on glia-to-neuron conversion

To understand the effect of each small molecule on glia-to-neuron conversion, we applied each one individually to treat cultured human astrocytes. Although certain small molecules alone induced a morphological change to the cultured human astrocytes, no obvious neuronal conversion was observed at the end of chemical treatment (data not shown). In addition, we detected the gene expression changes induced by individual molecules. Interestingly, CHIR99021 or VPA alone could increase expression of the proneural factors NGN2 and NEUROD1 (Fig. 3-9 A-B). However, no single molecule was able to down-regulate glial gene expression (Fig. 3-9 C).

Figure 3-9. Gene expression induced by individual molecules

(A–B) Real-time PCR revealed transcriptional regulation of neural TFs NGN2 (A) or NEUROD1 (B) by nine master conversion molecules or by each individual small molecule, at day 4 after small-molecule treatment. The fold changes are normalized to control D0 expression level. Only the nine molecules together or CHIR99021 alone could activate NGN2. VPA alone could activate NEUROD1.

(C) Real-time PCR revealed transcriptional regulation of astrocytic gene GFAP by the nine master conversion molecules or by each individual small molecule, at day 4 after small-molecule treatment. The fold changes are normalized to control D0 expression level. Only the nine molecules together significantly inhibited GFAP expression
Next, to determine the most critical small-molecule combination for glia-to-neuron conversion, we withdrew individual compound from our cocktail pool to see the removal of which drug would affect the induced neuronal number. When human astrocytes were sequentially exposed to the nine-molecule cocktail, a significant amount of NeuN-positive neurons were generated at D14 (160.5 ± 2.1, P < 0.0001, ***, n = 3 batches) (Fig. 3-10 B, K). Removal of DAPT led to the greatest reduction of NeuN+ cell number (40.2 ± 3.9, P < 0.0001, ***, n = 3 batches) (Fig. 3-10 C, K). Removal of CHIR99021 also remarkably impaired neurogenesis (60.0 ± 3.6, P < 0.0001, ***, n = 3 batches) (Fig. 3-10 D, K). Removing either SB431542 (Fig. 3-10 E, K) or LDN193189 (Fig. 3-10 F, K) resulted in a significantly reduced NeuN+ cell number (86.5 ± 6.0, 93.7 ± 8.0 respectively, P < 0.0001, ***, n = 3 batches). Removal of both SMADs inhibitors further reduced neurogenesis (67.0 ± 4.6, P < 0.0001, ***, n = 3 batches) (Fig. 3-10 K).

Unexpectedly, in spite of the dramatic effect shown on neuronal gene induction, the removal of VPA (Fig. 3-10 H, K) only affected the neuronal number slightly (130.3 ± 9.2, P < 0.05, *, n = 3 batches). One possible explanation could be that VPA may not be necessary to neuronal reprogramming, but could facilitate the conversion efficiency. Similar to VPA, removal of SAG and purmorphamine (Fig. 3-10 G, K) sacrificed the neuronal conversion mildly (123.2 ± 8.4, P<0.005, **, n = 3 batches). Interestingly, removing TTNPB (Fig. 3-10 J, K) or thiazovivin (Fig. 3-10 I, K) had no significant effect on neurogenesis (160.8 ± 8.8, 138.7 ± 7.6, respectively, n = 3 batches, P > 0.05, N.S).

Thus, these data suggested that SB431542, LDN 193189, CHIR 99021 and DAPT functioned as the indispensable compounds for astrocyte-to-neuron conversion. These four small molecules are hereafter called the core molecules.
Figure 3-10. Evaluating the essential role of each individual small molecule during astrocyte-to-neuron reprogramming

(A) Human astrocytes treated with 1% DMSO as a control. NeuN, green; MAP2, red.

(B) A defined combination of nine small molecules induced a massive number of neurons (14 days post initial small-molecule treatment, the same for the removal experiments).
(C–F) Individual removal of DAPT (C), CHIR99021 (D), SB431542 (E) or LDN193189 (F) from the nine small-molecule pool significantly reduced the number of converted neurons.

(G) Removal of sonic hedgehog agonists SAG and Purmo together slightly reduced the number of converted neurons.

(H) Removal of VPA also slightly reduced the neuronal number.

(I-J) Removal of thiazovivin (I) or TTNPB (J) did not affect the neuronal conversion.

Scale bars: 20 μm.

(K) Quantitative analyses showing that DAPT is the most potent reprogramming factor, followed by CHIR99021, SB431542, and LDN193189. * P < 0.05; ** P < 0.01; *** P < 0.001; one-way ANOVA followed with Sidak’s multiple comparison test. N = 3 batches.

We further investigated whether the core molecules were sufficient to reprogram human astrocytes into neurons. Not surprisingly, we observed a large number of cells that were immunopositive for the neuronal markers Tuj1, MAP2 and NeuN (Fig. 3-11 A-C), when human astrocytes were treated with the core molecules for 6 days. Compared to sequential exposure of the 9-small-molecule cocktail, the core molecules induced a larger amount of NeuN+ neurons at 2 weeks after small-molecule treatment (Fig. 3-11 D). Therefore, these data suggested the core molecules could reprogram human astrocytes into neurons with higher efficiency.
Figure 3-11. Four core molecules are sufficient for highly efficient reprogramming of human astrocytes into neurons

(A-C) Representative images showing the cells induced by four core molecules (SB431542, LDN193189, CHIR99021, DAPT) were immunopositive for neuronal markers Tuj1 (A, cyan), MAP2 (B, cyan) and NeuN (C, green). Scale bars = 20 µm.

(D) Quantitative analysis revealed a higher neuronal conversion efficiency induced by the four core molecules (N = 3 batches, *** P < 0.0001, one-way ANOVA followed by Dunnett’s test).

Next, we asked whether the signaling pathways on which the molecules targeted were indeed inhibited during our small-molecule treatment. We performed a series of immunostaining experiments against phosphorylated SMAD1/5/9 (Fig. 3-12 A), Notch intracellular domain (NICD) (Fig. 3-12 B), and phosphorylated GSK3β (Fig. 3-12 C). Our results showed that the BMP/TGFβ, Notch, and GSK3β signaling pathways were significantly inhibited (Fig. 3-12 D-G) after small-molecule treatment, and the inhibition of those signaling pathways was further confirmed by western blots (Fig. 3-13). Our data suggest a close link between the inhibition of these signaling pathways and the astrocyte-to-neuron conversion.
Figure 3-12. Signaling pathways in small-molecule-mediated reprogramming

(A and D) Representative images (A) showing the reduced level of phosphorylated SMAD1/5/9 (green) in nuclei after 2-day small-molecule treatment. (D) Quantitative analyses of the fluorescent intensity of p-SMAD1/5/9 indicating that the BMP signaling pathway was inhibited.

(B and E) Representative images and quantitative analysis illustrating the reduced level of Notch intracellular domain (NICD) (green) at D6 after the initial small-molecule treatment, indicating that the Notch signaling pathway was inhibited.

(C and F) Representative images and quantitative analysis illustrating the increased level of phosphorylated GSK3β (green) after 6 days of treatment with small molecules, indicating that GSK3β was inactivated.

(G) In parallel with the inhibition of signaling pathways, GFAP was significantly reduced at 6 days after small-molecule treatment.

** P < 0.001; *** P < 0.0001; one-way ANOVA followed by Dunnett’s multiple comparison test. N = 3 batches. Scale bars = 10 μm.

Data are represented as mean ± SEM.
Figure 3-13. Signaling pathways in chemical reprogramming revealed by western blot

(A) Western blot analysis showing the protein levels of phosphorylated SMAD 2, SMAD 3 and SMAD1/5/9 after 2 days of small-molecule treatment.

(B) Western blot analysis showing the protein level of phosphorylated GSK 3β and NICD at day 6 of small-molecule treatment.

(C–E) Quantitative analyses revealed the reduced levels of p-SMAD 2 (C), p-SMAD 3 (D) and p-SMAD1/5/9 (E), indicating that the BMP and TGF β signaling pathways were inhibited.

(F) Quantitative analyses revealed the increased p-GSK 3β, indicating that the GSK 3β signaling pathway has been inhibited.

(G) Quantitative analyses revealed the reduced level of NICD, indicating that the Notch signaling pathway has been inhibited.
3. 6 Summary

Our results suggest the neurons were directly converted from human astrocytes in the absence of a transient stem cell stage. Activation of neuronal genes and inhibition of glial genes at both the transcriptional and translational levels contributed to the glia-to-neuron conversion. Epigenetic regulations also played essential roles in neuronal reprogramming. In addition, we identified four core molecules (SB431542, LDN193189, CHIR99021, and DAPT) that are indispensable for the neuronal conversion.
Chapter 4

Application of small molecules in mouse brain promotes cell fate switch towards neuronal lineage

In this chapter, I applied the small-molecule compounds to mouse brain and found that they enhanced the neurogenic capability of mouse brain. Ningxin Ma helped with immunostaining and primary cell cultures. Part of the work was published in *Cell Stem Cell* (Zhang et al, 2015).

4.1 MCM-converted human neurons survive and integrate into mouse brain

We transplanted chemically converted human neurons into mouse brain to determine if the converted neurons could survive and integrate into mouse brain. To better visualize and distinguish the transplanted neurons from host mouse neurons, we infected the human astrocytes with high-tittered lentiviruses encoding EGFP. The majority of human astrocytes were labeled by GFP before chemical treatment. After 14 days of small-molecule treatment, cells containing both converted neurons and unconverted astrocytes were collected and injected into the lateral ventricles in newborn mouse brain (postnatal day 1, P1) (Fig. 4-1 A). At 7 days post-cell injection (DPI), we found clusters of GFP+ cells inside the lateral ventricles or in the subventricular zone (n = 6 mice), which were immunopositive for human cell–specific marker human nuclei (HuNu) (Fig. 4-1 B), indicating that they originated from the injected human cells. Many GFP+ cells showed neuron-like morphology with small soma and long processes. Consistently, immunostaining revealed those neuron-like GFP+ cells were
immunopositive for the neuronal markers DCX, NeuN and MAP2 (Fig. 4-1 B-D), suggesting that the human astrocyte–converted neurons survive in mouse brain in vivo.

Figure 4-1. In vivo survival of small molecule-converted human neurons in the mouse brain

(A) Schematic drawing showing the transplantation of small-molecule-converted human neurons into the mouse brains at postnatal day 1.

(B) GFP-positive cells were identified around lateral ventricles at 7 days post cell injection (7 DPI). Many GFP-positive cells were also positive for DCX (red), and all of
the GFP-positive cells were immunopositive for human nuclei (HuNu, blue), indicating their human cell identity. N = 6 mice.

(C) At 11 DPI, some GFP-positive cells were immunopositive for MAP2 (red), indicating the survival and growth of human neurons in mouse brain. N = 6 mice.

(D) Some GFP-positive human neurons, which were immunopositive for NeuN (red) and HuNu (cyan), migrated into the adjacent striatum areas and extended long neurites at 11 DPI.

Scale bars = 20 µm.

Even 1 month after cell transplantation, we were still able to identify clusters of GFP+ neurons in brain areas adjacent to the lateral ventricles, such as the thalamus and the striatum (n = 2) (Fig. 4-2 A), suggesting that the human astrocyte–converted neurons might have migrated out of the lateral ventricles and integrated into the local neural circuits. Converted neurons were immunopositive for VGluT1 (Fig. 4-2 B), indicating their glutamatergic neuronal identity. At 1 month after cell injection, many GFP-labeled human neurons extended elaborate neurites to connect with the surrounding mouse neurons. We also observed numerous SV2 puncta along the MAP2-labeled dendrites of GFP+ human neurons (Fig. 4-2 C). These data suggested that the grafted human neurons established synaptic connections with host mouse neurons. We concluded that the chemically converted human neurons could survive in mouse brain and integrate into host neuronal circuits.
Figure 4-2. Chemically reprogrammed human neurons integrate into host neural circuits

(A) Human neurons, labeled by NeuN (red) and HuNu (blue), survived for more than 1 month inside the mouse brain and were surrounded by mouse neurons (NeuN-positive but HuNu-negative). N = 2 mice.

(B) Small molecule–converted human neurons were immunopositive for Vglut1 in vivo, suggesting their glutamatergic identity.

(C) GFP-positive human neurons were innervated by surrounding neurons as indicated by many synaptic puncta (SV2, red) along the GFP-positive neurites (inset), suggesting the synaptic integration of the transplanted human neurons into the local neural circuits. N = 2 mice. Scale bars: 20 μm.
4. 2 Dedifferentiation of mouse astrocytes into neural stem cells using small-molecule cocktail

We attempted to reprogram mouse astrocytes into neurons with our master conversion molecules both *in vitro* and *in vivo*. Although we found a few cells immunopositive for Tuj1 and NeuN, the conversion efficiency was quite low (Fig. 4-3 A). We also injected the core molecules or vehicle solution daily via brain cannula into the cortex of GFAP::GFP mouse; in the mouse brain, the astrocytes were labeled green (Fig. 4-3 B, n = 3 mice). Although DCX immunoreactivity was increased in astrocytes, no clear DCX-positive neurons were observed (Fig. 4-3 C), indicating the core molecules are not sufficient to achieve the glia-to-neuron conversion in mouse brain. The differences in human and mouse astrocytes were huge in terms of gene expression, signaling pathways, and physiological functions. To convert mouse astrocytes into neurons, a new small-molecule protocol needs to be developed.
Figure 4-3. Core-molecule cocktail is not sufficient for converting mouse astrocyte to neurons

(A) Immunostaining revealed that only a small number of cells immunopositive for Tuj1 and NeuN could be induced from cultured mouse astrocytes in vitro.

(B) Brain cannula was installed in GFAP::GFP mouse cortex, in which mice, astrocytes were labeled green. The core molecules were perfused through cannula daily for 7 days. Chemical treated mice were sacrificed for immunostaining 2 weeks after small-molecule treatment.

(C) Immunostaining revealed increased DCX expression in GFAP-expressing astrocytes. No neuronal morphology was observed.

Scale bars = 20 µm.

Interestingly, we observed significantly increased Nestin expression in cortical and striatal mouse astrocytes treated with master conversion molecules in vivo (Fig. 4-4 A, B, D, *** P < 0.0005, student t-test, n = 3 mice), indicating they have gained stem cell properties. In addition, we also observed slightly increased DCX immunoreactivity around the injection site without clear neuronal morphology (Fig. 4-4 C).
Figure 4-4. Injection of small molecules in mouse brain induces Nestin expression

(A) Mouse cortical astrocytes (GFAP, red) showed dramatic changes in morphology and expressed high level of Nestin (green) at 6 dpi of small molecules, including SB431542 0.1 nmol, LDN193189 0.01 nmol, CHIR99021 0.03 nmol, DAPT 0.1 nmol, SAG 0.01 nmol and TTNPB 0.01 nmol (mixed in a total volume of 2 µl, N = 4 animals).

(B) Quantitative analyses showing increased Nestin expression in small-molecule-treated mouse astrocytes in vivo (Student t-test, *** P < 0.0001).

(C) Immunostaining revealed some DCX-(cyan) expressing cells were observed around the injection site. N = 4 animals.

(D) Mouse striatal astrocytes (GFAP, red) expressed a high level of Nestin (green) at 6 dpi of small molecules, including SB431542, LDN193189, CHIR99021, DAPT, SAG and TTNPB (mixed in a total volume of 2 µl). N = 3 animals.

Scale bars = 20 µm.

Since Nestin is highly expressed in neural stem cells, we hypothesized that our small-molecule cocktail could dedifferentiate mouse astrocytes into neural stem cells. Therefore, we dissected the cortical brain tissue surrounding the small-molecule injection site and cultured in vitro (Fig. 4-5 A). The brain tissues were dissociated into single cells and suspended in low-affinity culture dish (Fig. 4-5 A). Compared to DMSO-treated cells, the small-molecule-treated brain tissue gave many more neurospheres, which is the characteristic 3D cluster when neural stem cells are cultured in suspension culture system (Fig. 4-5 B-D, *** P < 0.0005, student t-test, n = 3 batches). When dissociated from primary neurospheres, small-molecule-treated single cells exhibited self-renewal capability to form secondary neurospheres (Fig. 4-5 E-G, ** P < 0.005, *** P < 0.0005, student t-test, n= 3 batches). Of note, almost no neurospheres formed when cells were isolated from non-treated mouse cortex, indicating that normal astrocytes were not able to proliferate and form neurospheres. In contrast, the small-molecule-treated astrocytes obtained stem cell properties and became self-renewable.
Figure 4-5. Small molecule-treated cortical astrocytes form neurospheres in vitro

(A) Small-molecule-treated cortical tissues were isolated and cultured in vitro.

(B) Cells with self-renewal capability formed primary neurospheres in suspension culture system.

(C–D) Quantitative analyses showing small-molecule-treated cortical tissues generating more neurospheres (C) and with bigger sizes (D). Student t-test, *** P < 0.0001.

(E) Primary neurospheres were subcultured and seeded as single cells. The highly proliferative single cells kept dividing to form the secondary neurospheres in suspension culture 3 days after initial seeding.
(F–G) Quantitative analyses showing more secondary neurospheres (F) with larger sizes (G) formed in the small-molecule-treated group. Student t-test, ** P < 0.001, *** P < 0.0001.

Scale bars = 200 μm, N = 3 mice.

In agreement, the dissociated neurospheres in monolayer culture were immunopositive for neural stem cell markers, such as Nestin and Sox2 (Fig. 4-6 A-C, n = 3 batches). In addition to self-renewal, neural stem cells can differentiate into multiple cell types within a neuronal lineage. When cultured in proper culture medium (Fig. 4-6 A), small-molecule-treated cells differentiated into Tuj1-expressing neurons, GFAP-positive astrocytes and CNPase-labeled oligodendrocytes (Fig. 4-6 D, E), suggesting the small-molecule-treated astrocytes obtained multipotency.

Figure 4-6. Small-molecule-treated cortical astrocytes obtain neural stem cell properties
(A) Cells derived from the secondary neurospheres were cultured in monolayer and induced to differentiation into neurons, astrocytes, and oligodendrocytes.

(B–C) Immunostaining revealed that small-molecule-treated cells were immunopositive for neural stem cell markers Sox2 (green) and Nestin (red) (C). Very few cells without small-molecule treatment attached in monolayer culture (B).

(D–E) Cells derived from secondary neurospheres were differentiated into neuronal cells (Tuj1, green) in neuronal differentiation medium, oligodendrocytes (CNPase, red) or astrocytes (GFAP, green) in glial medium (E). No differentiated neuronal cells were observed in the control group without small-molecule treatment. 

N = 3 batches. Scale bars = 20 μm. Data are represented as mean ± SEM.

In summary, although the MCM protocol is not sufficient to reprogram mouse astrocytes into neurons, our results suggest that in vivo treatment with small molecules changes the properties of mouse astrocytes towards those of neural stem cells.

4.3 Small molecules promote adult neurogenesis in the dentate gyrus

Neurogenesis has been identified in the dentate gyrus of the hippocampus throughout the adulthood. Newly generated neurons integrate into existing circuitry by migrating to the granule layer of the hippocampus and make crucial contributions to hippocampal functions. Interestingly, lineage-tracing data suggest that a large number of newborn neurons originated from GFAP-expressing radial glia-like cells (Ming et al., 2011) in the SGZ. In addition, growing evidence suggests the neural precursors and neuroblasts residing in adult neurogenic brain regions share many similarities with radial glial cells (Dhaliwal et al., 2011). This led us to hypothesize that our small-molecule cocktail could modulate adult neurogenesis in a way similar to how it acts on glia-to-neuron conversion.
Therefore, we injected a single dose of our small-molecule cocktail directly into the dentate gyrus of 3-month-old adult mice (Fig. 4-7 A). The small-molecule compounds included in the cocktail were SB431542 (100 µM), LDN193189 (5 µM), CHIR99021 (30 µM), DAPT (100 µM), TTNPB (10 µM), and SAG (2 µM). Six days after injection, we observed significantly increased Nestin expression in small-molecule-treated mouse SGZ (Fig. 4-7 B-D, * P < 0.05, student t-test, n = 3 mice). Moreover, the number of DCX+ newborn neurons was greatly enhanced (Fig. 4-7 B, C, E, ** P < 0.005, student t-test, n = 3 mice).

**Figure 4-7. Small molecules enhance neurogenesis in young adult mouse hippocampus**

(A) Illustration showing the single injection of small molecules directly into the dentate gyrus could enhance the adult neurogenesis in the hippocampus.

(B–C) Master conversion molecules (C) or DMSO (B) were injected into the dentate gyrus of 3-month-old adult mice. Immunostaining revealed the increased Nestin (red) expression induced by small molecules. More DCX+ newborn neurons were observed in small-molecule-treated mouse hippocampus.

(D–E) Quantitative analysis of Nestin intensity (D) and DCX+ cell number (E). * P < 0.01, ** P < 0.001. Student t test. N = 3 mice.
Considering a 3-month-old mouse is still in adolescence, the young brain possesses active plasticity. The aim was to test whether small molecules could also enhance the adult neurogenesis in mature mouse brain where neurogenesis has been much inhibited. Indeed, when we delivered the core-molecule compounds (SB431542, LDN193189, CHIR99021, and DAPT) into the hippocampus of 1-year old mice, seven days after small-molecule treatment, we observed a significantly increased number of DCX-positive cells in the dentate gyrus (Fig. 4-8 A, B, E, ** P < 0.005, *** P < 0.0005, one-way ANOVA with Dunnett’s test, n=3 mice). Interestingly, not only the amount of DCX+ neuroblasts in SGZ was increased (Fig. 4-8 A, B, F, ** P < 0.005, one-way ANOVA with Dunnett’s test, n = 3 mice), but also the number of DCX+ newborn neurons in granular layer was greatly enhanced (Fig. 4-8 A, B, G, ** P < 0.005, one-way ANOVA with Dunnett’s test, n = 3 mice). In contrast, only very limited numbers of new neurons were observed in DMSO-treated hippocampus (Fig. 4-8 A). These data suggest that the small-molecule cocktail dramatically enhanced the neurogenesis in the hippocampus, probably by expanding the neural precursor pool and promoting neural differentiation.

Next, we investigated whether the neurogenic effect remained if we reduced the number of compounds. We injected either dual SMADs inhibitors SB431542 and LDN193189 (Fig. 4-8 C), or GSK3 inhibitor CHIR99021, together with Notch inhibitor DAPT (Fig. 4-8 D). Intriguingly, the DCX+ cell number was greatly enhanced, even though only two compounds were applied (Fig. 4-8 C-E), suggesting two compounds were sufficient to promote neurogenesis. Of note, although the dual SMADs inhibitors induced a slightly more DCX+ neuroblasts in SGZ (Fig. 4-8 F), only the combination of four molecules led to the optimized neurogenic effect in the entire dentate gyrus.
Figure 4-8. Small molecules enhance neurogenesis in the adult mouse hippocampus

(A) When 4% DMSO was injected into the hippocampus of a 1-year-old mouse, very few DCX$^+$ newborn neurons were detected in the hippocampus.
(B–D) Injection of small molecules into the dentate gyrus with (B) the core molecules, (C) CHIR99021 and DAPT, or (D) SB431542 and LDN99021 significantly increased the number of DCX$^+$ cells in the subgranular layer (under the dashed line) and in granular layer (above the dashed line). Scale bars = 20 μm.
(E–G) Quantitative analysis revealed significantly more DCX$^+$ cells induced by small molecules in the entire dentate gyrus (E), subgranular layer (F) and granular layer (G).
Together, our data suggest that the small-molecule compounds are strong neuronal inducers that can greatly enhance neurogenesis in young and mature mouse brain.
In the mammalian CNS, cell fates are tightly controlled. Neurons and astrocytes are terminally differentiated somatic cells. Although astrocytes might dedifferentiate and acquire stem cell properties in the injured or Alzheimer’s brain (Magnusson et al., 2014; Sirko et al., 2013), transdifferentiation directly from astrocytes to neurons has rarely been reported in physiological or pathological conditions.

Here we demonstrate that a combination of nine small molecules can efficiently convert human astrocytes into functional neurons. These chemically converted human neurons survive in long-term culture and in mouse brain. Moreover, the chemically reprogrammed neurons form elaborate neuronal networks in culture and integrate into mouse neural circuits. Mechanistically, the small-molecule cocktail may act by epigenetic silencing of glial genes and transcriptional activation of neural TFs, such as NEUROD1 and NGN2. When injected into the mouse brain, small molecules de-differentiate astrocyte towards neural stem cells, and in addition, enhance neurogenesis in adult mouse brain.

5.1 Identification of small molecules capable of reprogramming astrocytes into neurons

The development of the CNS is under precise temporal and spatial control by both intrinsic genetic programs and external signals, such as FGF, TGFβ, SHH, BMP, Notch, RA and Wnt (Faigle and Song, 2013; Hur and Zhou, 2010; Miller and Gauthier, 2007). To identify the small-molecule compounds for astrocyte-to-neuron conversion, we searched for small molecules targeting the pathways that are crucial in gliogenesis and neurogenesis, including noggin, BMP, TGFβ, GSK3β, Wnt, retinoic acid, Notch, Shh, and cAMP. Since epigenetic regulations may facilitate cell fate remodeling by modulating the gene transcription, small
molecules targeting DNA methylation and histone modifications were also included for testing. After preliminary tests, nine small molecules (i.e., LDN193189, SB431542, TTNPB, thiazovivin, CHIR99021, DAPT, VPA, SAG, and purmorphamine) were capable of reprogramming human astrocytes into neurons. However, adding these nine molecules together would trigger massive cell death, maybe due to the simultaneous disturbance of too many signaling pathways.

Therefore, we investigated a stepwise strategy to apply those compounds sequentially for better cell survival and reprogramming. To initiate the glia-to-neuron conversion, we suppressed the glial signaling pathways, such as BMP and TGFβ, and their downstream molecules, SMADs (Krieglstein et al., 2011), for the first 2 days by dual SMAD signaling inhibitors SB431542 and LDN193189. The glial inhibition effect was confirmed by downregulation of glial gene transcription and GFAP protein expression. Next, we induced the neuronal properties by activating neural TFs. Inhibiting GSK3β regulates cell fate specification through phosphorylation of NGN1/2 (Li et al., 2012). Modulation of Wnt signaling may lead to the activation of NeuroD1 and the subsequent promotion of adult neurogenesis (Kuwabara et al., 2009). In addition, Notch inhibition in mouse subventricular zone might induce Ascl expression (Magnusson et al., 2014). HDAC inhibitors also induce adult neurogenesis via activation of NeuroD1 (Hsieh et al., 2004). Thus, we applied the Notch inhibitor DAPT, the GSK3β inhibitor CHIR99021, and the HDAC inhibitor VPA to induce neurons from day 3 to day 6. Consequently, transcriptional and translational levels of Ascl1, Ngn2 and NeuroD1 were significantly upregulated. Because long-term exposure to the HDAC inhibitor may lead to the unstable chromatin structure, VPA was withdrawn after 2 days. At day 6, the number of astrocytes was largely reduced due to the neuronal conversion. Since neuronal maturation may be affected without sufficient astrocyte support, we applied sonic hedgehog agonists SAG and purmorphamine, which may promote astrocytes proliferation. By treating the cells with SAG and purmorphamine for 2 days, the number of astrocytes was greatly increased, and the maturation of chemically converted neurons was also enhanced.

By withdrawing each individual molecule from the nine-molecule pool, we found that DAPT has the most significant role in chemical reprogramming, followed by CHIR99021,
SB431542, and LDN193189. By treating with these four core small molecules, human astrocytes can be efficiently converted into functional neurons. Therefore, we conclude that the Notch, GSK3, TGFβ and BMP signaling pathways play crucial roles in cell fate switch from astrocytes to neuron.

Notch signaling is initiated by the Notch ligands (Delta and Jagged) binding with the Notch transmembrane receptor. The heterodimeric Notch undergoes proteolytic cleavage by tumor necrosis factor-α-converting enzyme (TACE) and Y-secretase, leading to the release of NICD from cell membrane to cytoplasm. The translocation of NICD to nucleus results in the transcriptional activation of effector genes, such as the inhibitory basic helix-loop-helix (bHLH) genes Hes. The Hes proteins initiate astrogenesis by inhibiting neurogenic bHLH factors (e.g., Ngn1, Ngn2) and by activating the JAK-STAT pathway (Kamakura et al., 2004). A great deal of evidence has shown that the Notch pathway is critical for glial cell fate determination and maintenance (Malik et al., 2014; Miller and Gauthier, 2007). On the other hand, inhibition of Notch is important in neurogenesis (Breunig et al., 2007). When the Notch pathway was genetically disturbed in astrocytes after stroke, more Asc1-expressing neuroblasts were found in mouse brain adjacent to SVZ (Magnusson et al., 2014). Moreover, DAPT could enhance neuronal differentiation from neural stem cells, through the blockage of Notch signaling pathway (Crawford and Roelink, 2007).

CHIR99021 is a potent and highly selective inhibitor of GSK3s. Growing evidence has suggested GSK3s function as key regulators in a broad spectrum of neurodevelopmental processes, by targeting a variety of substances, including β-catenin, Gli proteins, cAMP-responsive element-binding protein (CREB), mTOR, Smad1 and bHLH proneural factors. Of note, inhibition of GSK3 promotes Ngn2 activity and, hence, modulates early neocortical neurogenesis (Li et al., 2012). Another important substrate of GSK3 is the microtubule-associated proteins (MAPs). Suppression of GSK3 activity promotes axon growth through both Par3/Par6/PCK ξ and PI3K/AKT signaling pathways (Hur and Zhou, 2010). In support of this notion, the CHIR99021-treated human astrocytes exhibited dramatically morphological changes towards neurons, suggesting a reorganization of cytoskeleton proteins.

TGF-β superfamily members include TGF-βs, BMPs, and activins. The TGF-β signal is initiated by ligands binding to TGF-β type II receptors and the type I receptor ALK5. The
signals are subsequently propagated via phosphorylation of Smad2 and Smad3. By binding to ALK1, 2 or 6, BMPs transduce the signal via the activation of Smad1, 5 and 8. Upon activation, Smad-1, 2, 3, 5 or 8 form heteromeric complexes with Smad4 in the cytoplasm and translocate to the nucleus, where they regulate the target gene expression (Krieglstein et al., 2011). TGFβ and BMP signaling pathways are highly active in mature astrocytes with critical roles in the proper development of astroglial cells (Kohyama et al., 2010; Nakashima et al., 2001). On the other hand, suppression of TGFβ/BMP signaling pathways promotes early neurogenesis (Wachs et al., 2006). Although evidence showing either TGFβ inhibitor SB431542 or BMP inhibitor LDN193189 alone is sufficient to induce neural differentiation from stem cells, combining these dual SMADs inhibitors further strengthens the neuron inductive effect (Chambers et al., 2009). Notably, given the pivotal role TGFβ signaling plays in central synapses, continuous application of dual SMAD inhibitors may hamper neuronal maturation and synapse formation (He et al., 2014). Therefore, we exposed human astrocytes to SB431542 and LDN193189 for an initial 2 days and then replaced them with CHIR99021 and DAPT afterward.

5.2 Cell fate is determined by spatial and temporal modulation of signaling pathways

Frequently, the same signaling pathway operating in two neighboring cells or in the same cell at different times causes completely different cellular effects, presumably due to the varying availability of effector molecules and epigenetic states of the target genes.

Since the activity of a signaling pathway varies greatly among different cell types, manipulation of the same pathway may lead to very different effects. In support of this notion, although some of the small molecules identified in our study appear to be important in inducing neural differentiation from human stem cells (hSCs) (Chambers et al., 2009; Chambers et al., 2012; Li et al., 2011), the properties of induced neurons varied greatly. Our astrocyte-converted neurons are deep layer cortical neurons or hippocampal neurons, possibly, because our human astrocytes originated from the cortex and thus bears the cortical lineage trace. In contrast, the pluripotent hSCs treated with five small molecules (LDN + SB + CHIR
+ DAPT + SU5402) acquired spinal cord neuron properties (Chambers et al., 2012). An independent study reported that treatment with three small molecules (CHIR, SB, and compound E, a γ-secretase inhibitor) induced hPSCs into self-renewing neuroepithelial cells that could be further differentiated into midbrain and hindbrain neurons (Li et al., 2011). Another study by Ladewig et al. (2012) showed that CHIR, SB and noggin enabled the neuronal reprogramming induced by ectopic expression of Ascl1 and Ngn2 from human fibroblasts. The reprogrammed neurons were a mixture of GABAergic, glutamatergic, serotonin and dopaminergic neurons. These studies, together with our own, suggest different neuronal subtypes will be obtained when the different cell types are used for reprogramming. This finding is supported by our own observation that the MCM-protocol only works for human astrocytes of brain origin, but not for human spinal cord astrocytes. In addition, some signaling pathways prominent for their pro-neural differentiation function were tested as dispensable for astrocyte-to-neuron conversion. RA and hedgehog pathways are the major organizing signals in the brain and spinal cord development (Dessaud et al., 2008; Lai et al., 2003; Sirko et al., 2013). They are widely used for neuronal differentiation from stem cells. However, removal of the RA receptor agonist and Shh activator did not affect the neuronal conversion, indicating they are not absolutely required for astrocyte-to-neuron conversion. Therefore, the mechanism of reprogramming astrocytes into neurons clearly differs from that of neural development or differentiation. One possible explanation is that neural development or differentiation starts from neural stem/progenitor cells, whereas our reprogramming process starts from astrocytes, which are the progeny of neural stem cells. RA and Shh may be upstream of astrogial fate determination and therefore not required for astrocyte-to-neuron reprogramming.

An additional and equally pivotal mechanism for small-molecule-based cell fate determination is temporal patterning. The output of the same signaling pathway will be quite different when the timing is shifted. Since the signaling effect is modulated by signaling pathway cross talks, TFs, and epigenetics, the same ligand binding on the same type of cell at a different time may or may not lead to the same effect. For instance, activation of Notch signaling in early cortical development induces radial glial generation, whereas the late activation of the same pathway drives astrogial differentiation from radial
glia (Freeman., 2010). In late cortical development, NICD and Notch downstream TF RBPJ form a transcriptional activation complex on the GFAP promoter to promote GFAP transcription (Ge et al., 2002; Takizawa et al., 2001). In contrast, the inaccessibility of glial genes caused by DNA hypermethylation of the GFAP promoter abolishes the Notch effect on astrogliogenesis in early cortical development (Takizawa et al., 2001). In this context, the efficient glia-to-neuron reprogramming may be attributed to the precise temporal control on signaling pathways. In support of this notion, we observed a much-decreased neuronal conversion when the order of small-molecule groups was rearranged, indicating that the glial cell fate could only be reprogrammed to neurons when the right signaling pathways are manipulated at the proper time.

5.3 Transcriptional regulation contributes to cell fate determination

To fulfill their functions, extracellular stimuli need to coordinate with the intracellular network and rely upon TFs to activate or repress gene transcription. In the developing and postnatal brain, proneural bHLH factors are key regulators of neurogenesis, coordinating a generic neuronal fate and a specific subtype identity (Imayoshi and Kageyama, 2014). Overexpression of proneural factors in non-neuronal cells results in neuronal differentiation of transduced cells (Berninger et al., 2007a; Vierbuchen et al., 2010). Conversely, loss of proneural genes leads to a severe deficit in neural development (Yun et al., 2002).

Ascl1 expression has been characterized in both neuroprogenitors and differentiated neurons (Imayoshi and Kageyama, 2014). In neuroprogenitors, Ascl1 induces numerous TFs known for neural differentiations, such as Sox4, Dlx2, Ebf3, Gli3, and initiates neural differentiation. In differentiating neurons, Ascl1 regulates genes for cell-cycle arrest, as well as proteins regulating a wide spectrum of molecular functions (e.g., signal transduction, neurite morphogenesis and cytoskeleton regulation) (Castro et al., 2011; Wapinski et al., 2013).

Ngn2 is expressed in early neural development, indicating its initiating role in CNS patterning. Interestingly, transient expression of Ngn2 in NPCs induces the subsequent
expression of bHLH gene cascades, such as NeuroD1/2/4/6, bHLHb5, N scl1/2, and Math6 (Imayoshi and Kageyama, 2014), suggesting a hierarchy in TF-regulated neural development.

Expressed in late development, NeuroD1 has been identified as a strong neuronal inducer, specifying neural fate during hippocampal neurogenesis (Roybon et al., 2009). The effect of NeuroD1 on neurogenesis may be achieved by directly regulating neuronal genes, such as DCX (Steiner et al., 2006).

Coincidentally, the expression of proneural factors during astrocyte-to-neuron conversion also follows a hierarchy pattern. In treating with small molecules, Ascl1 expression was observed as early as day 2, and sustained the expression by the end of chemical reprogramming. Ngn2 expression also appeared early at D2 and peaked at D4 in concert with NeuroD1 expression. Right after NeuroD1 expression peak, neuronal proteins such as DCX and NeuN were gradually turned on, indicating the neuronal fate had been decided. Taken together, the dynamic and hierarchy expression of proneural genes indicate that a tightly controlled genetic cascade is involved in chemical reprogramming from astrocytes to neuron.

Differing actions by glial and neural genes also contribute to the transition from neurogenic-to-astrogenic fate switch. To maintain glial fate, glial cells express neural inhibitory bHLH factors Hes1 and Hes5, which directly repress transcription of the proneural bHLH genes Ascl1 and Ngn2 by recruiting corepressor Transducin-like E (TLE1-4)/Groucho-related gene (Grg), at the carboxy-terminal WRPW domain (Grbavec and Stifani, 1996). A similar mechanism is observed in neural fate restriction: the expression of proneural factors such as Ascl1 and Ngn1/2 antagonizes glial bHLH factors and inhibits transcription of the glial-specific genes (GFAP and S100β) by preventing the binding of phosphorylated STATs with glial specific promoters (Sun et al., 2001). The elaborate balance between proneural and glial genes is important in controlling the timing of the neurogenic-to-gliogenic fate switch. In concert with this notion, we observed the down-regulation of Hes related bHLH factor Hey1 in the middle of small-molecule treatment. Interestingly, in coincident with the abolishment of glial factors, the proneural factors Ascl1 and Ngn1/2 were greatly up-regulated. In addition, the evidence that the elevated Ngn1/2 expression is concurrent with decreased GFAP expression during the astrocyte-to-neuron conversion suggests the inhibitory effect of
Ngn1/2 on glial genes.

Figure 5-1. Cartoon illustration showing the transcriptional regulation during chemical reprogramming

(A) In astrocytes, glial-specific bHLH TFs are highly expressed and activate glial genes, such as GFAP, and inhibit proneural bHLH factors, such as Ascl-s and Ngn2, to ensure an astrocyte fate.

(B) After small-molecule treatment, levels of proneural bHLH TFs are greatly increased and trigger the neural gene cascade. On the other hand, glial bHLHs are inhibited both by small molecules and by proneural bHLHs. The dominant expression of proneural genes leads to cell-fate switch to neurons.

Taken together, our data suggest the activation of proneural TFs amplifies the
proneural effect by activating downstream neural genes and, in the meantime, repressing glial genes. On the other hand, suppression of glial genes further enhances proneural gene expression. As a result, the dominant expression of neural genes facilitates the astrocyte-to-neuron conversion.

5.4 Epigenetic regulation is involved in cell fate determination

Growing evidence suggests that epigenetic events control responsiveness of cells to fate-modulating external signals and govern cell fate determination. During neural cell fate restriction, epigenetic modulations determine the DNA and histone accessibility of critical genes, regulate the expression of TFs or neurogenic genes, and shape the transcriptome landscape (Hirabayashi et al., 2009; Yao et al., 2014). In terminally differentiated cells, developmental genes are repressed by epigenetic modifications to secure the cell identity.

One well-characterized epigenetic modification for gene repression is DNA methylation, which occurs on the gene promoter or enhancer domain (Fig. 5-2). Methylation of cytosine in CG dinucleotides typically induces gene silencing by attenuating TFs binding and recruiting methylcytosine-binding proteins, such as MeCP2 and repressive histone modifiers (Cedar and Bergman, 2009). By use of this mechanism, terminally differentiated cells repress genes related to other lineages, ensuring cell identity. For instance, in neurons, the GFAP gene is hypermethylated in the promoter region and distal regulatory elements (Fan et al., 2005; Teter et al., 1994a; Teter et al., 1994b; Teter et al., 1996).
Figure 5-2. Cartoon illustration showing the role of DNA methylation on transcription

(A) By binding to gene promoters, TFs activate the gene transcription.
(B–C) DNA methylation in gene promoters typically induces gene silencing by attenuating TFs binding (B) and recruiting inhibitory epigenetic regulators (C) (e.g. MeCP2 and repressive histone modifiers), to create an inhibitory environment for transcription.

In concert with this notion, we found that the GFAP promoter region was demethylated in human astrocytes culture and underwent methylation after small-molecule treatment. Notably, DNA demethylation in GFAP gene within STAT-binding element has a crucial role in promoting gliogenesis (Fan et al., 2005; Takizawa et al., 2001). Methylation of the STAT-
binding element impairs association of the STAT complex with the GFAP promoter, thus attenuating GFAP transcription. In this context, we observed hypermethylation in the flanking regions of the STAT-binding elements, coincident with the decreased GFAP expression after small-molecule treatment.

Conversely, DNA was demethylated at the promoter region of neuronal genes NeuroD1 and NEFM, which may partially explain the dramatic increase in neuronal gene expression. Of note, although NGN2 gene expression was increased to 200-fold during conversion, the DNA sequence at NGN2 promoter was hypomethylated, which is consistent with previous reports (Hirabayashi et al., 2009). One underlying possibility could be the NGN2 gene promoter contains CpG island with highly frequent CpG dinucleotides, which normally remain unmethylated (Covic et al., 2010).

In addition to DNA methylation, chromatin conformation is another epigenetic determinant for gene transcription. A nucleosome is formed by winding 147 base pairs of DNA around a histone core. Modifications in N-terminal tails of histone proteins provide binding docks for gene activators and repressors, which modulate the chromatin structure and determine the accessibility of wrapped DNA sequence. H3K4me3 and H3Ac generally correlate with the activated transcriptional environment, while H3K27me3 is known as the repressive mark (Fig. 5-3).
Figure 5-3. Cartoon illustration showing the role of histone modifications on transcription

(A) Repressive histone marks such as histone H3 trimethyl Lys27 (H3K27me3) and histone H3 trimethyl Lys9 (H3K9me3) are highly correlated to heterochromatin, which is transcription inhibitory.

(B) Active histone marks such as histone H3 trimethyl Lys4 (H3K4me3) and Histone 3 acetylation (H3Ac) are highly correlated to euchromatin, which is transcription-permissive.

Levels of active chromatin marks H3Ac and H3K4me3 in the Ngn1/2 promoter are increased in neurogenic NPCs. In addition, inhibition of histone deacetylation, which is catalyzed by HDAC, could increase H3Ac level and promote neuronal differentiation of adult hippocampal NPCs, by inducing proneural factors, such as Ngn1/2 and NeuroD1 (Hirabayashi et al., 2009; Hsieh et al., 2004). Conversely, the increased H3K27me3 at the Ngn1/2 locus restricts gene expression and contributes to neurogenic-to-gliogenic switch in late neocortical development (Hirabayashi et al., 2009). During the process of glia-to-neuron reprogramming, the histone modifications also had important roles. After small-molecule
treatment, we observed the active epigenetic marks H3K4me3 and H3Ac accumulated in the Ngn2 promoter region, and the repressive mark H3K27me3 was reduced in Ngn2 TSS. Accompanied by these modifications, significantly elevated expression of Ngn2 gene was also observed, indicating the correlation between histone modifications and proneural gene expression.

To maintain the cell fate of a terminally differentiated cell, a large number of epigenetic events, including DNA methylation and histone modifications, are required to form a unifying framework for elaborate gene regulation. Serving as key conduits, DNA-binding proteins can bridge enzymes for epigenetic modulations with specific DNA motifs.

MeCP2 restricts expression of astrocyte-specific genes, such as GFAP, by binding to their highly methylated regions (Kohyama et al., 2008; Setoguchi et al., 2006). Essentially, methylated DNA in the GFAP promoter binds with MeCP2, which further recruits histone modification enzymes, such as histone H3 lysine9 methyltransferases and HDACs, which further induces inactive chromatin remodeling (Lunyak et al., 2002). In MeCP2-deficient neurons, astroglial genes were abnormally up-regulated (Colantuoni et al., 2001). On the other hand, overexpression of MeCP2 in NSCs in vitro results in promoted neuronal differentiation, indicating a pivotal role of MeCP2 in neuronal fate restriction. Intriguingly, we observed a gradual increase of MeCP2 expression during chemical reprogramming from astrocyte to neurons, suggesting that MeCP2 might play a role in astro-to-neural fate switch.

Another well-studied DNA binding protein REST (RE1 silencing transcription factor) orchestrates numerous epigenetic modifications and represses a large network of neuronal genes containing an RE1 (repressor element 1) motif (Chong et al., 1995; Schoenherr and Anderson, 1995). In terminally differentiated non-neural cells, REST binds to CoREST, which recruits H3K9 methyltransferases, MeCP2, H3K4 demethylase and the HDACs. Thereby, REST induces long-term silencing of its target neuronal genes, such as NeuroD1, BDNF, synaptotagmin IV and Calbindin 1, through H3K9 methylation, DNA methylation and H3K4 demethylation (Ballas and Mandel, 2005). In neurons, REST expression is suppressed, and a class of neuronal genes are released to be transcribed (e.g., Ascl1) (Ballas et al., 2005). We observed a gradual reduction of REST protein expression, accompanied by increased expression of neuronal markers during the chemical reprogramming. Therefore, REST may
have a role in cell fate switch from astrocyte to neurons.

Epigenetic mechanisms can be modulated by external stimuli, such as extracellular signaling molecules. REST is regulated by various signals. Activation of the BMP signaling pathway could lead to REST up-regulation and, thus, contribute to the maintenance of astrocytic identity (Kohyama et al., 2010). Therefore, inhibition of BMP pathway by LDN193189 and SB431542 may contribute to the loss of REST during glia-to-neuron conversion. Conversely, RA signaling could regulate REST in a negative pattern. Ballas et al. (2005) identified the RA receptor and its co-repressor N-CoR in the REST promoter, which associates with the RA-induced neurogenesis from NSCs. Moreover, Jepsen et al. (2007) showed that activation of the RA pathway directly induces H3K27me3 demethylase in cortical NPCs and leads to neuronal differentiation. In agreement with this notion, treatment of RAR agonist TTNPT to human astrocytes is accompanied by decreased REST expression and reduced H3K27me3 hallmarks at NGN2 locus.
Figure 5-4. Cartoon illustration showing the possible mechanisms underlying small-molecule-induced astrocyte-to-neuron conversion

(A) In astrocytes, glial signaling pathways, such as TGFβ, BMP and Notch, are active, leading to the epigenetic and transcriptional activation of glial genes. On the other hand, REST binds to and restricts the neuronal genes by recruiting co-repressors and creating an epigenetically inhibitory environment for neuronal gene transcription. In addition, neuronal bHLH factors are inhibited by glial specific bHLH TFs, contributing to the inactivation of neuronal genes.

(B) Small molecules inhibit the glial pathways and activate the neuronal pathways, resulting in the release of neuronal genes from REST. The increased neuronal bHLH factors and permissive epigenetic events, such as DNA demethylation, histone acetylation and H3K4me3 imprint, also contribute to the activation of neuronal gene transcription. On the other hand, glial genes are repressed as a result of the inhibition of glial TFs, the suppressive effect by neuronal bHLHs, together with the inhibitory epigenetic events. The activation of
neuronal genes, together with the glial gene inhibition, leads to the astrocyte-to-neuron reprogramming.

5.5 A different chemical formulation is needed for reprogramming of mouse astrocytes into functional neurons

Although the MCM compounds converted human astrocytes into neurons with high efficiency, our data suggest a different chemical protocol is necessary for neuronal reprogramming of mouse astrocytes.

This notion is supported by the fact that human astrocytes differ from mouse astrocytes in various aspects. Morphologically, human astrocytes are much larger and more complex than their murine counterparts are (Oberheim et al., 2009). On the molecular level, whole-brain transcriptional data demonstrated that the differential gene expression between human and mouse brain is largely focused on the astroglial genes (Zhang and Barres, 2013). Research in our lab, comparing the human and the mouse astrocyte gene profiles, also revealed the huge differences in gene expression, including signaling molecules. In addition, functional studies have shown that transplantation of human astrocytes in mouse brain improves learning and memory (Han et al., 2013), indicating the distinct difference in functions between these two cell types. Therefore, it is safe to speculate that the response to external signals by human and mouse astrocytes are widely different.

In fact, different TFs are required for neuronal reprogramming of human and mouse cells. The data generated in Dr. Wernig’s lab suggested that, by combining three transcriptional factors (i.e., Ascl1, Brn2, and Myt11), mouse fibroblasts can be directly converted into neuronal cells (Vierbuchen et al., 2010). However, NeuroD1 is necessary to synergize with the three factors for neuronal conversion from human fibroblasts (Pang et al., 2011). Similarly, in our case, additional small molecules might be critical for conversion of mouse astrocytes to functional neurons.
5.6 Small molecules promote adult neurogenesis

Active neurogenesis has been identified in the adult mammalian hippocampus. Over the past years, a large number of analyses have explored the role of new neurons on the cellular, circuitry and behavioral levels (Deng et al., 2010; Lazarini and Lledo, 2011). In hippocampus, newborn neurons are generated from neural precursors and neuroblasts in SGZ. They gradually migrate into granular layer, where they receive input from the entorhinal cortex and send their axonal projections to CA3 area. Moreover, the impact of adult neurogenesis on rodent behavior has been illustrated by many studies. Essentially, blockage of hippocampal neurogenesis could affect the mouse performance in behavioral tasks related to hippocampus-dependent learning and memory (e.g., eye-blink conditioning and fear conditioning) (Deng et al., 2010).

Considering the critical contributions of adult neurogenesis to hippocampal function, reduced adult neurogenesis in aging brain or under pathological conditions is often seen in parallel with deficits in learning and memory. Abolishing adult neurogenesis leads to disrupted long-term potentiation (LTP) and synchronization of dentate neuron firing (Lazarini and Lledo, 2011). On the other hand, physical exercise and seizures, which enhance neurogenesis in the young adult hippocampus, could partially reverse age-induced decline in cognitive function and impairments in learning and memory (Kronenberg et al., 2006; Lugert et al., 2010).

New neurons develop in distinct steps (Ming and Song, 2011). Neurogenesis is initiated by the activation of quiescent neural stem cells, which share many properties with astrocytes, so were given the name radial glia-like cells. According to the cell morphology and marker expression, adult neurogenesis can be divided into four phases: 1) a precursor cell phase, when intermediate neural progenitors undergo fast proliferation and express stem cell markers such as Nestin; 2) an early survival phase, when immature neurons exit cell cycle and express early neuronal markers such as DCX. Of note, only a small portion of newborn neurons could survive several days after birth; 3) a postmitotic maturation phase, when new neurons integrate local circuitry by establishing functional connections and express mature neuronal markers such as NeuN; and 4) a late survival phase representing a
period of fine-tuning. It takes around 7 weeks to produce a fully mature new granule neuron in circuitry.

After the small-molecule treatment, we observed increased Nestin expression in the SGZ. Concurrently, a greatly increased number of DCX+ cells were found in the SGZ, clustering together and showing neuroblast-like morphology with round soma and short processes. In addition, many of those DCX+ progenitors can be labeled by Ki67, a proliferative marker (data not shown). Collectively, these data suggest the activation and expansion of the neural progenitor pool. Many signaling pathways could contribute to this process. For instance, Shh signaling is required for maintenance of radial glia-like precursors (Breunig et al., 2008; Han et al., 2008). In this context, the activation of Nestin-expressing precursors may be attributed to the Shh agonist SAG in our small-molecule cocktail. Inhibition of GSK3β also leads to the promotion of neural progenitor proliferation (Mao et al., 2009). In support of this notion, our data suggest that the proliferation of neural progenitors can be greatly enhanced by combining the Shh agonist SAG and the GSK3 pathway inhibitor CHIR99021.

Accompanied by the expansion of neural progenitors, the number of DCX+ cells increases in the granular layer. These cells displayed nice dendritic structure and were devoid of Ki67, which we then identified as immature neurons at the early survival phase. NeuroD1 is a strong neuron-inducer during hippocampal neurogenesis (Roybon et al., 2009) and can be mediated by the GSK3 inhibition (Faigle and Song, 2013). Therefore, it might be reasonable to speculate that GSK3 inhibitor CHIR99021 promoted neural differentiation from neuroblasts via activation of NeuroD1 expression.

Our data suggest that, by modulating multiple signaling pathways simultaneously by small molecules, adult hippocampal neurogenesis can be greatly enhanced. Later time points need to be included for exploration of whether those newborn neurons could survive for a prolonged period. In addition, the behavioral consequences of enhanced neurogenesis need to be evaluated. Moreover, in the future, we plan to test the possibility of using small molecules to enhance neurogenesis in brains with learning and memory deficits (e.g., aged brains or Alzheimer’s brains).
5.7 Challenges of small-molecule-mediated reprogramming

To generate new neurons in supplement of the lost ones after CNS injury, our lab and others recently reprogrammed reactive glial cells into neuroblasts or functional neurons in the mouse brain by ectopic expression of TFs (Guo et al., 2014; Addis et al., 2011; Berninger et al., 2007a; Heinrich et al., 2014; Heinrich et al., 2010; Liu et al., 2015; Niu et al., 2015; Niu et al., 2013; Su et al., 2014; Torper et al., 2015; Torper et al., 2013). The transcription factor–mediated in vivo reprogramming requires brain surgery and intracranial viral delivery.

In contrast, our small-molecule-mediated reprogramming represents a new approach to avoid sophisticated viral production and invasive neurosurgical procedures. In addition, the ease of temporal and spatial control of small molecule–mediated reprogramming may avoid the transgene integration, rendering it the more reliable method (Li et al., 2013).

Despite promising results, there are certain variabilities in the current study that must be addressed. 1) The main cell types used for glia-to-neuron conversion are derived from human fetal tissues. Although we proved the purity of astrocyte culture and promoted the astrocytic maturation by FBS, the cultured fetal astrocytes may not be able to fully mimic the adult human astrocytes (Zhang et al., 2016). In the future, we plan to work on the isolation and culture of adult human astrocytes for neuronal conversion. 2) Most of the work on neuronal reprogramming was done in cell culture. Since the genetic and epigenetic landscape of cultured cells may vary significantly from the cells in vivo, it is worthwhile to try in vivo conversion of human astrocytes into neurons by small molecules. To achieve this, we will transplant human astrocytes into the mouse brain and deliver the small-molecule compounds into the CNS for neuronal conversion. 3) Although we observed the enhanced adult neurogenesis induced by the core molecules, we have not linked this phenomenon with any functional consequences.

For future applications, several challenges must be overcome before this chemical glia-to-neuron reprogramming can be translated into clinical practice.

Firstly, an effective method must be developed to deliver the small molecules across the BBB to the injured or diseased brain areas. Most chemical compounds cannot penetrate the BBB, which leads to the failure of many clinical trials on chemical compounds in treating
CNS diseases (Pardridge et al., 2005). During the past decades, extensive research on drug delivery through BBB has been conducted. For instance, the novel technologies utilizing pharmacological agents or ultrasound to disrupt the BBB have shown promise for improved drug delivery into the CNS (Fan et al., 2014; Burgess et al., 2016). Recently, growing evidence emerged on the success of drug transportation across the BBB via absorptive-mediated transcytosis (Banks, 2016). Molecular modifications on chemical compounds, such as pegylation, the addition of fatty acid, production of nanoparticles and glycosylation with glucose or other sugars have achieved certain success in animal models (Banks, 2016). In addition, receptor-mediated drug transport system might be employed for selective delivery of compounds into target brain regions (Chen et al., 2011). The optimal effect can be achieved when several methods are combined together. Of note, in the case of brain disorders such as Alzheimer’s disease, the permeability of the BBB is increased, which is advantageous for drug transportation (Abbott et al., 2006).

Secondly, since small molecules may affect signaling pathways broadly, side effects from the drug metabolism need to be carefully monitored. Take CHIR99021 as an example. The disturbance of GSK3 pathway and Wnt pathway may affect the blood glucose level and promote oncogenesis (Cohen and Goedert., 2004). Notch inhibitors could also induce gastrointestinal toxicity (Olsauskas-Kuprys et al., 2013) and other peripheral problems, including skin issues and weight loss (Doody et al., 2013). These side effects may be due to the strong inhibition of Notch signaling in all tissues (Strooper. 2014). In 2013, semagacestat, a γ-secretase inhibitor, failed in Phase III clinical trial in treating Alzheimer’s disease, mainly due to the lack of efficacy and the significant side effects (Doody et al., 2013). In the CNS, the core molecules may potentially exhibit an off-target effect on neuronal morphology, functions, and activity. For instance, blockage of GSK3 may inhibit neurite outgrowth in the cerebellum and dorsal root ganglion (DRG) (Alabed et al., 2010). Likewise, disturbance of Notch signaling will attenuate synaptic plasticity and dendritic complexity (Ables et al., 2011). TGFβ signaling is also required to maintain the excitatory/inhibitory balance in the hippocampus. Manipulation of TGFβ signaling leads to hyperactive behavior in mouse homecage and open-field tests (Sun et al, 2010). To avoid the off-target side effects, it is important to deliver the drugs directly into reactive astrocytes without influencing the other
cell types. One possibility is to develop novel nanoparticles with specific affinity for reactive glial cells.

Thirdly, specific small-molecule protocols for generating desired neuronal subtypes are required for repairing specific regions of the CNS.

Regardless of the challenges, our studies demonstrate the feasibility of chemical reprogramming of human astrocytes into functional neurons, which possesses the potential to be developed into a virus-free strategy for future CNS repair.
Chapter 6
Materials and Methods

6.1 Human astrocyte culture

Human astrocytes were purchased from ScienCell (HA1800, California) or Gibco (N7805-100). Human astrocytes were primary cultures obtained from human fetal brain tissue. They were isolated and maintained in the presence of 10% fetal bovine serum (FBS), which will essentially cause any progenitor cells to differentiate. Human astrocytes were subcultured when they were over 90% confluences. For subculture, cells were trypsinized by TrypLE™ Select (Invitrogen), centrifuged for 5 min at 900 rpm, resuspended, and plated in a culture medium 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). Cells were maintained at 37°C in humidified air with 5% CO₂.

6.2 Reprogramming human astrocytes into neurons

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). The cells were cultured in human astrocyte medium until 90% confluence. At day 0 before reprogramming, half of the culture medium was replaced by N2 medium of DMEM/F12 (Gibco), penicillin/streptomycin (Gibco) and N2 supplements (Gibco). The following day (Day 1), the culture medium was completely replaced by N2 medium supplemented with small molecules, or with 1% DMSO in control group. For most of the experiments using the nine molecules for reprogramming (MCM treatment), astrocytes were treated with TTNPB (0.5 µM, Tocris #0761), SB431542 (5 µM, Tocris #1614), LDN193189 (0.25 µM) and Thiazovivin (0.5 µM, Cayman #14245) for 2 days. At day 3, the culture medium was replaced with a different set
of small molecules, including CHIR99021 (1.5 µM, Tocris #4423), DAPT (5 µM, Sigma #D5942), VPA (0.5 mM, Cayman #13033) and thiazovivin (0.5 µM). At day 5, VPA was withdrawn by replacing medium containing only CHIR99021 (1.5 µM), DAPT (5 µM) and thiazovivin (0.5 µM). At day 7, medium was replaced containing SAG (0.1 µM, Cayman #11914), purmorphamine (Purmo, 0.1 µM, Cayman #10009634) and thiazovivin (0.5 µM). At day 9, medium was completely replaced with neuronal differentiation medium (NDM), including DMEM/F12 (Gibco), 0.5% FBS (Gibco), 3.5 mM glucose (Sigma), penicillin/streptomycin (Gibco), and N2 supplement (Gibco). Every week, 200 µl neuronal differentiation medium was added into each well to keep the osmolarity constant. To promote synaptic maturation of converted neurons, brain-derived neurotrophic factor (BDNF, 20 ng/mL, Invitrogen), insulin-like growth factor 1 (IGF-1, 10 ng/ml, Invitrogen) and neurotrophin 3 (NT-3, 10 ng/ml, Invitrogen) were added in neuronal differentiation medium at day 9 and were refreshed every 4 days until day 30 (Song et al., 2002).

To determine if our human astrocytes contained any neural stem cells, we cultured human astrocytes in neuronal differentiation medium supplemented with BDNF 20 ng/ml, NT3 10 ng/ml and NGF 10 ng/ml for 1 month. The growth factors were refreshed every 3–4 days.

Human neuroprogenitors (NPCs) derived from human pluripotent stem cells were a gift from Dr. Fred Gage. The NPCs were cultured in poly-L-ornithine and laminin-coated coverslips with neuronal proliferation medium, including DMEM/F12, penicillin/streptomycin, B27 supplement, N2 supplement and FGF2 (20 ng/ml) (Gibco).

### 6.3 Immunocytochemistry

For brain section staining, the mice were anesthetized with 2.5% Avertin and perfused with ice-cold artificial cerebral spinal fluid (ACSF), including 124 mM NaCl, 26 mM NaHCO₃, 10 mM glucose, 1.3 mM MgSO₄, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 2.5 mM CaCl₂. The brains were removed and post-fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Brains from young mice (< 1 month old) were dehydrated with 30% sucrose for 2 days and
cut into 50-µm sections by a cryostat (Leica). Brains for adult mice (> 1 month old) were cut at 45-µm sections by a vibratome (Leica). Coronal brain sections were incubated in 2.5% normal goat serum, 2.5% normal donkey serum and 0.3% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 2 hours, followed by incubation with primary antibody overnight.

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 with PBS and then incubated in 2.5% normal goat serum, 2.5% normal donkey serum and 0.1% Triton X-100 in PBS for 30 min. 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 washings in PBS, the samples were incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488, Alexa 546, Alexa 647 (1:800, Molecular Probes), FITC, TRITC, 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 analyzed with epifluorescent microscope (Keyence BZ-9000) or a confocal microscope (Olympus FV1000). Z-stacks of digital images were acquired and analyzed using FV10-ASW 3.0 Viewer software (Olympus).

A complete list of primary antibodies can be found in the supplemental data.

6.4 Electrophysiology

For human astrocyte-converted neurons, whole-cell recordings were performed using Multiclamp 700A patch-clamp amplifier (Molecular Devices, Palo Alto, CA) as described recently (Guo et al., 2014). The recording 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 (4–6 MΩ) and filled with a pipette solution consisting of 10 mM KCl, 125 mM K-Gluconate, 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 10–25 MΩ. For voltage-clamp
experiments, the membrane potential was typically held at -70 mV, except the recording of IPSCs when the holding potential was set at 0 mV. Drugs were applied through a gravity-driven drug delivery system (VC-6, Harvard Apparatus, Hamden, CT). To monitor gap junctions between human astrocytes, 2 mM sulphorhodamine B (SRB) dye (559 Da) was added in the pipette solution.

Data were acquired using pClamp 9 software (Molecular Devices), 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 (22–24 °C).

### 6.5 RNA extraction

A Macherey-Nagel NucleoSpin® RNA kit was used to extract RNA from human cortical astrocytes during the chemical treatment at D0, 2, 4, 6, 8, and 10. For each well of a 24-well plate, 350 μl of lysis buffer were added, and cell lysates were collected. RNA purification was conducted with NucleoSpin® RNA Column, and pure RNA was eluted with 40 μl of RNase-free H\(_2\)O, yielding RNA concentration ranging of 100–300 ng/μl per well. NanoDrop was used to measure RNA concentration and to check RNA quality. All isolated RNA had an A\(_{260}/A_{280}\) ratio of 2–2.1, which indicates RNA purity. Isolated RNA was stored at -80 °C.

### 6.6 cDNA synthesis and quantitative Real time PCR

For quantitative real-time PCR (qRT-PCR), cDNA synthesis was done using Quanta Biosciences qScript™ cDNA SuperMix. For each sample, 1 μg RNA was used per 20 μl of total reaction volume. The reaction mix was incubated at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and held at 4 °C. The cDNA product was diluted fivefold with
RNase/DNase-free H$_2$O. Primer sets were designed using Applied Biosystems Primer Express software and listed in Table 2. RT-qPCR was done using Quanta Biosciences PerfeCTa™ SYBR® Green SuperMix, ROX™. Real-time cycler Applied Biosystems® StepOnePlus™ was used. The final reaction volume of 25 μl included 5 μl of cDNA corresponding to 1 μg of total RNA. Forty PCR cycles of 95 °C for 15 s and 65 °C for 45 s were done for amplification. Melt-curve analyses were done after the PCR cycles. Comparative Ct method was used for quantification and calculation of gene expression fold changes. GAPDH was used as internal control gene, and relative gene expression was analyzed with respect to gene expression at Day 0 for control human astrocyte group. RT-qPCR data had three replicates of PCR reaction for each sample.

6.7 PCR array

RT$^2$ Profiler PCR Array (Qiagen, PAHS-404ZC-12) was conducted on human astrocytes before (D0) and after small-molecule treatment (D4 and D8). QIAGEN RT$^2$ First Strand Kit (Qiagen #330401) was used to synthesize cDNA from isolated RNA using NucleoSpin® RNA kit. For each 96-well PCR array plate, 0.5 μg of total RNA was mixed with 19.5 μl of reverse-transcription mix and incubated at 42 °C for 15 min followed by 95 °C for 5 min. Then 20 μl cDNA product was diluted with 81 μl RNase-free H$_2$O. For each 96-well PCR array plate, 101 μl diluted cDNA was mixed with RT$^2$ SYBR Green qPCR mastermix (Qiagen #330522) to reach a total volume of 2700 μl. 25 μl qPCR mixture was transferred to each well of PCR array plate. Real-time cycler Applied Biosystems® StepOnePlus™ was used for PCR reaction and data collection. 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min were conducted and followed by melting curve analysis. Threshold for genes was set at the same value for all RT$^2$ Profiler PCR Array runs in the same analysis. QIAGEN RT$^2$ Profiler PCR Array Data Analysis software version 3.5 was used for quantification. Gene expression at D0 was set as control.
6.8 Virus production

The pCAG::GFP-IRES-GFP retroviral vector was a gift from Dr. Fred Gage (Salk Institute, CA). The human GFAP promoter gene was subcloned from hGFAP promoter-Cre-MP-1 (Addgene) and replaced the CAG promoter to generate pGFAP::GFP-IRES-GFP retroviral vector (Guo et al., 2014). The mouse LCN2 promoter sequence was subcloned from mouse genome and replaced the CAG promoter to generate pLCN2::GFP-IRES-GFP retroviral vector. The FUGW-EGFP lentiviral vector was generously provided by Dr. Roger Nicoll (University of California, San Francisco). Retroviral particles were packaged in gpg helperfree HEK (human embryonic kidney) cells to generate VSV-G (vesicular stomatitis virus glycoprotein)-pseudotyped retroviruses as described (Guo et al., 2014; Tashiro et al., 2006). Lentiviral particles were packaged in HEK 293T cells as described (Naldini et al., 1996). The titers of viral particles were about $10^8$ particles/ml, determined after transduction of HEK cells.

6.9 Time-lapse imaging

Human astrocytes cultured in T25 flasks were transduced with 1 µl pCAG::GFP-IRES-GFP retroviral suspension. Two hours after virus transduction, cells were dissociated with TrypLE and plated on poly-D-lysine-coated coverslips at a density of 50,000 cells per coverslip in 24-well plates. At day 0, only one or two GFP-positive cell clusters could be found in each well. One GFP-positive cluster was imaged under epifluorescent microscope (Nikon TE-2000-S) at days 0, 2, 4, 6, 8 and day 10 without or with small-molecule treatment, which was the same as described above. To visualize the reprogramming process induced by sequential application of nine molecules, images were taken at each time before changing the medium containing the next group of small molecules.
6.10 Lineage tracing experiment

Human astrocytes were cultured in poly-D-lysine coated coverslips and infected with 2 µl of pGFAP::GFP-IRES-GFP retroviral suspension for overnight. For infection with pLCN2::GFP-IRES-GFP retroviruses, cultured human astrocytes were pretreated with 100 ng/ml lipopolysaccharide (LPS) to make them reactive and expressing LCN2. Cells infected with retroviruses were then treated with small molecules or 1% DMSO. Cells were cultured for 18 days before fixed for immunostaining.

6.11 BrdU birth dating assay

At 1 day before small-molecule treatment, human cortical astrocytes were incubated with 5-bromo-2-deoxyuridine (BrdU) with a final concentration of 10 µM for 12 hours. The following day, BrdU containing medium was completely removed and fresh human astrocyte medium was added in culture well. About 70–80% human astrocytes were labeled by BrdU at D0. The human astrocytes labeled by BrdU were treated with small molecules and fixed at day 30 after initial small-molecule treatment. In another group, 10 µM BrdU was added in neural differentiation medium at day 10 after small-molecule treatment and was refreshed every 3–4 days until day 30. At day 30, cells were fixed with 4% PFA for 15 min at room temperature, followed by 20 min treatment with 2 M HCl at 37 °C for DNA denaturation. After five washes with PBS, cells were blocked in blocking buffer (2.5% normal donkey serum, 2.5% normal goat serum, 0.1% triton in PBS) for 1 h at room temperature and incubated with primary anti-BrdU antibody (Dako, 1:500) at 4 °C overnight.

6.12 Calcium imaging

Calcium indicator Fura-2 AM (Life Technology) was loaded into the cells by incubating the human astrocyte-converted neurons in culture medium containing Fura-2 AM
(2 µg/ml) for 30 min in an incubator (37 °C). Calcium concentrations within the soma were monitored using a Nikon 20x Super Fluor objective (N.A. 0.75), a Hamamatsu ORCA-ER digital camera (Hamamatsu, Iwata City, Japan), and a Sutter DG5 optic switcher (Sutter Instrument, Novato, CA) for fast changing excitation wavelengths. Simple PCI software from Hamamatsu was used for data acquisition and analyses.

6.13 Methylated DNA immunoprecipitation (MeDIP) and high-throughput sequencing

MeDIP experiments were performed according to the manufacturer’s protocol (Active Motif). The enriched methylated DNA was purified by Qiagen DNA purification kit for library preparation using the NEBNext ChIP-Seq Library Prep Reagent Set for Illumina according the manufacturer’s protocol. In brief, 25 ng of input genomic DNA or experimental enriched DNA were utilized for each library construction. 150–300 bp DNA fragments were selected by AMPure XP Beads (Beckman Coulter) after the adapter ligation. An Agilent 2100 BioAnalyzer was used to quantify the amplified DNA, and qPCR was applied to accurately quantify the library concentration. 20 pM diluted libraries were used for sequencing. 50-cycle single-end sequencings were performed using Illumina HISeq 2000. Image processing and sequence extraction were done using the standard Illumina Pipeline.

6.14 Targeted BS-seq

The DNA samples were applied to EpiTect Bisulfite Kit (Qiagen), following the supplier’s instruction. PCR amplicons were then purified by Ampure XP bead, and eluted in 50 µl of H2O. The concentration was quantified with a Qubit High Sensitivity kit and then pooled together in equal molar for each sample. Mixed amplicons were then subjected to library preparation and Miseq deep sequencing (100X or above), following standard
procedures recommended by Illumina. Image analysis and base calling were performed with the standard Illumina pipelines.

To determine the DNA methylation status at GFAP transcription start site, genomic DNA was treated with sodium bisulfite with a EZ DNA Methylation-Gold Kit (Zymo Research), according to manufacturer’s instruction. Bisulfite-converted DNA was amplified using nested PCR (see sequences of primer set in Table 2). Purified PCR amplicons were then ligated into TOPO-TA vector (Invitrogen). Reconstructed plasmids were purified and individual clones were sequenced. Ten clones were randomly picked from each time point. Data presented were from two independent experiments.

### 6.15 Bioinformatics analyses

Bioinformatics analysis for MeDIP-seq was as described (Szulwach et al., 2011; Yao et al., 2014). Briefly, FASTQ sequence files were aligned to HG19 reference genome using Bowtie (Langmead et al., 2009). Peaks were identified by Model-based Analysis of ChIP-Seq (MACS) software (Zhang et al., 2008).

For BS-seq, paired-end reads were first preprocessed to remove adaptor sequences, as well as low-quality sequences on the 3’ and 5’ ends using Trimmomatic 0.20. Preprocessed reads were then aligned to both C to T and G to A converted sequences at the loci of our interest using Bowtie 0.12.9 (-m 1 -l 30 -n 0 -e 90 -X 550). Only uniquely mapping reads were retained, and PCR duplicates were removed using MarkDuplicates (Picard Tools 1.82). To avoid counting reference positions covered by overlapping paired-end reads, overlapping regions were clipped, keeping the region of the overlap with higher quality. The original computationally converted Cs and Gs were reverted, and for each reference cytosine position, the number C reads and T reads were counted using SAMTools mpileup.
6.16 Chromatin immunoprecipitation (ChIP)-quantitative PCR

ChIP experiments were performed essentially as described with minor modifications (Wang et al., 2004). Briefly, cultured human astrocytes before or after small-molecule treatment were fixed with 1% formaldehyde for 10 min and quenched by 0.125 M glycine for 5 min. The chromatin was sonicated to a range of 300–500 basepair fragments with a Bioruptor (Diagenode). Following the ChIP procedures, the eluted DNA samples were purified using the DNA clean and concentration kit (Zymo research). Enrichment was determined by qPCR and normalized to total input.

6.17 Transplantation of small-molecule-converted human neurons in vivo

In vivo experiments were conducted with wild-type C57/BL6 mice. Mice were housed in a 12-h 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.

Human astrocytes cultured in T25 flask were transduced with 10 µl of FUGW-GFP lentiviral suspension for high-efficiency infection. One day after virus transduction, cells were dissociated with TrypLE and plated on poly-D-lysine-coated coverslips at a density of 50,000 cells per coverslip in 24-well plates. When cells reached 90% confluence, about 70% cells were GFP-positive. After GFP infection, human astrocytes were treated with small molecules, according to the protocol described above. At day 14 after initial small-molecule treatment, the mixture of human astrocytes and converted neurons was dissociated with Accutase (Gibco) and resuspended with 20 µl of neuronal differentiation medium supplemented with 10 ng/ml BDNF, 10 ng/ml NT3 and 10 ng/ml IGF-1. A cell suspension containing 2x10^5 cells was injected into the lateral ventricles of newborn mouse pups (postnatal day 1, P1), with 2 µl injected into each hemisphere. Cells were injected 1.5 mm anterior and 1.5 mm lateral from the lambda, with a depth of 1 mm using a stereotaxic device (Hamilton). Brains were collected at 7, 11, and 14 days and 1 month after injection for analysis.
6.18 Stereotaxic injection of small molecules into mouse brain

Brain surgeries were performed on 2-month-old wild-type C57BL6 mice. The mice were anesthetized by injecting 20 mL/kg 0.25% Avertin (a mixture of 25 mg/ml of tribromoethylethanol and 25 μl/ml T-amyl-alcohol) into the peritoneum and then placed in a stereotaxic device. 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 one injection in cortex (coordinates: AP 1.25 mm, ML 1.4 mm, DV -1.5 mm), or in the striatum (coordinates: AP 0.5 mm, ML 2.4 mm, DV -3.1 mm) or in the dentate gyrus (coordinates: AP -2.06 mm, ML 1.5 mm, DV -2.1 mm) of small-molecule mixture or PBS containing 6% DMSO with a 2 μl syringe and a 34 gauge needle. The injection volume and flow rate were controlled as 2 μl at 0.2 μl/min. After injection, the needle was kept for at least 5 additional minutes and then slowly withdrawn.

6.19 In vitro cell suspension culture

At 6 dpi of the small molecules, the animals were sacrificed by exposure to CO₂. The brains were dissected out, and the cortical brain tissues ~1.5 mm around the injection site were isolated and chopped into 0.1x0.1 mm pieces and treated with 0.5% trypsin (Gibco) for 30 min at 37 °C, followed by centrifugation at 900 G for 8 min. The cell pellet was resuspended with neuronal proliferation medium supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF and ~100 cells in 10 ml medium were seeded in 6-well plate with an ultra-low attachment surface (Corning #3471). The growth factors were refreshed every 2–3 days. One week after initial seeding, neurospheres were observed and counted under 10x microscope (Nikon). For subculture, 1-week old primary neurospheres were collected by centrifuge at 900 G for 3 min, and incubated with accutase (Gibco) for 5 min at 37 °C. The cell pellet was spun down at 900 G for 5 min and triturated into single cells and suspended in neuronal proliferation medium. At 3 days after subculture, secondary neurospheres were observed and counted under 10x microscope. For monolayer culture, 4-day-old secondary neurospheres
were trypsinized and resuspended, according to the above-mentioned protocol. Single cells were seeded on poly-L-ornithine/laminin-coated coverslips and cultured with neuronal proliferation medium with 20 ng/ml FGF2 and 20 ng/ml EGF. When cells reach 60–70% confluence, cells were fixed with 4% PFA or induced differentiation with neuronal differentiation medium or glial medium containing DMEM/F12, 5% FBS, 50 mg/ml NaHCO$_3$ and penicillin/streptomycin.

6.20 Data and statistical analysis

Cell counting was performed by taking images at several randomly chosen fields per coverslip and analyzed by Image J software. The fluorescence intensity was analyzed by Image J software. Data were represented as mean ± SEM. Student’s $t$ test was used for the comparison between two groups of data. One-way ANOVA and post hoc tests were used for statistical analyses of data from multiple groups.

6.21 Antibody list

The following primary antibodies were used in this study

Polyclonal anti-green fluorescent protein (GFP, chicken, 1:1000, Abcam, AB13970), polyclonal anti-GFAP (rabbit, 1:1000, Abcam, Z0334), polyclonal anti-GFAP) chicken, 1:1000, Millipore, AB5541), monoclonal anti-S100β (mouse, 1:800, Abcam, ab66028), polyclonal anti-vesicular glutamate transporter 1 (vGluT1, rabbit, 1:1000, Synaptic Systems), polyclonal anti-vesicular glutamate transporter (SV2, mouse, 1:2000, Developmental Studies Hybridoma Bank, Iowa City), polyclonal anti-microtubule-associated protein 2 (MAP2, chicken, 1:2000, Abcam, AB5392), polyclonal anti- T-box, brain, 1 (Tbr1, 1:300, rabbit, Abcam, AB31940), polyclonal anti-Prox1 (rabbit, 1:1000, ReliaTech GmbH, 102-PA32), polyclonal anti-Musashi-1 (rabbit, 1:500, Neuromics, RA14128), monoclonal anti-SRY (sex determining region Y)-box 2 (Sox-2, mouse, 1:500, Abcam, AB79351), polyclonal anti- SRY
(sex determining region Y)-box 2 (Sox-2, rabbit, 1:500, Millipore, AB5603), monoclonal anti-βIII tubulin (Tuj1, mouse, 1:1000, COVANCE, MMS-435P), polyclonal anti-Doublecortin (DCX, rabbit, 1:500, Abcam, AB18723), polyclonal anti-NeuN (rabbit, 1:1000, Millipore, ABN78), monoclonal anti-NG2 (mouse, 1:200, Abcam, AB50009), monoclonal anti-pan-axononal neurofilament marker (SMI 312, 1:1000, mouse, Covance, SMI-312R), polyclonal anti- Glial Glutamate Transporter GLT-1 (EAAT2) (Glt1, Guinea pig, 1:2000, Millipore, AB1783), monoclonal anti-NeuroD1 (mouse, 1:1000, Abcam, ab60704), monoclonal anti-Human Nuclei (HuNu, mouse, 1:1000, Millipore, MAB1281), monoclonal anti-synaptophysin (mouse, 1:800, Millipore, MAB368), polyclonal anti-CDP (Cux1, rabbit, 1:500, Santa Cruz, sc-13024), monoclonal anti-Ctip2 (rat, 1:600, Abcam, ab18465), anti-Otx1 (mouse, 1:200, Developmental Studies Hybridoma Bank, otx-5F5), anti-HoxC9 (mouse, 1:200, Developmental Studies Hybridoma Bank, 5B5-2), anti-HoxB4 (mouse, 1:200, Developmental Studies Hybridoma Bank, 112 anti Hoxb4), polyclonal anti-FoxG1 (goat, 1:1000, Abcam ab3394) polyclonal anti-vesicular acetylcholine transporter (VACHT, guinea pig, 1:800, Millipore, AB1588), monoclonal anti-GAD67 (mouse, 1:1000 Millipore, MAB5406), anti-Is1 (mouse, 1:200, Developmental Studies Hybridoma Bank, 39.4D5) monoclonal anti tyrosine hydroxylase (TH, mouse, 1:600, Millipore, MAB318), polyclonal anti neurogenin2 (Ngn2, rabbit, 1:600, Abcam, ab26190), monoclonal anti-NeuroD1 (mouse, 1:800, Abcam, ab60704), polyclonal anti-MASH1/Acheate-scute homolog1 (Ascl1, rabbit, 1:800, Abcam, ab74065), monoclonal anti Nestin (mouse, 1:800, Neuromics, MO15056), polyclonal anti Ki67 (rabbit, 1:800, Abcam, ab15580), monoclonal anti N200 (mouse, 1:1000, Sigma, N0142), monoclonal anti BrdU (mouse, 1:500, Dako, 074401-8), monoclonal anti glutamine synthetase (GS, mouse, 1:800, Millipore, MAB302), monoclonal anti phosphor-GSK-3β (Ser9)(5B3) (rabbit, 1:100, Cell signaling, 9323), monoclonal anti phosphor-Smad1(Ser463/465)/Smad5 (Ser463/465)/Smad9 (Ser465/467) (D5B10) (rabbit, 1:600, Cell Signaling, 13820), monoclonal anti cleaved Notch1 (Val1744) (D3B8) (rabbit, 1:200, Cell Signaling, 4147), monoclonal anti CNPase (mouse, 1:800, Abcam, ab6319), polyclonal anti-Lipocalin-2/NGAL (LCN2, Goat, 1:1000, R&D, AF1857).

The following antibodies were used for DNA pull-down in the CHIP assay: polyclonal anti-acetyl-histone H3 (rabbit, Millipore, 06-599); polyclonal anti-trimethyl-histone H3
125

(Lys27) (H3K27Me3, rabbit, Millipore, 07-449); and polyclonal anti-H3K4me3 (rabbit, Active Motif 39159).

Table 6-1. Primer list

Bisulfite PCR - deep sequencing

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<th>Primer Sequence</th>
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