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Physiological, Pathological, and Engineered Cell Identity Reprogramming in the Central Nervous System

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Abstract

Multipotent neural stem cells persist in restricted regions of the adult mammalian central nervous system. These proliferative cells differentiate into diverse neuron subtypes to maintain neural homeostasis. This endogenous process can be reprogrammed as a compensatory response to physiological cues, traumatic injury, and neurodegeneration. In addition to innate neurogenesis, recent research has demonstrated that new neurons can be engineered via cell identity reprogramming in non-neurogenic regions of the adult central nervous system. A comprehensive understanding of these reprogramming mechanisms will be essential to the development of therapeutic neural regeneration strategies that aim to improve functional recovery after injury and neurodegeneration.

Introduction

Multipotent neural stem cells (NSC) shape the architecture of early neural networks using molecular patterning cues to control neurogenesis and differentiation. This process rapidly generates billions of neurons, but continues only in discrete areas of the adult mammalian central nervous system. Although tightly regulated by genetic and epigenetic factors, lineage-specific NSC differentiation can be significantly influenced by environmental and pathological cues.

Adult neurogenesis is critical to homeostatic maintenance in neural tissues. However, the small populations of neurons generated by endogenous NSC pools in response to traumatic injury or disease-related neurodegeneration are not sufficient to regenerate functional neural circuits. Therefore, engineered methods for *in vitro* somatic cell and *in vivo* glial cell reprogramming are being investigated as potential approaches to neural network

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reconstruction. These engineered reprogramming methodologies have enabled researchers to investigate neurodegenerative diseases and the mechanisms of neural differentiation in specific disease-relevant genetic models.

Overall, neural reprogramming processes in the adult central nervous system regulate homeostatic maintenance of neural networks, promote degeneration-responsive neural repair, and inspired the development of *in vitro* disease models and engineered therapeutics.

ADULT NEUROGENESIS OVERVIEW

Neurogenesis in the adult brain

As the central nervous system matures, neurogenesis becomes restricted to localized regions of the adult mammalian brain. The subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricle retain NSCs with the ability to proliferate and differentiate into diverse glial and neuronal lineages throughout adulthood (Figure 1)¹⁻⁶.

The SGZ is located at the interface of the granule cell layer and the hilus of the dentate gyrus. Here, slow-dividing glial fibrillary acidic protein (GFAP)- and nestin (NES)-positive radial glia-like NSCs (type 1 cells) give rise to non-radial precursor cells (type 2A and type 2B cells)⁷⁻⁹. These transiently amplifying type 2B precursor cells become neuroblasts that ultimately differentiate and mature into functional neurons¹⁰⁻¹³. SGZ neuroblasts adopt glutamatergic granule neuron identity and a small population exhibit GABAergic identity^{10, 13}.

The SVZ is a neurogenic region lining the lateral ventricle that harbours the largest population of proliferating cells in the adult brain¹⁴⁻¹⁷. Independent of SGZ neurogenesis, quiescent NSCs (type B cells) become activated and produce fast-dividing multipotent progenitors (type C cells), which ultimately give rise to neuroblasts (type A cells)¹⁸⁻²⁰. These neuroblasts migrate through the rostral migratory stream into the olfactory bulb and predominantly differentiate into GABA-positive granule cells²¹⁻²⁴.

In addition to the localized SGZ and SVZ niches, adult neurogenesis has been reported in the amygdala²⁵, cerebellum^{26, 27}, hypothalamus²⁸, substantia nigra²⁹, and various other brain regions. Consistent detection of stem cell populations in these adult brain regions has proven challenging³⁰. Importantly, an examination of markers for DNA replication and neuronal differentiation in human post-mortem neural tissues confirmed that neurogenesis similarly persists in discrete regions of the adult human brain^{6, 31}.

Neurogenesis in the adult spinal cord

Unlike the adult brain, it is unclear whether the adult spinal cord maintains neurogenesis into adulthood. Cells isolated from adult spinal tissue retain certain NSC hallmarks and exhibit multi-lineage differentiation *in vitro*³²; however, these proliferating progenitors exclusively generate glial cells *in vivo*³³. DCX-positive cells, a marker for neural precursor cells, immature neurons, certain reactive glial cells, and dorsal root ganglion sensory neurons³⁴, were reported in dorsal spinal cord. However, these cells do not develop into mature TUBB3-positive or RBFOX3-positive neurons under physiological conditions³⁵. This

suggests DCX might not be an effective stand-alone marker for neurogenesis. Furthermore, multiple related studies have failed to detect adult-born neurons in the developed spinal cord^{33, 36, 37}.

PHYSIOLOGICAL NEURAL REPROGRAMMING

Plasticity of adult neurogenesis

Diverse environmental stimuli and endogenous signalling cues modulate the rate of adult neurogenesis in the SGZ and SVZ. This plasticity has been associated with changes in behaviour, motor function, and the onset of neurodegenerative disease (Table 1).

The rate of neurogenesis in the SGZ of adult female rats occurs at a higher rate than in male counterparts. Ovariectomy and bromodeoxyuridine incorporation collectively implicated ovarian hormone production as a causative factor in this gender-specific difference in the rate of neurogenesis³⁸. Increased corticosteroid production in both aging male and female mice also reduces the total population of proliferating neural progenitors in the SGZ and SVZ³⁹⁻⁴¹. In addition to these gender-specific and age-related mechanisms, physical exercise has been implicated in the proliferation rate and survival of SGZ neural cells⁴². Exercise-induced increases in SGZ neurogenesis are present during periods of activity, but return to a basal rate during rest periods⁴².

Energetic and hormonal homeostasis is a complex whole-organism process directly influenced by circadian rhythm and sleep duration. Prolonged sleep deprivation negatively affects SGZ neural progenitor proliferation, survival, maturation, and differentiation suggesting an important role for sleep in the regulation of adult hippocampal neurogenesis^{43, 44}. Interestingly, short periods of sleep deprivation do not seem to impact SGZ neurogenesis⁴⁵ and appear to temporarily increase cell proliferation by accelerating the cell cycle⁴⁶. Similar to prolonged periods of sleep deprivation, mouse models of physical and psychological stress indicate external environmental stressors negatively affect the production of adult born neurons^{47, 48}.

Neuron identity and neurotransmitter switching

Neuronal subtype is often designated based on neurotransmitter synthesis and characteristic electrical properties. However, throughout early development, immature neurons often switch between the production of excitatory and inhibitory neurotransmitters⁴⁹. Functional maturation eventually biases neurons toward a principal neurotransmitter that defines the lineage-specific identity of each particular neuron⁴⁹.

The acquisition of lineage-specific identity does not irreversibly lock interneurons of the rat hypothalamus into exclusively dopamine- or somatostatin-positive fates. Alterations in day length, short- or long-day photoperiods, drive changes in the expression of postsynaptic dopamine receptor expression, but do not affect somatostatin receptor expression⁵⁰. Pharmacological inhibition of identity-switching dopaminergic neuron activity increased anxiety and depression phenotypes⁵⁰. Exposure to long-day photoperiods phenocopied this pharmacological inhibition and short-day photoperiods rescued these behavioural

phenotypes⁵⁰. These interesting findings suggest that environmental stimuli directly influence behaviour via neurotransmitter responsivity⁵⁰.

PATHOLOGICAL NEURAL REPROGRAMMING

The complexity of neuronal networks in the adult brain and spinal cord affords the mammalian nervous system an unparalleled level of cellular communication and information processing. Disruptions in this interconnected network by traumatic injury or degeneration often impart detrimental functional loss. Although neuronal death is one consequence of these destructive events, endogenous NSCs and progenitor cells respond through reparative mechanisms that synergize with reactive gliosis and glial scar formation to promote neural repair (Table 2).

Traumatic neural injury

Neurogenesis induced by traumatic brain injury—Traumatic brain injury activates the proliferation of endogenous neural progenitors. These cells migrate into the hippocampus, differentiate into neurons, and functionally integrate into local brain circuitry in an attempt to regenerate disrupted neuronal networks⁵¹. Intraventricular infusion of epidermal growth factor and fibroblast growth factor 2 after ischemic brain injury promotes this production of adult-born neurons⁵¹. Adult rodent models exhibit significant increases in SGZ and SVZ neurogenesis in response to traumatic brain injury with proliferation and migration of neural progenitors playing a critical role in functional recovery^{52, 53}.

In addition to changes in neurogenesis, striatal astrocytes have been shown to reprogram following transient middle cerebral artery occlusion⁵⁴. A subpopulation of striatal astrocytes traced by *GFAP*-driven green fluorescent protein adopted a PAX6- and NES-positive progenitor state within one week of injury⁵⁴. These cells expressed the mature neuron markers TUBB3, RBFOX3, and MAP2 within two weeks, as well as, specific markers of cholinergic or GABAergic identity⁵⁴. In response to stroke injury, SVZ NSCs give rise to a subpopulation of cortical reactive astrocytes that can be induced to adopt neuronal identity via forced *in vivo* overexpression of *Ascl1*⁵⁵.

Interestingly, the inhibition of Notch signalling in the absence of injury is also sufficient to reprogram striatal astrocytes into functional neurons⁵⁶. These Notch signalling pathways are integral to brain injury-response mechanisms and instruct quiescent ependymal cells lining the lateral ventricles to generate neuroblasts and astrocytes in response to acute injury⁵⁷.

Neurogenesis induced by spinal cord injury—Neural progenitor cells migrate into regions of neural damage following traumatic spinal cord injury^{58–60}. These *Nes-LacZ* and bromodeoxyuridine-traced cells co-localize with TUBB3 and RBFOX3 indicating that active neurogenesis might have a reparative role in injury-induced neuronal dysfunction events⁶¹.

Proliferative ependymal cells lining the central canal were first discovered in a funiculus incision model of spinal cord injury⁵⁸. Dil pre-labelling was used to trace proliferating ependymal-derived cells to injury sites and revealed these cells primarily differentiate into scar-forming astrocytes⁵⁸. Furthermore, Dil-labelled ependymal cells generated RBFOX3-

positive neuron-like cells with functional properties resembling immature neurons in an experimental model of autoimmune encephalomyelitis⁶². *Nes-CreER* or *FoxJ1-CreER* genetic tracing mouse lines revealed that migrating ependymal cells also differentiate into astrocytes and myelinating oligodendrocytes⁶³.

NG2 cells are another progenitor-like cell population in the adult spinal cord⁶⁴. These injury-responsive cells rapidly proliferate in response to injury. When transduced with retrovirus encoding *NG2* promoter-driven green fluorescent protein, these proliferative cells gave rise to glial scar-forming astrocytes and myelin-producing oligodendrocytes following injury⁵⁹. When dissociated and cultured *in vitro*, NG2 cells isolated from spinal cord injury models retained the potential to form neurospheres differentiable to astrocyte, neuron, and oligodendrocyte fates⁶⁵. Furthermore, these cells produce a small population of HuC/D and MAP2-positive immature neurons⁶⁵. The production, survival, and maturation of these neurons can be significantly enhanced by retroviral expression of *NEUROG2* or exposure to growth factors⁶⁵.

The meninges of the spinal cord retain a third population of cells that exhibit progenitor-like properties following spinal cord injury⁶⁰. The proliferation rate and population size of NES-DCX double-positive cells increases in meninges following injury⁶⁰. Lentiviral labelling of these migratory cells revealed populations in the spinal cord parenchyma and glial scars⁶⁰. Recent work utilizing *Glast-CreER* transgenic mice demonstrated that scar-forming stromal cells arise from a pericyte subpopulation and inhibition of this process negatively affects glial scar formation⁶⁶.

These studies clearly demonstrate a role for neural reprogramming in the injured adult spinal cord and highlight the innate ability of multiple cell types to dedifferentiate, proliferate, and produce scar-forming glial cells (Figure 2). However, lesion-induced spinal neurogenesis is an extremely rare event and in most injury models this process is not observed^{36, 58, 64}.

Neurodegenerative disease

Alzheimer disease—Alzheimer disease is an age-related neurodegenerative disease that impairs cognition, reduces memory recall, and disrupts activities of daily living^{67, 68}. In an adult mouse model of Alzheimer disease, the accumulation of amyloid-beta plaques within the dentate gyrus correlated with reductions in neurogenesis and neural proliferation^{69, 70}. Importantly, this observed decline in proliferation has not been uniformly observed in all Alzheimer disease studies⁶⁹⁻⁷¹.

Genome-wide association studies identified APOE4 as a predictor of Alzheimer disease onset. APOE4 knockout mice exhibit deficits in SGZ neurogenesis and neural precursor cells become biased toward rapid glial differentiation⁷². This may result from imbalanced intrahippocampal neurotransmitter release⁷³. Specifically, amyloid-beta aggregation might preferentially induce GABAergic neurotransmission relative to glutamatergic signalling in human amyloid precursor protein transgenic mice and disrupt mechanisms that regulate neurogenesis in the Alzheimer disease brain⁷⁴. Furthermore, adult born granule cells in this model exhibit impaired morphological and functional maturation suggesting deficits in neurogenesis accompany Alzheimer disease onset⁷⁴.

Amyotrophic lateral sclerosis—Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease caused by the degeneration of motor neurons resident to the motor cortex, brain stem, and spinal cord⁷⁵. The progressive degeneration of these neurons triggers fasciculation, hyperreflexia, and a reduced ability to initiate voluntary movement⁷⁵.

Cellular tracing of spinal neural precursor cells in a rodent model of ALS was achieved using *NES* promoter-regulated reporter expression in *SOD1-G93A* transgenic mice⁶¹. These neural precursors, derived from the ependymal zone surrounding the central canal, increased both proliferation rate and migration in response to motor neuron degeneration⁶¹. However, these cells lost proliferation potential upon migration, which limited the total number of reparative cells relocated to sites of degeneration⁶¹. These precursor cells migrated into the dorsal and ventral horn regions of the spinal cord following ALS onset. Importantly, disease onset increased *de novo* neurogenesis and enhancement of this process might be beneficial to the development of ALS therapies⁶¹.

Huntington disease—Huntington disease is an autosomal dominant genetic disorder that results in progressive loss of striatal medium spiny neurons^{76, 77}. This degeneration manifests as deficits of motor coordination, physical instability, involuntary movement, emotional outbursts, and cognitive impairment⁷⁶. Huntington disease-afflicted individuals exhibit increased subventricular cell division⁷⁸ and TUBB3-positive neural precursor cells near the caudate nucleus⁷⁹. In parallel with these findings, migratory striatum-bound neuroblasts were observed in transgenic mice that model rapid Huntington disease onset and disease progression^{80, 81}. Cellular tracing revealed these cells originate in the SVZ³¹; however, these neuroblasts were surprisingly unable to differentiate into functional neurons after relocation into the striatum^{80, 81}. Interestingly, mouse models of slow progressing Huntington disease exhibit reduced SGZ neural proliferation^{82, 83}, while SVZ proliferation remains relatively unchanged^{31, 78-81}.

Multiple sclerosis—Multiple sclerosis is a neuroinflammatory disease characterized by axon degeneration, demyelination, neural inflammation, and acquired neurological dysfunction^{84, 85}. Experimental models of autoimmune encephalomyelitis revealed that DiI-labelled ependymal cells proliferate and migrate into areas of inflammation⁶². These relocated cells then differentiate into TUBB3-positive, RBFOX3-positive neuron-like cells⁶². Whole-cell patch-clamp recordings from freshly isolated DiI-labelled cells demonstrated that these cells fire action potentials with properties similar to immature neurons⁶². This work demonstrated the first evidence that neurogenesis might occur in neuroinflammatory lesions within the adult spinal cord⁶².

Parkinson disease—Parkinson disease is an adult-onset chronic neurodegenerative disorder characterized by bradykinesia, gait disturbance, resting tremor, and rigidity⁸⁶. Deficits in motor function are primarily the result of dopaminergic neuron death in the substantia nigra and, in later stages, the basal forebrain and cortex⁸⁷. Individuals diagnosed with Parkinson disease generally exhibit deficits of neural proliferation in the SVZ evidenced by a reduction in post-mortem staining of proliferating cell nuclear antigen⁸⁸.

Reduced dopaminergic innervation in animal models of Parkinson disease might lead to deficits in dopaminergic signalling pathways with governing roles in the regulation of SVZ neural proliferation⁸⁹⁻⁹⁴. However, contrary immunocytochemical analyses of proliferation markers in the Parkinson disease-afflicted brain suggest dopaminergic neuron death does not directly contribute to reduced neural proliferation following disease onset⁹⁵. Although a consensus has not yet emerged on the severity of impact that dopaminergic neuron death has on neuroproliferation in the adult brain, reductions in olfactory bulb volume and SGZ neuron populations suggest that Parkinson disease does negatively impact adult neurogenesis^{89, 90, 96}. This reduced neurogenic potential might underlie behavioural symptoms such as depression and impaired logic⁹⁷.

ENGINEERED NEURAL REPROGRAMMING

Induced pluripotent stem cells

Foundational discoveries: nuclear transfer, teratocarcinoma cells, and embryonic stem cells—Lineage-specific differentiation and cellular identity are a consequence of intracellular signals that modulate transcription. The concept that targeted modification of the transcriptome can be used to engineer cellular identity emerged from early nuclear transfer and pluripotency research. The transplantation of *Rana pipens* nuclei from blastula cells into oocytes induced pluripotency and revealed that the nucleus dominantly regulates cell identity⁹⁸. Expanding this work, nuclei from *Xenopus laevis* epithelial cells were transplanted into enucleated oocytes, which developed into viable embryos⁹⁹. The remarkable discovery that nuclei from differentiated cells retain a potential to generate functional organisms was highlighted with the birth of lambs from oocytes carrying adult sheep mammary cell nuclei¹⁰⁰.

Somatic cell nuclear transfer demonstrated functional pluripotency *in vivo*; however, *in vitro* models of pluripotency relied on teratocarcinoma cells derived from murine tumors. The isolation of these stem cell-like cells provided the first *in vitro* model of pluripotency¹⁰¹. The subcutaneous engraftment of single teratocarcinoma cells into healthy mice resulted in teratoma formation and confirmed that these multipotential cells can differentiate into multiple somatic lineages¹⁰². However, this model exhibited limited differentiation potential relative to embryo-derived stem cells.

Breakthrough cell culture techniques next enabled the *in vitro* isolation of mouse and human embryonic stem cell (ESC) lines, which provided a physiological model of cellular differentiation and lineage specification¹⁰³⁻¹⁰⁵. Importantly, ESCs engineered to express a β -galactosidase reporter contributed to all parts of the central nervous system when implanted into a developing mouse blastocyst¹⁰⁶. Moreover, neural lineage-biased ESCs transplanted into the rat brain differentiated into astrocytes, neurons, and oligodendrocytes¹⁰⁷. These fundamental investigations into the genetic regulation of pluripotency and neural specification laid the groundwork for somatic cell engineering and reprogramming technologies (Figure 3).

Induced pluripotency—In a discovery that reshaped the definition of terminal differentiation, the viral overexpression of KLF4, MYC, POU5F1, and SOX2 transcription

factors induced embryonic and adult mouse fibroblasts to revert into a pluripotent state¹⁰⁸. Similarly, these factors were sufficient to reprogram adult human fibroblasts into induced pluripotent stem cells (iPSCs)¹⁰⁹. The genetic regulatory mechanisms underlying iPSC reprogramming were teased apart in studies demonstrating three factor induced pluripotency¹¹⁰ and even POU5F1-induced pluripotency under defined conditions¹¹¹. Providing a direct link between extracellular cues and intracellular transcription, a cocktail of seven chemical compounds was used to induce mouse somatic cells toward a pluripotent fate *in vitro*¹¹².

The immense therapeutic potential for patient-specific iPSCs and iPSC-derived cell types in neurodegenerative diseases inspired diverse applications to Alzheimer disease, ALS, Huntington disease, multiple sclerosis, and Parkinson disease. Adult human fibroblast-derived iPSCs differentiate into glia and diverse neuronal subtypes when cultured under lineage restrictive conditions^{108, 113} (Figure 4). The motor neuron-specific differentiation of 16 iPSC lines derived from healthy and ALS-afflicted individuals enabled novel modeling of disease progression and targeted drug discovery¹¹⁴. Specific to Huntington disease, PPP1R1B-positive medium spiny neurons were generated from iPSCs using SHH and DKK1 in combination with bone morphogenetic protein and transforming growth factor-beta inhibitors¹¹⁵. In addition to neurons, glial-specific iPSC differentiation protocols yielded astrocyte progenitors and immature astrocytes¹¹⁶. Although potentially valuable as a source of neural progenitors or differentiated neurons, the direct induction of iPSC cells *in vivo* results in teratoma formation in multiple organ tissues¹¹⁷. This drawback to induced pluripotency suggests that more restrictive neural progenitor cells might be better suited for therapeutic applications and *in vitro* modeling of neural differentiation.

Induced neural progenitors

Temporal restriction of POU5F1 expression to the first five days of reprogramming combined with constitutive SOX2, KLF4, and MYC expression converted fibroblasts into neural progenitor-like cells with properties similar to endogenous NSCs¹¹⁸ (Figure 4). The tight regulation of POU5F1 activity is critical as prolonged expression results in the adoption of pluripotent properties. NSC specification was also achieved with an expanded set of transcription factors that included KLF4, MYC, POU3F4, SOX2, and TCF3¹¹⁹. The induction of NSC-like progenitor cells using diverse combinations of proteins, RNAs, and microRNAs highlights how the modulation of protein, RNA, and microRNA expression offers multiple entry points for engineering cellular identity. For instance, FOXG1 and SOX2 are sufficient to induce a bipotent neural precursor identity from mouse fibroblasts¹²⁰. The co-expression of a third factor, POU3F2, further endows these reprogrammed cells with tripotent differentiation potential¹²⁰. Strikingly, mouse embryonic fibroblasts, mouse tail-tip fibroblasts, and human urinary cells exposed to valproic acid, CHIR99021, and Repsox under hypoxic culture conditions reprogram into multipotent neural precursors¹²¹.

In vivo multipotency—Acquired multipotency enables iPSC-derived neural precursor cells to differentiate into astrocytes, neurons, and oligodendrocytes¹¹⁸. Surprisingly, SOX2 itself is sufficient to impart multipotency in cultured human fibroblasts¹²². *SOX2*-transduced fibroblasts directly transform into self-renewing NSCs that form neurospheres and

differentiate into mature functional neurons following transplantation into the mouse cortex¹²².

Independent *in vivo* studies demonstrated that *SOX2*-encoding lentivirus transforms striatal astrocytes into neuroblasts poised to differentiate into functional neurons upon treatment with valproic acid¹²³. These reprogrammed astrocytes pass through an *ASCL1*-dependent progenitor state and require the nuclear receptor *TLX* for acquisition of neuronal identity^{124, 125}. Closely related work in the spinal cord demonstrated that *GFAP* promoter-regulated *SOX2* overexpression in astrocytes imparts a proliferative, but non-tumorigenic neuroblast identity capable of producing multiple neurons from each reprogrammed astrocyte³⁶ (Figure 2). In an attempt to demonstrate the clinical utility of reprogrammed neurons, iPSC-derived neural progenitor cells were transplanted into a primate model of Parkinson disease¹²⁶. These cells persisted for at least six months in the neural microenvironment and differentiated into astrocytes, functional neurons, and myelinating oligodendrocytes¹²⁶. These cell transplantation and *in vivo* reprogramming studies demonstrate that engineered cells exhibit significant regenerative potential in cases of traumatic neural injury and age-related neurodegeneration (Table 3).

Transdifferentiation

Transdifferentiation factors and neural subtype—The direct induction of functional neurons from mouse fibroblasts by *in vitro* overexpression of *ASCL1*, *POU3F2*, and *MYT1L* demonstrated a fundamental plasticity in the identity of lineage-committed somatic cells¹²⁷ (Table 4). The complexity of transcription and dynamic regulation of cell-specific gene sets provide multiple points of entry for targeted cell reprogramming. For instance, certain microRNAs can replace transcription factors to promote neuronal reprogramming of human fibroblasts^{128, 129}. Surprisingly, activation of small molecule-driven signal transduction pathways is sufficient to transdifferentiate mouse fibroblasts¹³⁰, human fibroblasts¹³¹, and human astrocytes¹³² into functional neurons. Highlighting the significant roles for signal transduction activators in neural reprogramming, the pro-neural transcription factor *NEUROG2* converts human fibroblasts into functional neurons with nearly 90% efficiency only in the presence of forskolin, a cyclic AMP synthesis activator, and dorsomorphin, a bone morphogenic protein type 1 receptor inhibitor¹³³.

The pro-neurogenic properties of *NEUROG2* have enabled the induction of neurons from fibroblasts¹³³, as well as, mouse cortical astrocytes¹³⁴. While fibroblasts specifically differentiated into cholinergic neurons, *in vitro* reprogrammed cortical astrocytes adopt a glutamatergic identity. This divergence in neuronal subtypes suggests that cell-of-origin identity influences reprogramming potential. In addition to excitatory glutamatergic neurons, *DLX2*-driven reprogramming generates GABAergic inhibitory neurons from mouse cortical astrocytes¹³⁴. The precise specification of striatal inhibitory medium spiny neurons from human fibroblasts utilized *DLX2* in combination with *miR-124*, *miR-9/9**, *MYT1L*, *BCL11B*, and *DLX1*¹³⁵. These *in vitro* generated neurons exhibited action potentials and expressed characteristic GABA, GAD1, and *PPP1R1B* molecular markers¹³⁵.

Lineage-specific induction of neurons is critical to the development of disease models and therapeutics. With relevance to Parkinson disease, mouse and human fibroblasts transduced

with defined combinations of pro-dopaminergic transcription factors reprogrammed into functional dopaminergic neurons¹³⁶⁻¹⁴⁰. These induced neurons exhibited mature synaptic properties and neurotransmitter release. Transplantation of these neurons into a 6-hydroxydopamine lesioned rodent model of Parkinson disease modestly improved deficits of motor function¹³⁷⁻¹³⁹.

Parkinson disease and ALS predominantly affect dopaminergic and motor neuron subpopulations, respectively. Expanding on the discovery that ASCL1, POU3F2, and MYT1L drive fibroblast-to-neuron reprogramming, these three pro-neural factors were co-expressed with LHX3, ISL1, MNX1, NEUROG2, and NEUROD1 to generate functional motor neurons *in vitro* with low efficiency¹⁴¹. When combined with growth factors and small molecules, ISL1, LHX3, NEUROG2, and SOX11 induced functional spinal motor neurons with high efficiency from adult human skin fibroblasts¹⁴². These motor neurons formed functional neuromuscular junctions with skeletal myotubes and exhibited disease-specific pathological features when generated from skin fibroblasts of ALS-afflicted individuals. Remarkably, these functional deficits were rescued by exposure to the small molecule kenpaullone during reprogramming¹⁴². These findings highlight the potential utility of induced neurons in disease modelling and drug identification.

The ability to directly *in vitro* transdifferentiate functional subtype-specific neurons from somatic and glial cells has enabled focused investigations into the progression of development and disease. Further, the genetic reprogramming factors and small molecules identified in these screens have guided *in vivo* neural reprogramming strategies.

In vivo transdifferentiation—To investigate whether somatic cells can be directly converted into neurons *in vivo*, human fibroblasts and astrocytes engineered to express doxycycline-inducible pro-neural transcription factors were transplanted into the rat striatum¹⁴³. This precise control over ASCL1, POU3F2, and MYT1L expression enabled transdifferentiation within the neural microenvironment. Both transplanted human fibroblasts and astrocytes reprogrammed into functional, synapse-forming interneurons and exhibited long-term survival post-doxycycline withdrawal. The *in vivo* application of these findings enabled researchers to directly convert resident non-neuronal glia of the adult mouse striatum into neurons using lentiviral-mediated expression of ASCL1, POU3F2, and MYT1L¹⁴³.

In parallel, *NEUROG2*-encoding retrovirus and local administration of growth factors following focal injury were used to induce glutamatergic and GABAergic neurons from non-neuronal cells in the neocortex and striatum, respectively¹⁴⁴. This divergence in cell identity suggests that *NEUROG2* activates a pro-neural transcriptional program while the neural microenvironment modulates subtype-specific differentiation.

Reactive astrocytes of the mouse cortex rapidly transform into functional neurons when transduced with *NEUROD1*-encoding retrovirus¹⁴⁵. While astrocytes adopted a strictly excitatory glutamatergic identity, NG2 glia converted into heterogeneous populations of glutamatergic and GABAergic interneurons¹⁴⁵. Interestingly, reactive astrocytes reprogrammed with higher efficiency in a mouse model of Alzheimer disease than control

reactive astrocytes¹⁴⁵. Furthermore, alternative strategies have demonstrated the *in vivo* reprogramming of astrocytes and NG2 glia into neurons using variable combinations of SOX2, ASCL1, LMX1A, and NR4A2¹⁴⁶⁻¹⁴⁸.

Similar to the transdifferentiation of adult glia and somatic cells, *in vivo* reprogramming can be used to influence the lineage-specific differentiation of early postmitotic neurons. The electroporation of neural progenitors that produce GABAergic medium spiny neurons in the lateral ganglionic eminence with the transcription factor FEZF2 resulted in differentiation toward a glutamatergic identity¹⁴⁹. Similarly, the *in vivo* overexpression of FEZF2 in early post-mitotic callosal projection neurons and layer IV interneurons induces glutamatergic corticofugal projection neurons^{149, 150}. These studies indicate that individual transcription factors can dictate the subtype-specific development of neurons and the fine-tuned expression of these factors is essential to the production of localized neuron populations in the developing central nervous system (Table 5). While *in vitro* and *in vivo* transcriptomic modification can induce functional changes in cellular identity, these cells often retain cell-of-origin genetic hallmarks not acquired during developmental differentiation.

Characterization of engineered cells

Identity: clearance of cell-of-origin identity and induction of lineage-specific neurons—The specification of neurons throughout development relies on stepwise shifts in gene expression that restrict cells to a defined lineage. These shifts in gene expression are tightly controlled by DNA methylation, histone modifications, and regional chromatin condensation. An analysis of global gene expression in ASCL1, POU3F2, and MYT1L reprogrammed neurons demonstrated that, although these pro-neural transcription factors bias the mouse embryonic fibroblast transcriptome toward a neuronal identity, induced neurons do not completely abolish fibroblast-specific gene expression¹⁵¹. Genetic studies using iPSC-derived neurons revealed that reprogramming occurs in waves of transcription accompanied by altered histone acetylation and methylation¹⁵².

Transcription is directly activated or repressed by the binding of transcription factors to gene enhancer and promoter regions. The methylation of cytosine nucleotides in these regions can reduce the affinity of DNA binding proteins for these sequences. Genome-wide methylation analysis in somatic cell-derived iPSCs demonstrated that induced pluripotency does not completely reset the genomic methylation signature in reprogrammed cells to remove marks characteristic of the cell of origin¹³⁸. A detailed understanding of the molecular mechanisms responsible for neural reprogramming will enable further advances in the robust generation of dopaminergic neurons¹³⁶, medium spiny GABAergic neurons¹³⁵, cholinergic neurons¹³³, and motor neurons^{141, 142}.

Function: single neuron electrical properties and network integration—

Neurotransmitter synthesis, synapse formation, and action potential kinetics are functional properties that characterize how a neuron will integrate and function within the local network. Single-cell electrophysiology demonstrated that fibroblast-derived neurons acquire electrical potential *in vitro*¹⁵³. The more complex *in vivo* conversion of astrocytes to calretinin-positive striatal neurons confirmed that induced neurons functionally integrate

directly into the local neuronal network¹²⁴. Four predominant types of neurons were generated from SOX2-transduced astrocytes and each exhibited unique electrical properties¹²⁴. This indicates that astrocyte identity and cues within the microenvironment directly impact functional maturation of induced neurons¹²⁴.

The *in vivo* generation of neurons from cortical and striatal glia highlights the genetic dominance of pro-neural factors in neural cell identity and maintenance^{36, 121, 123, 124, 143-146, 148}. The outgrowth of axons and dendrites during reprogramming enabled these cells to form synapses with neighboring endogenous neurons. However, whether these neurons meaningfully contribute to the cognitive or motor function remains untested. The transplantation of fibroblast-derived dopaminergic neurons into a mouse model of Parkinson disease demonstrated that reprogrammed neurons not only functionally integrate into local neural networks, but also improve motor coordination recovery following disease onset¹³⁸. These discoveries demonstrate that transcription factors are master regulators of cell identity and an understanding of the genetic mechanisms regulating neural reprogramming is critically important to the clinical development of these technologies.

Clinical applications: efficiency of neuron production and tumorigenicity—The clinical utility of engineered neurons relies on high efficiency production of neurons with stable identity. However, initial transdifferentiation methods produced neurons with efficiencies under 10% of the starting cell population. Similarly, human fibroblasts transduced with *NEUROG2*-encoding and *SOX11*-encoding lentivirus convert with low efficiency; however, exposure to forskolin and dorsomorphin dramatically enhance conversion above 90% efficiency¹³³. This small molecules-based approach might enable large-scale production of transplantable neurons and patient-specific pharmaceutical screening. Another approach to this problem is increasing the total number of reprogrammed neurons that survive into functional maturity. Astrocyte-derived neuroblasts proliferate upon reprogramming and yield multiple neurons that persist *in vivo* for at least 14 weeks¹²³.

The safe induction of new post-mitotic neurons is another hurdle in the translation of this technology to the clinic. The division of pluripotent stem cells and neural progenitors is regulated by molecular signals that control proliferation to prevent tumor formation. *In vivo* reprogramming toward an iPSC fate indicates deficits in these mechanisms and results in teratoma formation in multiple organ tissues¹¹⁷. However, the generation of neural-specific progenitors and *in vivo* transdifferentiation of neurons is not limited by tumorigenicity^{36, 123, 145}. In addition, genome-wide sequencing of iPSC lines uncovered both genetic and epigenetic mutations acquired upon reprogramming that have established roles in cancer progression¹⁵⁴. Further refinement of high-efficiency, non-tumorigenic reprogramming protocols will be required for engineered neurons to find breakthrough applications in the clinical setting (Figure 5).

Conclusion

The plasticity of cell identity is modulated throughout the developing and adult central nervous system by environmental, pathological, and therapeutic factors. Physiological

changes in neurotransmitter production, neurogenic proliferation post-traumatic injury, and degeneration-induced neurogenesis highlight the dynamic nature of neural identity within the mammalian nervous system. Although limited neurogenesis in the adult brain hinders regeneration following traumatic injury and disease-related degeneration, state-of-the-art engineered neuronal reprogramming methods might enable the transplantation or *in vivo* generation of new functional neurons within the human nervous system. As an early-stage biomedical technology, neuronal reprogramming currently faces numerous challenges that limit clinical utility. As constraints to reprogramming efficiency, factor delivery, and safety are overcome, we believe neuronal engineering will offer a new paradigm in patient-specific neural regeneration.

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Figure 1. Neurogenesis in the adult brain

The subgranular zone (SGZ) of the dentate gyrus and subventricular zone (SVZ) of the lateral ventricle (LV) retain the capacity for neurogenesis into adulthood. SGZ NSCs (type 1 cells) generate non-radial precursor (type 2) cells, which proliferate and develop into neuroblasts and ultimately differentiated granule neurons within the dentate gyrus. By contrast, active SVZ NSCs (type B cells) first generate fast-dividing transit amplifying progenitor (type C) cells, convert into neuroblasts, and migrate through the rostral migratory stream (RMS) into the olfactory bulb (OB) for differentiation into GABA-positive olfactory neurons. EP, ependymal cells; GCL, granule cell layer; ML, molecular layer.

Figure 2. Neural reprogramming in the adult spinal cord

Multiple cell types in the adult spinal cord reprogram in response to injury or engineered stimuli. In response to traumatic injury or neuroinflammation, resident spinal cells dedifferentiate, proliferate, and produce multiple glial lineages. However, the existence of endogenous lesion-induced neurogenesis remains debated. The *in vivo* generation of mature neurons has been achieved using both neuroblast-based and direct reprogramming strategies.

Figure 3. Historical timeline of pluripotency research

This timeline highlights eleven milestone events that defined our understanding of developmental and engineered pluripotency.

Engineered Cell Identity: Pluripotency

Engineered Cell Identity: Progenitors

Engineered Cell Identity: Transdifferentiation

Figure 4. Engineered cell identity reprogramming

Cell identity can be engineered using pluripotency-based, progenitor-based or transdifferentiation strategies. Pluripotency-based reprogramming strategies involve the dedifferentiation of a somatic cell and re-specification of the desired cell type from the resultant iPSC. Progenitor-based reprogramming converts a somatic cell into an expandable neural progenitor that further differentiates into the desired neural lineage. Transdifferentiation enables the direct generation of functional neurons from differentiated non-neuronal cell types.

Figure 5. Clinical barriers to engineered reprogramming

Numerous safety- and efficacy-related hurdles have hindered the clinical application of neural reprogramming technologies. Viral-based gene delivery, limited success of *in vivo* genetic correction technologies, iPSC tumorigenicity, and high-efficiency production of functional neurons with homogenous identity have limited the utility of reprogramming technologies in the clinic. Solutions to these issues such as small molecule-based reprogramming and *in vivo* transdifferentiation are currently under development.

Table 1

Physiological effectors of neurogenesis and innate reprogramming

Physiological effectors	Change in Neurogenesis	
	<u>SGZ</u>	<u>SVZ</u>
Aging	Decrease ³⁹⁻⁴¹	Decrease ³⁹⁻⁴¹
Long-Period Sleep Deprivation	Decrease ⁴⁴	
Short-Period Sleep Deprivation	Increase ^{45, 46}	
Ovarian Hormone Production	Increase ³⁸	
Physical and Psychosocial Stress	Decrease ^{47, 48}	
Physical Exercise	Increase ⁴²	

Table 2

Pathological effectors of neurogenesis and innate reprogramming

Pathological Effector	Change in Neurogenesis	
	<u>SGZ</u>	<u>SVZ</u>
Alzheimer Disease	Increase and Decrease ^{69, 70}	
Huntington Disease	Decrease ^{82, 83}	Increase ^{78, 79}
Parkinson Disease	Decrease ⁸⁹⁻⁹⁴	Decrease ^{89, 90, 96}
Traumatic Brain Injury and Stroke	Increase ⁵¹	Increase ⁵²
Amyotrophic Lateral Sclerosis	Enhanced Spinal Cord Neurogenesis ⁶¹	
Multiple Sclerosis	Spinal Cord Neurogenesis ⁶²	

Table 3Engineered reprogramming: *in vivo* and *in vitro* induction and differentiation of neural progenitors

Species	Origin Identity	Engineered Identity	Reprogramming Factors
Mouse ^a , <i>in vivo</i>	Spinal Astrocyte	Neuroblast ³⁶	SOX2
Mouse ^a , <i>in vivo</i>	Striatal Astrocyte	Neuroblast ¹²³⁻¹²⁵	SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	Bipotent NPC ¹²⁰	FOXG1, SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	Tripotent NPC ¹²⁰	FOXG1, POU3F2, SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	Tripotent NPC ¹²¹	CHIR99021, Repsox, Valproic Acid
Human ^u , <i>in vitro</i>	Urinary Cell	Tripotent NPC ¹²¹	CHIR99021, Repsox, Valproic Acid
Human ^f , <i>in vitro</i>	Fibroblast	NSC ¹²²	SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	NSC ¹²²	SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	NSC ¹¹⁸	KLF4, MYC, POU5F1, SOX2
Mouse ^e , <i>in vitro</i>	Fibroblast	NSC ¹¹⁹	KLF4, MYC, POU3F4, SOX2, TCF3

Note: If known, the developmental stage of cell origin is indicated.

^a
adult^e
embryonic^f
fetal^u
unknown.

Table 4Engineered reprogramming: *in vitro* transdifferentiation

Species	Origin Identity	Engineered Identity	Reprogramming Factors
Human ^a	Cortical Astrocyte	Neuron ¹³²	Nine small molecules
Human ^a	Fibroblast	Neuron ¹²⁸	miR-124, MYT1L, POU3F2
Human ^{p,a}	Fibroblast	Neuron ¹²⁹	ASCL1, miR-9/9*, miR-124, NEUROD2, MYT1L
Human ^a	Fibroblast	Neuron ¹³¹	Seven small molecules
Mouse ^e	Fibroblast	Neuron ¹³⁰	CHIR99021, Forskolin, ISX9, SB431542
Mouse ^e	Fibroblast	Neuron ¹²⁷	ASCL1, MYT1L, POU3F2
Human ^f	Fibroblast	Cholinergic Neuron ¹³³	Dorsomorphin, Forskolin, NEUROG2
Human ^a	Fibroblast	Cholinergic Neuron ¹³³	Dorsomorphin, Forskolin, FGF2, NEUROG2, SOX11
Human ^{e,p}	Fibroblast	Dopaminergic Neuron ¹³⁶	ASCL1, FOXA2, LMX1A, MYT1L, POU3F2
Mouse ^{e,a}	Fibroblasts	Dopaminergic Neurons ¹³⁷	ASCL1, LMX1A, NR4A2
Human ^{f,a}			
Mouse ^a	Fibroblasts	Dopaminergic Neurons ¹³⁸	ASCL1, EN1, FGF8, FOXA2, LMX1A, NR4A2, PITX3, SHH
Human ^f	Fibroblasts	Dopaminergic Neurons ¹³⁹	ASCL1, NEUROG2, NR4A2, PITX3, SOX2
Mouse ^e	Fibroblasts	Dopaminergic Neurons ¹⁴⁰	ASCL1, LMX1B, NR4A2
Mouse ^p	Astrocytes		
Mouse ^p	Cortical Astrocyte	GABAergic Neuron ¹³⁴	DLX2
Mouse ^p	Cortical NG2 Cell	GABAergic and Glutamatergic Neurons ¹⁴⁵	NEUROD1
Human	Cortical Astrocyte	Glutamatergic Neuron ¹⁴⁵	NEUROD1
Mouse ^p			
Mouse ^p	Cortical Astrocyte	Glutamatergic Neuron ¹³⁴	NEUROG2
Human ^e	Fibroblast	Motor Neuron ¹⁴¹	ASCL1, ISL1, LHX3, MNX1, MYT1L, NEUROD1, NEUROG2, POU3F2
Mouse ^e			
Human ^a	Fibroblasts	Motor Neuron ¹⁴²	Dorsomorphin, Forskolin, FGF2, NEUROG2, SOX11, ISL1, LHX3
Human ^p	Fibroblast	Striatal Medium Spiny Neuron ¹³⁵	BCL11B, DLX1, DLX2, miR-9/9*, miR-124, MYT1L

Note: If known, the developmental stage of cell origin is indicated.

^a adult^e embryonic^f fetal^p early postnatal.

Table 5Engineered reprogramming: *in vivo* transdifferentiation

Species	Origin Identity	Engineered Identity	Reprogramming Factors
Mouse ^{p, a}	Cortical Astrocyte	GABAergic and Glutamatergic Neurons ¹⁴⁸	ASCL1
Mouse ^a	Cortical Astrocyte	Glutamatergic neuron ¹⁴⁵	NEUROD1
Mouse ^a	Spinal Astrocyte	GABAergic and Glutamatergic Neurons ³⁶	SOX2, Valproic Acid
Mouse ^a	Striatal Astrocyte	Calretinin Neuron ^{123, 124}	SOX2, Valproic Acid
Mouse ^a	Striatal Astrocyte	Neuron ¹⁴³	ASCL1, MYT1L, POU3F2
Mouse ^a	Cortical NG2 Cell	GABAergic and Glutamatergic Neurons ¹⁴⁵	NEUROD1
Mouse ^a	Cortical NG2 Cell	Neuron ¹⁴⁶	SOX2
Mouse ^a	Striatal NG2 Cell	GABAergic and Glutamatergic Neurons ¹⁴⁷	ASCL1, LMX1A, NURR1
Mouse ^a	Non-Neuronal Cell	GABAergic and Glutamatergic Neurons ¹⁴⁴	EGF, FGF2, NEUROG2
Mouse ^p	LGE Neural Progenitor	Cortical Projection Neuron ¹⁴⁹	FEZF2
Mouse ^a	Spinal Neural Progenitor	GABAergic neuron ⁶⁵	BDNF, EGF, FGF2, NEUROG2
Mouse ^{e, p}	Callosal Projection Neuron	Glutamatergic Corticofugal Projection Neuron ¹⁵⁵	FEZF2
Mouse ^p	Spiny Neuron	Pyramidal Neuron ¹⁵⁰	FEZF2

Note: If known, the developmental stage of cell origin is indicated.

^a adult^e embryonic^p early postnatal.