Treating Parkinson’s disease by astrocyte reprogramming: Progress and challenges

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Parkinson’s disease (PD), the second most prevalent neurodegenerative disorder, is typified by both motor and nonmotor symptoms. The current medications provide symptomatic relief but do not stimulate the production of new dopaminergic neurons in the substantia nigra. Astrocyte reprogramming has recently received much attention as an avenue for increasing functional dopaminergic neurons in the mouse PD brain. By targeting a microRNA (miRNA) loop, astrocytes in the mouse brain could be reprogrammed into functional dopaminergic neurons. Such in vivo astrocyte reprogramming in the mouse model of PD has successfully added new dopaminergic neurons to the substantia nigra and increased dopamine levels associated with axonal projections into the striatum. This review deliberates the astrocyte reprogramming methods using specific transcription factors and mRNAs and the progress in generating dopaminergic neurons in vivo. In addition, the translational potential, challenges, and potential risks of astrocyte reprogramming for an enduring alleviation of parkinsonian symptoms are conferred.

INTRODUCTION

Parkinson’s disease (PD), the most prevalent neurodegenerative disorder after Alzheimer’s disease, afflicts ~6 million people worldwide (1). The incidence of PD is rare in young adults up to 40 years but increases considerably in both genders more than 60 years of age, with a mean age of diagnosis at ~70. The incidence of PD generally stabilizes after 80 (2). A recent analysis estimated, the prevalence of PD in United States residents more than 45 years old as 572 per 100,000 people (3). A meta-analysis of PD cases suggested a male:female ratio of 1.46, with a substantial difference in the gender ratio for individuals from 50 to 59 years old and an increased mortality rate in males (4). Multiple factors likely play roles in gender differences, including biomarkers, genetics, and therapeutic management (4). For example, lower urate concentrations have been shown to predict poorer PD prognosis in men, whereas leucine-rich repeat kinase 2 (LRRK2) mutation, a genetic factor that causes PD, is predominantly seen in women (4). It has also been suggested that more prolonged estrogen exposure in females leads to a decreased PD risk. However, there is conflicting data on estrogen’s effect on dopamine (DA) and PD (4).

PD is classically characterized by the loss of dopaminergic (DA-ergic) neurons in the substantia nigra pars compacta (SNpc), although the disease has a much more complex pathology involving more than the SNpc. Braak and colleagues (5) proposed a hypothesis that broke down PD into a six-stage process looking at the localization and spread of neurodegeneration. Braak stage 1 starts with degeneration of the olfactory bulb and olfactory nucleus. Stage 2 shows progression into the lower brainstem, the region from which nonmotor symptoms of PD arise. Typical motor symptoms emerge in stages 3 and 4 when the neurodegenerative processes have reached the SNpc. Stages 5 and 6 are characterized by the presence of Lewy bodies (5), which are intracellular inclusions of alpha-synuclein composed of radiating fibrils in DA-ergic neurons of the SNpc. However, it should be noted that the Braak stages reflect a proposition that have not been fully validated. The SNpc also has a high content of DA-ergic neurons, containing neuromelanin, a by-product of DA oxidation. Neuromelanin is a dark pigment that colors the SNpc black on gross examination. This pigmentation fades in PD, signifying a loss of the neuromelanin containing SNpc DA-ergic neurons (6). The SNpc is implicated in movement and motor control, and loss of DA-ergic neurons in this area leads to many of the classic motor symptoms seen in PD. It is believed that 50 to 80% of DA-ergic neurons need to be lost for the motor symptoms to appear (5).

In addition to DA-ergic neuron loss, there are other neurotransmitters involved in PD. There is a complex system of neurotransmitter interactions with nigrostriatal neurons influencing motor and nonmotor symptomology. DA and acetylcholine balance in the striatum is necessary for motor function, and when this balance is disturbed, motor symptoms arise. Glutamatergic and GABA-ergic neurons affect DA activity in the nigrostriatal neurons as well (7). Furthermore, a nonlinear loss of serotonin transporters is seen during PD progression, with a different neurodegeneration pattern than the DA-ergic neuron loss. Notably, the loss of serotonin transporters has been seen in patients with tremor-predominant PD, implicating serotonin’s role in such pathogenesis. Loss of serotonin could also be linked to depression and other nonmotor symptoms in PD (8). Lewy bodies in the midbrain are also pathognomonic for PD (9), which differs from Lewy bodies’ predominant occurrence in the cerebral cortical neurons in Lewy body dementia.

CURRENT TREATMENT OPTIONS FOR PD AND THEIR LIMITATIONS

PD has both motor and nonmotor symptoms; clinical presentation depends on the progression of the disease. The cardinal motor symptoms are rigidity, bradykinesia, and a resting tremor. Bradykinesia is always present, but not every patient with PD has a resting tremor. Patients may also have postural instability (10). The nonmotor symptoms are often called invisible symptoms, as they are non-descript and can occur in various stages of the disease. Patients may have autonomic dysfunction, depression, anxiety, sleep disturbances, mood, and cognitive changes. The Braak stages suggest that the nonmotor symptoms occur in the preclinical stages 1 and 2 when the disease is mostly restricted to the olfactory bulb and lower brainstem (11).
There are many prescription medications for the symptomatic treatment of PD. Levodopa (L-DOPA) is the most potent, which acts as a precursor to DA. L-DOPA crosses the blood-brain barrier (BBB), gets converted into DA by aromatic L-amino acid decarboxylase (AADC) and becomes bioavailable for the DA-deprived neurons. Many different forms of L-DOPA exist to increase bioavailability; one of the most popular is Sinemet. Sinemet is a combination drug with L-DOPA and carbidopa. Carbidopa prevents the peripheral conversion of L-DOPA into DA and increases the amount of central DA. DA agonists such as ropinirole and pramipexole also mimic DA and bind onto the DA receptor. These drugs could be used alone or combined with Sinemet (12). Catechol-O-methyltransferase (COMT), monoamine oxidase (MAO), and aldehyde dehydrogenase (ALDH) inhibitors reduce the breakdown of levodopa and DA (13). These drugs increase carbidopa/L-DOPA activity but conversely enhance side effects due to levodopa’s augmented effects (13). Anticholinergic drugs, trihexyphenidyl, and benztropine are not effective for bradykinesia but ease rigidity, dystonia, and tremor (13). The nonmotor symptoms, such as depression, can be treated as they would be in non-PD patients, with tricyclic antidepressants, serotonin-noradrenaline reuptake inhibitors, serotonin reuptake inhibitors, or cognitive behavioral therapy (13).

The most prominent side effect of sustained L-DOPA therapy is drug-induced dyskinesias, an involuntary movement disorder characterized by repetitive and irregular motions. Starting the treatment with DA agonists such as ropinirole and pramipexole reduces dyskinesia risk. However, once the disease progresses, L-DOPA needs to be added when monotherapy with a DA agonist becomes inadequate for controlling the symptoms. The risk for dyskinesias then increases in patients with PD taking L-DOPA. Current PD treatment involves establishing a balance of appropriate disease symptom control and limiting drug-induced side effects through the addition or subtraction of multiple drugs. As described above, anticholinergic drugs, COMT inhibitors, and MAO-ALDH inhibitors can decrease the amount of L-DOPA needed to control symptoms. Nevertheless, once the disease progresses and more DA-ergic neurons are lost, the patient requires higher L-DOPA dosing, enhancing the risk of dyskinesias and other side effects (14). There are surgical therapies available, as well. One such therapy is deep brain stimulation (DBS), which involves the stimulation of targeted electrodes in different regions of the brain. DBS of the ventral thalamus results in entrainment of local neurons of the globus pallidus interna and alterations in the neuronal firing of the subthalamic nuclei (STN) (15). Low-frequency stimulation of the mostly cholinergic pedunculopontine nucleus leads to improvements in gait-related motor function in PD. Also, high-frequency DBS of the STN has been shown to ameliorate the major motor symptoms of PD and slow down neurodegeneration, likely through inhibition of STN output (15). Other DBS targets include the prelemniscal radiations (a funnel of fibers in the posterior subthalamus) and caudal zona incerta to treat tremors (15). Medication plus DBS is far more superior to medical therapy alone (15). Duopa therapy is another surgical alternative where carbidopa/L-DOPA is given as an enteral suspension. A stroma is made in the stomach where a percutaneous endoscopic gastro-jejunal (PEG-J) tube is placed; a pump then delivers Duopa directly into the jejunum. Such administration avoids the stomach, which increases the bioavailability of carbidopa/L-DOPA (16). Nonetheless, currently available treatments do not stem the degeneration of the SNpc in PD. The medications and therapies provide symptomatic relief to patients and do not significantly slow down the disease’s progression, which results in worsening of symptoms over time. PD may even regress to a point where the disease becomes refractory to medications and other therapies (17). Thus, alternative and more efficient therapies are needed to improve the quality of life of patients with PD.

**IN VIVO ASTROCYTE REPROGRAMMING AS AN ALTERNATIVE THERAPY FOR PD**

Many alternative therapies are currently in development and are being tested in preclinical models of PD and clinical trials. These include gene therapy with LRRK2 inhibitors, glutamic acid decarboxylase, aromatic AADC, glucocerebrosidase moderators, vaccines against alpha-synuclein, and stem cell therapy (18–24). This review focuses on discussing the promise of in vivo astrocyte reprogramming as an attractive alternative therapy for PD.

Astrocytes are specialized non-neuronal glial cells found in the entire central nervous system (CNS). There are two main subtypes: protoplasmic and fibrous. Protoplasmic astrocytes are located throughout the gray matter and have several branches that give rise to many more branched processes. Fibrous astrocytes, located in the white matter, have many long fiber processes as well. Protoplasmic astrocytes envelope neuronal synapses, whereas fibrous astrocytes come in contact with the nodes of Ranvier (25). Astrocytes have many complex functions to maintain the CNS. One of the critical functions is their role in the development of neurons in the CNS. The boundaries formed by astrocytes guide the developing axons and neuroblasts toward the correct location through astrocyte tenascin-C and other proteoglycans (26). Astrocytes also have processes contacting blood vessels forming part of the BBB and synapses to regulate the synaptic activity. Astrocytes control cerebrovascular flow through molecular mediators such as prostaglandins, nitric oxide, or arachidonic acid (27) and regulate synaptic activity by releasing glutamate, D-serine, and adenosine triphosphate that have feedback activity on neurons (28). Astrocytes also have a role in synapse function/formation through the regulated release of active molecules, including glutamate, purines, γ-aminobutyric acid (GABA), and D-serine (25). The release of these molecules occurs in response to neuronal activity and astrocytic excitability (25). Astrocytes exert long-term influences on synapses through the release of growth factors. In addition to releasing active molecules into the synapse, astrocytes also envelop the synapses to maintain ion, pH, and transmitter homeostasis in a way that is essential for synaptic transmission. For instance, astrocytes contain aquaporin-4 water channels and K⁺ channels, as well as Na⁺/H exchangers and bicarbonate transporters. The activity of these channels and proton shuttles present on astrocytes is essential for regulating healthy synaptic activity (25). Last, astrocytes aid in the reuptake of neurotransmitters from the synaptic cleft, which are then recycled into active neurotransmitter precursors for reuse in synaptic activity (25). Thus, astrocytes play a crucial role in CNS homeostasis and essential neuronal activities. Glial fibrillary acid protein (GFAP), an intermediate filament protein, is the prototypical marker of astrocytes. When an injured part of the CNS goes through reactive astrogliosis, there is an up-regulation of GFAP expression due to astrocyte hypertrophy and hyperplasia (29). GFAP expression is typically less intense in healthy CNS astrocytes or astrocytes that are further away from the injury site. However, it depends on the region examined, as strong
GFAP expression could be present in some areas of the healthy CNS (25). GFAP expression by astrocytes also seems to have regional variability based on the different astrocyte responses to injuries or pathologies in the CNS (25).

In the developing brain, neurogenesis occurs with type 1 neural stem cells (NSCs), which are radial glia producing intermediate neural progenitors. The radial glial cells also guide neuroblasts to appropriate locations. Glial cells, such as astrocytes, are formed later (30). Classically, it is believed that the developed CNS has a fixed number of neurons that can be permanently lost or damaged from neurodegenerative diseases like PD or Alzheimer’s disease. In rodents, studies have reported adult neurogenesis at a much lower rate in many brain regions except the hippocampus, where significant neurogenesis has been shown to continue throughout life (31–33). However, the occurrence of hippocampal neurogenesis in the adult human brain is still controversial (34). Notably, the brain regions relevant to PD (the SNpc and striatum) have very limited neurogenesis in the adult period, and hence, reprogramming of glial cells into DA-ergic neurons has recently received considerable interest in PD models. The details of reprogramming of reactive astrocytes in the substantia nigra and/or striatum into DA-ergic neurons and the efficacy of such an approach to ease PD symptoms are discussed in the next section.

**ASTROCYTE REPROGRAMMING METHODS**

Astrocyte reprogramming involves manipulating the genetic environment to bring the astrocyte to a dedifferentiated state, so then it can be redifferentiated into a mature cell of a different cell fate. Reprogramming can also go through a direct pathway, which foregoes the dedifferentiated, pluripotent state, and the astrocyte converts directly to a different cell. Dedifferentiation of astrocytes occurs endogenously in adults, usually in the presence of CNS injury (35–36), but most dedifferentiated astrocytes are pushed back into an astrocyte cell fate creating a glial scar. This process, reactive gliosis, creates more astrocytes around the injury site (37). In vitro studies have shown that a small number of astrocytes differentiate into neurons after CNS injury (38). Many pathways have now been found to drive dedifferentiated astrocytes into a neuronal cell fate. Two pathways and methods that will be focused on in this review are reprogramming astrocytes into neurons through transcription factors (TFs) and microRNAs (miRNAs) (Fig. 1).

Ruiz and associates (39) showed that retroviral transduction of TFs Oct-4, Sox-2, Klf4, and c-Myc in vitro could be used to reprogram mature human cerebral astrocytes into human induced pluripotent stem cells (hiPSCs). The astrocyte-derived hiPSCs then generated neural progenitor cells that differentiated into postmitotic, neuron-specific nuclear protein (NeuN)-expressing mature neurons and astrocytes. Since then, reprogramming using TFs has advanced, including single TF reprogramming in vivo. miRNAs are involved in the differentiation of adult NSCs into mature cells. Multiple miRNAs have also been investigated for promoting the dedifferentiation of astrocytes and their eventual differentiation into neurons. The most common miRNAs studied in this context are miR-9 and miR-124 (40). The ability to reprogram endogenous astrocytes into neurons has implications in the setting of neurodegenerative diseases that result in permanent neuron losses. Since astrocytes are prevalent and numerous in the CNS, they can be ideal targets for therapeutic interventions. In PD, DA-ergic neuron loss is one of the major causes leading to motor symptoms and death. Studies have shown that DA-ergic neurons could be generated through astrocyte reprogramming using TFs and/or miRNAs (41).

**Astrocyte reprogramming through TFs**

TFs are regulatory proteins that either up-regulate or down-regulate the transcription of specific genes. They bind to their target DNA and influence transcription by interacting with the RNA polymerase’s transcription complex. Alternatively, TFs can inhibit transcription by down-regulating other factors involved in stimulating transcription (42). By altering or manipulating TFs, downstream gene products would be changed markedly. TFs reprogram cells by mimicking the environment of embryonic stem cells (ESCs) in the developmental period. It was known from earlier studies that mature cells could be reprogrammed into ESCs by fusion with ESCs, but how this process occurs and which factors are involved were unknown. Takahashi and Yamanaka (43) found that Oct-4, Sox-2, and Nanog are core TFs that maintain the pluripotency of ESCs. Many oncogenic genes maintain pluripotency as well, such as c-Myc, Klf4, and β-catenin. Of these genes, Oct-3/4, Sox-2, c-Myc, and Klf4 were essential TFs for pluripotency and conversion of adult mouse fibroblasts into ESCs. Notably, these four TFs are also highly expressed in ESCs. The exact mechanism of reprogramming mature cells into ESCs is very complex and not fully understood, but it is believed that there are two waves of gene activation. c-Myc is an effective activator of the first wave that activates genes implicated in proliferation and inhibits developmental genes. This first wave occurs due to cells responding to Oct-4, Sox-2, c-Myc, and Klf4 exposure. Oct-4 and Sox-2 up-regulate the essential pluripotency genes for the second
wave, which occurs only in cells that are successfully progressing into pluripotency. Klf4 supports pluripotency by activating pluripotent genes during both waves, and combining the four TFs would mimic a genetic environment seen in endogenous ESCs (44).

Ruiz and colleagues (39) first infected human cerebellar astrocytes with retroviruses that encoded Oct-4, Sox-2, Klf4, and c-Myc to convert them into hiPSCs in vitro. Neural progenitor cells expressing nestin and Sox-2 were then generated from hiPSCs, which could be differentiated further into mature postmitotic neurons and astrocytes expressing beta-III tubulin or GFAP, respectively. Corti and associates (45) directly reprogrammed human cortical astrocytes into neural progenitor cells by expressing a single TF (Oct-4, Sox-2, or NANOG). This reprogramming strategy is a significant advance because it occurred by bypassing the hiPSC pathway. In this study, cortical astrocytes were cultured with lentiviral vectors encoding Oct-4, Sox-2, or NANOG to generate NSCs, with no intermediate iPSC stage, conferring a reduced risk for teratoma formation. Grafting into the lateral ventricles of the mouse brain resulted in significant neuronal differentiation of astrocyte-derived NSCs at 2 months after transplantation, with some graft-derived cells remaining as NSCs and some acquiring a glial phenotype (45).

Another study directly converted mouse astrocytes into mature neurons in vivo via lentiviral transduction of Sox-2 into the striatum. The resident mouse astrocytes were first converted with Sox-2 into neural progenitor cells and then differentiated into mature neurons that could integrate into the local mouse brain circuitry (46). Liu and associates (47) reprogrammed dorsal midbrain astrocytes into mature neurons by using an adeno-associated virus (AAV) encoding the TF Ascl1 driven by the GFAP promoter. All of the above experiments demonstrated astrocyte reprogramming into neurons either in vitro or in vivo at an early postnatal and young adult age. Zarei-Kheirabadi and collaborators (48) extended this technology to convert adult mouse cortical astrocytes into NSCs in vitro through the overexpression of TF Zfp521, which could then be differentiated into neurons, astrocytes, and oligodendrocytes. Furthermore, the neurons derived from such an approach expressed the appropriate glutamatergic and GABA-ergic markers. Thus, currently, the technology is in place to directly reprogram astrocytes into neurons in vivo by overexpressing only a single TF.

**Astrocyte reprogramming using miRNAs**

miRNAs are another way to reprogram astrocytes into neurons. They are noncoding sequences of RNA that regulate gene expression at a posttranscriptional level. As discussed above, TFs regulate gene expression at a transcriptional level. miRNAs target mostly protein-coding mRNA transcripts via RNA silencing. They act as guide molecules by binding to the mRNA sequence of interest, leading to RNA silencing. They are around 22 nucleotides long and produced by Drosha and Dicer proteins (49). miR-124 and miR-9 are two commonly used miRNAs in reprogramming. miR-124 is expressed in differentiating and mature neurons and is restricted to neurons. On the other hand, miR-9 is expressed in neural precursors (50). miR-124 and miR-9 can promote neuronal development through the repression of class IIa histone deacetylase (HDAC5) expression. HDAC5 is an inhibitor of neuronal differentiation in the CNS (51). miR-124 and miR-9 have also been implicated in controlling many other genes that regulate neuronal differentiation. Yoo and colleagues (52) demonstrated that human neonatal foreskin fibroblasts could be converted into neurons using the expression of miR-9 and miR-124. They transduced fibroblasts with a single lentiviral vector that expressed both miR-9 and miR-124 precursors, which resulted in neuron-like morphologies expressing microtubule-associated protein 2 4 weeks after transduction (52). However, the conversion rate was less than 5% with only the miRNAs. The addition of a neurogenic TF, neuronal differentiation 2 (NEUROD2), increased the conversion to ~50%. miR-9 and miR-124 also targeted Brd/Brn-associated factor 53a (BAF53a), a subunit of the BAF complex that is involved in neural development (53). When miR-9 and miR-124 repress BAF53a, other BAF subunits essential for neuronal maturation were found in the complex. Since BAF complexes control epigenetic changes across several sites of the genome in ESCs, it appeared that miRNAs could also reprogram cells by inducing epigenetic changes. miR-124 and miR-9 also targeted other genes such as RE1 silencing TF (REST), co-REST, and poly(A) ribonuclease tract binding protein 1 (PTBP-1) to facilitate neurogenic differentiation (52).

Ghasemi-Kasman and colleagues (53) transduced adult mouse astrocytes in the striatum and human astrocytes derived from an astrocytoma cell line with a lentiviral vector that expressed miR-302/367 cluster, which resulted in the conversion of astrocytes into neuroblasts 2 weeks after transduction. This conversion did not go through a pluripotent stage, so there were no concerns of teratoma formation. miR-302/367 cluster is implicated in early CNS development and expressed in NSCs. Pluripotency genes Oct-4, SOX2, and NANOG all increase the expression of miR-302/367, and miR-302/367 increases the expression of those pluripotency genes as a positive feedback loop. However, an efficient reprogramming of mouse astrocytes into neurons required valproic acid (VPA; an HDAC inhibitor), in addition to miR-302/367 (53). Human astrocytes, when grafted with miR-302/367, transformed into neuroblasts and then into neuron-like cells. Human astrocytes could be reprogrammed into neurons with miR-302/367 in the absence of VPA due to a lower HDAC activity in human cells (53). Thus, exogenous miRNAs can directly reprogram astrocytes into neurons.

Another way the miRNAs contribute to reprogramming is through the modulation of miRNA targets. For example, miR-124 can promote neuronal differentiation by targeting poly(A)tract binding (PTB) protein and its homolog, neuronal PTB (nPTB). PTB protein inhibits splicing of neuron-specific exons and is down-regulated in the nervous system, whereas nPTB is up-regulated during neuronal differentiation. PTB and nPTB undergo a programmed switch during neuronal differentiation controlled by miR-124, which reduces PTB, favoring neuronal-specific splicing events. Conversely, forced PTB expression will block miR-124 activity, thus blocking neuronal differentiation (54). Xue and collaborators (55) found that down-regulating PTB-induced TFs could differentiate mouse embryonic fibroblasts directly into functional neurons. This finding revealed how the REST complex functions to suppress neuronal genes. The REST complex suppresses many neuronal genes, such as miR-124, in nonneuronal cells. However, miR-124 targets and inhibits REST, synaptonemal complex protein 1 (SCP1), and co-REST, which activates neuronal genes and creates a regulatory loop. PTB can suppress the inhibitory activity of miR-124, so PTB is a critical target that miR-124 must down-regulate to express more neuronal genes. Therefore, by down-regulating PTB, one could induce functional neurons in a wide variety of cells by manipulating an miRNA target (55). Thus, astrocytes could be reprogrammed directly into functional neurons in vivo by using the PTB/nPTB loop.
Potential risks of in vivo reprogramming of astrocytes

There are many issues to overcome for a successful reprogramming of astrocytes into neurons in vivo using TFs and miRNAs. Some of the hurdles are resolvable, but several other issues need additional studies and advancements in reprogramming strategies. The resolvable issues comprise the potential for teratoma formation and immune reaction. The risk for teratoma formation is high if the reprogramming process involves an intermediate iPSC state as iPSCs have a higher propensity for generating teratoma (56). However, this issue has been addressed by new reprogramming techniques that avoid the intermediate iPSC state (45). Another issue is the extent of immune reaction to vectors used in reprogramming. The innate immune system has been shown to react to the AAV vector, which is frequently used for reprogramming using TFs. For instance, during endosomal AAV trafficking, Toll-like receptor 9 (TLR-9) could sense cytosine-phosphate-guanine (CpG) motifs on the vector, which may induce nuclear factor xB to activate the transcription of proinflammatory genes (57). Such activation of the TLR9-myeloid differentiation primary response gene 88 (MyD88) pathway has also been implicated in CD8+ T cell responses to AAV2 capsids and transgene products in mice, resulting in the formation of AAV-neutralizing antibodies (58). Transient immunosuppression via methylprednisolone could reduce such immune response against AAV capsid antigens. However, several other approaches could also be adopted to combat the immune response against the AAV. For example, inhibition of epidermal growth factor receptor protein tyrosine kinase can increase AAV2 transduction efficiency by decreasing the degradation of AAV by the proteasome (57).

Other significant complications need further attention. First, the reprogramming efficiency and survivability as well as maturation of converted neurons in vivo must be thoroughly analyzed at extended end points before clinical trials. Grande and colleagues (59) have demonstrated that regional differences and different injury conditions could influence the efficacy of reprogramming and the survival of newly transformed neurons. For example, varying degrees of efficacy and maturation were observed when non-neuronal cells were reprogrammed into neurons through exposure to growth factors and NeuroG2 in the striatum vis-à-vis neocortex. In the striatum, only a relatively small number of neurons were converted. In the neocortex, NeuroG2 alone was able to induce a large number of immature neurons early, but with time, a negligible number of mature neurons survived. Brain injury, such as ischemia in these brain areas, increased the production and maturation of reprogrammed neurons (59). Similarly, another study demonstrated the conversion of numerous NG2* oligodendrocyte precursors into neuronal progenitors in the adult neocortex with Pax6 expression and antagonization of Olig2, but the numbers of transformed neurons significantly waned 30 days later (60). Thus, in addition to the varying reprogramming efficacy in diverse brain regions, transformed neurons’ maturation and long-term survival are the major hurdles that need to be resolved before considering clinical trials. Also, the time points after neural injury or the stage of neurodegenerative disease at which reprogramming efficacy is highest need to be identified. Furthermore, the capability to repair or regenerate functional neuronal networks from newly reprogrammed neurons need to be assessed. The new neurons need to send out axons to accurate efferent sites to establish appropriate synapses with the target neurons to restore the neuronal networks to ultimately provide functional recovery after CNS injury or disease (61). Newly reprogrammed neurons that fail to grow into correct neuronal networks could form abnormal connections with neighboring neurons, leading to neuronal hyperexcitability in reprogrammed areas and may generate seizures. Thus, long-term studies testing the survival, maturation, function, and adverse effects of reprogrammed neurons are critically needed.

PROGRESS IN ASTROCYTE REPROGRAMMING INTO DA-ERGIC NEURONS

A recent study reprogrammed mouse midbrain and cortical astrocytes into functional DA-ergic neurons in vivo by manipulating the PTB/nPTB loop (62). In a mouse model of PD, the astrocyte-derived DA-ergic neurons alleviated several symptoms of PD. The reprogramming performed in this study varied from most examples described in the previous section, as the approach involved the removal or knockdown of TFs and miRNAs (62). Different types of cells express distinct components of the PTB/nPTB loop in higher amounts. For example, nonneuronal cells are prevented from developing into neurons by REST as it inhibits neuronal-specific factors (55). Furthermore, miR-124, a REST inhibitor, is also suppressed by REST in these cells. In neuronal induction, increased expression of miR-124 facilitates REST inhibition, which leads to increased expression of neuronal-specific genes (55). In fibroblasts, a higher level of PTB inhibits the expression of miR-124. Also, disinhibition of REST by PTB results in the suppression of neuronal genes and the repression of miR-124. When PTB is repressed, the inhibition of miR-124 is released, allowing miR-124 to inhibit REST and promote the expression of neuronal genes. However, PTB repression also promotes nPTB expression leading to the inhibition of Brain-2 (BRN2) and miR-9, which are key transcription activators and neuronal maturation genes (62). In mouse fibroblasts, nPTB is initially expressed but then diminished, so sole suppression of PTB adequately reprograms mouse fibroblasts into neurons. On the other hand, human fibroblasts persistently express nPTB, which inhibits the neuronal factors BRN2 and miR-9. Therefore, to reprogram human fibroblasts into neurons, both PTB and nPTB need to be suppressed (63). Dual repression of PTB and nPTB increases the expression of miR-124 and BRN2/miR-9, respectively, all of which facilitate neuronal differentiation.

Neurons display higher levels of miR-124, BRN2, and miR-9. Higher expression of miR-124 inhibits REST, which increases the expression of neuronal genes. The miR-124 expression also inhibits PTB, leading to increased expression of nPTB, but enhanced BRN2 and miR-9 keep nPTB at a lower level in neurons. Thus, higher levels of miR-124, BRN2, and miR-9 favor neuronal maturation and maintenance as they could suppress all inhibitory components of the PTB/nPTB loop (62). Astrocytes express lower levels of miR-124 due to a higher expression of REST, as seen in fibroblasts (Fig. 2). However, higher expression of miR-9 and BRN2 in astrocytes keeps nPTB at a lower level (Fig. 2). Such expression patterns allow the reprogramming of astrocytes into neurons with the sole knockdown of PTB. PTB knockdown would typically induce nPTB expression, but such induction was prevented because of higher expression of miR-9 in astrocytes (62).

Generation of neurons from astrocytes in vitro and in vivo

Qian and associates (62) initially explored the possibility of reprogramming mouse cortical and midbrain astrocytes into functional
Astrocytes

miR-124

PTB

REST

nPTB

BRN2

miR-9

Addition of:

AAV-shPTB

Effect of AAV-shPTB

Conversion to NeuN+ GFAP-

neuronal cells

Differentiation into TH+

DA-ergic neurons

Recovery of 1/3 DA-ergic neurons with outgrowth of TH+ fibers

Increased dopamine levels

Improved Parkinson’s disease symptoms

Unilateral 6-OHDA

Results

Mouse model of PD

Unilateral 6-hydroxydopamine (6-OHDA) injection into the substantia nigra (SN) resulted in the recovery of approximately one-third of the TH+ fiber density compared to the intact brain. Consistency for RFP and TH in the SN, resulting in the recovery of about one-third of the TH+ fiber density compared to the intact brain.

**Integration of astrocyte-derived DA-ergic neurons in a PD model**

Next, Qian and colleagues investigated the effects of astrocyte reprogramming into DA-ergic neurons in a mouse model of PD (Fig. 3). The PD prototype was induced in mice through a unilateral ablation of endogenous DA-ergic neurons using an injection of 6-hydroxydopamine (6-OHDA) into the SNpc. This mouse model does not recapitulate all features of PD, but significant DA-ergic neuron loss leads to several symptoms of PD that are quantifiable. As observed in the intact midbrain, injection of AAV-shPTB into the lesioned midbrain resulted in increased RFP+ and TH+ neuron numbers at 10 to 12 weeks after injection. Animals receiving 6-OHDA alone exhibited ~90% loss of TH+ fibers. However, animals receiving 6-OHDA and AAV-shPTB displayed transformed neurons positive for RFP and TH in the SN, resulting in the recovery of about a third of the TH+ fiber density compared to the intact brain. Considerable loss of TH+ fibers was apparent in animals receiving only 6-OHDA, but not in animals receiving 6-OHDA and AAV-shPTB. The latter group also showed a significant amount of axons positive
for RFP and TH in the caudate-putamen. Besides, the DA level in these animals was restored to 65% of control levels. Furthermore, DA neuron function was restored, which was apparent from DA release following the stimulation of the medial forebrain bundle. Behaviorally, animals receiving 6-OHDA and AAV-shPTB displayed time-dependent improvements in contralateral forelimb use. There was also the reversal of contralateral and ipsilateral rotational behavior in these animals, implying the integration of astrocyte-derived DA-ergic neurons (62). Thus, the study provided the proof of principle that reprogramming of midbrain astrocytes into DA-ergic neurons is a new avenue for improving brain function in PD.

**TRANSLATIONAL POTENTIAL OF ASTROCYTE REPROGRAMMING FOR TREATING PD**

The successful reprogramming of midbrain astrocytes into functional DA-ergic neurons in a mouse model of PD is an exciting development, as such an approach could potentially replace a significant amount of the lost DA-ergic neurons in PD. Since this study was performed in a mouse model of PD, it remains to be determined whether such a strategy is feasible for translation in patients with PD. There are quite a few issues that need to be addressed.

It is currently unknown whether midbrain astrocytes in middle-aged and aged animals are amenable to reprogramming. This is a vital issue, as the vast majority of patients with PD are 65 to 79 years old (2), and astrocytes in patients with chronic PD and in the elderly show an altered degree of activity (64, 65). Reactive astrocytes are diverse and display distinct transcriptome profiles in different neurodegenerative diseases (66). In PD models, it has been found that reactive astrocytes in the SNpc release an excessive amount of GABA, causing a neurotransmitter imbalance (67). Animal model studies have also reported complex changes in aging astrocytes, although there is not enough evidence to categorize them as true reactive astrocytes (66). Such modified astrocytes may significantly affect the reprogramming efficiency as well as the survival of astrocyte-reprogrammed neurons.

Furthermore, the long-term impact of local astrocyte depletion in the PD midbrain due to their reprogramming on the function of existing DA-ergic neurons is unknown (Fig. 3). For example, induction of stroke in a mouse model with astrocyte depletion resulted in rapid neuronal loss and severe motor deficits. Lack of astrocytes in this prototype also increased neuronal injury by oxidative stress due to reduced redox scavenging ability (68). Moreover, reactive astrocytes can mediate multiple beneficial effects relevant to brain repair.
Sofroniew and colleagues (69) demonstrated that when reactive astrocytes were ablated or made dysfunctional after brain injury, a prolonged increase in leukocyte infiltration, poor BBB repair, increased neurodegeneration and neuroinflammation, and decreased functional recovery ensued. These results implied that reactive astrocytes play roles in preventing the detrimental outcomes of CNS injury by providing a supportive microenvironment to the injured neurons, which likely ensures their survival and function (69). However, it remains to be established whether such beneficial effects of reactive astrocytes could also be present in chronic neurodegenerative conditions such as PD. Astrocytes also play an essential role in modulating neuronal circuits via Ca^{2+} signals (70, 71). Ca^{2+} signaling in astrocytes could also affect multiple signaling pathways relevant to the maintenance of neuronal circuits (70, 71). Thus, depletion of astrocytes could disrupt local interactions between astrocytes and neurons, leading to adverse effects on neuronal function. From these perspectives, it is clear that multiple factors need to be considered as further research is conducted on the effects of astrocyte reprogramming on CNS function, particularly in conditions such as brain injury and neurodegenerative diseases.

Another issue is, while the classic pathology of PD is the loss of DA-ergic neurons, PD also affects many other brain regions. A complex system of neurotransmitters is out of equilibrium in PD. While DA and acetylcholine are the neurotransmitters vital for maintaining motor function, DA activity in the SNpc is modulated by excitatory glutamatergic neurons and inhibitory GABA-ergic interneurons. Also, serotonin and DA interactions are involved in reward-related behaviors (7). The levels of these neurotransmitters are typically altered in PD. As seen in L-DOPA monotherapy, the sole increase of DA concentration causes dyskinesias, likely due to the alteration of different neurotransmitters in the corticobasal ganglia-thalamic loop responsible for fine motor control (72). While reprogramming of astrocytes into DA-ergic neurons did provide symptomatic relief in an animal model of PD (62), it remains to be seen whether the addition of reprogrammed DA-ergic neurons into neuronal networks would normalize other neurotransmitter levels (Fig. 3). Besides, the long-term impact of reprogrammed neurons on the leftover, frail, DA-ergic neurons in the SNpc of patients with PD is unknown. In PD, the leftover DA-ergic neurons in the SNpc display accelerated aging, likely due to a sustained demand on oxidative phosphorylation, which causes mitochondrial DNA damage from the production of reactive oxygen species and free radicals within the DA-ergic neurons (73). Therefore, it remains to be investigated in aged PD models whether the conversion of astrocytes into DA-ergic neurons in the aged brain further impairs the redox balance in the SNpc to induce rapid death of leftover DA-ergic neurons in the brain of a patient with PD (Fig. 3).

Rigorous investigations are also needed to confirm that TH^+ neurons derived through astrocyte reprogramming are functional DA-ergic neurons. The demonstration of marker proteins of DA-ergic neurons such as TH and DAT or other neuronal marker proteins such as TuJ-1 and MAP-2 is not enough to conclusively claim that the neurons generated through astrocyte programming are bona-fide DA-ergic neurons. Many features need to be considered while classifying a neuron to a particular functional category, including axonal geometry, projection patterns, and firing patterns (74). In this context, a patch-seq approach combining electrophysiological patch-clamp recordings, single-cell RNA sequencing, and morphological characterization is highly relevant. Using patch-seq, Cadwell and colleagues (74) generated electrophysiological and molecular profiles of 58 different neocortical neurons based on the above properties. Such a patch-seq approach will be needed in future studies to validate whether TH^+ neurons generated from reprogrammed astrocytes display morphological, electrophysiological, and molecular features akin to endogenous DA-ergic neurons (74). Thus, in vivo conversion of astrocytes into functional DA-ergic neurons has the promise to be a disease-modifying treatment. Nonetheless, astrocyte reprogramming efficacy, the consequence of astrocyte depletion, the side effects of elevated DA levels, validation of TH^+ neurons derived from astrocytes into definitive DA-ergic neurons, and the effects of reprogramming on leftover DA-ergic neurons need to be ascertained before considering the clinical trials.

In conclusion, currently, there are no therapies that regenerate DA-ergic neurons in the PD brain. L-DOPA and other medications provide only symptomatic relief and attempt to slow down the disease progression but do not regenerate the lost DA-ergic neurons. Approaches such as gene therapy targeting LRRK2, GAD, AADC, and glucocerebrosidase, vaccines against PD-specific epitopes, and stem cell−derived DA-ergic precursor cell grafting have considerable promise, but they are still in clinical trials or undergoing additional preclinical testing. From this perspective, the ability to reprogram astrocytes into functional DA-ergic neurons in the preclinical prototype is a new advancement for PD treatment. Astrocyte reprogramming could also be used to generate specific populations of neurons lost in other neurodegenerative diseases, including Huntington’s disease, stroke, and Alzheimer’s disease. Astrocytes are plentiful throughout the CNS, so they are an ideal target for reprogramming to replenish the lost neurons in these diseases. However, as discussed in the previous section, several issues need to be addressed before in vivo reprogramming of astrocytes can be brought to human trials. Notably, studies that investigate the feasibility of astrocyte reprogramming in middle-aged and aged PD prototypes, the long-term survival and function of reprogrammed neurons as bona fide DA-ergic neurons, and the effect of astrocyte depletion on the function of neurons and ongoing neuroinflammation in PD need to be addressed. Also, PD is a multifactorial disease that affects much more than just the DA-ergic neurons. Complete systemic and disease-modifying treatments would need to address all neurotransmitter imbalances that contribute to PD symptoms. As discussed, imbalances between acetylcholine and DA could lead to motor symptoms, whereas serotonin is implicated in some nonmotor symptoms such as depression in PD. Astrocyte reprogramming has been shown to increase DA levels, but further research is needed to determine whether such an approach would normalize other neurotransmitter deficits and adversely affect the survival of leftover DA-ergic neurons in PD.

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8 of 10


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