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Review article

Cell reprogramming approaches in gene- and cell-based therapies for Parkinson's disease

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ABSTRACT

Degeneration of dopamine (DA) neurons in the *substantia nigra pars compacta* is the pathological hallmark of Parkinson's disease (PD). In PD multiple pathogenic mechanisms initiate and drive this neurodegenerative process, making the development of effective treatments challenging. To date, PD patients are primarily treated with dopaminergic drugs able to temporarily enhance DA levels, therefore relieving motor symptoms. However, the drawbacks of these therapies including the inability to alter disease progression are constantly supporting the search for alternative treatment approaches. Over the past years efforts have been put into the development of new therapeutic strategies based on the delivery of therapeutic genes using viral vectors or transplantation of DA neurons for cell-based DA replacement. Here, past achievements and recent advances in gene- and cell-based therapies for PD are outlined. We discuss how current gene and cell therapy strategies hold great promise for the treatment of PD and how the use of stem cells and recent developments in cellular reprogramming could contribute to open a new avenue in PD therapy.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder involving genetic defects, exposure to environmental toxins, trauma, and aging in its etiology. These etiological variables cause progressive neuronal damage and formation of α-synuclein-containing Lewy bodies *via* multiple pathogenic mechanisms including intracellular stress, excitotoxicity, altered proteolysis and neuroinflammation. The pathological hallmark of PD is well characterized by the progressive degeneration of dopamine (DA) neurons in the *substantia nigra pars compacta* [1–3]. These subset of DA neurons innervate the striatum and give rise to the nigrostriatal pathway [4], (see also Box 1 and Fig. 1). Degeneration of nigrostriatal DA neurons results into depletion of striatal DA and impaired DA signaling in the basal ganglia, a pivotal neural circuitry involved in the regulation of movement [5]. This in turn eventually causes the primary clinical motor symptoms such as tremor, rigidity and bradykinesia [3].

PD therapeutic approaches should be of neurorestorative as well as neuroprotective nature to slow down, reverse or even prevent disease progression. However, currently available treatment approaches including pharmacological interventions and neurosurgical procedures (*e.g.* levodopa medication, deep brain stimulation) do not alter disease progression [6, 7]. Currently available treatment only provide symptomatic relief to PD patients and it is clear that these are associated with drawbacks and limitations over time [7–10]. For instance, levodopa medication, the mainstay DA replacement treatment for PD, loses efficacy as disease progresses [8, 10]. Over the past two decades promising therapeutic strategies using gene and cell replacement therapies have emerged. Despite this, research in this field has been facing difficult hurdles and none of these treatment approaches have moved forward towards clinical use yet [11, 12]. Recent advances made in cellular reprogramming technology, however, may become a turning point for gene- and cell-based therapy for PD, bringing these therapeutic approaches back to clinical trials. In this review an overview of current gene- and cell-based therapy strategies for PD is outlined, while considering their potential and limitations. We then take the opportunity to discuss recent progresses made in the use of stem cells and cellular reprogramming and their prospects for the treatment of PD.

2. Past achievements in viral vector-based gene therapy for PD

Gene therapy strategies involve the delivery of therapeutic genes using viral vectors into desired target regions. Currently, viruses including adeno-associated viruses (AAVs) and lentiviruses (LVs) are being used for the delivery of therapeutic genes with a relatively acceptable safety profile. Thus far, three major therapeutic strategies

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Box 1
The dopaminergic system in the brain

Dopamine is one of the major neurotransmitters in the brain that spreads throughout multiple regions via dopaminergic pathways. The two major dopaminergic pathways are the mesocorticolimbic and nigrostriatal pathways, originating from two distinct regions of the midbrain (Fig. 1). The mesocorticolimbic pathway consists of dopaminergic neurons projecting from the ventral tegmental area to the nucleus accumbens and cortical regions. Conversely, the nigrostriatal pathway comprises of dopaminergic projections from the substantia nigra pars compacta to the striatum (caudate-putamen) [4]. Degeneration of the nigrostriatal neurons is the primary pathological hallmark in PD. This leads to impaired dopaminergic signaling to the striatum, a subcortical nucleus part of the basal ganglia circuitry, and motor dysfunction ([3, 5]).

based on the delivery of genes using viruses have been clinically evaluated in PD patients.In this section, these gene therapy strategies are discussed and their potential as future treatments are weighted.

3. Restoration of striatal DA synthesis

DA biosynthesis is mediated by three major enzymes tyrosine hydroxylase (TH), GTP cyclohydrolase-1 (GCH-1) and dopa decarboxylase (DDC; also known as AADC, aromatic amino acid decarboxylase). Both TH and GCH-1 are crucial for the conversion of tyrosine to levodopa of which the latter is subsequently converted to DA by DDC [13]. In PD, progressive degeneration of nigrostriatal DA neurons disrupts DA production and release in the striatum. Therefore, creating a new DA-producing source in the striatum is an appealing strategy to replace lost striatal DA. This has been attempted by expressing genes encoding the enzymes required for DA synthesis in non-degenerating striatal medium spiny neurons (MSNs). The feasibility of this concept is supported by a number of successful preclinical studies [14–20]. Thus far, four phase I clinical studies have assessed the safety and efficacy of bilateral AAV-DDC delivery to the *putamen* in moderate to advanced PD patients. Viral vector-based gene transfer of DDC was well-tolerated and ameliorated motor complications with improved total and part III motor Unified PD Rating Scale (UPDRS; see Box 2/Table 1) score [21–23]. Furthermore, a long-term follow-up study reported sustained transgene expression for over four years [24]. In line with these results, a phase I/II trial confirmed the safety and efficacy of bilateral LV vector-based intraputaminal delivery of DDC, TH and GCH-1 (ProSavin) in moderate to advanced PD patients. ProSavin improved off-medication part III motor UPDRS scores for up to 12 months in all PD patients [25]. Taken together, the data gathered so far support the therapeutic potential of these strategies for the treatment of PD. Despite this, an overestimation of the beneficial effects due to a 'placebo effect' cannot be excluded because of the open-label design of these clinical studies. Pursuit of randomized placebo-controlled clinical studies would be the next step in order to support current positive findings. However, it remains debatable whether non-degenerating striatal MSNs are the right candidate for targeting. Striatal MSNs lack the ability to store DA in vesicles and to regulate its release. In combination with unregulated and sustained transgene expression, this can lead to continuous high cytosolic and extracellular DA levels, which in turn causes intracellular stress that further induces neurodegeneration [26–28]. This raises concerns for the long-term effect of this gene therapy strategy and the use of viruses for continuous gene expression under unregulated conditions.

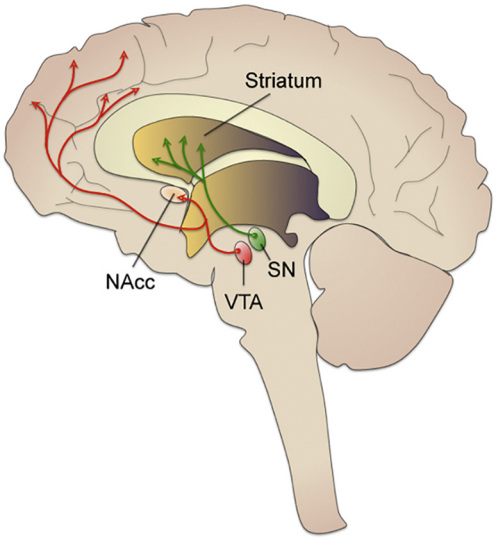

Fig. 1. Sagittal cross-section of the brain showing the two major dopaminergic pathway of the dopamine neuron system. Red arrows: mesocorticolimbic pathway; Green arrows: nigrostriatal pathway (note: the nigrostriatal pathway projects primarily from the *pars compacta* region of the *substantia nigra*). Abbreviations: NAcc = nucleus accumbens; VTA = ventral tegmental area; SN = *substantia nigra*.

4. Modulation of subthalamic nucleus neuronal activity

The basal ganglia circuit comprises of a number of subcortical nuclei including the striatum, the subthalamic nucleus, the *substantia nigra* and the *globus pallidus*. In PD neuronal activity within these subcortical nuclei is altered as described in Box 3 and Fig. 2 [5]. Briefly, striatal DA depletion results in disinhibition of the subthalamic nucleus, subsequently leading to glutamatergic overstimulation of the *globus pallidus interna* and the *substantia nigra pars reticulata* (GPi-SNr), the basal ganglia output nuclei. This further triggers an increased inhibitory drive from GPi-SNr to the thalamus, leading to reduced motor cortex stimulation and subsequently motor impairment. Therefore, controlling neuronal activity within the subthalamic nucleus and its excitatory glutamatergic outflow towards the GPi-SNr could restore lost motor cortex stimulation and improve motor dysfunction in PD. This has been done by viral vector-based gene transfer of glutamate decarboxylase (GAD) into neurons of the subthalamic nucleus. GAD is a key enzyme that exists in two isoforms, GAD65 and GAD67. Both are involved in converting glutamate to GABA (gamma-aminobutyric acid), which are

Box 2
Unified Parkinson's Disease Rating Scale

The Unified Parkinson's Disease Rating Scale (UPDRS) is an extensively used clinical rating scale for Parkinson's disease (PD). In 2007, the Movement Disorder Society published a revised UPDRS (MDS-UPDRS) in effort to overcome the limitations of the original UPDRS [147, 148]. The MDS-UPDRS comprises of four categories (Table 1).

Table 1
MDS-UPDRS.

Categories	Key purpose
Part I Non-Motor Experiences of Daily Living	MDS-UPDRS Part I subscale consists of 13 items designed to assess the presence and severity of non-motor symptoms (*e.g.* sleep, mood disorder, cognitive impairment, apathy, psychosis) in Parkinson's disease patients.
Part II Motor Experiences of Daily Living	MDS-UPDRS part II subscale consists of 13 items designed to assess motor aspects of disability on activities of daily living (*e.g.* speech, eating, handwriting) and quality of life in Parkinson's disease patients.
Part III Motor Examination	MDS-UPDRS part III subscale consists of 18 items designed to assess the presence and severity of motor symptoms (*e.g.* tremor, rigidity) in Parkinson's disease patients.
Part IV Motor Complications	MDS-UPDRS part IV subscale consists of 6 items designed to assess the presence and severity of motor complications related to treatment (*e.g.* motor fluctuations, dyskinesias)

Each part consists of a number of items (*i.e.* questions, clinical assessments) which are scored using a 0–4 rating scale, where 0 = normal, 1 = slight, 2 = mild, 3 = moderate, and 4 = severe.

Box 3
Pathophysiology of motor dysfunction in Parkinson's disease

The striatum is innervated by dopaminergic and glutamatergic neurons of the *substantia nigra pars compacta* and the cortex, respectively. Excitatory glutamatergic input from the cortex results in the stimulation of striatal medium spiny neurons. These are neurons of which neurotransmission involves the release of the primary inhibitory neurotransmitter GABA (gamma-aminobutyric acid). Striatal medium spiny neurons are found in two types that project to different downstream targets, giving rise to the direct and indirect pathway of the basal ganglia. The direct pathway comprises of striatal medium spiny neurons projecting to the main output nuclei *globus pallidus interna* and *substantia nigra pars reticulata* (GPi-SNr). Conversely, in the indirect pathway striatal medium spiny neurons project to the *globus pallidus externa*. Activity of the direct and indirect pathway is modulated by dopaminergic signaling from the *substantia nigra pars compacta*. Importantly, dopamine facilitates and inhibits neuronal activity in the direct and indirect pathway, respectively (Fig. 1) [5].

the primary excitatory and inhibitory neurotransmitters in the brain. Early preclinical studies have demonstrated that AAV-GAD delivery to the subthalamic nucleus facilitated and reduced its local GABAergic neurotransmission and excitatory outflow towards the GPi-SNr, respectively [29, 30]. Following these encouraging preclinical results, two clinical studies have been completed so far. In an open-label phase I study unilateral delivery of AAV-GAD to the subthalamic nucleus in advanced PD patients was well tolerated with improvements in part III motor UPDRS score [31]. A subsequent randomized double-blinded and sham-controlled phase II trial evaluated the therapeutic value of bilateral gene delivery of AAV-GAD but only modest improvement in off-medication part III motor UPDRS score was reported from baseline up to 6 months [32]. Overall, current clinical findings are positive and support the safety of AAV-GAD therapy for PD but the clinical effect found was only modest. We have to take into consideration that this therapeutic strategy solely modulates neuronal activity in the subthalamic nucleus and its downstream targets to a certain extent, while nigrostriatal DA neurons progressively degenerate over time. This illustrates that delivering genes for GAD to the subthalamic nucleus may only provide a modest way to improve motor function in PD.

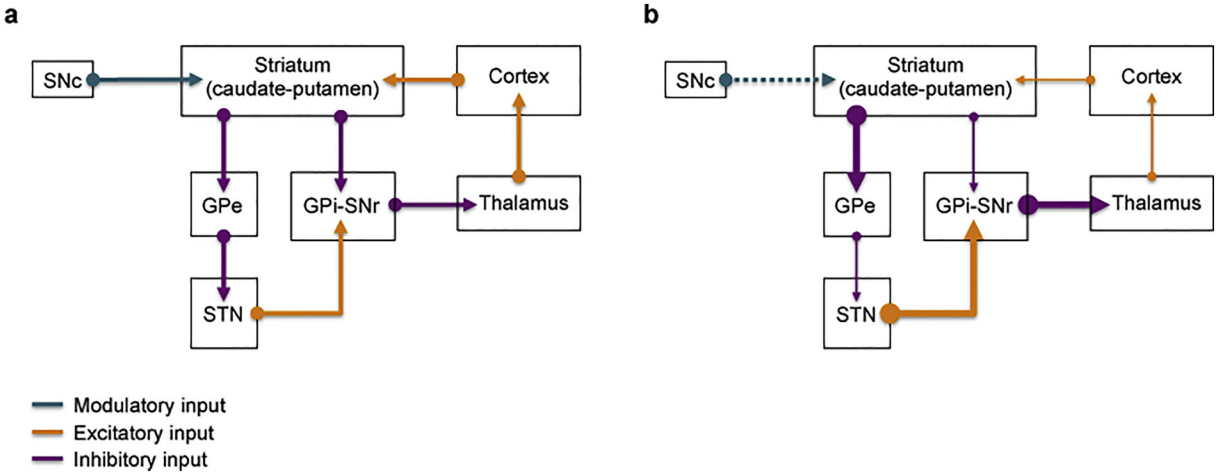

Fig. 2. The basal ganglia circuitry. (a) Under physiological conditions the indirect pathway complements the direct pathway. Activation of striatal medium spiny neurons of the direct pathway suppresses GPi-SNr activity. This in turn reduces the inhibitory outflow from the GPi-SNr to the thalamus, leading to motor cortex stimulation. At the same time, activation of the indirect pathway stimulates neuronal activity within the GPe, leading to the inhibition of the STN. This results in reduced excitatory glutamatergic transmission from the STN to the GPi-SNr, leading to decreased neuronal activity in the GPi-SNr. (b) In PD, progressive degeneration of dopaminergic neurons in the SNc results in striatal dopamine depletion and subsequently loss of the modulatory function of dopamine on the basal ganglia circuitry. Activity of striatal medium spiny neuron is reduced in the direct pathway, while increased in the indirect pathway. The latter causes overstimulation of the GPi-SNr, following increased inhibition of the GPe and disinhibition of the STN. This enhances inhibition of the thalamus, leading to reduced motor cortex stimulation and therefore motor dysfunction. Abbreviations: SNc = *substantia nigra pars compacta*; GPe = *globus pallidus externa*; GPi-SNr = *globus pallidus interna* and *substantia nigra pars reticulata*; STN = subthalamic nucleus.

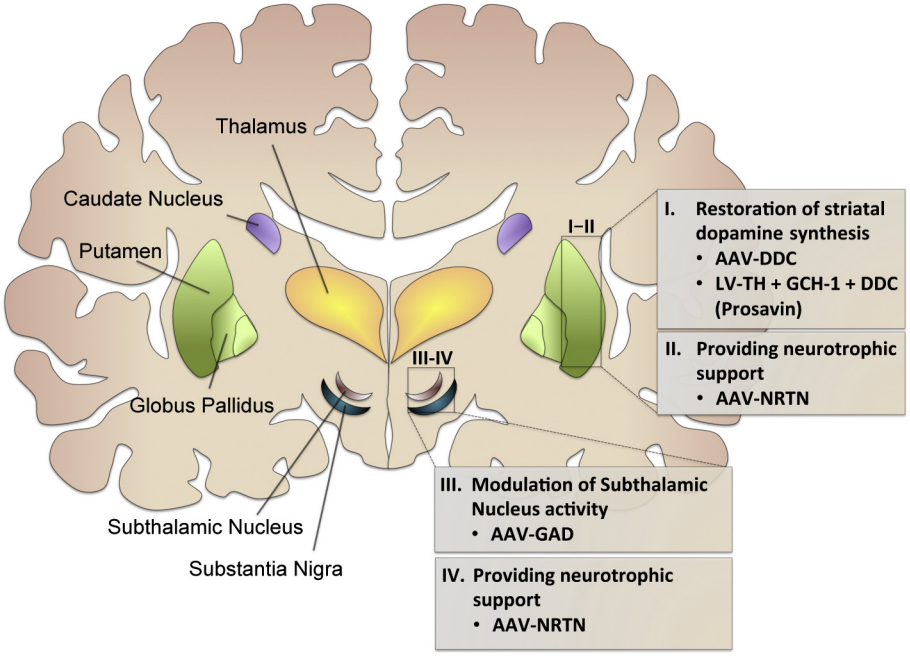

Fig. 3. Current gene therapy strategies for the treatment of PD. Gene therapy strategies to date use viral vectors for target-specific delivery and continuous expression of therapeutic genes. They intend to (I) restore striatal dopamine synthesis, (II and IV) provide neurotrophic support to promote neuronal survival at sites of neurodegeneration or (III) modulate excitatory neuronal activity in the subthalamic nucleus (STN). Abbreviations: AAV = adeno-associated virus; LV = lentivirus; DDC = dopa decarboxylase; TH = tyrosine hydroxylase; GCH-1 = GTP cyclohydrolase-1; GAD = glutamate decarboxylase; NRTN = neurturin.

5. Providing neurotrophic support

Modulating neurodegenerative process in PD is a key strategy to alter disease progression. Neurotrophic factors including glial-cell-line-derived neurotrophic factor (GDNF) and neurturin (NRTN) are pivotal in promoting survival of DA neurons [33]. Based on our current understanding of the pathology of PD, neuroinflammation is associated with loss of neurotrophic support [2]. Therefore, restoring neurotrophic support is an appealing approach to protect and preserve degenerating nigrostriatal DA neurons. Supported by promising results from preclinical studies [34–37], four human clinical trials have now assessed the therapeutic value of viral vector-based gene delivery of NRTN. In an open-label phase I study, bilateral intraputaminal delivery of AAV-NRTN in advanced PD patients improved motor function with reduced off-medication part III motor UPDRS score at 12 months [38]. In contrast to this, a following double-blinded and sham-controlled phase II study, only found substantial improvement after 18 months according to off-mediation part III motor UPDRS scores. Results imply that the delayed clinical effect could be due to defects in axonal transport of the therapeutic gene from the *putamen* to the *substantia nigra* as a consequence of extensive neurodegeneration of the nigrostriatal pathway [39]. Two subsequent clinical studies evaluated the safety and efficacy of AAV-NRTN gene delivery to both the *putamen* and the *substantia nigra* in advanced PD patients [40, 41]. Remarkably, contrary to earlier observation, no significant improvement in motor function was observed in the treatment group in comparison to the control group [40, 41]. These findings suggest that the clinical effects of this gene therapy strategy depends on the extent of degeneration of nigrostriatal DA neurons. Future clinical studies may therefore require strict patient selection in which for instance only 'early stage' PD patients can participate. Nevertheless, PD is often only diagnosed after the occurrence of first motor symptoms when more than half of the nigrostriatal DA neurons are already lost. This underlines the need to identify markers that would allow early diagnosis, as neuroprotective effect may not work in patients with extensively degenerated nigrostriatal pathway. Despite this, it has been stressed that uncontrolled and continuous overexpression of neurotrophic factors could be detrimental resulting in excessive sprouting of DA fibers into other areas such as the *globus pallidus* and the *substantia nigra reticulata* [42–44]. Current gene therapy strategies use viruses to promote long-term gene expression but under unregulated conditions. Alternative delivery strategies including direct neurotrophic factor protein delivery may overcome current limitations of viral vector-based gene therapy. Earlier attempts with direct infusion of recombinant GDNF protein using pumps have achieved little clinical success [45–47] but new drug delivery systems such as intranasal delivery may open a new opportunity for direct and non-invasive delivery of biomolecules including neurotrophic factors [48, 49].

In summary, to date, three viral vector-based gene-based therapeutic strategies have been pursued for PD (Fig. 3) showing a potentially clinical benefit for PD patients. Nevertheless, many open points need to be tackled before gene therapy could be used as alternative treatment for PD. Some of the most crucial points are linked to the use of viral vectors to deliver genes under unregulated conditions.

An alternative therapeutic approach that could overcome gene therapy drawbacks is represented by cell therapy. PD cell-based DA replacement is based on the transplantation of a DA-producing source in the striatum. Several potential DA cell sources have been investigated including human fetal ventral mesencephalic (fVM) tissues (Fig. 4a) [11].

6. Past achievements in cell-based DA replacement therapy for PD

Encouraging results from preclinical animal studies and a series of open-label human studies supported the therapeutic potential of fVM tissue transplantation for PD [11, 50, 51]. However, the prospects for cell-based DA replacement therapy using fVM tissues has dimmed following two randomized sham-controlled phase II clinical trials [52, 53]. Remarkably, in both studies no significant improvement in motor symptoms was observed in PD patients after fVM tissue transplantation in the *putamen*, while significant axonal outgrowth from the graft and increased DA uptake in the striatum was reported after post-mortem examination. Furthermore, patients who received fVM tissue transplantation frequently experienced adverse effects including dyskinesia, which is now referred to as graft-induced dyskinesia (GID) [52–54]. It is now proposed that the occurrence of GID, which may have contributed to the lack of efficacy of fVM tissue transplantation, is attributed to several factors. These factors include imbalanced DA release from the graft [54], pre-existing levodopa-induced dyskinesia [55, 56], placement and capability of graft to reinnervate the target area [57, 58], immunological rejection of graft due to discontinuation of immunosuppressant therapy [52, 53, 59] and the heterogeneity of the graft [60, 61]. Furthermore, it appears that disease progression is a crucial factor as well. Indeed, clinical effect was reported for a subset of younger patients with less severe PD who had no levodopa-induced dyskinesia [52, 53]. However, whether fVM tissue transplantation is a

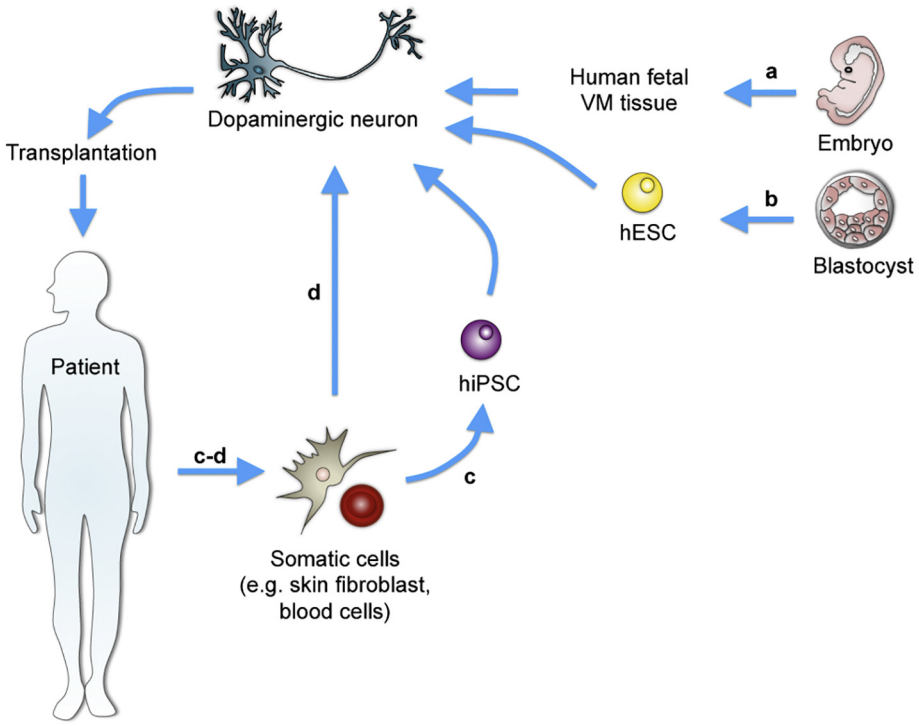

Fig. 4. Sources of dopaminergic neurons for replacement therapy in PD. (a) Human fetal VM have been used as dopaminergic source in transplanted PD patients. (b) hESC are isolated from blastocysts and can be differentiated into functional dopaminergic neurons. (c-d) Patient-specific somatic cells can be (c) reprogrammed into hiPSC and differentiated into dopaminergic neurons or (d) can be directly converted into dopaminergic neurons through direct cell lineage reprogramming. Abbreviation: VM = ventral mesencephalic; hESC = human embryonic stem cell; hiPSC = human induced pluripotent stem cell.

compatible source for cell-based DA replacement therapy in PD is debatable as tissue availability and ethical issues remain an ongoing problem [62]. Recent progress in stem cell technology and cellular reprogramming could overcome at least the issues linked to the limited source of human DA neurons and to the immunological compatibility.

7. DA neurons derived from pluripotent stem cells

The first step towards stem cell-based DA replacement therapy for PD was made after functional midbrain DA neurons were successfully differentiated from mouse embryonic stem cells (ESCs) [63]. Upon transplantation in the striatum of parkinsonian rats, midbrain-specific mouse ESCs-derived DA neurons showed good survival and this was linked to improvement in behavioral motor deficits [64]. Based on this, others have attempted to generate midbrain-specific DA neurons from human ESCs by co-culturing strategies with either stromal feeder cells [65–68] or astrocytes [69]. At first, these approaches successfully produced TH-positive neurons *in vitro* [66–71]. Remarkably, *in vivo* transplantation of these neurons in the striatum of parkinsonian animals have yielded inconsistent results with some experiments resulting in improvement in motor function [67, 69], while others did not [65, 68, 71]. Negative findings were later reported to be linked to poor *in vivo* survival of the cell transplant and 'unstable' human ESC-derived DA neurons with incorrect phenotypic characteristics [65, 68, 71] Furthermore, human ESC-derived cell population contained undifferentiated pluripotent cells, which led to formation of tumors [65, 67, 69]. This underlines a significant safety issue surrounding the use of human ESC-derived DA neurons.

A few years later a new procedure described the successful derivation of stable and functional midbrain-specific DA neurons from human ESCs further exploiting developmental principles (Box 4) [72–75]. In contrast to previous studies, *in vivo* transplantation of these neurons resulted in significant motor improvement without tumor formation as demonstrated in toxin-based animal models of PD [74, 75]. Recent preclinical studies further support the therapeutic potential of human ESC-derived DA neurons as an alterative source for cell replacement therapy for PD [76, 77]. Currently, teams from USA, Europe and Japan (G-Force PD, http://www.gforce-pd.com/) are moving forward to use human ESC-derived DA neurons in new clinical trials for cell-based DA replacement therapy in PD. Several steps need to be taken before understanding the value of this therapeutic approach as widely used clinical solution.To date, G-Force PD teams are focusing on several

Box 4

Current concept in the generation of dopaminergic neurons *in vitro*

Protocols describing the differentiation of dopaminergic neurons from pluripotent stem cells are based on principles derived from embryonic development of DA neurons in mice. First differentiation protocols involved the use of stromal feeder cells or astrocytes and the activation of genes for sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF-8) [65–69]. Remarkably, although TH-positive neurons were generated, they lacked expression of crucial DA neural markers for FOXA2 and LMX1A and showed poor survival upon *in vivo* transplantation [65–71] It is now well accepted that midbrain DA neurons arise from floor plate cells and their further development is regulated by WNT, SHH and FGF signaling pathways [72, 73]. Pluripotent stem cells can be induced to become neural floor plate cells through the dual inhibition of the SMAD signaling pathway at the same time under activation of SHH. Following this, activation of WNT signaling pathway through treatment with GSK3B (glycogen synthase kinase-3 beta) inhibitors facilitate conversion of floor plate cells to dopaminergic neural cell fate. Further differentiation yields stable and functional TH-positive dopaminergic neurons expressing neural markers for FOXA2 and LMX1A [75]. Based on this, similar protocols have been developed by others, resulting also in dopaminergic neurons of correct phenotype [74, 82, 149, 150].

Table 2
Current direct reprogramming approaches to convert somatic cells to functional dopaminergic neurons.

Reference(s)	Cell source	Transcription factor cocktail
Caiazzo et al. [99]	Mouse and human fibroblasts	*Ascl1, Nurr1, Lmx1a*
Kim et al. [100]	Mouse fibroblasts	*Ascl1, Nurr1, Lmx1a, Pitx3*
Pfisterer et al. [102]	Human fibroblasts	*Ascl1, Foxa2, Lmx1a, Brn2, Myt1l*
Liu et al. [101]	Human fibroblasts	*Ascl1, Nurr1, Pitx3, Ngn2, Sox2*
Addis et al. [98]	Mouse Astrocytes	*Ascl1, Nurr1, Lmx1b*
Jiang et al. [103]	Human fibroblasts	*Ascl1, Nurr1, Lmx1a, mir-124*
Rivetti di Val Cervo et al. [111]	Mouse astrocytes (*in vivo*)	*Ascl1, Neurod1, Lmx1a, mir-218*

aspects linked to the definition of a proper immunosuppressive regime, patient selection parameters, patient assessment protocols and trial design. Hopefully, the results of these combined efforts would lead to start clinical trials within 2020 and therefore clinical readouts few years after, [78, 79] (Fig. 4b). However, the use of ESCs still remains an issue of ongoing ethical debate [62] but cell reprogramming technologies couldhelp to overcome this difficulty (Fig. 4c).

The feasibility to promote pluripotency in somatic cells with cellular reprogramming was first reported a 12 years ago [80, 81]. These reprogrammed cells known as induced pluripotent stem cells (iPSCs) share comparable features with ESCs but they are derived from patient-specific somatic cells, eventually overcoming immunological graft rejection upon transplantation and ethical issues. Successful generation of DA neurons from iPSCs and their functional survival as well as therapeutic potential in preclinical models of PD have been reported [82–87]. However, the report of tumor formation upon *in vivo* transplantation of iPSC-derived DA neurons still raises some questions regarding the proposed safety [87].

In order to induce pluripotency in somatic cells, forced overexpression of transcription factors including *Oct3/4*, *Sox2*, *c-Myc* and *Klf4* is required. These are delivered using retroviral or LV vectors which are capable of random integration into the host genome, potentially leading to alteration in genomic structure and unregulated transgenes expression [80, 81]. It has been reported that reactivation of these transgenes, in particular the oncogene *c-Myc*, induced tumor formation [88, 89]. This stresses the need for alternative strategies to transduce transcription factors. A number of new strategies to reprogram somatic cells have been implemented in the last years. These strategies use non-integrating adenoviral vectors [90, 91], direct protein-based delivery [92, 93], DNA- or RNA-based delivery [94–96], or Cre-recombinase-mediated excision of transgenes for cell reprogramming [97].

Another factor that triggers tumor formation is the presence of undifferentiated pluripotent cells in the generated cell populations [85, 93]. These could lead to continuous and extensive outgrowth of the graft upon transplantation. Considerable progress has been made to remove undifferentiated pluripotent cells and other contaminants and enrich iPSC-derived DA neurons using cell sorting strategies including magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) [82, 86]. Thus far, cell sorting using neural markers such as $CORIN^+$ or $NCAM^+/CD29^{low}$ have reduced the number of contaminants and increased the number of TH-positive and midbrain-specific DA neurons in iPSC-derived cell populations [82, 86]. Further optimization of these approaches could refine clinical safety issues surrounding the use of iPSCs as a new cell source for cell replacement therapy for PD (Fig. 3c). However, nowadays, other reprogramming approaches based on direct lineage conversion are also available to generate DA neurons without supporting the risk of tumor generation (Fig. 4d).

8. DA neurons derived from direct cell lineage conversion of somatic cells

Direct cell lineage conversion (also referred to as transdifferentiation) involves fast and direct conversion of somatic cells into functional neurons including midbrain-specific DA neurons skipping any intermediate pluripotent stage. This cellular reprogramming strategy involves viral vector-based delivery of defined combination of transcription factors essential for the direct generation of midbrain DA-specific characteristics in somatic cells (Table 2) [98–103]. Through this way, functional DA neurons with correct phenotype, currently referred to as induced DA (iDA) neurons, were directly derived from somatic cells. iDA neurons share comparable morphological features with resident brain DA neurons and are also capable of producing as well as releasing DA [99, 101, 104].

In vivo transplantation of iDA neurons in the striatum of parkinsonian rats reduced drug-induced rotational motor behavior and were reported to survive and innervate the targeted area [100, 105]. Moreover, Dell'Anno and colleagues showed the *in vivo* functionality of iDA neurons and further proposed the benefits of using the pharmacogenetic tool Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to advance functional cellular control [105]. DREADDs are engineered G-protein coupled receptors activated by small pharmacological ligands (*e.g.* clozapine-N-oxide (CNO)) that provide reversible and non-invasive manipulation of neuronal activity [106, 107]. The use of DREADDs in iDA neurons provides a novel and efficient way to modulate these neurons for the fine-tuning of their therapeutic efficiency (*i.e.* release of DA) upon transplantation.

The prospect of iDA neurons for cell-based DA replacement therapy are thus far promising. Several issues surrounding the use of pluripotent stem cells including ethical problems, immunological rejection of grafts and potential tumor risk are avoided with direct cell lineage conversion. Although reprogramming strategy still uses viral vectors capable of random genomic integration, alternative transcription factor delivery approaches have been proposed as mentioned earlier. Despite this, current direct conversion strategy using human somatic cells is hindered by low conversion efficiency, implying limitations in scalability which needs to be addressed before translation to therapy [99, 101, 102]. However, advances are being made in improving efficiency of direct conversion [103, 104, 108].A very promising advance in cell reprogramming technology is represented by the implementation of microRNA (miRNA). The first evidence was provided by using the well-known neural miRNAs mir-9/9* and mir-124 to improve the transdifferentiation of fibroblasts into functional neurons [108, 109]. Very interestingly, a recent work showed that mir-34b/c is able to increase the efficiency of transdifferentiation from fibroblasts to induced DA neurons [110] and mir-218 has been proven to improve *in vivo* DA transdifferentiation [111].

These recent advances further contribute to envision a future in

which direct conversion of human somatic cells into compatible DA neurons could provide a safe and unlimited patient-specific source for PD cell-based DA replacement therapy (Fig. 4d).

Taken together, advances made in the use of stem cells and cellular reprogramming opened a new avenue for cell therapy in PD by introducing the possibility of differentiating pluripotent stem cells or somatic cells functional DA neurons. Intriguingly, cell reprogramming technologies are quickly improving in the ability to generate glial neural cell types with neuromodulatory action such as astrocytes and microglia, opening the way for new therapeutic strategies for PD.

9. Glial cells as new key players in cell reprogramming approaches for cell-based gene therapy for PD

Glial cells are the most abundant cell type in the brain and are represented by microglia, astrocytes and oligodendrocytes. Microglia and astrocytes are multifaceted and could be either of neuroprotective or neurodegenerative nature depending on their state of activation. In PD, dysregulated microglia and astrocytes activation enhance neurodegeneration. This is associated with the release of pro-inflammatory and neurotoxic factors as well as loss of beneficial neuroprotective function [112]. Therefore, providing and modulating lost glial cell function is an attractive therapeutic approach as will be outlined below. At the same time, new strategies are arising from the combination of gene- and cell-based therapy such as cell *in vivo* direct reprogramming of resident astrocytes [113].

10. Prospects of microglial cell-based gene therapy for PD

Microglia are resident macrophages of the brain and they play a crucial role in mediating immunological and homeostatic processes by tightly surveying the extracellular environment for pathogenic elements or tissue injury [112]. Under pathological conditions, albeit due to inflammation or tissue damage, microglia are activated and can enter two different phenotypic states defined as M1 and M2. Importantly, microglia of M1 phenotype promote inflammation by releasing pro-inflammatory mediators and cytotoxic factors, while M2 microglia suppress inflammatory response and facilitate tissue repair with the release of anti-inflammatory and trophic factors. Emerging evidence indicate microglia are primarily in their M2 state playing a beneficial role during early stages of inflammation, while over time they switch to a M1 phenotype inducing inflammation [114, 115]. Normally, inflammation ceases upon homeostasis is reached, however, in PD, inflammatory response is uncontrolled with the presence of reactive microglia [116], indicating microgliosis. The continuous production of pro-inflammatory and cytotoxic factors from reactive microglia is detrimental to DA neurons. Subsequently, degenerated DA neurons release aggregated and misfolded proteins as well as reactive oxygen species, which in turn further promote inflammation and drive the vicious cycle of neurodegeneration [112, 115]. Thus, sustained neuroinflammation favoring the activation of pro-inflammatory microglia trigger neurodegenerative process.

An appealing therapeutic strategy could consider to compensate for dysfunctional microglia. Earlier studies revealed that systemically infused bone marrow stem cells (BMSCs) give rise to microglia in the brain. BMSC-derived microglia precursors are recruited and travel to sites of inflammation and neurodegeneration in which they subsequently differentiate into microglia [117, 118]. Many have used this cellular migration and differentiation mechanism to their advantage [119]. For PD, BMSC microglia precursors have been genetically modified *ex vivo* to produce neurotrophic factors and systemic delivery of these engineered BMSCs in parkinsonian mice resulted in preservation of functional nigrostriatal DA neurons with no significant motor dysfunction [120–122]. This experiments support the use of BMSC-derived microglia precursors for cell-based gene therapy for PD. However, it has to be taken in consideration that microglia derives from the embryonic yolk sac leaving doubts on whether BMSC-derived microglia could be defined as 'true microglia'. Moreover, it is uncertain to what extent BMSC-derived microglia contribute to and integrate into the resident microglia population in the brain [117].

In light of this, attempts have been made to generate a new source of microglia from pluripotent stem cells. Only recently microglia cells were successfully derived from yolk sac progenitor cells using human ESCs and iPSCs [123]. This new source of microglia shares similar genetic and molecular patterns with 'true microglia'. Although PSC-derived microglia are primarily of great interest for disease modeling or therapeutic drug screening, they could also be employed as cellular delivery systems for therapeutic genes. In this context, it is of particular interest to use patient-specific microglia derived iPSCs. These could be genetically modified to produce trophic factors or anti-oxidants and compensate for dysfunctional microglia upon transplantation. However, a remaining major drawback of using iPSCs is the risk of tumor formation but implementation of alternative reprogramming approaches and cell sorting may reduce this risk. Future developments in direct cell lineage reprogramming could be an alternative approach but this remains unexplored to date.

11. Prospects of astroglial cell-based gene therapy for PD

Several lines of evidence suggest that astrocytes are involved in neuroinflammation in neurodegenerative diseases [124]. Astrocytes are multifunctional glial cells involved in the regulation of brain homeostasis. They play a pivotal role in supporting neuronal survival and tissue repair by releasing trophic factors and mediating intracellular oxidative stress through the secretion of anti-oxidants. Additionally, astrocytes are capable of uptaking and degrading toxic molecules in the extracellular environment and are vital for ion and nutrient metabolism [124]. Under pathological conditions, astrocytes become reactive and in PD, astrocytes switch to their reactive state once the balance between uptake and degradation of toxic molecules (*e.g.* α-synuclein) is disrupted [125]. Astrocytes are also activated by pro-inflammatory cytokines released from reactive microglia [126]. Reactive astrocytes in turn release pro-inflammatory mediators and reactive oxygen species of which further amplify neuroinflammation and contribute to the vicious cycle of neurodegeneration [112, 125, 126].

Emerging evidence indicates that beneficial astroglial function in providing trophic support and mediating intracellular stress against toxic molecules is reduced during astrogliosis in PD [124, 127]. Therefore, as proposed earlier for microglia, compensating dysfunctional astrocytes is an appealing therapeutic option. Currently functional astrocytes can be generated from embryonic glial restricted precursor cells (GRPCs) [128] and human pluripotent stem cells [129, 130]. Embryonic GRPC-derived astrocytes are capable of releasing trophic factors and anti-oxidants and *in vivo* transplantation in the striatum of parkinsonian rats improved behavioral deficits and restored TH expression [128]. In a similar context, it can be envisioned to genetically modify human PSC-derived astrocytes in order to produce anti-oxidative agents or trophic factors.

Recently, direct cellular lineage conversion has allowed the differentiation of somatic cells into functional induced astrocytes (iAstrocytes) with functional and phenotypic features that are comparable to resident brain astrocytes [131]. Very interestingly, a recent report showed that fibroblasts can be directly converted into astrocytes by treatment with small compounds [132]. Although the therapeutic potential of iAstrocytes for glial cell-based gene therapy remains to be explored *in vivo*, they are of clinical interest as they could contribute as

safe and unlimited patient-specific source to glial cell-based therapy. Recently astrocytes have been proposed also as a potential *in vivo* target for neuronal generation by direct conversion. Direct *in vivo* lineage reprogramming involves the transdifferentiation of resident cells into other cell types through forced gene expression *in vivo*. Thus far, in the brain, functional subtypes of neurons have been directly generated *in vivo* from NG2$^+$ glial cells (oligodendrocyte progenitor cells), astrocytes and other types of neurons [133–140]. For PD, *in vivo* cell reprogramming has great therapeutic potential as it offer the possibility to restore nigrostriatal DA neurons by converting resident cells involved in the neurodegenerative process such as reactive glial cells. Successful reprogramming of these glial cells would have neurorestorative effect, while reducing the presence of reactive glial cells and at the same time altering disease progression. Astrocytes have efficiently been reprogrammed to midbrain DA neurons before *in vitro* [98], but to which extent this *in vitro* cell reprogramming approach can be expanded to *in vivo* reprogramming remains to be determined. *In vivo* reprogramming of microglia has not been done yet and most of the attempts to convert *in vivo* resident astrocytes did not give rise to DA neurons [134, 136, 139]. Very interestingly a recent work showed that mouse brain astrocytes can be converted *in vivo* into DA neurons also providing the first proof-of-principle evidence that this approach can be used to treat a PD animal model [111]. Nevertheless, as research in the field of direct *in vivo* reprogramming is still in its early stages, future developments may soon overcome current challenges to move this approach towards human applications (Fig. 5e).

Taken together, recent advances in cell reprogramming approaches offer a role for glial cells in new treatment strategies for PD (Fig. 5a-c). This opens a new avenue of therapies beyond cell-based DA replacement. As proposed before, *in vitro* reprogrammed microglia and astrocytes could be employed to compensate for dysfunctional glial cells in PD in addition to their role in other biomedical purposes such as disease modeling. This approach would consider to genetically modify *ex-vivo* iAstrocytes in order to secrete anti-oxidants and neurotrophic factors, that would be released, upon brain transplantation (Fig. 5d). Current advances in pharmacogenetics would facilitate the remote modulation of these cells and their therapeutic efficacy upon transplantation using DREADDs [141]. This has previously been demonstrated with *in vivo* transplanted iDA neurons in a rat model of PD [105] and its use could be expanded to astrocytes and microglia. DREADDs have already been used before to selectively and temporarily modulate cellular function of astrocytes and microglia *in vivo* [142, 143], supporting the feasibility to apply this approach (Fig. 5d).

12. Concluding remarks and future perspectives

Current PD treatment approaches primarily address motor dysfunction but are associated with significant drawbacks or decline in effectiveness over time. Over the past decades the focus of attention has been also pointed towards gene therapy strategies. Viral vector-based delivery of therapeutic genes became of great interests as it provides target-specificity as well as long-term transgene expression. Very interestingly viral delivery technology *via* AAVs is fastly improving as shown by the discovery of newly generated AAV9 serotypes with high specificity for the CNS that could allow systemic delivery for PD treatments [144–146].

On the other hand the successes in cellular reprogramming have supported the idea of patient-specific DA neurons directly from somatic cells. However, further developments are necessary to improve reprogramming efficiency of human somatic cells before translation in cell-based DA replacement therapy for PD. Importantly, we have to take into consideration that altering disease progression is of primary interest and that replacement of striatal DA alone does not necessarily have neurorestorative effect. Therefore, future research in this area

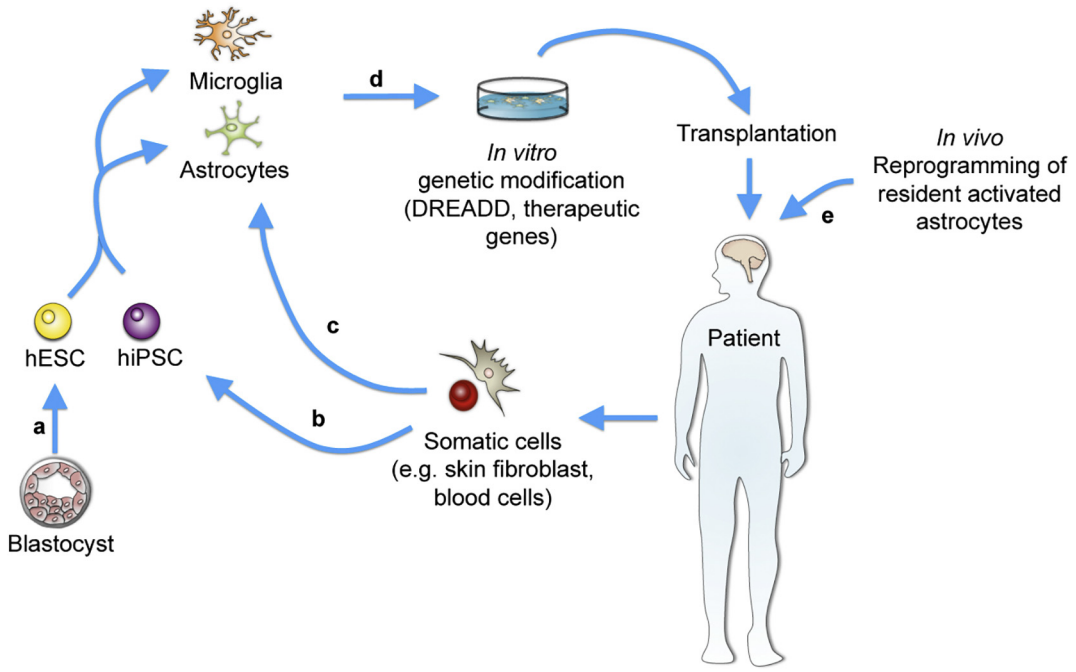

Fig. 5. Microglia and astrocytes as new key players in cell reprogramming approaches for cell-based gene therapy for PD. Reprogrammed microglia and astrocytes can be obtained from (a) hESCs or (b) hiPSCs. (c) An alternative approach to generate reprogrammed astrocytes is *via* direct *in vitro* reprogramming of somatic cells. (d) Reprogrammed glial cells can then be genetically modified *in vitro* to express therapeutic genes of interest (*e.g.* neurotrophic factors, anti-oxidants). Upon transplantation they can compensate for dysfunctional glial cells in PD. Furthermore, inducing the expression of DREADDs at the same time would allow the fine-tuning and remote control of activity and therapeutic effect of these reprogrammed glial cells upon transplantation. (e) Direct reprogramming of resident brain cells *in vivo* could be used to restore dopaminergic neurons at sites of degeneration without the need for transplantation. Resident (activated) glial cells are potential candidates for this therapeutic strategy. Abbreviations: hESC = human embryonic stem cell; hiPSC = human induced pluripotent stem cell.

should focus on how iDA neurons could be used as cell-based treatment for the regeneration of the nigrostriatal pathway in PD patients.

In order to alter disease progression, new therapies should also provide neuroprotection, pointing towards strategies to target pathogenic mechanisms that enhance neurodegeneration such as neuroinflammation. Dysfunctioning microglia and astrocytes remains an unresolved problem and contributes to the vicious cycle of neurodegeneration. With this in mind, we outlined potential cell reprogramming approaches for cell-based gene therapy for PD with glial cells as new key players. Another exciting perspective involving glial cells is the *in vivo* cell reprogramming to convert reactive microglia and astrocytes into functional DA neurons for neurorestorative purposes.

Overall, several efforts are currently put into the development of new PD therapies using our current knowledge in the use of stem cells and cell reprogramming to provide new symptomatic treatments and disease modifying cures to slow down, reverse or even prevent disease progression in PD patients.

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