Cell-Based Therapy and Genome Editing in Parkinson's Disease: Quo Vadis?

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3.1 Neurodegenerative Diseases: Urgent Need for Cell-Based Therapies

The incidence of neurodegenerative diseases is steadily increasing due to aging societies worldwide. Age-related neurodegenerative processes are hallmarked by a progressive loss of selectively vulnerable neural cells in the central nervous system (CNS). The most frequent neurodegenerative diseases are amyloid-, tau-, or synuclein-associated clinical entities defined by the pathological aggregation of the respective protein.[1] The broad spectrum of symptoms is mainly defined by specific CNS regions affected the most by neuronal dysfunction and consequent cell loss due to the continuous aggregation and spread of distinct protein species. The symptoms consist of a variable range of cognitive, motor, or neuropsychiatric

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[1] Dugger and Dickson (2017)

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deficits predominantly linked to distinct susceptible neurons and its corresponding neurotransmitter systems.[2,3] The majority of currently used symptomatic therapies aim to substitute or compensate the deficit of specific neurotransmitter systems in order to improve the clinical phenotype. However, besides causing adverse side effects in the long-term, previous studies showed that neurotransmitter-based symptomatic therapeutic approaches are not able to slow down, halt, or even reverse disease progression in these disorders.[4,5] Furthermore, the progressive dysfunction and loss of neurons have a tremendous impact on quality of life measures. Although the CNS maintains a pool of neural stem cells in some niches such as the hippocampus, these cells are not able to repopulate or even compensate the loss of neurons observed in age-related neurodegenerative diseases.[6] Almost a half century ago, the foundation to replace diseased neural cells by grafting neural cells into defined CNS regions has been laid by a group of scientists in Sweden.[7,8,9] Since the pharmacological substitution of neurotransmitters appeared promising to some degree, the idea to transplant specific neural cells secreting the respective neurotransmitter was considered as a promising long-lasting therapy to intervene in the course of these devastating neurodegenerative diseases. After the failure of randomized clinical trials grafting fetal dopaminergic cells in Parkinson's disease (PD), the development of technologies such as the generation of human-induced pluripotent stem cells (hiPSCs) and human cerebral organoids opened up new possibilities with respect to a revival for cell-based therapeutic approaches for the CNS.[10,11] Currently, therapeutic cell-based approaches are exclusively using cellular suspensions of hiPSC-derived neural cells. Up to now, the application of brain organoids into certain brain regions is limited due to the lack of a safe approach applying these macroscopic cell clusters. The transplantation of brain organoids might further damage the anatomical site of grafting due to the needle size required for the transplantation of an organoid. Therefore, with currently available protocols, brain organoids are rather suitable for preclinical disease modeling or testing of pharmacological compounds. The following chapter will summarize these cellular and molecular breakthroughs focusing on PD, the prototypical and most prevalent synucleinopathy. Furthermore, we will reflect and discuss very recent molecular gene editing advancements in integrating these innovative therapeutic strategies toward regenerative medicine.

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[2] Pereira, Ferreiro, Cardoso, & de Oliveira (CR732004), p. 97

[3] Rinne (1993), p. 31

[4] Heumann et al. (2014), p. 472

[5] Sharma (2019), p. 1479

[6] Gage (2000), p. 1433

[7] Olson and Seiger (1972), p. 175

[8] Olson and Seiger (1975), p. 141

[9] Seiger and Olson (1975), p. 325

[10] Lancaster and Knoblich (2014), p. 2329

[11] Takahashi et al. (2007), p. 861

3.2   Parkinson's Disease: Pathophysiology and Diagnosis

PD belongs to the group of synucleinopathies. These disorders are defined as a spectrum of age-related neurodegenerative disorders commonly characterized by an abnormal aggregation of the intracellular presynaptic protein alpha-synuclein (aSyn). The progressive aggregation of aSyn in PD results in the deposition of aSyn in the cytoplasm of neurons (Lewy bodies) and/or neurites (Lewy neurites[12];). In 85–90%, PD patients are affected sporadically with a late onset usually during the sixth decade of life. Besides sporadic PD, 10–15% of PD cases are linked to mutations in specific genes known as PARK loci. These loci harbor different types of mutations including multiplications of the entire gene locus of aSyn, the SNCA gene.[13] Monogenic forms of PD are characterized by an earlier onset of motor symptoms and in some instances associated with severe cognitive or other psychiatric deficits in comparison to sporadic PD.[14,15,16,17,18,19] Clinically, sporadic PD is hallmarked by cardinal motor symptoms such as bradykinesia, rigidity, and resting tremor.[20] The presence of these symptoms is primarily linked to the progressive loss of dopaminergic neurons within the substantia nigra pars compacta of the midbrain.[21] Diagnosing PD remains challenging in the clinical routine and is still based on the presence of the above-mentioned clinical symptoms; however the definitive diagnosis requires the demonstration of Lewy bodies in post mortem neuropathological examinations.

3.3   Current Therapies

Current pharmacological therapies for PD-related motor deficits consist of dopaminergic partial replacement using the dopamine precursor levodopa (L-Dopa), the most potent compound to restore motor functions in PD. The usage of L-Dopa in PD represents a major breakthrough in the treatment of age-related neurodegenerative movement disorders. Although Dr. G. Cotzias discovered L-Dopa already in 1967 as a very powerful and effective compound for treating PD symptoms, it is still the gold-standard up today. The major sequelae of long-term L-Dopa treatment is, however, the development of adverse effects called motor fluctuations such as hypo-, hyper- or dyskinesias becoming in particular more prominent within or after

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[12] Spillantini et al. (1997), p. 839

[13] Lesage and Brice (2009), p. R48

[14] Kiely et al. (2013), p. 753

[15] Kruger et al. (1998), p. 106

[16] Pasanen et al. (2014), p. 2180 e1–5

[17] Polymeropoulos et al. (1997), p. 2045

[18] Zarranz et al. (2004), p. 164

[19] Proukakis et al. (2013), p. 1062

[20] Jankovic (2008), p. 368

[21] Baba et al. (1998), p. 879

the first decade of therapy. In particular, patients start to suffer from other motor fluctuations, i.e., freezing of gait or a decreasing response to L-Dopa. To increase the efficacy and tolerability of L-Dopa during the long-lasting disease course, there are other compounds to increase the dopaminergic tone within the CNS such as dopamine receptor agonists and inhibitors of dopamine metabolizing enzymes such as the monoaminooxidase B or the catecholmethyltransferase.[22] Besides pharmacological approaches, deep brain stimulation (DBS) has been approved as an effective neurosurgical intervention in PD. The mode of action for DBS is based on the continuous electrical stimulation of anatomically well-defined CNS regions.[23] For instance, several electrodes are implanted into the thalamus, the pallidum, or the subthalamic nucleus resulting in the alleviation of distinct motor symptoms in PD patients.[23] Implanting these electrodes requires an invasive neurosurgical procedure by an interdisciplinary team. Despite these great therapeutic advances for patients suffering from PD, none of the aforementioned therapies is able to slow down the progression of the disorder. Thus, there is still an urgent need for novel innovative approaches more effectively modifying the course of the disease.

3.4   History of Cell-Based Therapy

The therapeutic concept of cellular transplantation into neuronal structures has a long history in translational neurosciences going back to the first transplantation studies in the 1970s. In 1972, *Olson and Seiger* set the basis for the transplantation of neural tissue.[7,8,9] In their initial experimental approach, they collected cerebral tissue consisting of monoaminergic neurons from newborn animals or fetuses further successfully transplanting this tissue in the anterior chamber of the adult rodent eye.[8] In a subsequent study, *Olson and Seiger* succeeded to transplant ganglion cells in combination with fetal cortical tissue resulting in a profound reinnervation of disconnected rodent eyes using similar monoaminergic neurons.[9] Noteworthy, these studies provided clear evidence to use fetal tissue for transplantation purposes based on findings such as the good cellular survival postgrafting and the potential for appropriate reinnervation.

After obtaining these encouraging findings in preclinical models, the transplantation of adrenal medullary tissue into the caudate nucleus of PD patients was initiated in 1985, however without resulting in clinical benefits.[24] Following these initial attempts in PD patients, a novel source for grafts was discovered: human fetal ventral mesencephalic (HFVM) tissue prepared from aborted fetuses. HFVM tissue consists of dopaminergic neurons,[25] thereby representing a "good cellular source" for transplantation into the putamen and caudate nucleus of PD patients. In contrast to the initial transplantation efforts using adrenal medullary tissue, two patients

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[22] Lindvall (2016), p.30

[23] Benabid (2003), p. 696

[24] Backlund et al. (1985), p. 169

[25] Kontur, Leranth, Redmond, Roth, & Robbins (CR471993), p. 172

demonstrated an impressive improvement of PD symptoms after receiving HFVM grafts in 1990.[26] These initial promising results encouraged neuroscientists to move forward with the concept of HFVM transplantation approaches in randomized clinical studies. This intention was further supported by the optimization of pre-existing transplantation procedures resulting in the positive outcome after neural graft transplantation.[27,28] However, despite all positive preliminary clinical studies, larger, randomized clinical studies testing the efficacy of fetal dopaminergic grafts in PD patients failed to show an overall significant clinical improvement postgrafting.[29,30,31] The lack of clinical efficacy observed in the randomized clinical trials after fetal grafting and the presence of graft-induced dyskinesia was a major setback for moving forward with this cell-based transplantation approach. More importantly, the presence of Lewy body pathology in the transplanted fetal grafts 10 years after transplantation hampered further the optimism in regard to long-term safety and feasibility of HFVM transplantation in PD patients.[32] Neither follow-up studies demonstrating that the majority of the grafted cells was unaffected by Lewy body pathology nor reports of a maintained clinical improvement after transplantation changed this initial view on HFVM transplantations.[33,34] Besides crucial ethical concerns, the major clinical disadvantage of HFVM grafting strategies is the need for permanent immunosuppression in order to decrease the host versus graft reaction aimed to improve graft survival.[35,36] Since neural fetal grafts derive from several allogenic fetuses (i.e., up to four pooled fetuses are needed for one hemisphere of a single PD patient), the host immune response may result in the rejection of the transplanted fetal grafts. In general, immunosuppressive therapies carry additional risks for further detrimental adverse effects in elderly patients such as PD patients.[37] In summary, these important clinical considerations raise crucial ethical and methodological concerns regarding transplantation of fetal grafts. However, these clinical studies in PD patients had very important implications for i) the better understanding of the underlying molecular pathogenesis in PD by implying the potential spreading of aSyn from the neighboring CNS tissue of the host into the grafted immature fetal dopaminergic neurons and ii) introducing significant

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[26] Lindvall et al. (1990), p. 574

[27] Kordower et al. (1998), p. 383

[28] Kordower et al. (1995), p. 1118

[29] Brundin et al. (2000), p. 1380

[30] Freed et al. (2001), p. 710

[31] Olanow et al. (2003), p. 403

[32] Kordower, Chu, Hauser, Freeman, & Olanow (2008)), p. 504

[33] Li et al. (2008), p. 501

[34] Li et al. (2010), p. 1091

[35] Frodl, Nakao, & Brundin (CR301994), p. 2393

[36] Nakao, Frodl, Duan, Widner, & Brundin (1994), p. 12408

[37] Wennberg et al. (2001), p. 1797

encouraging clinical efficacy data concerning neural grafting strategies in PD, however using other suitable cell sources.

3.5   Development of the Modern Era of Stem Cell Technology

Consequently, the basic and clinical research community was continuously searching for an alternate cellular source for this type of neural transplantation approach: a novel era started with the discovery of human embryonic stem cells (hESCs[38]). The development of the hESCs has been inspired by its murine analogue, the mouse embryonic stem cells (mESCs[39]). hESCs are derived from human blastocysts and show pluripotency allowing the differentiation into all germ layers and its cellular derivatives.[40,41] A major disadvantage for the clinical usage of pluripotent hESCs is their potential to form malignant embryonic tumors such as teratomas.[42,43] Thus, the preparation of hESCs for further clinical application requires very high safety profiling standards.[42] Nevertheless, hESCs raised the hope as a novel cellular source for grafting approaches in order to develop an alternate grafting strategy for PD. hESCs represent an unlimited cellular source with an overwhelming potential to differentiate into distinct mature human cells. Detailed protocols were immediately established for the differentiation toward various neuronal subtypes.[44,45] Moreover, preclinical studies highlighted the potential of hESC-derived neural progenitor cells (NPCs) as an ideal source for allogenic transplantation of human cells into animal models. hNPCs integrated into the host murine brain postgrafting and were able to differentiate into distinct neural lineages.[45,46] The motor phenotype in PD is closely linked to a progressive loss of dopaminergic neurons, thereby defining the need to establish specific, standardized, and safe differentiation protocols for human midbrain dopaminergic neurons (mDANs). Initial achievements were obtained by differentiating dopaminergic neurons derived from mESCs,[47] but the translation to hESCs remained challenging. Although human neurons with specific dopaminergic characteristics were obtained,[48] there was no significant symptomatic improvement

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[38] Thomson et al. (1998), p. 1145

[39] Evans and Kaufman (1981), p. 154

[40] Itskovitz-Eldor et al. (2000), p. 88

[41] Schuldiner, Yanuka, Itskovitz-Eldor, Melton, & Benvenisty (CR412000), p. 11307

[42] Hentze et al. (2009), p. 198

[43] Prokhorova et al. (2009), p. 47

[44] Reubinoff et al. (2001), p. 1134

[45] Zhang, Wernig, Duncan, Brustle, & Thomson (2001), p. 1129

[46] Englund, Fricker-Gates, Lundberg, Bjorklund, & Wictorin (2002), p. 1

[47] Kawasaki et al. (2000), p. 31

[48] Yan et al. (2005), p. 781

after transplantation in rodent PD models.[49,50] Furthermore, transplanted hESCs formed tumors after grafting into the CNS.[51] Although this procedure was not applicable for therapeutic approaches in patients, these studies significantly contributed to our current understanding of the molecular machinery driving the differentiation of pluripotent stem cells into a specific midbrain dopaminergic phenotype.[52]

In 2006, K. Takahashi and Yamanaka reported the first success in reprogramming somatic mouse fibroblasts into adult induced pluripotent stem cells,[53] followed by the reprogramming of adult human fibroblasts into hiPSCs one year later.[11] This was the beginning of a new era in stem cell biology. The generation of patient-derived cells revolutionized the entire stem cell research field regarding its scientific and therapeutic impact including specific ethical questions raised by this novel molecular and cellular technology.

3.6   Human-Induced Pluripotent Stem Cells: A Promising Cell Source

K. Takahashi and Yamanaka successfully generated for the first time embryonal-like stem cells by reprogramming adult mouse fibroblasts. Initially, a large set of transcription factors was tested for their potency to induce stemness in somatic cells until they identified a pool of candidate genes associated with pluripotency.[53,54] Further selection led to the identification of four transcription factors sufficient for reprogramming murine somatic cells to iPSCs: Klf4, Sox2, c-Myc, and Oct4.[53] Based on this breakthrough, one year later, K. Takahashi and colleagues generated hiPSCs derived from human somatic cells.[11] The hiPSC technology facilitates the generation of isogenic pluripotent cells harboring the genetic background of the individual from whom they were obtained.[55] Additionally, this technology provides a novel personalized cell source on a large-scale for research and therapeutic purposes. Upon the establishment of hiPSC cultures, new opportunities emerged for differentiating hiPSCs toward specified neural cells, such as neurons[56] or oligodendrocytes.[57] Recently, several studies provided optimized differentiation protocols for the generation of mDANs from hiPSCs of genetic PD patients and demonstrated the power of this tool for subsequent investigations of disease-associated

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[49] Barker, Drouin-Ouellet, & Parmar (2015), 492

[50] Park et al. (2005), p. 1265

[51] Roy et al. (2006), p. 1259

[52] Friling et al. (2009), p. 7613

[53] K. Takahashi and Yamanaka (2006), p. 663

[54] Tokuzawa et al. (2003), p. 2699

[55] Winner, Marchetto, Winkler, & Gage (2014), p. R27

[56] Sanchez-Danes et al. (2012), p. 56

[57] Hu, Du, & Zhang (2009), p. 1614

pathways.[58,59,60] Furthermore, hiPSC-technology-based in vitro models of PD indicated aSyn oligomers to be rather responsible for cellular toxicity than aSyn fibrils.[61] This rapid development of efficient differentiation protocols opened the window for novel strategies to model genetic or sporadic CNS disorders, but furthermore built the basis for developing innovative therapeutic strategies to treat age-related neurodegenerative diseases.

3.7   Adding a Dimension: 3D Human Cerebral Organoids

The advances in hiPSC generation and the continuous development of protocols to increase efficiency and reproducibility opened up new opportunities in the field of human in vitro systems: the generation of human cerebral organoids. Neural tissue originates from the ectodermal germ layer.[62] The ectoderm was reproducibly generated from structures called hiPSC-derived embryoid bodies (EBs[63]). Neural lineage commitment of these ectodermal-like cells was induced by specifically modifying in vitro conditions using chemically defined media.[64] Importantly, the generated neuroepithelium requires additional structural support to self-organize into a three-dimensional (3D) structure since the standard cell culture system is lacking a distinct basement membrane. Therefore, a system based on hydrogels was established to provide the neuroepithelial cells with a specific environment for 3D self-organization resulting in the formation of small neurogenic regions defined as cerebral organoids.[10] The use of the cerebral organoid model enables to recapitulate important aspects of CNS development as neural progenitor cells undergo self-organization and differentiation.[65] Human cerebral organoids demonstrate similar heterogeneity as the human brain in vivo during early development.[66] Previous research has already succeeded in modeling pathologic phenotypes in cerebral organoids, which enables the investigation of disease mechanisms more closely to the native state. This is of particular importance as cell–cell interactions in a 3D environment might significantly influence disease progression.[66] Furthermore, *Qian* et al. successfully generated brain-region-specific organoids displaying the identity of all six cortical layers, but also midbrain and hypothalamic organoids.[67] Overall, cerebral organoid technology provides a novel and highly innovative platform to

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[58] Brazdis et al. (2020), p. 1180

[59] Simmnacher et al. (2020), p. 113466

[60] Sommer et al. (2018), p. 123

[61] Prots et al. (2018), p. 7813

[62] Rubenstein (2013)

[63] Eiraku et al. (2008), p. 519

[64] Hu and Zhang (2010), p. 123

[65] Renner et al. (2017), p. 1316

[66] Lancaster et al. (2013), p. 373

[67] Qian et al. (2016), p. 1238

investigate disease mechanisms in an organ-like context. Additionally, human cerebral organoids represent a large-scale and renewable cell source for neurons and other CNS cell types.

3.8   The Evolution of Genome Editing

Evolving reprogramming and differentiation strategies advanced the usage of hiP-SCs in basic and translational research. Reprogramming of somatic cells with patient- and disease-specific genetic background offered the potential to gain further insights into disease pathomechanisms but also shifted the focus on developing molecular tools for genome editing as potential rescue strategy or for the manipulation of disease-associated genes. Consequently, initial gene editing tools emerged, the zinc finger nucleases (ZFN[68]). Zinc fingers are small-sized proteins capable of recognizing and binding specific nucleotide sequences of genes. The coupling with an endonuclease allows the cleavage of DNA in a site-specific manner.[69] Notably, the design of such ZFN is quite challenging and exceeds the expertise for the majority of laboratories. The major disadvantage using ZFNs is that the delivery of these nucleases is an irreversible process, thus potentially leading to serious off-target modifications. As a result, the need for efficient easy-to-handle gene editing tools increased. The discovery of transcription activator-like effector nucleases (TALENs[70,71]) offered a new DNA targeting tool, much "easier" in design and handling. Two variable adjacent amino acid repeats enable to recognize specific DNA sites.[71] The major challenge of TALENs is the correct combination of the variable adjacent amino acid repeats for specific targeting of DNA sites and the resulting immense increase in size of TALEN proteins. Due to the simplicity compared to ZFNs, TALENs were subsequently used for genome editing in stem cell-based disease models with initial promising results.[72,73,74] Since DNA-binding motifs are capable of binding homologous DNA sites, there is a minimal probability of non-desired genome modifications.[75] These novel promising gene-editing tools were replaced very rapidly after the discovery of the Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) initiating a novel dimension in genome editing.[76] CRISPR/Cas9 became rapidly a very powerful and state-of-the-art tool for genome engineering. The CRISPR system in combination with different CRISPR-associated genes (Cas) participates in the adaptive immune system of

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[68] Kim, Cha, & Chandrasegaran (CR461996), p. 1156

[69] Bibikova et al. (2001), p. 289

[70] Boch et al. (2009), p. 1509

[71] Christian et al. (2010), p. 757

[72] Bedell et al. (2012), p. 114

[73] Ding et al. (2013), p. 238

[74] Sun and Zhao (2014), p. 1048

[75] Yee (2016), p. 3239

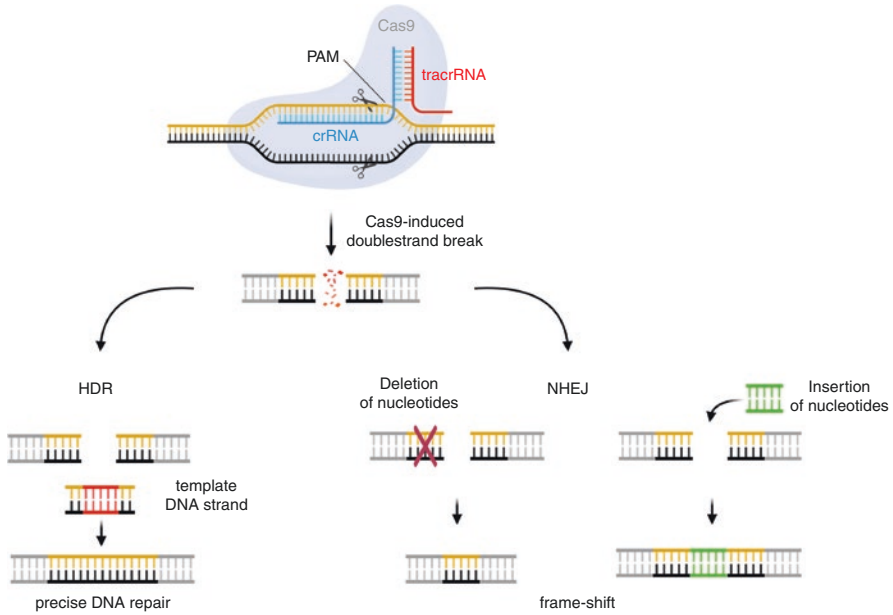[76] Doudna and Charpentier (2014), p. 1258096

Fig. 3.1 *Principle of CRISPR/Cas9.* The Cas9 endonuclease (gray) consists of two independent endonuclease domains capable of generating DSBs in a DNA site-specific manner directed by an sgRNA. The sgRNA is divided into a crRNA (blue) for complementary pairing with the target DNA site and a tracrRNA (red). In addition, the Cas9 also contains a PAM recognition subunit for PAM-dependent base pairing. By the Cas9-induced DSBs, two DNA repair mechanisms are potentially triggered. The homology-directed repair (HDR) is based on the existence of a template DNA strand with homology (red) to the edited DNA site. Using the template, the cell is capable of precisely repairing the edited DNA strand. The second pathway represents non-homologous end joining (NHEJ). NHEJ is not template-based resulting in deletions (red "X") or insertions of nucleotides (green) causing frame-shift mutations

prokaryotic organisms.[77] Components of the CRISPR operon could be repurposed for genome editing. The CRISPR-associated protein 9 (Cas9) is able to form a ribonucleoprotein complex (RNP) with the trans-activating CRISPR RNA (tracrRNA) and the CRISPR RNA (crRNA), both expressed at the CRISPR array recombined to a single guide RNA (sgRNA)[78] (Fig. 3.1). A 5′ stretch of the crRNA, the protospacer, can be reprogrammed to pair with complementary 20 nt specific target DNA sequence of the genome. The Cas9 scans the genomic DNA strand for a specific protospacer adjacent motif (PAM[79]). If the PAM matches, the protospacer will pair with the genomic sequence, and subsequently, the endodeoxyribonuclease RuvC and endonuclease domain HNH of the Cas9 initiate a process that results in the

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[77] Barrangou et al. (2007), p. 1709

[78] Deltcheva et al. (2011), p. 602

[79] Mali et al. (2013), p. 957

generation of a double-strand break (DSB) three to four nucleotides upstream of the PAM.

The ability to generate specific DSBs triggers several potential scenarios for genome editing. Employing the nonhomologous end joining pathway, it is possible to generate gene knockouts by inducing out-of-frame insertions and deletions (indels). By the addition of a homologous donor template containing the edit of choice, the homology-directed repair pathways allows the stable reversal of disease-causing mutations. The CRISPR/Cas9 system is based on the delivery of the endo-nuclease and the sgRNA by plasmids, viral transduction or as synthetic RNPs. The ability to program the CRISPR/Cas9 system simply by adapting the sgRNA renders CRISPR/Cas9 a far superior system than ZFNs or TALENs, which rely on protein–DNA interaction. Hence, CRISPR/Cas9 represents a very fast and easy "hands on" approach. A further tremendous advantage of using CRISPR/Cas9 technology is the possibility for targeting multiple genomic loci simultaneously allowing multiplex genome engineering.[80] Compared to TALENS, the probability modifying off-target sequences using CRISPR/Cas9 is marginally higher. The field of CRISPR/Cas9 is rapidly evolving, thus identifying continuously promising applications and new bacteria-derived endonucleases with different PAM specificities, allowing a broader range of host genome modification. Combined with the platform of hiPSCs, CRISPR/Cas9 represents a powerful tool to modulate disease-associated genes and provides novel functional data of pathways in health and disease.

3.9   A New Hope: Preclinical Stem Cell Replacement Therapies

Based on the outcome of previous studies using hESCs, protocols for differentiating hESCs and hiPSCs into a dopaminergic lineage were refined and optimized.[81] Initial transplantation studies using ESC approaches have been initiated already in 2008, called "therapeutic cloning."[82] In this study, all mice engrafted with ESC-derived dopaminergic neurons by autologous transplantation showed a significant attenuation of the PD-like phenotype in behavioral tests. Notably, the applied autologous transplantation approach revealed no graft rejection or an increased immune response in the host brain. The fundamental finding that dopaminergic neurons originate from a developmental structure called floor plate (FP) catalyzed the process of generation and specification of dopaminergic neurons.[83] Based on this finding, *Kriks and colleagues* established a protocol for effective transplantation of human-derived ESCs in nonhuman primates with a toxin-induced PD phenotype showing a robust survival of mDANs.[84] Analysis of the ESC-derived mDAN transplantation revealed

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[80] Cong et al. (2013), p. 819

[81] Chambers et al. (2009), p. 275

[82] Tabar et al. (2008), p. 379

[83] Ono et al. (2007), p. 3213

[84] Kriks et al. (2011), p. 547

an efficiency of the transplantation comparable to previous studies using HVFM transplanted grafts.[85]

In 2008, first studies of hiPSC transplantation succeeded in reproducing the findings from ESC transplantation approaches. The hiPSCs were differentiated into mDANs, analyzed for dopaminergic markers, and subsequently transplanted into the CNS of a PD rat model.[86] The mDANs successfully integrated into the host brain, formed synaptic contacts, and were electrophysiologically active. Rodents with grafts showed a symptomatic improvement although a continuous proliferation of these cells was detected postgrafting. Comparable results have been obtained by a similar strategy using a sorting approach of cells originating from a developmental structure (CORIN) important for the differentiation of mDANs.[87] CORIN+ cells are more suitable for dopaminergic differentiation. Transplantation of these cells resulted in a better survival of mDANs in conjunction with an improved functional outcome. The first autologous transplantation approaches of hiPSCs in a nonhuman primate PD model were performed in 2013.[88] This study showed that hiPSC grafts efficiently integrate into the host brain, but the authors did not observe a functional improvement. *Morizane and colleagues* initiated an autologous and allogenic transplantation of hiPSC-derived dopaminergic neurons comparing intragenomic retrovirally with nonintegrating episomally generated hiPSC grafts.[89] The authors performed this transplantation study in nonhuman primates, demonstrating a strong immune response by allografts, but a very limited by autografts. Furthermore, an improved survival of tyrosinhydroxylase (TH+)-expressing human neurons was observed in both the types of grafts, even with a higher number of TH+ human neurons in the autografts. These findings were confirmed by a follow-up study using optimized protocols for hiPSC generation and transplantation procedures.[90] A very crucial and relevant finding for further translation of autologous cell transplantation approaches to humans was the consistency and rigidity in regard to the observed symptomatic improvement in nonhuman primates with grafts.[91] The animals were screened over a period of 2 years after transplantation. A prolonged survival of the engrafted cells in conjunction with a sustained functional improvement was observed. Taken together, these landmark studies in nonhuman primates emphasized the therapeutic potential of autologous hiPSC transplantation by demonstrating an augmented survival of engrafted cells with a concurrent functional and biological relevant improvement of the disease course in broadly accepted preclinical nonhuman primate PD models. At this stage, it is very important to note that no immunosuppression was necessary to obtain these results after transplantation in contrast to allogenic transplantation approaches using HFVM or hESCs. Therefore,

[85] Grealish et al. (2014), p. 653

[86] Wernig et al. (2008), p. 5856

[87] Doi et al. (2014), p. 337

[88] Emborg et al. (2013), p. 646

[89] Morizane et al. (2013), p. 283

[90] Sundberg et al. (2013), p. 1548

[91] Hallett et al. (2015), p. 269

autologous hiPSC transplantation approaches represent the most promising platform for present and future clinical studies in the light to achieve an effective, long-term symptomatic treatment of PD patients without the necessity of immunosuppressive medication.

In the field of brain organoid transplantation, there is little published data about preclinical cerebral organoid transplantation. Two studies provide evidence of successfully engrafted hiPSC-derived brain organoids into mouse brain. The study of Mansour et al. revealed a vascularization of transplanted brain organoids in adult mice and the capability of neuronal maturation and differentiation, as well as axonal outgrowth and gliogenesis.[92] A second study observed similar findings in lesioned mouse cortex, confirming the potential of brain organoid transplantation as an alternate therapeutic cell-based approach.[93] However, there are currently no studies described in nonhuman primates further. Nonetheless, brain organoids represent a heterogeneous population of cells, thus consisting of pluripotent cell populations within the organoid, leading to an incompatibility with the current available protocols regarding safety for in-vivo approaches (see below). In addition, the transplantation process requires an invasive procedure for successfully transplanting organoids into the region of interest. Since organoids are of macroscopic nature, the use of a larger application device may result in additional tissue damage at the site of transplantation.

3.10   In-Human Studies: hiPSC-Based Cell Replacement in PD

In 2015, the international consortium named G-Force PD was founded focusing on novel cell-based therapies for treating neurological disorders in humans, especially patients with PD. In the framework of this consortium, four transplantation studies were initialized involving two hESC and two hiPSC transplantation studies.[94]

In 2018, the first clinical trial was initiated aiming to implant allogenic hiPSC-derived mDANs in Japan.[95] The hiPSCs were obtained from a single healthy donor carrying the most common human leukocyte antigen (HLA) type, as indicator for immunocompatibility, in Japan to minimize the risk of an immunogenic rejection of the transplant. The hiPSCs were obtained by reprogramming peripheral blood cells using episomal plasmid vectors containing the prototypical Yamanaka reprogramming transcription factors. Midbrain dopaminergic differentiation (Fig. 3.2) was performed according to the aforementioned protocols followed by a thorough screening for tumorigenicity, cell overgrowth, and survival in a PD rat model. Additionally, the behavioral parameters were evaluated to assess the potential clinical outcome after transplantation. The cells demonstrated no tumorigenic characteristics and a robust survival as well as adequate engrafting into the rat host

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[92] Mansour et al. (2018), p. 432

[93] Daviaud et al. (2018), p. ENEURO.0219–18.2018

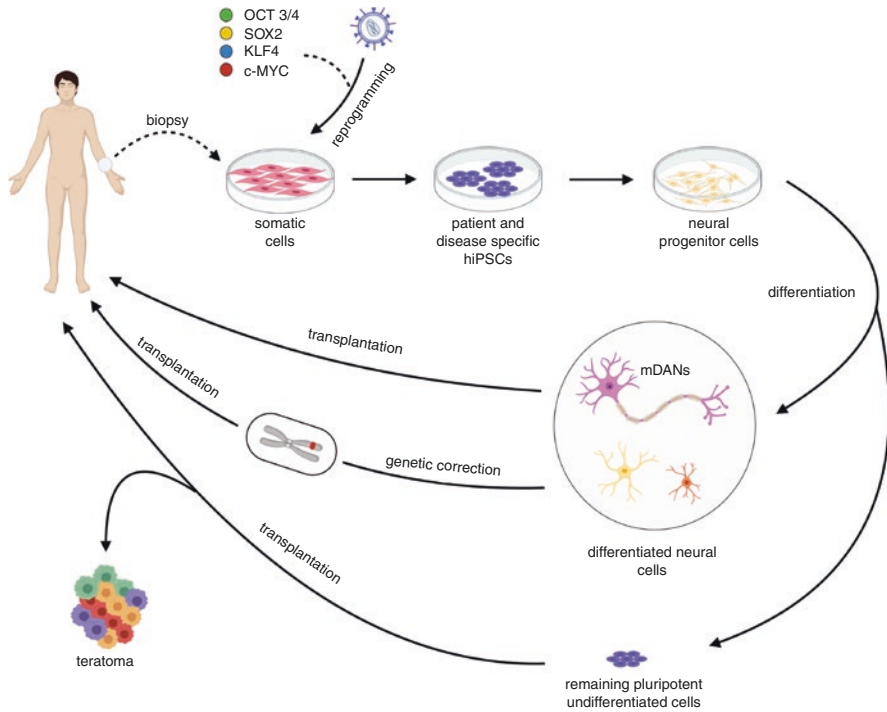[94] Barker et al. (2017), p. 569

[95] J. Takahashi (2020), p. 18

Fig. 3.2 Autologous transplantation of hiPSC-derived mesencephalic dopaminergic neurons (mDANs): Somatic cells are obtained by standard biopsy techniques and subsequently reprogrammed into hiPSCs by the ectopic overexpression of the Yamanaka reprogramming transcription factors (OCT3/4, SOX2, KLF4, c-MYC). Further cell fate-specific differentiation allows the generation of mDANs and other neural cell types. Differentiated mDANs are further utilized for transplantation into the patient's affected brain regions. The usage of genome editing systems enables the correction of genetic aberrations. The major concern of hiPSC transplantations refers to the potential of tumor formation (e.g., teratomas) due to remaining cells in a pluripotent state

brain. Moreover, grafted rats presented a solid motor improvement suggesting a high potential to translate these findings toward initiating a clinical trial using mDANs in PD patients. The consequent clinical trial enrolled seven PD patients in the range between 50 and 69 years of age and a disease duration exceeding 5 years. PD patients showed already motor symptoms not controlled by their oral medication. The patients received five million cells injected into the putamen as spheres using a stereotactic needle designed for transplantation purposes. Due to the allogenic origin of the grafts, patients underwent immunosuppression for a period of 12 months. The follow-up of this allogenic transplantation approach was envisioned at least 24 months after transplantation.

The second study performing autologous transplantation in PD patients was planned to start recruiting patients in 2019 (Summit for PD[94]). The inclusion criteria are almost identical to the clinical study headed by *J. Takahashi and colleagues*. The clinical follow-up was estimated to take place 1 year after transplantation.

Since the follow-up in both the studies is still pending, there is no explicit report thus far describing the current clinical status of the patients enrolled into both the studies. Overall, the very rigid preclinical work of the consortium G-Force PD is promising. Finally, a positive outcome of these ongoing clinical trials will represent a new milestone in the field of neurorestoration in PD.

Besides G-Force PD, to date, a single case report was recently published in the *New England Journal of Medicine* reporting a preliminary "blueprint" of an autologous transplantation of patient-derived mDANs.[96] The patient was a 69-year-old physician with a 10-year history of progressive, sporadic PD. Based on this report, he was continuously treated according to the present guidelines for the treatment of PD, however with poor outcomes, leading to a severe worsening of his symptoms. The patient received an autologous graft of mDANs progenitors in the right and left putamen, both the surgeries separated by a 6-month interval. The patient was not immunosuppressed after undergoing transplantation. To assess whether grafted mDANs are tolerated by the host CNS, cells were prescreened and initially implanted in patient-humanized mice, suggesting that the grafts will be immunologically tolerated by the patient brain. The patient was imaged up to 24 months after the first transplantation procedure. The analysis displayed an initial reduction of dopamine uptake in the putamen followed by a mild increase over a longer period, suggesting that the injected cells engrafted successfully into the host brain. The patient demonstrated improved motor symptoms showing a decline in the severity of symptoms, both with and without his standard medication. Furthermore, the patient reported an improved quality of life after 24 months. In addition, the dosage of the standard medication was reduced in comparison to the status prior to the transplantation, and no graft-related dyskinesias were observed. In summary, this first pilot study addressing the feasibility of autologous transplantation of hiPSCs showed the potential of this avenue for treating PD patients, but a detailed and robust double-blinded, randomized clinical trial must be performed in order to draw some meaningful and rigid conclusions.

The application of genome editing in hiPSC technology for therapeutic purposes is dramatically rising. By 2017, almost 2600 ongoing or completed trials using gene therapy approaches have been approved globally.[97] The overall aim of gene-based therapeutic strategies is the incorporation of plasmids or viral vectors to target proteins identified to cause diseases such as cancer, but also rare monogenic diseases.[98,99] Autologous transplantation of genetically altered cells is exclusively tested in sporadic PD thus far. However, there are also about 10–15% PD patients linked to monogenic mutations and thus representing a potential target population of genome editing efforts. Since hiPSC-derived mDANs resemble neural cells in a very early stage, the transplantation of such immature neurons still harboring mutant genes may result in less favorable outcomes compared to mDANs derived from sporadic

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[96] Schweitzer et al. (2020), p. 1926

[97] Ginn et al. (2018), p. e3015

[98] Hacein-Bey Abina et al. (2015), p. 1550

[99] Porter et al. (2011), p. 725

PD patients. One of the most prominent PARK locus, PARK4, is characterized by the duplication or triplication of the SNCA gene resulting in an aggregation-promoting overexpression of aSyn. Genetic manipulations allow removing additional alleles of the SNCA locus, thereby restoring the physiological level of aSyn expression in the patient-derived hiPSCs (Fig. 3.2).

The PARK1 locus refers to missense point mutation in the SNCA gene, resulting in gain- or loss-of-function events of aSyn. Similar to PARK4, it is possible to target the disease-causing mutations and replace the affected exon/gene, thus re-establishing the physiological function. In summary, the genetically modified hiPSCs may be further differentiated to mDANs and subsequently implanted as a genetically treated cell population in affected brain areas, such as the putamen in PD (Fig. 3.2).

Alternatively, gene-editing tools are also an appropriate tool to improve the therapeutic potential of hiPSCs by genetically improving cell survival after transplantation.[100] Overall, genome editing represents a powerful tool for the modulation of patient-derived cells but important aspects in terms of safety and bioethics must be considered prior to applying these genetically modified hiPSCs in patients. At present, there are no registered clinical trials using genetically edited hiPSCs for transplantation purposes in PD.

3.11 The Flip Side of the Coin: Safety and Social Concerns of hiPSC Technology

The discovery of hiPSCs revolutionized the field of stem cell research due to its individualized source and standardized procedures for scaling up, but moreover, by circumventing certain ethical and legal concerns, which have been raised in particular with the usage of hESCs. By "simply" obtaining somatic cells from an individual by a less invasive method such as a skin biopsy or drawing peripheral blood, hiPSCs overcome serious ethical concerns "to use" or "to consume" human blastocysts, embryos, or fetuses for therapeutic purposes. Moreover, autologous transplantation of hiPSC may allow circumventing lifelong immunosuppression since graft and host refer to the identical individual thus paving the way to immunocompatibility. So far, hiPSC circumvent ethical concerns of embryonal- or fetal-tissue-derived stem cell technology, but the term "pluripotency" implies the potential to form tumors.[101] Since the potency of teratoma formation is a gold standard to evaluate pluripotency, undifferentiated hiPSC populations in the engrafted cells pose the risk of tumor formation after transplantation. Besides this safety concern, an additional tumor-promoting characteristic refers to the genomic instability of hiPSCs, an important aspect hampering the usage of these cells for its application in humans.[102] Reprogramming technologies for somatic cells require the usage of oncogenic transcription factors such as c-MYC or the integration of retro- and lentiviral vectors

[100] Moradi et al. (2019), p. 341

[101] Lindvall (2015), p. 20140370

[102] Yoshihara et al. (2017), p. 7

potentially resulting in nontargeted mutagenesis.[103] Therefore, it is necessary to continuously develop and improve differentiation protocols not only to increase the purity of the desired cells but also to fulfill the highest safety standards to exclude the risk of tumor formation.

hiPSCs represent a powerful tool for disease modeling and drug discovery in a human-based in vitro model. However, despite the advantages of hiPSC, a large transcriptional variability between cells derived from the identical donor was observed,[104] resulting in a considerable heterogeneity of cells despite its identical "mother" cell.[105] Due to this transcriptional variability, the prediction in regard to the expected outcome of transplanted hiPSCs remains a huge challenge. Another arguable factor relates to the molecular strategy for reprogramming. As retro- and lentiviral-based reprogramming strategies involve the integration of defined reprogramming factors into the genome, an increased risk of intragenic mutations may occur. For a safe clinical application, the development of new molecular strategies such as integration-free transient vector systems is fundamental to lower the risk of mutagenesis. However, up to now, there is not sufficient knowledge regarding the safety of integration-free generated hiPSC.[103] Finally, the usage of genome editing strategies for hiPSCs imply other risks such as i) the delivery of bacterial endonucleases into hiPSCs and subsequent transplantation into the immunocompetent CNS, ii) the possibility of off-target mutagenesis by the Cas9 or triggered DNA repair mechanisms, iii) the potential of unknown mechanisms involving other genes in the pathogenesis caused by the known monogenic mutation (e.g., multiplication of a whole chromosome stretch in PARK4 patients involving additional genes).

Finally, the financial burden of these molecular and cellular procedures is a major obstacle for public health care systems to implement hiPSC transplantation technology for a disorder such as PD due to its increasing prevalence worldwide.[106,107] The aspect of health costs raises the serious question for society whether autologous hiPSC transplantation is affordable at all for healthcare systems.

3.12 Pay-to-Participate: The Slippery Slope of Scientific Integrity

In this review, we have outlined the advantages but also safety, ethical, and social concerns associated with the advancements of hiPSC technology. In the brief report of *Schweitzer and colleagues*,[96] the clinical assessment of the PD patient revealed a return of dopamine uptake to the baseline (*pretransplantation*) 24 months after autologous transplantation of hiPSC-derived mDANs. As a result, the patient reported improved motor symptoms as well as quality of life. Although this report

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[103] Volarevic et al. (2018), p. 36

[104] Liang and Zhang (2013), p. 149

[105] Carcamo-Orive et al. (2017), p. 518

[106] Beers et al. (2015), p. 113119

[107] Prescott (2011), p. 1575

appears promising for the future of hiPSCs transplantation technology as a new therapeutic approach for PD, several serious concerns of this study must be discussed. In fact, the grafted hiPSCs were characterized in previous studies;[108] however, the current safety protocols are not sufficient to exclude the above-mentioned tumor-promoting genomic instability of hiPSCs.[102] Therefore, a more detailed preclinical evaluation of the hiPSC properties in humanized animal models is required to ensure the safety for future patients.

From a clinical point of view on this single case published in one of the most relevant journals in medicine, there are several issues further to be considered. The patient had an intermediate course of PD offering the therapeutic option for him just by increasing his daily L-Dopa dosage to improve his motor symptoms since no L-Dopa-induced dyskinesias were observed yet. Moreover, he declined deep brain stimulation as an alternative therapeutic approach. By analyzing the pattern of the cerebral positron-emission tomography, it becomes evident that the dopamine uptake returned or minimally exceeded the initial baseline uptake. Notably, since PD is a progressing neurodegenerative disease, the putaminal dopamine uptake consequently decreases over the period of 24 months, thus indicating that the transplantation of human mDANs was able to halt disease progression at least based upon the levels of the initial dopamine uptake. The lack of an internal (sham surgery on the less affected side) or adding an external control further raises questions about the issue whether the restorative effects observed are linked to the grafted mDANs or to the procedure itself clinically well-known as placebo effect. Crucially, although PD is defined by prototypical motor symptoms, there is a plethora of nonmotor symptoms in PD frequently present prior to the onset of motors symptoms or throughout the course of the disease.[109] Thus, it is evident that dopamine replacement or substitution is not able to relief nonmotor symptoms such as cognitive deficits or depressive symptoms. In summary, the transplantation of mDANs may be a powerful and long-term restorative therapy to enhance the dopaminergic tone within the CNS of PD patient, but will never represent a causal cure of the disease.

The last and potential ambiguous aspect of this initial pilot study on the clinical application of hiPSC-derived mDANs to reflect on is the social and financial circumstances in a highly respected academic institution such as the Harvard Medical School. The transplanted PD patient is a wealthy former physician and businessperson. After receiving the diagnosis PD, the patient decided to fund the research on hiPSC transplantation technology to benefit from the findings of this research. In the present case, the patient funded a scientist investigating safety and efficiency of hiPSC transplantation after being declined for other public funding sources. Besides the preclinical research, he paid for the surgical procedure including the legal and ethical approval by the institutional review board and the Food and Drug Administration (FDA). This payment to researchers, administrators, and physicians directly involved in the preclinical and clinical procedures may result in a selection

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[108] Song et al. (2020), p. 904

[109] Chaudhuri et al. (2006), p. 235

bias leading to research and clinical decisions made in favor of the donator of funds than rigid science as a whole. Moreover, this type of pay-to-participate study[110] sheds an ambiguous light on scientists and clinicians who may apparently be bought from a single individual for his or her own purpose. A further questionable aspect was the selected FDA program for approval rather intended for patients with life-threatening conditions or no remaining therapeutic alternatives. It is noteworthy at this moment to reiterate that PD is not a fatal disease; furthermore, life expectancy has tremendously increased with new developments and optimizations of current therapeutic approaches. Due to this fact, FDA approval for the transplantation of hiPSC-derived mDANs is arguable in the present case since there is no necessity for this intervention in the light of alternate therapeutic options. Finally, this first case report of an autologous hiPSC transplantation was published in one of the most cited, high-impact medical journals eventually fostering false interpretations, hopes and overestimations of the prospect of this type of treatment.[111]

3.13 Conclusion

Since its discovery, the research field of stem cells and genome editing is developing continuously and rapidly.[53,11,38] Although promising results were obtained in pre-clinical models of rodents and nonhuman primates, the idea of self-derived transplantation requires precautious interpretations. Pluripotency is generally linked to unconditional potential to proliferate and differentiate, an immanent risk factor for tumorigenesis. Until sufficient safety data for hiPSC grafts are not yet fully established, the transplantation of hiPSC-derived neural cells in humans is very cautiously to be considered as an additional, but powerful symptomatic approach possibly halting the deterioration of distinct clinical symptoms in PD. For diseases in which multiple cell types are affected, brain organoids, as kind of "mini organs" may represent a powerful cell source in the future. However, similar to hiPSCs, no current protocols of organoid generation ensure the highest safety for transplantation in humans. Moreover, since brain organoids are macroscopic cellular clusters, there are major biotechnical concerns regarding invasiveness applying these clusters into patients. Additionally, self-funded research raises numerous concerns regarding scientific integrity. Therefore, the study of *Schweitzer and colleagues* is a hallmark for the entire research community and society to further discuss and develop stringent guidelines for this type of cutting-edge technology in modern medicine. In light of all considerations and results at present, autologous transplantation of hiPSCs offers the promise to restore CNS functions and potentially to increase the quality of life of thousands of patients suffering from age-related neurodegenerative diseases such as PD.

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[110] Grady (2005), p. 1681

[111] Jankovic et al. (2020), p. 1312

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