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Human induced pluripotent stem cell–derived therapies for regeneration after central nervous system injury

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From the Contents

Abstract

In recent years, the progression of stem cell therapies has shown great promise in advancing the nascent field of regenerative medicine. Considering the non-regenerative nature of the mature central nervous system, the concept that "blank" cells could be reprogrammed and functionally integrated into host neural networks remained intriguing. Previous work has also demonstrated the ability of such cells to stimulate intrinsic growth programs in post-mitotic cells, such as neurons. While embryonic stem cells demonstrated great potential in treating central nervous system pathologies, ethical and technical concerns remained. These barriers, along with the clear necessity for this type of treatment, ultimately prompted the advent of induced pluripotent stem cells. The advantage of pluripotent cells in central nervous system regeneration is multifaceted, permitting differentiation into neural stem cells, neural progenitor cells, glia, and various neuronal subpopulations. The precise spatiotemporal application of extrinsic growth factors *in vitro*, in addition to microenvironmental signaling *in vivo*, influences the efficiency of this directed differentiation. While the pluri- or multipotency of these cells is appealing, it also poses the risk of unregulated differentiation and teratoma formation. Cells of the neuroectodermal lineage, such as neuronal subpopulations and glia, have been explored with varying degrees of success. Although the risk of cancer or teratoma formation is greatly reduced, each subpopulation varies in effectiveness and is influenced by a myriad of factors, such as the timing of the transplant, pathology type, and the ratio of accompanying progenitor cells. Furthermore, successful transplantation requires innovative approaches to develop delivery vectors that can mitigate cell death and support integration. Lastly, host immune responses to allogeneic grafts must be thoroughly characterized and further developed to reduce the need for immunosuppression. Translation to a clinical setting will involve careful consideration when assessing both physiologic and functional outcomes. This review will highlight both successes and challenges faced when using human induced pluripotent stem cell-derived cell transplantation therapies to promote endogenous regeneration.

Key Words: axon regeneration; central nervous system regeneration; induced pluripotent stem cells; neurotrauma; regenerative medicine; spinal cord injury; stem cell therapy

Introduction

The human central nervous system (CNS) represents a crucial and distinctive phenomenon of evolution. Notably, the capacity for growth and repair in the CNS is greatly diminished in adulthood (Gotz et al., 2016). This presents a major barrier to overcoming neurological dysfunction, especially considering the devastating effects on the quality of life of those impacted. Neurologic injury or disease, affecting either the central or peripheral nervous system, impacts approximately 1 in 3 people worldwide (~3.4 billion) and represents the greatest burden among all disease. The spectrum of those affected comprises congenital and traumatic injuries, the majority of which have limited or no effective treatment options (GBD 2021 Nervous System Disorders Collaborators, 2024). Despite recent advancements in understanding these complex pathophysiologies, treatments for individuals with CNS injuries or diseases still fail to deliver significant improvements in quality of life.

CNS injury or disease can be broadly categorized as either traumatic or genetic/congenital. Neurotraumatic injuries, including spinal cord injury (SCI), traumatic brain injury (TBI), or cerebrovascular accidents, typically involve an initial mechanical or vascular insult that precipitates primary tissue damage. This primary injury is often followed by secondary

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damage mediated through prolonged inflammatory response, edema, and ischemic conditions (Aghili-Mehrizi et al., 2022). Moreover, neurologic dysfunction includes congenital and genetic degenerative conditions such as Alzheimer's disease and Parkinson's disease. While clinical manifestations of these neurodegenerative disorders vary, they universally involve progressive neuronal loss and disruption of effective neurotransmission pathways, leading to a decline in cognitive or motor functions (Lamptey et al., 2022). The profound impact of these conditions is further exacerbated by the limited growth and regenerative capacity of mature neurons. Accordingly, stem cell therapies were regarded as an ideal treatment option. While embryonic stem cells (ESCs) showed great promise in regenerative medicine, technical and ethical constraints largely impeded advancement from preclinical studies to clinical trials (Volarevic et al., 2018; Golchin et al, 2021). The Nobel-winning work by Takahashi and Yamanaka (2006) revitalized enthusiasm for stem cell therapies using human induced pluripotent stem cells (hiPSCs). This novel approach circumvents the ethical concern when sourcing ESCs, as somatic cells are reprogrammed to a pluripotent state.

The repair mechanisms for cell therapies that utilize hiPSCs have been reported to improve outcomes for some of these conditions, through providing neurotrophic support, anti-inflammatory cytokines, and pro-regenerative compounds that favorably alter the microenvironment for recovery. Through stimulating neurogenesis, axonal regeneration, or synaptic plasticity, hiPSC-derived therapies can ameliorate the symptoms and underlying pathogenesis of neuronal dysfunction (Černeckis et al, 2024). Comparatively, hiPSC-derived therapies have shown great regenerative potential, rivaling current treatment methods. Considering these advancements, this review will focus on the regenerative potential of hiPSC-derived treatment in CNS dysfunction.

Search Strategy

In June and July of 2024, a comprehensive search was conducted on Semantic Scholar using these initial terms: hiPSC, transplantation, and regeneration. The search results were limited from 2006 to 2024. We filtered studies that did not focus on CNS pathologies. The following terms were used to further filter through our initial results: Neural stem cells or NSCs, neural progenitor cells or NPCs, neurons, interneurons, cortical neurons, glia, astrocytes, oligodendrocytes, oligodendrocyte precursor cells or OPCs, xenograph, allograph, regeneration, spinal cord injury or SCI, traumatic brain injury or TBI, human leukocyte antigen or HLA, amyotrophic lateral sclerosis or ALS, Parkinson's disease or PD, Huntington's disease or HD, Alzheimer's disease or AD, biomaterials, and hydrogel. The listed search terms were used in various combinations to effectively and thoroughly retrieve relevant literature. Studies investigating the following topics were removed: hiPSC-derived cell transplantation for replacement therapy rather than for host regeneration or repair, transplantation outside the CNS, iPSCs derived from animals, and the use of studies examining ESCs was limited to use only as reference material. To supplement this search, we used the same search terms in Google, Google Scholar, and PubMed. Semantic Scholar is an AI-powered scientific literature search tool from the Allen Institute for AI (AI2).

Multipotent Stem Cells

Neural stem cells

During early development, CNS formation begins via symmetric division and expansion of neuroepithelial cells as they form the neural tube. These cells maintain an undifferentiated state and a high capacity for self-renewal. Neurogenesis begins as a pool of undifferentiated cells and then undergoes asymmetric division to give rise to one neuroepithelial cell and one radial glial cell. During mid to late embryonic development, radial glial cells become the primary form of neural stem cells (NSCs), acting as a scaffold for migrating neurons and giving rise to neural progenitors (Bergstrom and Forsberg-Nilsson, 2012; Homem et al., 2015). Broadly, neural stem cells are often used as a comprehensive category that encompasses a range of self-renewing neural cells including radial glial cells and neuroepithelial cells. Much of the current literature is conflicted on how to define NSCs and distinguish them from other progenitor cells. Many studies combine neural stem/progenitor cells (NS/PCs), therefore, this review will individually assess NSC and neural progenitor cell (NPC) transplantation, and the expression of key markers of the lineage will be reported. A summary of these markers can be seen in **Figure 1**.

The efficacy of cell transplantation therapy largely depends on the timing and severity of the injury. The milieu of inflammation, hypoxia, and scarring directly affects the viability and differentiation of transplanted stem cells (Cesare et al., 2022; Marques et al., 2023). Given the pathophysiologic role of hypoxia, particularly in neurotraumatic injury, it is probable the lack of oxygen in the injury microenvironment can suppress neuronal differentiation that precludes adequate proliferation of neural precursors and ultimately treatment efficacy. Moreover, embryonic development primarily occurs under hypoxic conditions. Consequently, NSCs are adapted to this environment through the regulation of hypoxia-inducible factors and hypoxia-responsive elements (Večeřa et al., 2020; Wu et al., 2021). It is well established that hypoxia-inducible factor 1α interacts with the Notch intracellular domain, which then acts as a primary driver of neuronal differentiation in development (Louvi and Artavanis-Tsakonas, 2006; Dengler et al., 2014; Ferrante et al., 2022). Notch-dependent inhibition is driven by pre- and post-natal hypoxic conditions to maintain a viable pool of NSCs. Concurrently, hypoxia-inducible factor 1α activates Hes1, a known neural repressor (Večeřa et al., 2020). However, hypoxia-responsive elements can be engineered to control the expression of various pro-regenerative factors. In a study by Wu et al. (2021), a hypoxia-responsive element sequence was engineered to repeat five times, incorporated into the promoter region of nerve growth factor, and then transfected into NSCs for transplantation following SCI. The repeat sequence increases the NSC sensitivity to the hypoxic environment seen in SCI, stimulating the release of nerve growth factor. Their results showed increases in nerve growth factor protected against excessive autophagy, reduced the size of the glial scar, and ultimately improved locomotor function. Linking neurotrophic expression to pathophysiologic changes is intriguing, as this can limit off-target effects; however, further study of this approach is needed to validate its regenerative potential.

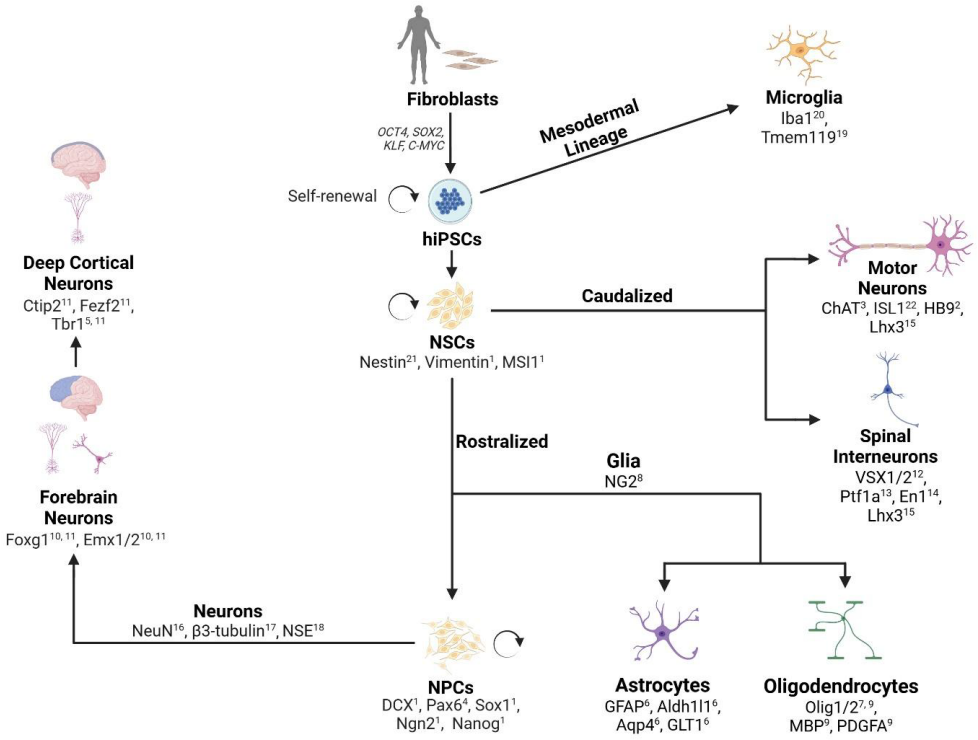

Figure 1 | **Differentiation and identification of iPSC-derived cells.**
Figure 1 depicts the differentiation pathways of the cells derived from human iPSCs covered in this text for regenerative medicine. 1: Oikari et al. (2016); 2: Arber et al. (1999); 3: Phillis (2005); 4: Hu et al. (2010); 5: Hevner et al. (2001); 6: Forrest et al. (2023); 7: Zhou and Anderson (2002); 8: Zhu et al. (2008); 9: Seeker and Williams (2022); 10: Varga et al. (2022); 11: Molyneaux et al. (2007); 12: Zholudeva et al. (2024); 13: Glasgow et al. (2005); 14: Falgairolle and O'Donovan (2019); 15: Thaler et al. (2002); 16: Duan et al. (2016); 17: Janke and Magiera (2020); 18: Babkina et al. (2024); 19: Ruan and Elyaman (2022); 20: Schwabenland et al. (2021); 21: Bernal and Arranz (2018); 22: Thiry et al. (2020). Created with BioRender.com. Aldh1l1: Aldehyde dehydrogenase 1 family member L1; Aqp4: aquaporin 4; ChAT: choline acetyltransferase; Ctip2: COUP-TF interacting protein 2; DCX: doublecortin; Emx1/2: empty spiracles homeobox 1/2; Fezf2: forebrain embryonic zinc finger-like protein 2; Foxg1: forkhead box G1; GFAP: glial fibrillary acidic protein; GLT1: glutamate transporter 1; HB9 (MNX1): homeobox 1 (motor neuron and pancreas homeobox 1); Iba1: ionized calcium-binding adapter molecule 1; iPSCs: induced pluripotent stem cells; ISL1: insulin gene enhancer protein 1; Lhx3: LIM homeobox 3; MBP: myelin basic protein; MSI1: musashi RNA binding protein 1; Ngn2: neurogenin 2; NSE: neuron-specific enolase; Olig1/2: oligodendrocyte transcription factor 1/2; Pax6: paired box 6; PDGFRA: platelet-derived growth factor receptor alpha; Ptf1a: pancreas transcription factor 1a; Sox1: SRY-box transcription factor 1; Tbr1: T-box brain transcription factor 1; Tmem119: transmembrane protein 119; TUBB3: β-3-tubulin; VSX1/2: visual system homeobox 1/2.

Understanding the epigenetic regulation of hiPSC-derived transplants is crucial for successful treatment. The complexity of multipotent cells necessitates a deeper understanding to successfully manipulate the epigenome for regeneration and repair. For example, studies by Tuszynski et al. (2014a, b) demonstrated that embryonic NSC grafts retain their growth and maturation patterns from their host origin, with maturation taking place over approximately 1.5 years. They reported functional recovery continued as long as 1 year post-transplantation (Lu et al., 2017). Although this study utilized ESCs, the rationale for using hiPSCs is that they emulate the pluripotency and genomic properties of ESCs and insight can be gained from this analogous work.

Another concern is the potential for cancer-associated mutations or unregulated proliferation in hiPSCs and their derivatives. The beneficial attributes that contribute to both NSC robustness and proliferative capacity also pose a risk of ectopic colonies and overall tumorigenicity. The remaining undifferentiated cells retain epigenetic memory that can be affected when reverting the cells back to a pluripotent state (Merkle et al. 2017; Yoshihara et al., 2019). To address this concern, several studies have developed strategies that target undifferentiated transplanted cells. In a rat model of TBI, Imai et al. (2023) used CRISPR/Cas9 genome editing to introduce a "suicide gene" into the transplanted hiPSC genome. Using yeast cytosine deaminase-uracil phosphoribosyl transferase as an enzyme-prodrug, the transfected NS/PCs then respond to the administration of 5-fluorocytosine, leading to dysfunctional RNA processing and ultimately cell death. Notably, only NS/PC viability and proliferation were affected, leaving intact the βIII-tubulin positive mature neurons (Imai et al., 2023). Other approaches to regulate proliferation or differentiation *in vivo* include designer receptors exclusively activated by designer drugs (DREADDs) (Armbruster et al., 2007; Urban and Roth, 2015; Ji et al., 2016). Their utility can also be exploited when investigating the immunogenicity of grafts and will be discussed in a later section of this review.

Along with utilizing ESC-derived NSCs, Tuszynski et al. (2014a, b) have employed hiPSCs as a source for NSC transplantation. Lu et al. (2014) transplanted hiPSC-derived NSCs embedded in a growth factor cocktail containing fibrin matrix into immunodeficient rats 2 weeks following C5 lateral hemisection injury. Analyses at 3 months post-transplantation revealed the survival of the graft and differentiation of the cells into

NeuN$^+$ MAP2$^+$ and Tuj1$^+$ mature neurons (71.2% ± 3.1%), and GFAP$^+$ astrocytes (17.7% ± 2.8%) with no teratoma formations and ectopic colonies reported in this study. However, other studies have reported ectopic colony formation following NSC grafting, suggesting the efficacy of NSC therapies continues to be a contested topic (Steward et al., 2014a, b; Tuszynski et al., 2014a, b; Kadoya et al., 2016). Lu et al. (2014) have also identified these grafted NSCs differentiating into VGlut1$^+$, ChAT$^+$, and 5HT$^+$ neurons, but found no expression of GABAergic markers. This demonstrates the potential of grafted NSCs to differentiate into several cell types and form functioning synapses, with some reportedly migrating as far as the olfactory bulb (Lu et al., 2014). However, the functional recovery and integration into host neural networks were not assessed when investigating these cells with a high differentiation potential. Further studies must focus on characterizing functional outcomes and synaptic integration to achieve a more comprehensive perspective on the therapeutic potential of NSCs.

Neural progenitor cells

In recent years, the focus has shifted from NSCs to NPCs. They maintain a high proliferative and differentiation potential, yet are lineage-restricted to region-specific subpopulations. Importantly, the capacity of self-renewal compared to NSCs is reduced. NPCs undergo asymmetric division, mitigating the risk of tumorigenesis associated with NSCs (Zhang and Jiao, 2015; Yabut and Pleasure, 2016). The unique regional patterning of NPCs can also be exploited when developing hiPSC-derived treatments for regeneration. Kajikawa et al. (2020) used NPCs to generate regionalized forebrain and spinal cord neurons for transplantation following SCI. Using a T10 (thoracic level 10) contusion injury paradigm, they transplanted both spinal cord-type (HOXB4$^+$) or forebrain-type progenitor cells (FOXG1$^+$) into the lesion epicenter at 9 days post-injury. Their results showed that only the spinal cord-type NPCs successfully integrated into the host corticospinal tract, showing pre- and post-synaptic colocalization between the host and graft (Kajikawa et al., 2020). Significantly, the corticospinal tract is the primary efferent control for voluntary muscle movement in humans and is often targeted in regenerative studies (Li and Lu, 2022). In a human clinical trial of Parkinson's disease, Schweitzer et al. (2020) transplanted midbrain dopaminergic progenitor cells into the putamen. While marginal functional improvements were observed, the autologous midbrain dopaminergic progenitor cells were transplanted without the need for immunosuppression. Although this study is classified as a replacement therapy, key insights can be gained from a human model. This variable success based on regionalized NPCs highlights the importance of further examining the unique properties of neuronal subpopulations.

In addition to the cellular composition of the graft, the injury core or diseased regions have a unique microenvironment that can influence NPC proliferation and differentiation (Marques et al., 2023). A key component of both healthy and injured microenvironments is the extracellular matrix (ECM). It provides critical structural support and promotes cell adhesion for migrating cells. Scar formation following neurologic injury is primarily composed of a dense ECM, surrounded by a glial scar. This protein-rich matrix has been shown to directly influence NSC and NPC proliferation and differentiation (Barros et al., 2011). In particular, targeting growth-promoting proteins within the ECM has been effective. For example, Andrews et al. (2009) had previously found that forced expression of the α9 integrin subunit promotes neurite outgrowth and regeneration following spinal cord injury. Tenascin-C is the primary glycoprotein of the CNS and is upregulated following injury. Binding of the α9 subunit promotes regeneration, however, neurons of the corticospinal tract downregulate the α9 integrin subunit following maturation. Forbes and Andrews (2019) utilized viral vectors to overexpress the α9 subunit in NPCs and found sustained expression up to 8 weeks following transplantation in neonatal rats. Despite the transplantation occurring in a developing sensorimotor cortex, this study offers a unique approach by leveraging endogenous signaling and intrinsic modifications, rather than modifying the extrinsic lesion environment.

Achieving control over NPC fate *in vivo* has remained a significant and ongoing challenge. hiPSC-derived grafts often contain some proportion of stem-like or progenitor cells, prompting concern about tumorigenicity. Importantly, methods to regulate *in vivo* differentiation must be clinically translatable, further limiting approaches such as genetic manipulation. One common technique seen in clinical treatment is the use of electrical stimulation. Using functional electrical stimulation to stimulate select muscle groups is often used during the rehabilitation of stroke or SCI patients (Ho et al., 2014; Howlett et al., 2015; Biasiucci et al., 2018; Luo et al., 2020). Considering this, Patil et al. (2023) transplanted spinal NPCs (TUJ1$^+$, NF200$^+$) 8 weeks after a T9 contusion injury in immunocompromised rats, modeling a chronic injury. Subsequently, tail nerve electrical stimulation took place 1 week following transplantation. Their results showed that tail nerve electrical stimulation increased the proportion of spinal NPCs that differentiated into oligodendrocytes. This resulted in improved myelination as well as increased axonal/dendritic projections both rostral and caudal to the lesion. In addition, serotonergic fiber expression was higher in the tail nerve electrical stimulation-treated group and demonstrated improvements in functional recovery (Patil et al., 2023). Activation of these serotonergic extensions can enhance motor recovery after SCI and aid in initiating locomotor activity (Sławińska et al, 2014). The idea that increased neurotransmission in the injured spinal cord can contribute to functional recovery is not just limited to electrical stimulation. Recognizing that physical rehabilitation is a routine treatment for neurologic injury and disease, Shibata et al. (2023) investigated its impact by combining NPC transplantation with treadmill training in immunocompromised rats. Using a T10 chronic contusion SCI model, they found heightened expression of brain-derived neurotrophic factor and neurotrophin 3, both recognized for their pro-regenerative properties (Keefe et al., 2017). As a result, the animals exhibited significant functional recovery and an increase in serotonergic (5-HT$^+$) expression, suggesting the treatment contributed to host regeneration and repair.

NPCs offer several advantages over NSC transplantation; however, they still maintain multipotency and require careful consideration to optimize their efficacy *in vivo*. Key factors must be considered both pre- and post-transplantation to regulate differentiation and promote regeneration or repair. Importantly, pre-clinical studies should strongly consider components of their treatment that can impact translation to a clinical setting for safe application in human injury and disease.

Glial Cells

Glial cells, particularly astrocytes and oligodendrocytes, are a crucial component of hiPSC-derived therapies due to their supportive functions within the CNS. In recent years, methodologies aimed at generating reliable glial populations from hiPSCs have become clarified and streamlined (Kamata et al., 2021). Consequently, hiPSC-derived glial cells are being explored increasingly as a tool for improving existing treatment options for CNS pathologies.

These cells are generally utilized in one of two ways: glial cell-replacement therapies or co-transplantation with hiPSC-derived neurons. In cell-replacement therapies, the primary goal is to restore the function of the CNS through the replacement of damaged or lost glial cells. The replacement of targeted glial populations allows for a high degree of precision in cases where a specific cell type is damaged or lost; it can also aid in restoring specific glial functions, e.g., remyelination of host tissue by transplanting oligodendrocyte progenitor cells along with hiPSC-NS/PCs (Kawabata et al., 2016) or neurotrophin production and inflammatory modulation by astrocytes (Nicaise et al., 2015; Yang et al., 2023). However, there is limited evidence suggesting that these transplantation strategies regenerate the host nervous system but rather act as a supporting factor to modulate the host microenvironment to subdue pathology. Current work mainly focuses on using hiPSC-glia for modeling disease in rodent models (Li and Shi, 2020; Stöberl et al., 2023), more research must focus on the potential of hiPSC-glia in inducing regenerative potential in the host.

In co-transplantation therapies, hiPSC-derived glial cells are transplanted in combination with hiPSC-NSCs or hiPSC-NS/PCs as a more comprehensive treatment approach, which relies on their synergistic relationship and ability to better mimic the environment of the CNS. For instance, Olmsted et al. (2022) transplanted fully mature spinal motor neurons (SMNs) derived from hiPSCs with hiPSC-derived oligodendrocyte progenitor cells and found robust survival following transplantation without further need for neuroprotective modifiers, such as brain-derived neurotrophic factor, NT3, or protective biomaterials (Lu et al., 2012). However, careful consideration must be taken to ensure an optimal ratio of neuronal and glial transplantation, as an overproduction of glial cells, particularly glial scar-forming astrocytes, can inhibit neuronal regeneration. Glial progenitor heterogeneity and regional specificity add another layer of complexity to combined hiPSC-derived neuron and glial transplants. Wei et al. (2023) report specifically on the heterogeneity of astroglia, elucidating distinct populations of GFAP-expressing progenitor cells that exist in a model of SCI both before and after injury. Their findings add to a body of work showing that glial progenitor heterogeneity needs to be carefully considered when developing combined hiPSC-derived neuron and glial treatments, as improperly differentiated glial populations are likely to disrupt neural networks and undermine the efficacy of the transplant.

Co-culturing hiPSC-derived neurons with glial cells is also a critical strategy employed in the development of organoids as models of neurologic conditions. Glial cells encourage the development of mature neurons, enhancing the electrophysiological properties of the 3D model and markedly improving its physiological relevance. Synaptic activity and plasticity are also improved in organoid models developed with co-cultured glial cells, further underscoring their importance in developing a model that is representative of actual nervous tissue. Kim et al. (2019) examined oligodendrogenesis in both ventral forebrain organoids and dorsal forebrain organoids and noted distinct temporal expression of OLIG2 between them. Further, they found that a fused forebrain organoid approach promoted oligodendroglia maturation, demonstrating the importance of accurately mimicking the architecture of the brain. The employment of human iPSCs also allows for the modeling of diseases that are historically inaccurate in rodent models such as microcephaly, thus allowing therapy discovery in a more human-like system (Lancaster et al., 2013). However, the lack of a neuroimmune system in organoids is a fundamental hurdle in developing organoid-based therapies and conducting accurate modeling. Microglia, the resident immune cells of the CNS, do not naturally appear in cerebral organoids, and work is being done to address this disparity (Quadrato et al., 2017; Qian et al., 2019). Microglia-like cells differentiated from hiPSCs through erythromyeloid progenitors have been co-cultured with cerebral organoids and shown to improve the network maturation and electrophysiological capabilities of the model (Fagerlund et al., 2021).

Neurons

A less explored approach to hiPSC-derived regenerative therapies is the use of post-mitotic, mature-like neurons. Their reduced capacity for growth relative to NSCs or NPCs had initially deterred interest in their ability to aid in the regeneration or repair of the CNS. However, a comprehensive evaluation of the efficacy of hiPSC-derived cell therapies has prompted researchers to explore neuronal transplantation. A notable early example of this is the work by Dimos et al. (2008), who derived motor neurons from a patient with amyotrophic lateral sclerosis (ALS). Although this particular study aimed to use autograft-generated motor neurons to replace the dead or dying neurons seen in ALS, their successful differentiation encouraged further investigation into the topic.

Aside from proliferative potential, transplantation of mature-like neurons faces several barriers. There is a diverse array of neuronal subpopulations, each with distinctive phenotypic signatures. Many of these neurons are intrinsically programmed to project local or long-distance axons while maintaining synaptic input from a distinctive combination

of neural circuits (Vanderhaeghen and Polleux, 2023). In the context of SCI, the corticospinal tract is composed of two neurons, the upper and lower motor neurons. The upper motor neuron cell body extends an axon that travels to the spinal level of its target innervation, and synapses onto a lower motor neuron where it then exits the CNS and innervates the target tissue. In humans, a single upper motor neuron axon can extend from the cortex to the lumbar cord before it synapses (Stifani, 2014; Vanderhaeghen and Polleux, 2023). The lower motor neuron, while also extending a large distance, varies greatly in its length depending on the region it exits. Additionally, lower motor neurons typically receive synaptic input from an upper motor neuron, interneuron, and a sensory neuron. Comparatively, the upper motor neuron integrates synaptic input from many cortical and subcortical neurons (Arber, 2012; Stifani, 2014). The innate epigenetic regulation of this extensive growth is imprinted on these cells and is critical to the proper development of the CNS. Considering this region-specific connectivity, it is logical that transplanting lower motor neurons (i.e., spinal motor neurons) into the spinal cord has a greater potential to integrate into the endogenous motor network. Yet, few studies have investigated the use of transplanted SMNs and achieved successful integration into host motor networks (Olmsted et al., 2022). A study by Lee et al. (2020) directly generated motor neurons from human fibroblasts. Briefly, fibroblasts were transduced to express the transcription factor *Oct4*, and then cultured in a neural induction medium for 14 days. The resulting motor neuron intermediate cells were transduced with human *LHX3*, with a medium change to motor neuron induction medium for 7 days (Days 14–21). At 21 days, the media was changed to motor neuron maturation media, and the cells were allowed to mature for another 7–14 days. The rats received a T9 crush injury and the induced motor neurons were transplanted 7 days later. Analyses showed an improvement in locomotor function and ensheathment by host oligodendrocytes *in vivo* and formation of neuromuscular junctions with myotubes *in vitro*. Another group has exploited the recent advancement of differentiation protocols, which allows further analysis of regionalized transplants. Olmsted et al. (2021) sought to transplant preformed neuronal networks consisting of SMNs, interneurons, and oligodendrocyte precursor cells. Using a combined neuromesoderm and neuroectoderm approach, cultures were ultimately transplanted as a whole SMN network and encapsulated in an alginate/hydrogel matrix forming a neural ribbon. This neural ribbon facilitated a much smaller number of cells transplanted compared to cell suspensions (~5000 *vs*. ~200,000), permitting a robust assessment of grafted cells. Their electrochemical properties and spatial distribution were assessed by microelectrode array, whole-cell patch clamp, and magnetic nanoparticles before transplantation, demonstrating successful and functional network formation *in vitro*. Subsequently, rats received a C4 hemi-contusion injury and neural ribbons were transplanted 15 days after the injury. At 6 weeks, the grafts were reported to have retained synapses with dense $SYN1^+$ staining, aligning along lesion edges with ramified oligodendrocyte progenitor cells, and $TUJ1^+$ fibers were interspersed among host fibers, suggesting successful integration into the endogenous network.

Considering the axon length and synaptic connectivity of cortical neurons, some studies have begun to assess the efficacy of these neurons in neurotraumatic injuries (Tornero et al., 2013; Doulames et al., 2024). Notably, there is a distinction between using hiPSC-derived neurons for cell replacement therapy as opposed to stimulating host regeneration or repair (Barker et al., 2018). hiPSC transplantation can show efficacy by either activating intrinsic repair mechanisms or replacing damaged or diseased neurons. Degenerative diseases, such as Parkinson's disease, have a distinct pathophysiology that typically necessitates the replacement of dysfunctional or dying neurons for complete recovery. However, neurotraumatic injuries such as TBI or SCI may better benefit from endogenous host regeneration that reconnects existing neural circuitry. Few studies have investigated the ability of transplanted mature-like neurons to functionally integrate into the host network in neurotraumatic models. Recently, groups have ventured to further characterize the potential of cortically fated transplants. In a rat model of stroke, Palma-Tortosa et al. (2020) transplanted hiPSC-derived neuroepithelial cells directly at the lesion site 48 hours after distal middle cerebral artery occlusion. Following transplantation, the graft and lesion were examined at 6 months post-injury and found that 41% of transplanted cells expressed cortical layer marker Satb2, demonstrating a maturing neuronal phenotype, while approximately 40% expressed the oligodendrocyte marker Sox10. Functionally, the distal middle cerebral artery occlusion-induced asymmetry was completely resolved in the group that received neuroepithelial cell transplantation. To determine if the observed functional improvements were the result of the graft, halorhodopsin-expressing hiPSC-derived neurons were inhibited via an LED light source. Interestingly, the observed recovery was not abolished with inhibition of the grafted cells. This suggests that the functional improvements were not directly the result of these hiPSC-derived neurons, but likely from local environmental changes induced by the transplant (Palma-Tortosa et al., 2020). Doulames et al. (2021) have also investigated the capacity of mature-like cortical neurons to stimulate host regeneration. Using a cervical model of SCI, they found that transplantation of hiPSC-derived deep cortical neurons into the acutely injured spinal cord promotes regeneration and significant functional recovery. The hiPSCs were directed toward a neuroepithelial fate via dual SMAD inhibition (Doulames et al., 2021). The resulting dorsal neural progenitor cells were then driven rostrally through Wnt inhibition and allowed to mature for 35 days. Mature-like deep cortical neurons were transplanted 2 weeks following a C6 hemisection SCI then administered to the lesion core, suspended in a fibrinogen/thrombin matrix. The subsequent functional tests demonstrated complete or near complete motor recovery at 12 weeks. A second cohort underwent further functional assessments until 30 weeks, after which diphtheria toxin was used to selectively ablate the graft. Diphtheria toxin binds to human cells with 100,000 times greater affinity than it does to rat cells, therefore, was used to determine the contribution of the graft to the observed recovery. A significant reduction in motor function was observed following graft ablation, suggesting the recovery

was indeed a result of the transplantation. In addition, axons extended 1.5 cm caudally and 9 mm rostrally from the lesion epicenter, maintaining their deep cortical neuron ($Tbr1^+$, $Ctip2^+$, $SatB2^{low}$) phenotype throughout the 1-year study (Doulames et al., 2021). The above studies highlight the early successes seen when transplanting post-mitotic neurons, including robust regeneration and substantial functional recovery. These approaches warrant further investigation, as this understudied approach offers a novel perspective in hiPSC-derived cell therapy.

hiPSC-derived spinal interneurons (SpINs) have been implicated in several facets of CNS recovery after transplantation and are understood to be important facilitators of neuroplasticity. SpINs comprise an array of neuronal cell types in the spinal cord which enable the integration and processing of sensory information and the transmission of the appropriate signals to motor neurons. Spinal interneurons are generally organized into "cardinal classes" based on their function and location within the spinal cord. Ziskind-Conhaim and Hochman (2017) provide an overview of these classes as well as the progenitor domains associated with various interneuronal subtypes, which have been clarified via careful examination of transcription factor expression. Identifying functionally discrete groups of interneurons is of particular interest to the field of SCI because of the role that SpINs play in central pattern generators, interconnected circuits of interneurons which are responsible for rhythm-generating and rhythm-coordinating networks (Calancie et al., 1994; Calancie, B., 2006; Nadeau et al., 2010; Ziskind-Conhaim and Hochman, 2017; Minassian et al., 2023). Ten progenitor domains have been identified, including V0–V3, which are generated in the ventral neural tube, and dI1–dI6, which are generated in the dorsal alar plate (Goulding, 2009; Ziskind-Conhaim and Hochman, 2017). Zholudeva et al. (2021) reviewed the heterogeneity of several populations of ventrally-derived SpINs, demonstrating their broad range of activity and providing an in-depth analysis of their role in the movement. However, many of the precise mechanisms by which SpINs allow for the integration of sensory information to influence motor activity have yet to be fully elucidated.

Developmental neurobiology has permitted the isolation of markers that can be used to identify different interneuronal progenitor domains. As a result, the development of neural precursor cells biased toward differentiation to SpINs has been explored, and protocols have been developed to improve the reliability of this technique. Gupta et al. (2021) developed differentiation protocols for deriving heat-mediating dI4/dI6 interneurons as well as proprioceptive dI3s and mechanosensory dI4s. These build on their previous protocols, which allow for the generation of dI1s, dI2s, and dI3s (Gupta et al. 2018). As these protocols have been refined, combination treatments including interneuronal subpopulations have been hypothesized to improve certain aspects of recovery after SCI. For example, V2a SpINs, which have been implicated with the phrenic motor circuit (Jensen et al. 2019), have been demonstrated to improve functional recovery when transplanted alongside neural progenitor cells in the injured cervical spinal cord (Zholudeva et al., 2018). More recently, the focus has narrowed to generate transplantable populations of VSX2-expressing spinal V2a SpINs with rostrocaudal specificity, which was hypothesized to further improve respiratory function but requires optimized protocols. In this study, Zholudeva et al. (2024) aimed to transplant optogenetically engineered cervical V2a interneurons into a sub-acute model of C4 contusion injury, with the goal of improving diaphragm function. Subsequently, pseudorabies virus tracing indicated synaptic connectivity and donor-to-host integration into the phrenic nerve circuit. Optogenetic activation also indicated functional integration of the transplanted V2a SpINs by supraspinal host axons, and postliminary histology showed extensive anatomical integration of the host injury site by donor cells. Most importantly, transplant recipients showed a significant improvement in baseline respiratory function as well as respiratory challenge 2 months post-transplantation.

hiPSC-derived interneurons show promise as complementary therapies to traditional transplantation methods, and an improved understanding of the interneuronal subpopulations responsible for plasticity post-injury may allow for the development of more appropriate therapies with improved regenerative potential.

Transplantation Substrates for Human Induced Pluripotent Stem Cells

Each approach of hiPSC-derived transplantation requires a method to deliver the cells to the target tissue. Importantly, a shared goal of these methodologies is to increase survivability and reduce graft migration *in vivo*. These vectors must support the transfer of the cells, while maintaining biocompatibility into the *in vivo* site of injury or disease. Most commonly, the techniques used for cell transplantation therapies include direct injection to the site of injury or disease or systemic venous administration (Amer et al., 2017; **Additional Table 1**). Each depends on the specific location where the cells are needed, but both often require delivery via a syringe. Relocating cells from a controlled *in vitro* environment to an injury or disease-state *in vivo* environment can induce cellular stress that has been captured at transcriptional and post-translational stages (Amer et al., 2017). As cells travel through a needle, shearing and mechanical stress on cell membranes can cause cell lysis or indirectly induce necrosis or apoptosis (Aguado et al., 2012; Amer et al., 2017; Wahlberg et al., 2018). The resulting transplantation will not only have a reduced number of viable cells, but all cells involved in the transplant can upregulate genes associated with cellular stress or other cytotoxic cell–cell communication.

Extracellular matrices and their derivatives are commonly used to supplement cell transplantation due to their innate ability to anchor cells while having side chains that can influence proliferation and regeneration (Pakulska et al., 2012; Suzuki et al., 2017). Indeed, ECM-based hydrogels have been used in various fields for cell therapies. Their application to stem cell therapies is now being explored and the results are encouraging. To address the cell shearing during transplant, groups have utilized biocompatible hydrogels. Furthermore, hydrogels can aid in reducing transplant migration away from the target region leading to a reduction in the overall spread

of the graft. Doulames et al., (2024) have developed a dual-component hydrogel that has been shown to increase graft viability and mitigate graft migration outside of the injury site. Termed SHIELD (shear-thinning hydrogel for injectable encapsulation and long-term delivery), this thixotropic hydrogel improves functional outcomes while protecting cells from shear forces. Additionally, the ECM-derived ligands promote cell adhesion and neurite outgrowth. Other studies have utilized biopolymers such as hyaluronic acid, alginate, and collagen that naturally regulate cell adhesion and proliferation, much like the ECM (Aguado et al., 2012; Yao and DeBrot, 2020; Olmsted et al., 2021). However, custom-engineered hydrogels provide the benefit of permitting more extensive modifications to resolve the multifaceted problem facing hiPSC transplantation.

Transferring cells from a carefully controlled *in vitro* environment to an injured or diseased region of the body requires a supportive medium to reduce cellular stress and increase graft viability. These delivery vectors must also be compatible with the new *in vivo* environment, providing a growth-supportive scaffold that promotes successful integration into the existing host neural networks. The current literature shows that some progress is being made regarding the transfer of hiPSC-derived cells from an *in vitro* to an *in vivo* environment; however, further work must be done to optimize these strategies and ensure biocompatibility with human biology.

Immunology & Human Leukocyte Antigen Matching

The existing literature has yet to fully address the immune response of hiPSC transplantation in animal models. While each application of hiPSC-derived cells has strengths and weaknesses, a shared concern is the immunogenicity of the grafts. Much of the current literature uses immunocompromised animal models as proof-of-concept during transplantation (**Additional Table 1**, Kajikawa et al., 2020; Palma-Tortosa et al., 2020; Patil et al., 2023; Shibata et al., 2023). While this will prevent graft rejection and permit the study of graft response *in vivo*, it is difficult to properly examine the species-specific responses to human tissue. This critical question is unanswered and can have a significant impact on a study's success in clinical trials.

An additional challenge to hiPSC-derived therapies is the availability of HLA-matched cells. While recent studies show promising results, the progression to clinical trials is encumbered by the need for immunosuppression (Liu et al., 2017; Deinsberger et al., 2020). Like immunosuppressive therapy following organ transplantation, this approach reduces the prospect of graft rejection by the host. However, patients must continue to receive immunosuppressive treatment, predisposing them to opportunistic infections, cancer, or other complications (Mika and Stepnowski, 2016; Robers and Fishman, 2021). Initially, a solution was thought to be the establishment of a global hiPSC bank. Individuals within genotypically similar populations can donate tissue to be reprogrammed back to a pluripotent state while maintaining the variety of necessary HLA isotypes (Taylor et al., 2012; Solomon et al., 2014; Steeg et al., 2020; Yoshida et al., 2023). Ideally, this approach can reduce or remove the need for immunosuppressive therapy and ensure the majority of those within a given region have access to hiPSC-derived therapy. Although this approach has achieved success in certain countries, a persisting challenge is the need to culture, differentiate, and mature the cells. In particular, neurotraumatic injuries often have a window of optimal treatment in the acute to subacute stages of injury (Sandean et al., 2020). The time needed to produce NSCs, NPCs, or mature-like neurons from hiPSCs can often exceed this window. In an effort to circumvent immunosuppression, Song et al. (2020) sought to achieve this through autologous derivation of midbrain dopaminergic neurons. Using a customized pipeline of fibroblast reprogramming, generation of midbrain dopaminergic neurons, and removal of residual undifferentiated cells, the group confirmed successful integration and behavioral improvement in mice. However, they acknowledge the barrier to widespread adoption is the cost of personalized, autologous transplantations. While this study proposes a cell replacement therapy, this novel approach to obviating the need for immunosuppression can provide a framework for translating to regenerative therapies. However, further optimization of culturing techniques is necessary to ensure cells are appropriately matured for transplantation.

While banking genotypically similar hiPSCs can reduce the chances of graft rejection, the consequence is underrepresentation of the diverse human genome and phenotypes. Estimates from whole genome sequencing report many hiPSC lines listed in the NIH registry are primarily composed of European ancestry (93%), while also making up approximately 67% of other large global cell banks (Merkle et al., 2017; Ghosh et al., 2022). In an attempt to balance the need for genetic diversity while maintaining the necessary haplotypes, smaller, region-specific hiPSC banks have emerged (Taylor, et al., 2012; Huang et al., 2019). This solution works for many regions, however, numerous others may not have the resources to develop such a cell bank.

Recently, Hu et al. (2024) have developed a strategy to generate hypoimmune hiPSCs. By utilizing CRISPR-Cas9 genome editing, they were able to inactivate *B2M* and *CIITA* gene expression to suppress lymphocyte recognition in non-human primates. To address macrophage and NK cell identification of a "missing self," the critical immune checkpoint inhibitor CD47 was overexpressed via lentiviral transduction. Notably, these "stealth hiPSCs" were differentiated into pancreatic islet cells to treat diabetes in immunocompetent mice (Hu et al., 2024). Although this differentiation was driven toward mesodermal and endodermal lineages, a neuroectodermal differentiation would likely retain the genetic modifications and maintain its pluripotent state (Han et al., 2019). Additional studies have confirmed this approach *in vitro* and again in mice (Han et al., 2019; Pizzato et al., 2023), demonstrating the success and growing interest in this methodology.

Importantly, these studies have also highlighted the risk associated with immune evasion. Removing the ability of the

immune system to identify and remove foreign cells permits teratomas and stochastic differentiation to go unchecked (Gonzalez et al., 2018; Pende et al., 2019). Further work is necessary to evaluate the overall impact of immune-evasive cellular therapies. One potential solution involves incorporating a built-in kill switch or suicide gene. Various approaches to this strategy exist and have their advantages and disadvantages. One approach is the use of DREADDs. Armbruster et al. (2007) engineered yeast muscarinic G protein-coupled receptors to be ectopically expressed, exclusively binding clozapine-N-oxide, a pharmacologically inert compound. This allows the expression of select receptors that are only activated by synthetic ligands, ameliorating the risk of off-target effects. Importantly, DREADDs can be either activating or inhibitory, expanding their utility to manipulate the *in vivo* environment. Groups have trialed DREADDs in hiPSC-derived therapies and found success in allogeneic graft models (Armbruster et al., 2007). Ji et al. (2016) attempted this by inducing hM4Di in NPCs and monitoring their differentiation via *in vivo* positron emission tomography (PET). In addition, they found that DREADDs can be used as a reporter for hiPSC-derived cells following transplantation using functional magnetic resonance imaging, which is routinely used in a clinical setting. This technique uses a radiotracer, often fluorodeoxyglucose, that accumulates in cells and tissue with a high metabolic turnover. Tanimoto et al. (2020) have sought to exploit PET using the 18 kDa translocator protein (TSPO) as a marker for neural stem and precursor cells. Following transplantation of the NS/PCs into the striatum of immunocompromised mice, they used PET to identify changes in TSPO expression at 9 and 42 days. While they were able to detect differences, TSPO is not exclusive to undifferentiated stem and progenitor cells, therefore, further work must be done to hone in on a more selective method. The significance of developing a reliable imaging methodology to identify transplanted cells cannot be understated. Considering the concern for stochastic differentiation, fate mapping these cells and tracking their spatial distribution *in vivo* is a crucial step in achieving hiPSC-derived therapies for clinical use.

Conclusions

The profound impact of CNS injury and disease continues to diminish the quality of life and overall lifespan, affecting approximately one-third of the global population (GBD 2021 Nervous System Disorders Collaborators, 2024). Current preclinical treatments offer limited restoration of sensory, motor, or cognitive deficits, although no current treatments exist that completely resolve these impairments. The pluripotency of stem cells has rekindled interest in potential cures for these conditions. Initially, ESCs showed early success in promoting CNS regeneration and repair. However, ethical and technical limitations have hindered the widespread adoption of ESCs as standard treatment. This led to the development of induced pluripotent stem cells and hiPSC-derived cell transplantation, which holds significant promise for advancing regenerative medicine.

Despite the significant potential of hiPSC-derived treatments, substantial work remains before they can be brought to clinical trials. A primary concern with early stem or progenitor cells is the risk of stochastic differentiation and teratoma formation. NSCs are particularly susceptible due to their ability to differentiate into any neuronal or glial population (Bergstrom and Forsberg-Nilsson, 2012; Homem et al., 2015). NPCs carry less risk but still retain multipotency (Zhang and Jiao, 2015; Yabut and Pleasure, 2016), thus, concerns remain regarding their use in humans. Various strategies, such as kill switches or DREADDs, have been developed to mitigate these issues (Armbruster et al., 2007; Urban and Roth, 2015; Ji et al., 2016; Imai et al., 2023). Although these methods have shown considerable success, NSCs and NPCs still possess the genotypic and epigenetic capacity to introduce problems later in life (Merkle et al. 2017; Yoshihara et al., 2019). Furthermore, treatments with NSCs and NPCs require the grafted cells to differentiate *in vivo* within a hostile microenvironment (Barros et al., 2011; Cesare et al., 2022; Marques et al., 2023). This environment can affect their differentiation and proliferation, potentially reducing the optimal window for synaptic integration and CNS regeneration.

Alternatives that are currently being explored to circumvent these problems include post-mitotic cells such as neuronal subpopulations and interneurons. They have the innate ability to incorporate synaptic input from various sources (Arber, 2012; Stifani, 2014). In particular, cortical neurons are intrinsically programmed to extend long axons (Tornero et al., 2013; Doulames et al., 2024), while interneurons naturally are critical components of motor networks in the spinal cord. It should be noted that interneurons may be better suited for the repair of relay pathways, such as the phrenic nerve respiratory circuit or spinal motor neuron reflexes (Zholudeva et al., 2018; Jensen et al. 2019). These approaches are fairly recent and will require further work to determine their efficacy in the future.

Key supplementary treatments to improve hiPSC-derived transplantation efficacy include glial cells and optimized delivery vectors. Glial cells, such as astrocytes or oligodendrocytes, naturally support neurons *in vivo* and can help promote the maturation of synapses (Kim et al., 2019). Administration of grafted cells requires an ideal medium to increase viability and promote adherence to the transplant. Several methods exist, with many based on ECM and ECM derivatives (Aguado et al., 2012; Liu et al., 2017; Yao and DeBrot, 2020; Doulames et al., 2024). Currently, hydrogels are showing positive results in improving viability and integration, although further work must investigate the human response to these biomaterials.

Xenogenic or allogeneic cell transplantation must overcome the additional barrier of immune rejection. While current research shows promise, most studies rely on immunocompromised animal models (Lu et al., 2017; Kajikawa et al., 2020; Palma-Tortosa et al., 2020; Doulames et al., 2021; Imai et al., 2023; Patil et al., 2023; Shibata et al., 2023; Doulames et al., 2024; **Additional Table 1**). Patients with neurologic injury or disease would be further disadvantaged by the need for immunosuppressive therapy. Therefore, more work must be done to determine the human immune response to allogeneic hiPSC-derived grafts. Genetic manipulation of hiPSC-derived grafts *in vitro* has

been attempted to mitigate this issue (Armbruster et al., 2007; Urban and Roth, 2015; Ji et al., 2016), yet it adds an additional barrier to clinical translation. Common approaches include the use of DREADDs and hypoimmunogenic cells. DREADDs, activated by pharmacologically inert compounds, show potential, but the human immune response is still unknown. Hypoimmune hiPSCs can evade the immune system and integrate into host circuitry, but immune evasion can also increase the risk of cancer (Pende et al., 2019; Dębska-Zielkowska et al., 2021; Pizzato et al., 2023; Hu et al., 2024). Additionally, long-term outcomes of hiPSC-derived cell transplantation therapies require longitudinal studies to fully assess their safety and efficacy. Addressing these challenges is crucial, as hiPSC-derived therapies hold the potential to transform the treatment of neurological dysfunction and provide a definitive path forward for regenerative medicine.

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Additional file:
Additional Table 1: *Summary of transplant studies.*

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