Chapter 13
Neural Stem Cell Dysfunction in Human Brain Disorders

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Abstract Neural stem cells (NSCs) give rise to the entire nervous system. Animal models suggest that defects in NSC proliferation and differentiation contribute to several brain disorders (e.g., microcephaly, macrocephaly, autism, schizophrenia, and Huntington's disease). However, animal models of such diseases do not fully recapitulate all disease-related phenotypes because of substantial differences in brain development between rodents and humans. Therefore, additional human-based evidence is required to understand the mechanisms that are involved in the development of neurological diseases that result from human NSC (hNSC) dysfunction. Human-induced pluripotent stem cells provide a new model to investigate the contribution of hNSCs to various neurological pathologies. In this chapter, we review the role of hNSCs in both neurodevelopment- and neurodegeneration-related human brain pathologies, with an emphasis on recent evidence that has been obtained using embryonic stem cell- or induced pluripotent stem cell-derived hNSCs and progenitors.

13.1 Introduction

Neural stem cells (NSCs) are multipotent cells that give rise to the entire nervous system during development and contribute to physiological neuron renewal in specific brain areas. The maintenance and expansion of the NSC pool by self-renewal and the timing and mechanism by which NSCs become committed to differentiation are tightly regulated and critical for proper development of the nervous system. Defects in NSC proliferation and differentiation have been shown to be responsible for brain pathologies in animal models of various neurodevelopmental disorders. The dysfunction of NSCs has been observed in other brain diseases that are typically linked to improper neuronal transmission (e.g., autism, epilepsy, schizophrenia, and bipolar disorder) and neuronal death

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(i.e., neurodegenerative disorders). Animal models of disease provide key insights into the pathogenesis of neurological disorders, but they do not fully recapitulate all phenotypes that are observed in humans because of substantial differences in brain development between rodents and humans. Therefore, additional human-based evidence is required to understand the mechanisms that are involved in the development of neurological diseases that result from human neural stem cell (hNSC) dysfunction. Recent advances in stem cell technologies (e.g., optimized protocols for cell reprogramming and differentiation) have provided new tools to investigate the contribution of hNSCs to various neurological pathologies. Combined with genome engineering and high-throughput methods to analyze gene and protein expression globally, patient-derived cellular models have begun to reveal the ways in which hNSCs are affected, not only in the course of neurodevelopmental diseases but also in other nervous system pathologies. In this chapter, we review the role of hNSCs in human brain pathologies, with an emphasis on recent evidence that has been obtained using embryonic stem cell (ESC)- and induced pluripotent stem cell (iPSC)-derived hNSCs.

13.2 Neurogenesis and Neural Stem Cell-Related Disorders

The mature nervous system is composed of several cell types (e.g., neurons and glia) that originate from NSCs. The transition from multipotent and proliferative NSCs to fully differentiated and functional neurons is called neurogenesis. The majority of neurons are generated during early embryonic development and early postnatal stages. In the human brain, as in the brains of other mammals, a few neurogenic niches remain active in adulthood and produce neurons throughout life, although less efficiently (Bergmann et al. 2015; Lim and Alvarez-Buylla 2016; Gonçalves et al. 2016). During embryonic development, the first neurogenic niche is formed after closure of the neural tube (Florio and Huttner 2014). The single layer of neuroepithelial cells that surround the lumen of the neural tube, known as the neuroepithelium, represents the first NSCs. These cells first undergo so-called symmetrical proliferative divisions that lead to transformation of the neural tube into multilayered tissue. As neurogenesis progresses, neuroepithelial cells begin to divide asymmetrically and generate radial glia. Radial glia reside in a layer that lines the ventricle, called the ventricular zone (VZ), and are able to undergo proliferative or differentiating division. During differentiating/asymmetric division, radial glia give rise to one radial glia cell and one neuron or one intermediate progenitor. Intermediate progenitors migrate to a layer basal to the VZ, called the subventricular zone (SVZ), and proliferate to increase the number of neurons (Florio and Huttner 2014). As brain development progresses, the production of new neurons by these stem cell niches declines, but in some brain areas the SVZ retains its neurogenic competence postnatally. In adult rodents, neurogenesis occurs in the SVZ, and newly born γ-aminobutyric acid (GABA)ergic neurons populate the olfactory bulbs (Lim and Alvarez-Buylla 2016). However, the neurogenic potential of the SVZ in humans

remains controversial (Bergmann et al. 2015). To date, there is no definitive evidence of the migration of new neurons from the human SVZ to olfactory bulbs, which are atrophied relative to the olfactory bulbs in rodents and other mammals that rely more heavily on olfaction. Some studies suggest that by 2 years of age, no new neurons are born in the SVZ (Sanai et al. 2011; Bergmann et al. 2012). However, recent studies suggest that such neurons exist but populate the striatum (i.e., an area adjacent to the SVZ) instead of the olfactory bulbs (Ernst et al. 2014). In addition to the VZ and SVZ, two additional neurogenic niches generate neurons during central nervous system (CNS) development: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and external granule cell layer of the cerebellum, which generate DG and cerebellar granule neurons, respectively. Dentate gyrus stem cells originate from the VZ and migrate toward the hilar region. Interestingly, the SGZ of the DG generates neurons that integrate into an existing circuit throughout life not only in rodents but also in humans (Bergmann et al. 2015). Although the role of this adult neurogenesis is still debated, rodent studies suggest that these adult-born neurons are needed for brain plasticity (Gonçalves et al. 2016).

Neurodevelopmental disorders often have a clearly established genetic cause, and brain tumors are the main CNS pathologies that are linked to NSCs and neuroprogenitor cell (NPC) abnormalities (Swartling et al. 2015; Prajumwongs et al. 2016). One example of the former group is primary microcephaly (MCPH), which is either an autosomal recessive or X chromosome-linked disease. The characteristic features of MCPH include a smaller head size (mostly attributable to a reduction of the cerebral cortex) and deficits in intellectual, language, and motor skills. Magnetic resonance imaging scans of MCPH patients revealed a smaller brain volume but normal brain organization, suggesting that MCPH is not a disorder of neuronal migration or organization but rather a disorder of neuronal number. Primary microcephaly is caused by different genes that encode proteins that are expressed in cortical NSCs/NPCs and are involved in regulating the cell cycle and the function of the centrosome or mitotic spindle orientation (e.g., *MCPH1*, *ASPM*, *CDK5RAP2*, *CENPJ*, and *STIL*). Cellular and animal model studies demonstrated that MCHP is related to the defective proliferation of NSCs/NPCs and disturbances in the balance or premature transition from symmetric to asymmetric neuronal progenitor cell division, resulting in a reduction of the progenitor pool, a decrease in the number of neurons, and reduced cell survival (Faheem et al. 2015). Another group of neurodevelopmental disorders comprises pathologies that are associated with the upregulation of mechanistic/mammalian target of rapamycin (mTOR) kinase signaling, including tuberous sclerosis complex (TSC), neurofibromatosis type 1 (NF1), and phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) mutation-related syndromes (Switon et al. 2017). Based on animal models and human-derived brain samples, these disorders were shown to be linked to NSC/NPC dysfunction (Switon et al. 2017). For example, the formation of brain tumors and macrocephaly in tuberous sclerosis was suggested to be caused by the improper proliferation and differentiation of NSCs (Switon et al. 2017). The observation that autism spectrum disorder (ASD) is common to several neurodevelopmental disorders (e.g., TSC, Fragile X syndrome [FXS]) prompted

the search for connections between ASD and hNSC pathologies in addition to analysis of disturbances of synapse formation and neural network function.

The link between aberrant neurogenesis and neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), is not often considered. Convincing evidence has shown that the human HD brain exhibits greater NPC proliferation that is proportional to the severity of the gene defect that is responsible for the disease and proportional to the severity of the pathology of the disease (Curtis et al. 2003). The data for PD are less consistent, but fewer proliferating cells in the SVZ were observed in postmortem human brain samples (Höglinger et al. 2004). Mice that expressed a mutation of leucine-rich repeat kinase 2 (*Lrrk2*; G2019S mutation), which is encoded by a PD susceptibility gene, had fewer proliferating cells in the SGZ and SVZ (Winner et al. 2011). Mice that overexpressed A30P α-synuclein, another PD susceptibility gene, did not present significant differences in the number of neurons that were generated in the SVZ, but the integration and survival of postnatally born dopaminergic neurons were affected (Neuner et al. 2014). Analyses of hNSCs and NPCs in mouse models of AD and patients' brain specimens yielded contradictory results, suggesting either increases or decreases in proliferation that depended on the animal model and stage of the disease (Liu and Song 2016; Tincer et al. 2016; Hollands et al. 2016). Additional disease models are needed to understand the primary effects of the disease on hNSCs and NPCs and their contribution to disease progression. Promising new models have emerged. In the following sections, we discuss these technical advancements in more detail.

13.3 Human Neural Stem Cells In Vitro: Patient in a Dish

For many years, animal models and postmortem human samples were the only source of accessible material to investigate the role of NSCs in human CNS diseases. Although both of these provided important information, they also have limitations. Disease models that consist of knockout animals often do not fully recapitulate all phenotypes that are observed in humans because of substantial differences in rodent and human neurogenesis. These problems could be potentially overcome by using human cell models in studies of neurological disorders. However, obtaining human brain tissue samples is limited by inaccessibility, difficulties in obtaining material, or poor status of the tissue samples. Thus, postmortem studies of neurological disorders are mainly conducted. This creates a problem for understanding disease etiology and progression because postmortem samples give only a "snapshot" of mainly the end stage of the disease that does not inform about the underlying mechanism of pathology. Pathological changes that are observed in these samples could be secondary or mask the primary causes of the disease. Therefore, knowledge of neuropathological abnormalities and their progression during the course of a human disease is limited, and the development of new human models that are based on defined cell populations that are affected by the disease (e.g., hNSCs and hNPCs) is important. Until recently,

in vitro-cultured hNSCs that are obtained either directly from the patient nervous system or as a result of the differentiation of hESCs were used to overcome the aforementioned obstacles. However, two recent discoveries substantially changed this situation. Yamanaka and colleagues showed that differentiated somatic cells can be reprogrammed to pluripotency, and several efficient protocols for the production and differentiation of iPSCs were developed (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Kulcenty et al. 2015). This allowed researchers to obtain human disease-specific iPSCs from which hNSCs and hNPCs could be derived. It also provided the opportunity to analyze the early stages of disease progression in cells with genomes that are prone or can lead to disorder development and investigate the molecular mechanisms that could be the origin of the disease. New tools for genome engineering (e.g., Clustered Regularly Interspaced Short Palindromic Repeats [CRISPR]-CRISPR-associated protein 9 [Cas9] technology) were developed that allowed the introduction of "disease-causing mutations" to already available hNSC lines. In Sect. 13.3.1, we describe iPSCs and their differentiation to hNSCs. Descriptions of CRISPR-Cas9 technology can be found in several extensive recent reviews (Hsu et al. 2014; Muffat et al. 2016; Komor et al. 2017).

13.3.1 Induced Pluripotent Stem Cells as a Source of Human Neural Stem Cells

Takahashi and Yamanaka were pioneers of the reprogramming method. They demonstrated that the combined ectopic expression of four transcription factors [octamer-binding transcription factor 4 (Oct-4; also known as POU5F1), sex-determining region Y box-2 (Sox-2), Kruppel-like factor 4 (Klf-4), and proto-oncogene c-Myc (c-Myc)] was sufficient to reprogram mouse fibroblasts back to the pluripotent state. The newly obtained cells were called iPSCs (Takahashi and Yamanaka 2006; Fig. 13.1). Shortly thereafter, this approach was repeated using human fibroblasts, resulting in the generation of human iPSCs (hiPSCs). Human iPSCs resemble human ESCs (hESCs) in many aspects, including morphology, proliferation, pluripotency markers, gene expression profiles, and the ability to differentiate into three germ layers (Takahashi et al. 2007). Soon after, iPSCs were obtained from somatic cells of patients who suffered from a variety of neurological disorders, including both neurodevelopmental and neurodegenerative disorders (e.g., Rett syndrome, FXS, HD, and PD; Park et al. 2008; Han et al. 2011; Dolmetsch and Geschwind 2011; Marchetto et al. 2011). Numerous studies subsequently established many protocols for the differentiation of hiPSCs into hNSCs/hNPCs with variable efficiency and quality of the obtained cells. The traditional protocol required the generation of embryoid bodies (EBs) by culturing iPSC colonies under non-adherent conditions in iPSC culture medium without basic fibroblast growth factor (bFGF) (Fig. 13.1). In some protocols, to increase induction of the neural linage, retinoic acid was added for a couple of the last days of EB formation, or the

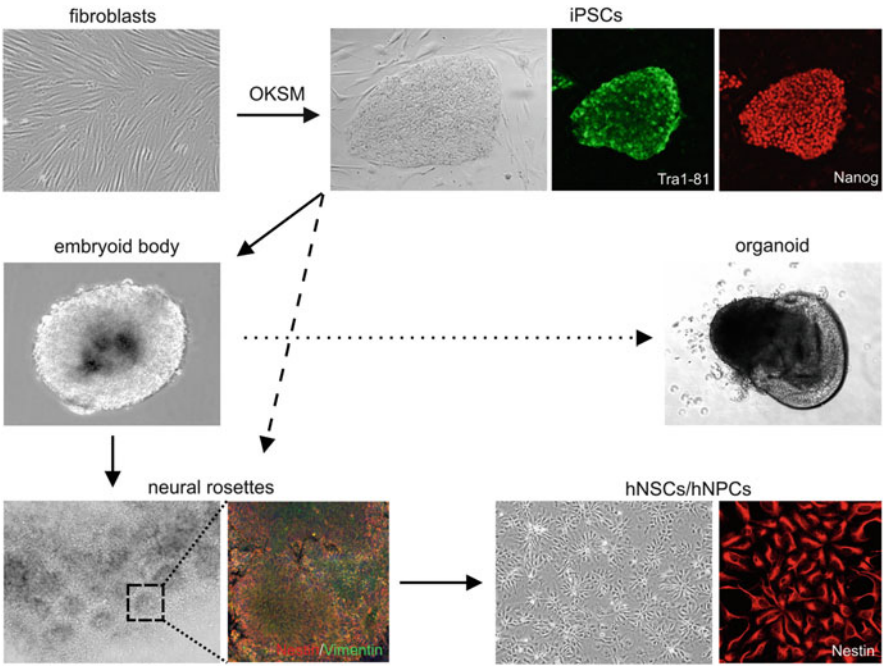

Fig. 13.1 Application of reprogramming technology for hNSC/hNPC production. Differentiated cells (e.g., fibroblasts) can be reprogrammed to induced pluripotent stem cells (iPSCs), which express pluripotency markers such as Tra1-81 and Nanog. iPSCs can be next differentiated via embryoid bodies or directly (e.g., by dual SMAD inhibition protocol; dashed line arrow) to neural rosettes resembling neuroepithelium expressing nestin and vimentin, markers of NSCs. From neural rosettes NSCs lines can be established. Using spinning bioreactors embryoid bodies can be also used to produce 3D brain organoids (dotted line arrow). *See text for more details*

EBs were transferred to serum-free medium that was supplemented with bFGF and epidermal growth factor (EGF; Yuan et al. 2013). The EBs were then plated on matrigel- or laminin/poly-L-ornithine-coated dishes and cultured to form neural rosettes that were reminiscent of early neural tube organization (Fig. 13.1). The composition of the medium that was used for rosette formation varied between protocols (e.g., serum-free medium supplemented with bFGF/EGF or retinoic acid (Salimi et al. 2014). The rosettes were then manually collected and dissociated. The NSCs that were obtained could be further cultured as neurospheres or as a monolayer in serum-free medium supplemented with bFGF and EGF; some protocols also supplemented the medium with brain-derived neurotrophic factor (BDNF). Further steps depended on the particular experimental needs (i.e., required population of neurons). The simplest approach for NSC differentiation requires the withdrawal of bFGF and EGF from the culture medium. In a few weeks, iPSC-derived NSCs could be differentiated into a mixture of different classes of neurons and astrocytes (Fig. 13.1). The enrichment of a particular neuronal type could be obtained by additional treatment with growth factors, small molecules, or inhibitors of certain

signaling pathways during the differentiation process or cell fractioning based on live staining for protein markers (Mertens et al. 2016). A widely used method for the derivation of NSCs/NPCs from hiPSCs is currently a dual Sma and Mad Related proteins (SMAD) inhibition protocol that bypasses the EB step (Fig. 13.1). This protocol is based on the synergistic action of two inhibitors of SMAD signaling, for example, Noggin (antagonist of *bone morphogenetic protein* [BMP] signaling) and SB431542 (inhibitor of transforming growth factor β [TGFβ] receptor), which efficiently and rapidly induce neural conversion in an adherent monolayer of hiPSCs (Chambers et al. 2009). The SMAD inhibitors were also successfully used to modify the conventional protocol of NSC derivation from hiPSCs, in which they were added during the steps of EB and rosette formation.

13.3.2 Brain Organoids

hESC- and hiPSC-derived NSCs, NPCs, and neurons have already been shown to be useful for modeling both human neurogenesis and disease (Han et al. 2011; Dolmetsch and Geschwind 2011; Marchetto et al. 2011). However, two-dimensional (2D) culture models lack important information on cell positioning within tissue, morphogen gradients, and complex interactions between different cell types in the nervous system. This is a serious limitation to modeling the dysfunction of such a complex environment as the brain. These limitations can be overcome by using organoids. Organoids are miniature three-dimensional (3D) cell cultures that are derived from pluripotent cells, in which the cytoarchitecture of particular organs is at least partially recreated, based on the ability of ESCs/iPSCs to self-organize and differentiate. In 2013, the first cerebral organoids were obtained from iPSC-derived EBs that were cultured in spinning bioreactors (Lancaster et al. 2013). The first organoids were heterogeneous and contained several brain microregions per organoid. Analyses of protein marker expression revealed that these microregions resembled such structures as the dorsal cortex, hippocampus, choroid plexus, and ventral telencephalon (Lancaster et al. 2013). The initial protocol had relatively low efficiency and was quite expensive, but subsequent improvements to the protocol overcame some of these drawbacks (Hartley and Brennand 2016). Importantly, organoids cytoarchitectonically resemble the developing brain. Analyses of single-cell gene expression and epigenetic markers confirmed that the genetic program that was activated in cerebral organoids was very similar to the fetal cortex (Camp et al. 2015; Luo et al. 2016). Therefore, organoids or other 3D types of cultures are receiving more attention in modeling neurodevelopmental brain disorders, such as genetic or ZIKA virus-induced microcephaly (Lancaster et al. 2013; Garcez et al. 2016; Qian et al. 2016), lissencephaly (Bershteyn et al. 2017), non-syndromic ASD (Mariani et al. 2015), and neurodegenerative disorders (e.g., AD; Raja et al. 2016) (Table 13.1).

Table 13.1 Use of organoids to model neuropathology

Neuropathology/ manipulation	3D culture type	NSC/NPCs phenotype	References
Microcephaly (*CDK5RAP2* mutation)	iPSC-derived cerebral organoids	• Premature differentiation to neurons	Lancaster et al. (2013)
Microcephaly (ZIKA virus infection)	iPSC-derived forebrain-specific organoids	• Preferential NCS infection • Lower NCS proliferation rate • NCS cell death	Garcez et al. (2016) and Qian et al. (2016)
Miller–Dieker syndrome	iPSC-derived cerebral organoids	• Increased apoptosis of NSCs • Increased number of horizontal divisions of NSCs • Outer radial glia cytokinesis delay	Bershteyn et al. (2017)
CRISPR-Cas9 *CHD8* deletion (ASD model)	iPSC-derived cerebral organoids	• Not analyzed	Wang et al. (2017)
Idiopathic ASD	iPSC-derived telencephalic organoids	• Decreased cell cycle length in day 11 organoids • Enhanced differentiation toward GABAergic neurons	Mariani et al. (2015)
CRISPR-Cas9 *PTEN* deletion	hESC-derived cerebral organoids	• Enhanced proliferation of NSC/NPCs • Transient delay in NPCs differentiation • Organoid surface folding • Increased organoid size	Li et al. (2017b)
AD (*APP duplication*)	iPSC-derived organoids	• Not analyzed	Raja et al. (2016)
PD (*LRRK2* G2019S)	3D human ectodermal spheres	• Not analyzed	Son et al. (2017)

13.4 Neural Stem Cell-related Disorders: Lessons from In Vitro Modeling

13.4.1 Neurodevelopmental Disorders and Autism

The iPSC-based modeling of neurodevelopment appears to be an ideal tool for studying disorders of CNS development. The protocols that are used to differentiate ESCs and iPSCs into hNSCs/hNPCs and organoid cultures allow the reliable reproduction of certain stages of neurogenesis and gliogenesis that resemble those in the developing human brain. Such protocols also provide the opportunity to study disorders that cannot be easily modeled in rodent systems because of an unknown

genetic cause or differences between human and rodent brain development (e.g., during cortical development). Consequently, the iPSC technology has been extensively used to model several neurodevelopmental diseases, including MCPH, FXS, Timothy syndrome, TSC, Rett syndrome, Williams–Beuren syndrome, Williams–Beuren region duplication syndrome, and several non-syndromic ASD cases (Prajumwongs et al. 2016; Wen et al. 2016; Ben-Reuven and Reiner 2016) (Table 13.2). One important feature that emerged from these studies was that gene expression was significantly dysregulated in NSCs in a majority of these disorders (Table 13.2). The affected genes are known to regulate neurodevelopment, differentiation, cell adhesion, inflammation, and oncogenesis. The dysregulation of certain effector genes/processes is common to several datasets, even if the initial cause of the disease and intermediate signaling hubs are different (e.g., the dysregulation of GABA neuron differentiation may depend on FoxoG1 or CHD8 in nonsyndromic autism; Mariani et al. 2015; Wang et al. 2017). The findings of these studies are too numerous to be described in detail herein, but several recent articles extensively reviewed the modeling of neurodevelopmental disorders in vitro (Wen et al. 2016; Young-Pearse and Morrow 2016; Ben-Reuven and Reiner 2016). In the following sections, we discuss only a few of these studies as examples of the ways in which "brain in the dish" technology helps reveal NSC/NPC pathology in neurodevelopment.

Microcephaly is characterized by a marked reduction of brain size. Several genes that cause this disease have been identified. However, mice that carry mutations of these identified genes have failed to recapitulate the severe reduction of brain size that is seen in humans, which substantially limits studies of the cellular and molecular mechanisms that lead to disease development. Lancaster et al. (2013) were the first to apply cerebral organoid technology to model this neurodevelopmental disorder. These authors showed that a heterozygous mutation of *CDK5RAP2* (i.e., a newly identified microcephaly-related gene) led to the premature differentiation of NPCs into neurons, which likely prevented the generation of a sufficient number of cells that are needed to attain a proper brain size. Li et al. (2016b) analyzed the mechanism of primary microcephaly that was caused by a mutation of citron kinase and found that the duration of cytokinesis may play a role in brain undergrowth.

Hypertrophy of the brain or specific brain regions has been observed in many neurodevelopmental disorders, which is often linked to hyperactivation of the mTOR pathway (Switon et al. 2017). A greater head circumference is associated with a higher risk for ASD. Li et al. (2017b) generated cerebral organoids with mutated *PTEN*. The loss of *PTEN* increases activity of the mTOR signaling pathway and may cause a variety of disorders, including Cowden syndrome, Lhermitte–Duclos disease, Bannayan–Riley–Ruvalcaba syndrome, and various cancers (Endersby and Baker 2008; Pilarski et al. 2013). *PTEN* mutations were also described in approximately 1% of human ASD patients. The majority of patients with *PTEN* mutations exhibit macrocephaly, often with polymicrogyria. Macrocephaly can be modeled in rodents (Switon et al. 2017), but polymicrogyria cannot. Brain organoids that are derived from PTEN-deficient cells present a larger size and form gyri-like structures through extensive organoid surface expansion that is caused by an extended time of NPC proliferation (Li et al. 2017b). Similar effects of Akt hyperactivation in cerebral organoids were observed (Li et al. 2017b).

Table 13.2 Use of hESC/iPSC-NSC/NPCs to model neurodevelopmental disorders

Disease	Starting cell type	NSC/NPCs phenotype	References
ASD	hiPSC	• Decreased cell cycle length	Mariani et al. (2015)
ASD (*CHD8* knockdown)	hiPSC	• Transcriptional program alteration	Sugathan et al. (2014)
ASD (*TRPC6* loss)	hiPSC	• Calcium signaling alteration • Transcriptional program alteration	Griesi-Oliveira et al. (2015)
ASD (Timothy syndrome)	hiPSC	• Changed calcium signaling • Calcium-dependent transcriptional • Decreased lower cortical layer neuron generation	Paşca et al. (2011)
MCPH (*CIT*; c317g>T; c376A>C; c689A>T)	hiPSC	• Abnormal cytokinesis with delayed mitosis • Multipolar spindles • Increased apoptosis	Li et al. (2016b)
Down Syndrome	hESCs	• Increased apoptosis • Downregulation of forebrain developmental genes	Halevy et al. (2016)
Williams–Beuren syndrome (7q11.23 CNV - deletion)	hiPSC	• Transcriptional program alteration	Adamo et al. (2015)
Williams–Beuren region duplication syndrome (7q11.23 CNV - duplication)	hiPSC	• Transcriptional program alteration	Adamo et al. (2015)
Fragile X Syndrome	hESCs	• Gradual silencing of *FMR1* • Delayed differentiated to neurons • Inappropriate balance between Sox2/Sox9 levels	Telias et al. (2013, 2015)
Fragile X Syndrome	hiPSC	• Inappropriate neuronal differentiation	Sheridan et al. (2011)
Rett syndrome	hiPSC	• Increased rate of L1 retrotransposition	Muotri et al. (2010)
Bipolar disorder	hiPSC	• Transcriptional program alteration suggesting changes in hNCS differentiation to different neuronal subtypes	Chen et al. (2014)
Bipolar disorder	hiPSC	• Transcriptional program alteration	Madison et al. (2015)
Schizophrenia (idiopathic)	hiPSC	• Decreased migration • Accelerated neural differentiation, • Increased canonical Wnt signaling • Increased abundance of translational machinery	Brennand et al. (2015), Topol et al. (2015a, b, 2016)

(continued)

Table 13.2 (continued)

Disease	Starting cell type	NSC/NPCs phenotype	References
		• Increased expression of miRNA-9 • Elevated oxidative stress • Mitochondrial damage • Transcriptome alterations	
Schizophrenia (idiopathic)	hiPSC	• High variability in stress responses to environmental/extracellular stressors on a single cell level	Hashimoto-Torii et al. (2014)
Schizophrenia (4-bp deletion in *DISC1*)	hiPSC	• Increased expression of miR-219 • Reduced proliferation	Murai et al. (2016)
Schizophrenia (15q11.2 CNV, loss of *CYFIP1*)	hiPSC	• Adherens junctions disruption • Loss of apical polarity	Yoon et al. (2014)
Schizophrenia (heterozygous deletion of *CNTNAP2;* loss of exons 14-15)	hiPSC	• Decreased migration • Changes in *CNTNAP2* isoform expression	Lee et al. (2015)
TSC (*TSC2*$^{-/-}$; deletion by zinc finger nuclease)	hESC	• Increased ectodermal rosettes area • Decreased number of differentiated neurons • Increased differentiation toward astroglial lineage	Costa et al. (2016)
TSC (*TSC2*$^{-/-}$; deletion by zinc finger nuclease)	hESC	• Reduced neuronal maturation • Increased astrogliosis • Transcriptional and translational program alterations	Grabole et al. (2016)
TSC (*TSC2*$^{+/-}$; c.1444-2A>C)	hiPSC	• High proliferation rate of NSC	Li et al. (2017a)
Paroxysmal kinesigenic dyskinesia (*PRRT*$^{+/-}$; c.487C>T; c.573dupT)	hiPSC	• Impairment of neuronal differentiation by dual SMAD inhibition protocol • Transcriptional program alterations	Li et al. (2016a)

As shown in Table 13.2, several studies investigated the pathology of NSCs and NPCs in ASD. For example, Paşca et al. (2011) analyzed hiPSCs-NSCs/NPCs that were obtained from Timothy syndrome patients. Timothy syndrome is caused by a missense mutation of the L-type calcium channel Cav1.2 (Splawski et al. 2004). Although cell proliferation and migration and neuron formation from NPCs appeared to be intact in Timothy syndrome cells, changes in calcium signaling led to profound changes in calcium-dependent transcription. This in turn caused subtle changes in neuronal differentiation. Gene expression analysis at the level of single cells revealed a significant decrease in the proportion of neurons that expressed lower cortical layer markers. According to the authors, this phenomenon may be responsible for fewer interhemispheric connections via the corpus callosum that are

observed in Timothy syndrome and other ASD patients. Another study investigated organoids from non-syndromic ASD patients and found subtle changes in neuronal differentiation and a greater number of GABAergic neurons (Mariani et al. 2015).

In vitro-cultured hESC- and iPSC-derived hNSCs/hNPCs provide interesting insights into FXS. Fragile X syndrome is caused by inactivation of the *FMR1* gene, which is attributable to expansion of the CGG-triplet repeat in the 5-′-untranslated region. In hESCs, derived from blastocysts identified via preimplantation genetic diagnosis as carrying *FMR1* mutation, *FMR1* gene silencing occurred only upon ESC differentiation (Telias et al. 2013, 2015). Neuronal differentiation from hESCs was shown to recapitulate the gradual loss of FMRP, an *FMR1* product (Telias et al. 2013, 2015). In contrast, hiPSCs have already an inactivated *FMR1* locus as the "parental" cells from which they were derived. In some cases, however, random unsilencing can occur which makes the iPSC-based model less predictable (Sheridan et al. 2011). Fragile X syndrome has been considered to be a disease of neuronal networks, but studies have demonstrated that the pathology begins already in the hNPC stage. The differentiation of hESCs-NPCs in FXS was shown to be delayed because of an inappropriate balance between the levels of Sox2 (differentiation inhibitor) and Sox9 (differentiation activator) proteins (Telias et al. 2015).

13.4.2 Schizophrenia

The potential contribution of hNSC dysfunction to schizophrenia, bipolar disorder, and ASD has been investigated (Table 13.2). This section focuses on recent studies of schizophrenia, which is likely the most intensively studied psychiatric disorder with regard to hNSCs/hNPCs. Changes that are observed in postmortem schizophrenia patient specimens include ventricle enlargement, brain size/weight reduction, heterotopias through cortical areas, and reductions of dendritic arbors and dendritic spines (Harrison and Weinberger 2005). The data suggest that the pathogenesis of schizophrenia begins during prenatal brain development. The knockout of schizophrenia-related genes (e.g., Disrupted in schizophrenia 1 [*DISC-1*]) in mice causes aberrant postnatal and adult neurogenesis (Duan et al. 2007; Kim et al. 2009). The lower proliferation of NSCs has also been reported in the DG in schizophrenia patients, based on the number of cells that express Ki67, a marker of proliferating cells (Reif et al. 2006). Schizophrenia is a mental disorder that is characterized by disturbances in social behavior and problems differentiating reality from hallucinations. Both environmental and genetic factors have been linked to a higher risk for this complex psychiatric disorder (Lewis and Levitt 2002; Harrison and Weinberger 2005). Schizophrenia affects up to 1% of the human populations. There is an urgent need for models that better recapitulate this human disease to further understand the mechanism of pathogenesis and for potential drug screening.

Several lines of iPSCs have been developed from sporadic schizophrenia patients and schizophrenia patients with a clear genetic cause (Table 13.2). Initial studies focused on analyzing differentiated neurons, revealing deficits in neuronal

connectivity that could be "cured" by the antipsychotic drug loxapine (Brennand et al. 2011). This study was followed by several analyses of hiPSCs-NPCs that were derived from sporadic schizophrenia patients, which revealed several differences from control cells that were obtained from healthy people. These differences included a decrease in NPC migration, accelerated neural differentiation, an increase in canonical Wnt signaling, an increase in the abundance of translational machinery, higher expression of microRNAs (e.g., miRNA-9), elevated oxidative stress, and mitochondrial damage (Brennand et al. 2015; Topol et al. 2015a, b, 2016). The increase in miRNA-9 expression correlated with deficits in migration, and the downregulation of miRNA-9 effectively reversed this phenotype (Topol et al. 2016). Another interesting observation at the single-cell level was that schizophrenia-derived hiPSCs-NPCs, unlike control cells, had very high variability in response to environmental/extracellular stressors, which is consistent with the significant impact of different types of prenatal stress on the development of schizophrenia (Hashimoto-Torii et al. 2014). Notably, none of the studies cited above reported changes in the rate of proliferation of NSCs/NPCs. Such changes have been reported in schizophrenia patients (Allen et al. 2016).

In addition to NSCs/NPCs that are obtained from sporadic schizophrenia patients, cells from patients with genetically driven cases have been analyzed (Table 13.2). Yoon and coworkers suggested an interesting link between schizophrenia and the presence of cortical heterotopias in patients. Heterotopic neurons may result from either inappropriate NPC/neuron migration or premature differentiation. Schizophrenia that was related to the 15q11.2 copy number variant provided insights into the way in which such a phenotype can be induced by NSC defects (Yoon et al. 2014). An analysis of neural rosettes that were derived from patients' iPSCs revealed defects in apical polarity and the formation of adherens junctions of NSCs that stemmed from *CYFIP1* haploinsufficiency. Subsequent analyses in a mouse model revealed that the lack of CYFIP1 led to the detachment and mispositioning of radial glia, followed by the aberrant positioning of NPCs and neurons in the developing cortex. Notably, however, such changes were specific to cells that were derived from patients with the 15q11.2 copy number variant but not those whose schizophrenia was attributable to the loss of *DISC1*, further confirming the complex etiology of schizophrenia (Yoon et al. 2014). hNPCs that were derived from patients with mutated *DISC1* have been analyzed. Murai et al. (2016) reported that such iPSCs-hNPCs exhibit a significant decrease in proliferation and increase in neuronal differentiation compared with control cells. According to these authors, such changes resulted from the higher expression of miR-219. These observations appear to be corroborated by a previous study of neurogenesis deficits in *Disc-1* knockdown mice (Mao et al. 2009) but are in striking contrast to the work of Srikanth et al. (2015). In the latter study, using hNPCs with *DISC-1* that was edited to mimic the disease-related mutation, these authors observed an increase in proliferation and a decrease in differentiation. Further work needs to be done to explain this discrepancy. To date, most standard iPSC-based models have not proved significant changes in the proliferation of hNSCs in schizophrenia.

13.4.3 Neurodegenerative Diseases

Most studies of neurodegenerative diseases that have used human stem cells (embryonic or reprogrammed) focused on disease phenotypes that are observed in mature neurons in affected patients. However, some observations of hESC or hiPSC differentiation and analyses of postmortem material prompted speculations that hNSCs can be affected much earlier in neurodegenerative disorders before actual symptoms of neurodegeneration are observed (Table 13.3). The pathological functioning of hNSCs can also make some people more vulnerable to neurodegeneration or at least explain some of the disease symptoms that cannot be explained simply by neuronal cell death.

To date, most progress in revealing the role of hNSCs in neurodegenerative disorders has been made in the case of HD. Huntington's disease is an autosomal dominant genetic disorder that affects six in 100,000 people. The best known symptoms of HD are erratic, random, and uncontrollable movements. As the disease progresses, physical symptoms become more severe and include rigidity, posture abnormalities, and the loss of muscle control. Several behavioral symptoms are also observed in HD, including irritability, apathy, anxiety, depression, and obsessive/compulsive behaviors. Huntington's disease is caused by a mutation of the *Huntingtin* gene (*HTT*). The mutation encompasses the expansion of CAG repeats that encode polyglutamine tracts. The *HTT* gene in unaffected individuals has fewer than 26 such repeats. The disease manifests when the number of repeats exceeds 35, the onset and severity of which depend on the number of repeats. With more than

Table 13.3 Use of hESC/iPSC-NSC/NPCs to model neurodegenerative disorders

Neuropathology/ manipulation	starting cell type	NSC/NPCs phenotype	References
Huntington disease	hiPSC	• Lower number of MAP2-positive cells	Chae et al. (2012)
Huntington disease	hIPSC	• Vulnerable to cell death caused by BDNF withdrawal • NeuroD1 downregulation • Transcriptional program alteration	Mattis et al. (2015 and Lim et al. (2017)
Huntington disease	hESC	• Transcriptional program alteration • Mitotic spindle mispositioning	Lopes et al. (2016)
Parkinson disease (LRRK2 G2019S)	hIPSC	• Decreased proliferation at latter passages • Lost ability for differentiation at latter passages • Changes in nucleus shape	Liu et al. (2012)
SPG11	hIPSC	• Decreased proliferation • Decreased differentiation • Transcriptional program alteration	Mishra et al. (2016)

60 CAG repeats, symptoms may occur as early as 20 years of age. *HTT* encodes huntingtin protein, which has several cellular functions (e.g., intracellular transport, cell division, and transcription; (Saudou and Humbert 2016). Mutated huntingtin forms potentially toxic aggregates in cells, but cellular damage begins before that. Cellular symptoms of HD likely stem from a combination of the gain of function of mutated HTT and the partial loss of function of wild-type HTT. Additionally, mutated *HTT* RNA may contribute to the HD phenotype and disease progression (Fiszer and Krzyzosiak 2013; Martí 2016).

The first iPSCs from HD patients were obtained in 2012 and differentiated into neurons (The HD iPSC Consortium 2012; Jeon et al. 2012; Chae et al. 2012). Patient-specific neurons presented greater vulnerability to cellular stress that was caused by glutamate, prolonged culture, trophic factor withdrawal, and oxidative stress (the HD iPSC Consortium 2012; Jeon et al. 2012). However, the pathology was not restricted to diseased neurons; it also occurred in hNSCs. For example, Chae et al. (2012) used a classic differentiation protocol and found that HD-derived cells gave rise to significantly fewer MAP 2-positive cells, suggesting that HD hNSCs might have deficits in proper differentiation. Recent work that focused specifically on hNSCs that were derived from early-onset HD patients revealed additional findings (Mattis et al. 2015; Lim et al. 2017). Although HD hNSCs could be efficiently differentiated into neurons and glia, a substantial number of nestin-expressing neural progenitors were preserved compared with control hNSCs. The HD hNPCs were vulnerable to cell death that was caused by BDNF withdrawal and subsequent glutamate toxicity. This phenotype could be prevented by the specific inhibition of mutated HTT expression. Similar results were found with NPCs that were obtained from embryos in a mouse model of HD. High-throughput analyses of mRNA and protein in HD hiPSCs that differentiated into a neural lineage revealed prominent changes in the expression of genes and proteins that are related to neuronal development and neuronal function (e.g., NeuroD1 downregulation) compared with control cells (Lim et al. 2017). Comparisons with the gene expression profiles of the normally developing striatum suggested that the differentiation of HD hNSCs is indeed either delayed or abnormal. Intriguingly, the small molecule Isx-9, which is known to upregulate NeuroD1 expression, reversed the changes in gene expression in HD-hNSCs and their vulnerability to BDNF withdrawal (Lim et al. 2017). Isx-9 also reversed behavioral phenotypes in an R6/2 mouse model of HD. The work of the HD iPSC Consortium revealed that HD pathology begins much earlier than when visible neurological and physical symptoms occur, likely during neural development, and can be at least partially reversed by small-molecule compounds. Recently, Lopes et al. (2016) suggested that aberrant gene expression that is related to neurogenesis and neuronal function may contribute to abnormalities that are seen in HD NSCs. These authors reported the improper mitotic spindle orientation of hESCs-NSCs from HD patients. Thus, in HD, NSCs might be affected before neurodegeneration occurs, but unclear are which and how particular symptoms of HD are linked to this hNSC defect. One speculation is that a similar defect in NSCs appears in HD during adult neurogenesis, thus contributing to the production

of new striatal neurons. If so, then patients would have a limited capacity to cope with the greater loss of these neurons, which may aggravate HD symptoms.

The proper function of adult NSCs may also be compromised in PD. Parkinson's disease is a neurodegenerative disorder that primarily affects motor function and causes shaking, rigidity, and problems with walking. The non-motor symptoms of PD include depression, anxiety, and hyposomia. More than 90% of PD cases do not have a clear genetic cause (i.e., idiopathic PD). The remaining ~10% of cases are caused by mutations of different genes, including α-synuclein, parkin, *LRRK2*, and *PTEN*-induced putative kinase 1 (*PINK1*). The proteins that are encoded by these genes have different cellular functions. The ultimate result of such gene mutations is the death of dopaminergic neurons. Such cell death is likely caused by a decrease in the clearance of cellular protein deposits, increases in oxidative stress, and mitochondrial dysfunction (Calì et al. 2011). Several studies have reported the successful generation of iPSCs from Parkinsonian patients (Soldner et al. 2009; Hargus et al. 2010; Nguyen et al. 2011; Liu et al. 2012). In most of these studies, no defects in NSC proliferation or differentiation have been reported. However, early passages of hNSCs were used in these studies. Liu et al. (2012) aged hiPSCs-NSCs that were derived from PD patients with a *LRRK2* mutation (G2019S) by serial passaging, and the disease phenotype appeared. Up to passage 14, no significant differences were found in the number of colonies that formed or neuronal differentiation between wild-type and PD hNSCs. However, after passage 14, "diseased" hNSCs lost the ability to efficiently proliferate and generate adult neurons. This loss was accompanied by changes in the shape of cell nuclei. Interestingly, similar changes in nuclear shape were found in neurogenic areas in PD patients' brains (Liu et al. 2007). Thus, in PD, adult hNSC dysfunction may progress and lead to non-motor PD symptoms.

Similar to PD, most AD cases are sporadic, the underlying cause of which is unknown. The smaller proportion of cases is caused by mutations in genes that encode amyloid precursor protein (APP) and presenilins 1 and 2. These mutations lead to the greater production of β-amyloid (Aβ42), which is neurotoxic and responsible for neurodegeneration. Neuronal loss eventually leads to progressive dementia. Postmortem brain analyses and various in vitro and in vivo models have led to two contradictory conclusions: AD is linked to either an increase or decrease in hNSC proliferation and/or differentiation (Liu and Song 2016; Tincer et al. 2016). In vitro experiments showed that the exposure of hNPCs to Aβ42 decreased their proliferation and neuronal differentiation in in vitro cultures (Haughey et al. 2002). However, in several AD patients, no deficits in the proliferation or differentiation of iPSC-derived hNPCs have been reported (Yagi et al. 2011; Israel et al. 2012; Kondo et al. 2013; Duan et al. 2014; Sproul et al. 2014). There may be several explanations for these discrepancies. For example, similar to NSCs in PD, the AD phenotype may become apparent after in vitro aging. Another possibility is that the levels of Aβ42 that are produced by hNSCs in culture may be too low to affect function. Therefore, more studies are needed to clarify whether neurogenesis and NSC/NPC function are affected in AD.

Recently, hNPC dysfunction has been reported in another neurodegenerative disorder, hereditary spastic paraplegia (SPG), which is caused by a mutation of

spastic paraplegia gene 11 (*SPG11*). In addition to a neurodegenerative phenotype that is caused by the degeneration of axons in corticospinal tracts, mutated SPG11 causes cortical atrophy and thinning of the corpus callosum. This particular form of SPG may be associated with both neurodegeneration and problems in neurodevelopment. Gene expression analyses of patient hiPSCs-NPCs revealed changes in the expression of genes that are related to neurogenesis and differentiation (Mishra et al. 2016). Subsequent analyses of the proliferation and differentiation of patients' NPCs confirmed that these cells proliferated and differentiated less efficiently. Interestingly, this phenotype could be reversed by GSK3 inhibitors (Mishra et al. 2016).

13.5 Future Directions

Over the last decade, tremendous progress has been made in the generation of human-derived models of neurological disorders using iPSCs. Undoubtedly, the list of diseases that can be modeled by iPSCs will expand further. Some important points should be considered with regard to existing and future models. In many cases, cells have been obtained from insufficient cohorts of patients, which limits generalization of the findings. Moreover, insufficient details of analyses of NSC/NPC phenotypes have been reported. The results of very detailed transcriptomic studies were not tested with regard to functional significance. Finally, genetic correction has rarely been performed to demonstrate that defects in NSCs/NPCs indeed depend on a particular mutation. Further efforts should be made to address these shortcomings, and 2D cultures should be validated in 3D settings.

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