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Application of induced pluripotent stem cells to understand neurobiological basis of bipolar disorder and schizophrenia

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The etiology of neuropsychiatric disorders, such as schizophrenia and bipolar disorder, usually involves complex combinations of genetic defects/variations and environmental impacts, which hindered, for a long time, research efforts based on animal models and patients' non-neuronal cells or post-mortem tissues. However, the development of human induced pluripotent stem cell (iPSC) technology by the Yamanaka group was immediately applied to establish cell research models for neuronal disorders. Since then, techniques to achieve highly efficient differentiation of different types of neural cells following iPSC modeling have made much progress. The fast-growing iPSC and neural differentiation techniques have brought valuable insights into the pathology and neurobiology of neuropsychiatric disorders. In this article, we first review the application of iPSC technology in modeling neuronal disorders and discuss the progress in the accompanying neural differentiation. Then, we summarize the progress in iPSC-based research that has been accomplished so far regarding schizophrenia and bipolar disorder.

Key words: bipolar disorder, induced pluripotent stem cells, neural stem cells, neurodevelopmental disorders, schizophrenia.

IT HAS BEEN 10 years since the Yamanaka group developed human induced pluripotent stem cell (iPSC) technology. In 2006, Shinya Yamanaka and colleagues in Japan found that artificially activating four transcription factors (i.e., Oct3/4, Sox2, c-Myc, and Klf4) in mouse fibroblasts can generate new cells with pluripotency.[1] One year later, the Yamanaka group in Japan and the Thompson group in the USA validated the reprogramming approach in human skin fibroblast cells.[2,3] The induced human iPSC are very similar to the human embryonic stem cells (ESC) in morphology, proliferation, cell surface antigen, pluripotency gene expression and epigenetic status, and telomerase activity. More recently, urine-derived cells and blood cells have also been demonstrated as being able to generate iPSC.[4,5]

Although these reprogrammed iPSC can hardly be applied for transplantation – at least at present – due to immune barriers, they can be used to monitor the adaptive responses and evolutionary processes of human tissues through comparing the transcription, genetics, and functions of human cells to those of other species. A second important application is that the iPSC derived from patients can be used as cell models to monitor the cell biology of inheritable and sporadic human diseases, such as neuropsychiatric and neurodegenerative diseases. In addition, combined with research techniques at the single-cell or molecular level, iPSC technology yields a powerful platform for the identification of potential drug targets, the screening of new drugs, and the

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development of new therapeutic strategies for these diseases.[2]

DEVELOPMENT OF iPSC-BASED RESEARCH ON NEURONAL DISORDERS

iPSC technology quickly drew great attention from neuroscientists focusing on neuronal disorders. In 2008, the first iPSC model of a neuronal disorder was established for amyotrophic lateral sclerosis by Dimos *et al.*[6] In 2009, the first iPSC-based study for a neuronal disorder was carried out for spinal muscular atrophy (SMA).[7] In the same year, an iPSC model of familial dysautonomia was established, for the first time, to validate candidate drugs.[8] Soon, iPSC-based research expanded to a variety of neuronal diseases, such as Huntington's disease,[9] Rett syndrome,[10] and schizophrenia (SCZD).[11–13] All of these studies have presented convincing evidence that iPSC modeling can provide important insights into the neurobiology and therapy-development of diseases.

The iPSC modeling strategy is particularly useful for the study of polygenic neuropsychiatric disorders, which usually involve defects in multiple genes or signaling pathways. Although gene knockout and knockin techniques have made the mouse one of the most useful models for biomedical research, the complexity of the clinical symptoms of neuropsychiatric diseases often cannot be ideally recapitulated in animals.[14] This is also true for the limitations in the current gene editing techniques that can simply modify one or two genes. Another important reason is that despite the high developmental conservation of genomes, humans and mice separated on the tree of evolution millions of years ago and thus have very different genetic characteristics. As a result, the developmental and physiological features have diversified a lot in the two species.[15] The second conventional approach for research on neuropsychiatric disorders is post-mortem analysis. Although this approach is based on brain tissues that carry the natural genetic background of the patients, the tissue samples have a highly stringent requirement for acquisition and maintenance, and cannot be investigated under flexible experimental conditions. Importantly, changes in the brains of the patients may be secondary to long-term medication treatment and consequently cannot represent the brain deficits of the disease. Compared to these methods, the neurons derived from the patients' iPSC not only retain the original genetic information of the disease, but also can be studied under flexible conditions. Moreover, although the gene expression in the patients' brains can be easily influenced by the therapeutic drugs, the genomes of fibroblasts used for reprogramming are unlikely to be affected, which enables the derived iPSC and neurons to remain in the untreated stage.

Following the first application of iPSC technology in the modeling of several neurodegenerative diseases, iPSC models for SCZD, the most commonly known neuropsychiatric disorder, were first constructed in 2011. Since then, a considerable number of stem cell models has been established for a variety of neuropsychiatric disorders, such as autistic spectrum disorders (ASD), bipolar disorder (BD), and attention-deficit hyperactivity disorder (ADHD), to facilitate understanding the mechanisms of these diseases.

NEURAL DIFFERENTIATION OF iPSC AND ESC

The cause for the behavioral abnormalities of psychiatric disorder patients often involves changes in multiple types of neurons. A brain consists of multiple types of neural cells. Hence, to better investigate the diseases, it is necessary to develop techniques to differentiate patient iPSC into a specific type of neural cell. The development of the techniques to differentiate iPSC/ESC into different types of neural cells started booming almost at the same time as the reprogramming approaches appeared. Before 2007, the progress of research to differentiate mouse or human ESC into brain cells was slow, with only a limited number of methods available for the generation of spinal motor neurons and oligodendrocytes.[16–18] Excited by the broad prospects of iPSC application, divergent methodologies were generated to efficiently differentiate the iPSC/ESC into a variety of subregional neurons, including cortical neurons, midbrain dopaminergic neurons, striatal medium spiny neurons, GABAergic interneurons, basal forebrain cholinergic neurons, and hindbrain serotonergic (5-HT) neurons, as well as non-neuronal astrocytes and oligodendrocytes. The timely developments of these neural differentiation approaches have evoked and continue to promote the passion and effort of research into the biology of neuropsychiatric disorders.

Differentiation of iPSC and ESC into subtypes of neurons is based upon the principles learned from the study of brain patterning, which is determined by the temporary and spatial expression of morphogens along the anterior–posterior (A-P) and dorsal–ventral (D-V) axes (see Fig. 1).[19] The main morphogens influencing the A-P patterning are fibroblast growth factors (FGF), WNT, and retinoid acid (RA). For instance, WNT exhibit an increasing gradient of expression from the forebrain to the midbrain and further to the hindbrain, ending at the anterior spinal cord. Hence, the activation of the WNT pathway in a dose-dependent manner would determine the differentiation of iPSC to forebrain, midbrain, hindbrain, and anterior spinal cord progenitor cells.[20] WNT signaling is not only essential for A-P patterning, it also plays an important role in the D-V patterning process and thus the WNT-signaling pathway is efficient to caudalize neural progenitors.[21,22] In addition to WNT, the morphogens involved in the D-V patterning include sonic hedgehog (SHH) and bone morphogenetic proteins (BMP). SHH determines the ventral fate of the neural progenitor cells, whereas inhibition of the WNT and BMP pathways can promote the dorsal fate.[23,24] As the identity of neural progenitor cells is defined by the morphogen combinations at its A-P and D-V location, ESC and iPSC can be technically differentiated into specific types of neurons through the precise application of morphogens and accessory-activating or -inhibiting molecules to simulate the regional differentiation pathways in the brain.

The classic way to differentiate iPSC or ESC in a neural pathway often involves the formation of

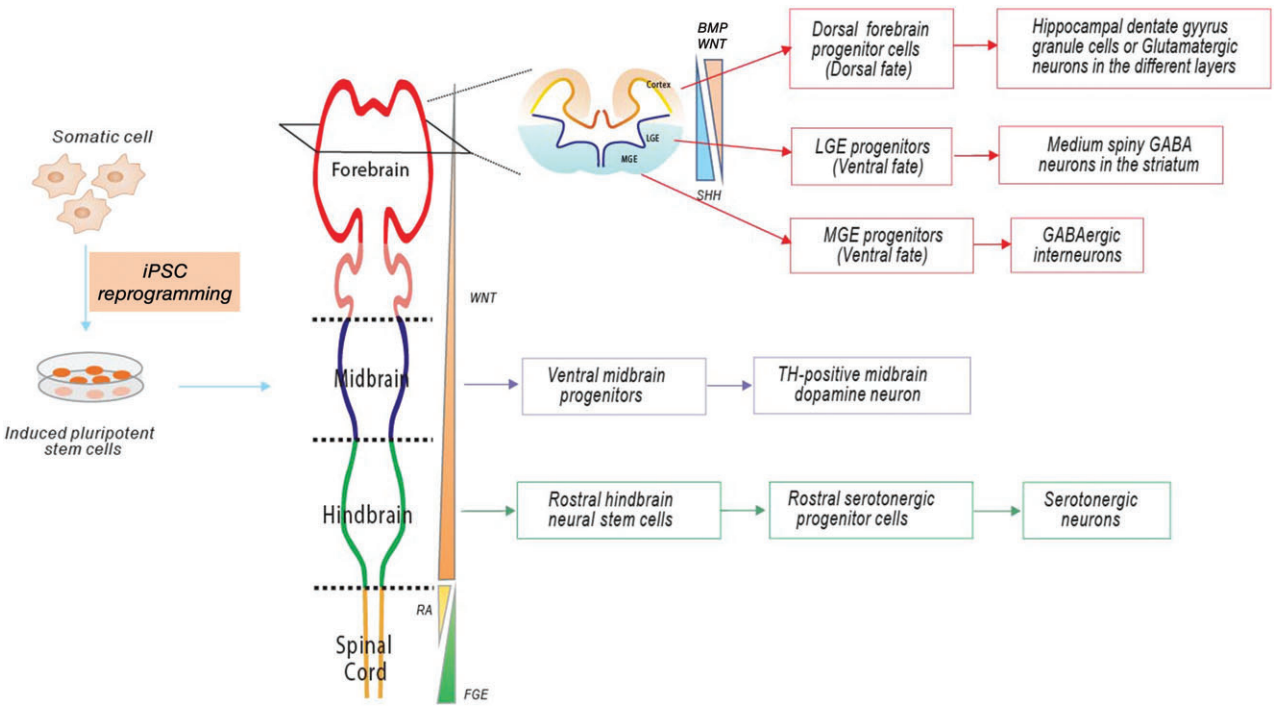

Figure 1. Differentiation of multiple types of neural cells from induced pluripotent stem cell (iPSC). Differentiation of iPSC into neurons mimics neuronal development and maturation *in vivo*. The main morphogens regulating the anterior–posterior (A-P) patterning include WNT, fibroblast growth factors (FGF), and retinoid acid (RA). The increasing gradient of WNT induces iPSC to differentiate into specific type of neural progenitor cells (NPC) of forebrain (red), midbrain (purple), hindbrain (green), and anterior spinal cord; the gradient of FGF and RA determine the spinal cord (yellow) segmentation. The morphogens regulating the dorsal–ventral (D-V) patterning include sonic hedgehog (SHH), WNT, and bone morphogenetic proteins (BMP). SHH determines the ventral fate of NPC, whereas inhibition of the WNT and BMP pathways determines the dorsal fate. The differentiation principle of multiple types of neural cells is shown. Red arrows and boxes indicate the differentiation process of multiple types of forebrain neurons. Purple arrows and boxes indicate the midbrain dopamine neurons. Green arrows and boxes indicate the hindbrain serotonergic neurons. LGE, lateral ganglionic eminence; TH, tyrosine hydroxylase.

embryoid bodies, which are aggregates of the PSC. To promote differentiation towards the neuronal fate, a 'dual SMAD inhibition' method was developed to treat the iPSC or ESC with inhibitors for the BMP- and TGF-β-signaling pathways, such as Noggin (or LDN193189, a small molecule analog to Noggin) for the former pathway and SB431542 for the latter one. The neural cell fate can be identified by the high expression of PAX6 in the differentiated cells.[25,26] Based on this dual SMAD inhibition strategy, a number of differentiation protocols were then generated to differentiate human iPSC or ESC into subtypes of neurons with high efficiency.

Differentiation of ESC/iPSC into dopaminergic neurons

So far, deficits in dopaminergic (DA) neurons have been suggested to be involved in a variety of neuropsychiatric disorders, such as SCZD, BD, and ADHD. The DA neurons reside mainly in the ventral midbrain areas, such as the substantia nigra and the ventral tegmental area, as well as the posterior hypothalamus.[27] Based on the dual SMAD inhibition strategy, Chambers *et al.* supplemented the differentiation recipe with the application of SHH and FGF8 to promote ventral midbrain fate, and eventually obtained approximately 30% progenitor cells that can differentiate into tyrosine hydroxylase (TH)-positive midbrain dopaminergic neurons.[26] As activation of the WNT-signaling pathway would facilitate the generation of caudal neural progenitor cells, Kriks *et al.* later supplemented the application of a WNT activator to the dual SMAD inhibition method with SHH enhancement.[28] This approach improved the efficiency of DA neuron differentiation to an 80% rate of $FOXA2^+/TH^+$ achievement. Kriks *et al.* transplanted these ESC-derived DA neurons in a 6-OHDA-lesioned animal model of Parkinson's disease (PD), and demonstrated that the human neurons were incorporated into the animal neural network and consequently rescued the behavioral deficits of the PD animals.[28] This study has also been regarded as evidence to show that ESC/iPSC-derived neurons can be used for the treatment of neuronal disorders.

Differentiation into forebrain glutamatergic neurons

Excitatory glutamatergic neurons are the most important subtype of neurons in the central nervous system, as glutamatergic synaptic transmission is indispensible for the transduction of nerve impulses within the neural network, which encodes all aspects of brain functions. Dysfunctions in the pyramidal neurons and granule cells within the neocortex and limbic system have been suggested as being involved in many neuropsychiatric disorders, including SCZD and BD. One representative method for cortical pyramidal neuron differentiation was reported in 2012 by Shi *et al.*[29] Progenitor cells with cortical glutamatergic neuronal fate can be identified by the expression of specific transcription factors, such as FOXG1, PAX6, OTX1/2, EMX1, and TBR2. Shi *et al.* demonstrated that supplementing the dual SMAD inhibition with activation of the retinoic acid signaling pathway would facilitate the differentiation towards the dorsal forebrain fate but not the ventral or caudal fate. This was evidenced by the high expression of these transcription factors in >95% of the ESC- or iPSC-differentiated progenitors cells. By comparing the combination of the expression of cortical layer-specific makers TBR1, CTIP2, and BRN2, the authors further investigated the temporal pattern of cortical neuron differentiation over a 70-day observation window, and found that the time-dependent appearance of neuronal subtypes can represent the sequential formation of the cortical layers. When cortex is formed, neurons in the deep layers appear first, then the new neurons pass through the deep layer along the dendrites of the radial glial cells and consequently form upper layers.[30] The glutamatergic neurons in the different layers can be distinguished by the combined expression of different transcription factors, such as TBR1.[31] For instance, layer-6 corticothalamic projection neurons express TBR1 but not CTIP2,[32] while layer-5 subcortical projection neurons are opposite,[33] and layer-2–4 neurons are characterized by the expression of CUX1, BRN2, and SATB2.[34–36] This study therefore demonstrated that glutamatergic neuron differentiation from iPSC based on the dual SMAD inhibition method and retinoid signaling enhancement can recapitulate the important stages in the development of the cortex. In parallel, another group also independently developed an approach to differentiate iPSC into cortical glutamatergic neurons through supplementing the dual SMAD inhibition with WNT signaling suppression and FGF2 application.[37] Based on this method, Yu *et al.* developed a protocol to differentiate iPSC into hippocampal dentate gyrus (DG) granule cells.[38] In this protocol, in addition to

the combined inhibition of BMP, TGF-β, and WNT-signaling pathways, an antagonist of the SHH pathway, cyclopamine, was also applied to get a high ratio of dorsal forebrain progenitor cells.[38,39] The iPSC-derived neural progenitor cells (NPC) were treated with Wnt3a, a WNT protein important for the maintenance of hippocampal progenitor fate and the potential to elicit differentiation into DG granule cells,[40–44] as well as brain-derived growth factor, a neurotrophic factor that can promote hippocampal neurogenesis throughout life.[45,46] This *in vitro* differentiation protocol recapitulated important aspects of hippocampal neurogenesis *in vivo*. After 10 days of differentiation, markers specific for hippocampal NPC, including EMX2, FOXG1, PAX6, and NeuroD1, showed strong expression.[47–54] Following 20 days of differentiation, the hippocampal NPC showed a high percentage of DG granule cells, as evidenced by the prominent expression of Prox1, a marker specific for DG granule cells.

Differentiation into midbrain GABAergic neurons

GABAergic neurons endow the neural network with the ability to encode signals for complex brain functions, and have been recently suggested as being involved in multiple neuropsychiatric disorders, such as SCZD.[55] Medium spiny GABA neurons in the striatum come from progenitors in the lateral ganglionic eminence (LGE) of the telecephalon,[56,57] and GABAergic interneurons mostly arise from the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE), which belongs to the ventral part of the forebrain.[58–61] The MGE progenitor cells are featured with high expression of NKX2.1. As a ventralizing morphogen, SHH shapes the primitive neuroepithelial cells to MGE progenitor cells. Liu *et al.* first reported that by applying high doses of SHH and its agonist purmorphamine, over 90% of ESC can be differentiated into NKX2.1-expressing MGE progenitors, which can be further differentiated into GABAergic interneurons.[59] Following 25 days of differentiation, the progenitor cells not only showed expression of the ventral markers ISL1, OLIG2, and ASCL1 and the forebrain marker FOXG1, but they also exhibited high expression of GABAergic interneuron markers LHX8 and LHX6,[62,63] which solidly validated this approach for GABAergic interneuron acquisition from ESC and iPSC. Almost at the same time, another two groups applied the SHH-enhancing and WNT-suppressing strategy based on the dual SMAD inhibition method and also succeeded in acquiring a high percentage of MGE progenitor cells from ESC or iPSC,[60,61] and such method later was further strengthened by activation of FGF8, a rostralizing signaling factor.[58]

Differentiation into hindbrain serotonergic neurons

Serotonergic neurons are involved in a variety of neuropsychiatric disorders, such as SCZD, anxiety, BD, and major depressive disorder.[64] Serotonergic neurons are largely located in the raphe nucleus of the hindbrain and arise during development from ventral hindbrain progenitors,[65] which are specified by gradient concentrations of WNT along the A-P axis.[19,20] In 2016, Lu *et al.* first reported differentiation of human iPSC and ESC into serotonin neurons by activating the WNT-, SHH-, and FGF4-signaling pathways based on dual SMAD inhibition.[66] The iPSC and ESC were differentiated with administration of 1.4 μM CHIR99021, a glycogen synthase kinase-3β (GSK-3β) inhibitor to enhance the WNT-signaling pathway, to become rostral (r2-3) hindbrain neural stem cells expressing hindbrain marker HOXA2. With a 1-week treatment of high concentration SHH, the r2-3 hindbrain NSC were then ventralized into rostral serotonergic progenitor cells that express NKX2.2 and NKX6.1 but not OLIG2.[67] As SHH cooperates with FGF4 to promote the serotonergic cell fate,[68] the serotonergic progenitor cells were then treated with FGF4 for 1 week together with SHH, which eventually led to a >60% differentiation rate of serotonergic neurons that express key serotonin markers, including serotonin, TPH2, GATA3, GATA2, PET1, LMX1B, SERT, AADC, and VMAT2.[69,70]

Three-dimensional cerebral organoids

Two-dimensional neural differentiation cultures can provide important insights into the neurobiology of diseases. However, the brain is an extremely complicated structure with subtypes of cells organized in 3-D arrangements, which interact to determine cell identity and function. The iPSC- or ESC-derived 3-D organoid culture systems have recently been developed to accurately recapitulate defects in early development of the brain in diseases. The pluripotency within the ESC or iPSC should endow the cells with the capability to form structurally

and functionally intact tissues or organs.[71–74] In 2012, Mariani *et al.* differentiated human iPSC (hiPSC) towards the forebrain fate in the presence of FGF2 and inhibitors of the BMP, WNT, and TGF-β pathways.[37] Following the transfer of the derived cell aggregates onto a coated surface, telencephalon-like multilayered structures were generated. These tridimensional structures were composed of radial glial cells, intermediate progenitor cells, and a spectrum of layer-specific cortical neurons, which were arranged in a pattern similar to their organization *in vivo*.[37] In parallel, Nasu *et al.* revealed that the application of a laminin–entactin extracellular matrix in the cultured medium could promote 3-D telencephalic tissue formation and maintenance.[75] In 2013, Lancaster *et al.* developed a jelly-like iPSC culture system by embedding the growing embryoid bodies into pure Matrigel, and successfully built cerebral organoids.[76] Recently, Qian *et al.* successfully differentiated iPSC into forebrain organoids that contain all six cortical layers using a self-developed miniaturized spinning bioreactor system.[77] These organoid systems not only show identical organization of cells to the cortex *in vivo*, but they can also recapitulate the sequential formation of cortical layers during cortical development, as evidenced by the observation that the earlier-born neurons were labeled by deep-layer markers and located underneath the later-born neurons.[75–78] Such organoid technique endows researchers with powerful abilities to precisely study human brain developmental deficits involved in neuronal disorders. The first attempt to model human neuronal disorders using the organoid technique was for microcephaly. In the study of Lancaster *et al.* compared to healthy people, the patient organoids showed a smaller size and imperfect neuronal differentiation,[76] which is consistent with the clinical phenotype of the disease. Qian *et al.* used the forebrain organoid system to model microcephaly caused by Zika virus infection, and found that infection of neural progenitor cells with Zika virus led to increased cell death and reduced proliferation, as a result of which the volume of the layers within the organoid was decreased, thus resembling microcephaly.[77]

In summary, iPSC technology and the following *in vitro* and *ex vivo* neural differentiation approaches provide powerful tools to model the development and diseases of human brains, as well as the screening of new chemical compounds aimed at these diseases.

RESEARCH OF SCZD BASED ON iPSC MODELS

Pathophysiology of SCZD

Schizophrenia is probably the most commonly known neuropsychiatric disorder, affects about 1% of the worldwide population,[79] and has been intensively studied using iPSC technology (see Table 1).[11,12,38,80–90] Patients with SCZD suffer three categories of symptoms: (i) positive symptoms, including hallucinations, delusions, and thought disorganization; (ii) negative symptoms, including deficits in affect and social life; and (iii) cognitive impairments, such as memory and language deficits.[91,92] Microarray comparison of transcripts between SCZD patients and healthy people has revealed significant decrease in genes involved in synaptic function,[93] which leads to the hypothesis that SCZD is a disease of the synapse. In this hypothesis, deficits in synapse formation and/or pruning in specific brain regions or neural circuits during neurodevelopment in childhood and adolescence are essential for the clinical onset of the disease. One example is the deficits in dopaminergic transmission in the prefrontal cortex, which have been suggested to cause both the positive and negative symptoms and the pathophysiology of SCZD.[94–97] The dopamine activity in the prefrontal cortex has an inhibitory effect on the dopaminergic transmission in the limbic region, such as the hippocampus.[98] On the one hand, the atrophy in the prefrontal cortex attenuated local dopaminergic transmission, which is probably the cause for the negative symptoms of the SCZD patients; on the other hand, the consequent deinhibition of the dopamine activity in the limbic area is thought to be the cause for hallucinations and delusions.[99] In the meanwhile, the hypoactive function of glutamatergic neurotransmission, especially the N-methyl-D-aspartate receptor (NMDAR) activity in the cortex, is also thought to contribute to psychotic symptoms. Administrating mice with phencyclidine and ketamine, two NMDAR antagonists, would induce psychotic hallucinations in the mice, whereas NMDAR co-agonists, such as D-serine, cycloserine, and glycine, can generate modest antipsychotic actions.[100,101]

Epidemiological research aiming at the etiology of SCZD has revealed that SCZD is an inheritable disorder with a genetic risk of around 80%.[102,103] A high penetrance of the translocation in the Disrupted in Schizophrenia (*DISC1*) gene locus was

Table 1. Summary of hiPSC-based studies of SCZD

Pathological factors	Genetic mutation	Patients	Types of investigated cells	Relevant phenotypes or major findings	References
Synaptic dysfunctions	Sporadic and familial	One childhood-onset and three familial SCZD patients	Induced neurons by pan-differentiation protocol	(i) Reduced neuronal connectivity; (ii) synaptic dysfunctions; (iii) reduced expression of WNT and cAMP-signaling pathways	Brennand *et al.*[11]
	Sporadic and familial	Same as above	Hippocampal DG granule neurons	(i) Reduced rate of synaptic vesicle exocytosis; (ii) confirmed reduction in synapse number	Yu *et al.*[38]
	A frameshift mutation in *DISC1*	A patient family (one SCZD patient and one major depressive disorder patient, and two in-family controls)	Forebrain neurons	(i) Decreased presynaptic bouton number and neurotransmitter release; (ii) dysregulated genes related to synaptic transmission, neurodevelopment and dendritic spine formation	Wen *et al.*[80]
	15q11.2 copy number variants	Three individuals carrying 15q11.2 del and five individuals without the CNV	Rapid induced-primitive neural stem cells	(i) Deficiencies in adherent junctions and apical polarity; (ii) insufficient expression of CYFIP1; (iii) CYFIP1 contributes to the susceptibility of SCZD	Yoon *et al.*[81]
	Sporadic	SZ1: four SZ fibroblasts and six controls; SZ2: nine patients and eight controls	Forebrain neurons and excitatory induced-neurons	(i) Elevated $STEP_{61}$; (ii) $STEP_{61}$ inhibition rescues the deficits in spontaneous neurotransmitter release; (iii) restores the behavioral and cognitive deficits of the animals	Xu *et al.*[82]
Mitochondrial dysfunction	Sporadic	One SCZD patient and H9 female human embryonic stem cells	NPC	Deficient mitochondrial functions	Paulsen *et al.*[12]
	Sporadic	Three SCZD patients and two controls	Dopaminergic neurons and glutamatergic neurons	(i) Deficits in neuronal differentiation; (ii) impaired mitochondrial functions	Robicsek *et al.*[83]
	Sporadic	Four patients and six controls	Forebrain NPC	(i) Abnormal expression of genes related to cytoskeletal remodeling and oxidative stress; (ii) abnormal migration of cells; (iii) increased oxidative stress	Brennand *et al.*[84]
	Sporadic	Four SCZD patients and five controls	Forebrain NPC	Higher variability in the activation level of heat shock factor 1	Hashimoto-Torii *et al.*[85]

Table 1. (*Continued*)

Pathological factors	Genetic mutation	Patients	Types of investigated cells	Relevant phenotypes or major findings	References
microRNA	22q11.2 microdeletion	Six SCZD patients and six controls	Glutamatergic and GABAergic neurons	miRNA showed significantly increased expression	Zhao *et al.*[86]
	22q11.2 deletion	Two SCZD patients with 22q11.2 deletion and three controls	Neurons and astrocytes	Downregulated expression of miRNA belonging to miR-17/92 cluster and miR-106a/b drive gliogenic differentiation of iPSC	Toyoshima *et al.*[87]
	Sporadic	Three SCZD patients and three controls	Adult hippocampal NPC	miR-19 showed abnormality	Han *et al.*[88]
	Sporadic	Four SCZD patients and six controls	Forebrain NPC	Significant downregulation of miR-9	Topol *et al.*[89]
L1 retrotransposon	22q11 deletions	Three SCZD patients and three controls	NeuN-positive Neurons	(i) Increased L1 expression; (ii) enhanced L1 insertions into the synapse- and SCZD-related genes	Bundo *et al.*[90]

CNV, copy number variants; DG, dentate gyrus; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; L1, long interspersed element-1; NPC, neural progenitor cells; SCZD, schizophrenia.

identified in a Scottish pedigree.[104] Interestingly, *DISC1* gene disruption in this family is not only a feature of SCZD patients, but it also labels those family members showing BD and major depressive disorder,[105] indicating that *DISC1* may be involved in multiple psychiatric disorders. *DISC1* has been suggested to play a 'hub' role in neurodevelopment as its functions are involved in multiple signaling cascades of maturation of neurons and synapses.[106] For instance, *DISC1* interacts with the components of the dynein and kinesin motor complexes, thus playing fundamental roles in the migration of pyramidal neurons during cortical development and dendritic arborization.[107,108] *DISC1* can regulate the WNT-signaling pathway through interaction with GSK-3β and thus determine the differentiation and proliferation of neural progenitor cells.[109,110] Moreover, synaptically localized *DISC1* plays essential roles in synapse formation and synaptic plasticity.[111–115] In addition, *DISC1* also contributes to mitochondrial functions[116,117] and the cAMP-signaling pathway.[111,118,119]

Recent genome-wide association studies have discovered a number of susceptibility genes for SCZD carrying either single-nucleotide polymorphisms or copy number variants (CNV).[120] Many of these identified genes play central roles in synaptic functions. For instance, NRXN1, Neuregulin1/ErbB4,[121] ARC,[122–124] and CNTNAP2[125] are involved in synapse formation and organization; Voltage-gated Ca^{2+} channels, including CACNA1C and CACNB2,[126,127] are subunits of L-type Ca^{2+} channels and thus are fundamental for Ca^{2+} signaling; *GRIA1*, *GRIA4*, *GRIN2B*, and *GRM5* encode glutamate receptors, and *GAD1* encodes the GABA synthetic enzyme, all of which are essential for neurotransmission;[128,129] and *FMR1* plays important roles in mRNA transport and regulation.[130] The Voltage-gated Ca^{2+} channel genes are also identified in people with bipolar disorder and *FMR1* encodes a fragile X mental retardation protein and thus is responsible for fragile X syndrome. All these genes are critical for synaptic organization and transmission, suggesting that SCZD is a developmental disorder with deficits in synapses.

Synaptic dysfunction in SCZD iPSC-derived neurons

SCZD is the first neuropsychiatric disorder being studied with iPSC technology. In 2011, three groups

independently established iPSC models for SCZD. Particularly, Brennand *et al.*[11] studied the pathophysiological changes of neurons derived from the iPSC established from one childhood-onset and three familial SCZD patients. The neurons derived from patient iPSC using a pan-differentiation protocol showed remarkable attenuation in the neuronal connectivity within the neural network, which is essentially comprised of glutamatergic neurons. The authors further elegantly demonstrated synaptic dysfunctions in the SCZD patient neurons, including reduced dendritic spine number, PSD-95 expression, and glutamate receptor expression. Transcriptome analysis using microarray revealed reduction in the expression of genes involved in the WNT and cAMP-signaling pathways, which is consistent with the past findings in mouse models of SCZD. Further, applying the antipsychotic loxapine to the patient iPSC-derived neurons, the authors demonstrated improvement in the deficits of neuronal connectivity and expression of key genes. Using the same iPSC model, Yu *et al.*[38] generated hippocampal dentate gyrus granule neurons and found a remarkable reduction in the rate of synaptic vesicle exocytosis in the disease neurons, thus confirming the reduction in the synapse number in the SCZD neural network. In 2014, Wen *et al.* studied a patient family with *DISC1* frameshift mutations using iPSC technology.[80] The two selected people carrying this mutation include one SCZD patient and one major depressive disorder patient. Researchers generated the iPSC from these two patients and two in-family controls. The differentiated forebrain neurons with *DISC1* deficits also showed a decreased presynaptic bouton number and consequently reduced neurotransmitter release. RNA-seq analysis revealed dysregulated expression of genes related to synaptic transmission, neurodevelopment, and dendritic spine formation. If the synaptic defects originated from neuronal developmental deficits, the NPC from the disease subjects might have disease-related cellular phenotypes. Yoon *et al.* investigated the NPC derived from the iPSC of SCZD patients carrying 15q11.2 deletion.[81] The 15q11.2 microdeletion has been identified as one prominent CNV susceptible for SCZD in a number of studies.[131–135] One gene affected by this deletion is *CYFIP1*, a subunit gene of the WAVE complex, which affects the migration of radial glial cells during cortical development in mice through regulating the dynamics of the intracellular cytoskeleton. The authors found that the patient NPC showed deficiencies in adherent junctions and apical polarity, which was caused by insufficient expression of *CYFIP1*. Re-introduction of *CYFIP1* into the patient iPSC lines would generate NPC with normal phenotype, suggesting that CYFIP1 regulates neural stem cell physiology to contribute to the susceptibility to SCZD. Another iPSC-studied synapse-regulating protein is striatal-enriched protein tyrosine phosphatase (STEP), which acts to increase NMDAR internalization through dephosphorylating GluN2B and ERK1/2 to baffle synaptic potentiation. Xu *et al.*[82] recently reported that $STEP_{61}$, one isoform of STEP, was elevated in SCZD patient iPSC-derived forebrain neurons and induced excitatory neurons, which is consistent with their observation in the cortical lysates of the $Nrg1^{+/-}$ mouse model of SCZD. Treating the iPSC-derived neurons with neuroleptics would inhibit STEP activity, which consequently increased the phosphorylation of GluN2B and ERK1/2. As a functional result, the deficits in spontaneous neurotransmitter release in the SCZD neurons were rescued by $STEP_{61}$ inhibition. Behavioral tests based on $Nrg1^{+/-}$ mice suggested that $STEP_{61}$ inhibition can restore the behavioral and cognitive deficits of the animals.

Mitochondrial dysfunctions in SCZD iPSC-derived neurons

Mitochondrial dysfunction is probably a substantial pathological factor in SCZD. Clinical studies revealed that patients with mitochondrial abnormalities harbor a high risk of psychiatric disorders, including SCZD and BD.[136,137] Post-mortem analysis using the SCZD patient brain tissues has detected cell respiration abnormality and oxidative stress response in the patient tissues.[138,139] In 2012, Paulsen *et al.*[12] established iPSC for a SCZD patient and found that the NPC of the patient consumed more oxygen and generated more reactive oxygen species compared to the control. This difference in mitochondrial function was rescued by valproic acid, a mood stabilizer that has been used to treat BD. This evidence suggests that mitochondrial deficits are probably associated with defective neurogenesis in SCZD. In 2013, Robicsek *et al.* generated iPSC from hair follicle keratinocytes, which share the common ectodermal origin with neurons, and further differentiated the iPSC into dopaminergic neurons and glutamatergic neurons.[83] Both types of progenitor

cells showed difficulty differentiating into mature neurons. Further analysis of mitochondrial function also revealed a series of mitochondrial deficits in the SCZD cells, including impaired mitochondrial respiration, reduced membrane potential, and perturbations in the mitochondrial network structure and connectivity. These results indicate that abnormalities in mitochondrial functions play an essential role in the deficits of neurodevelopment in SCZD. In 2015, Brennand *et al.* studied the gene expression pattern of SCZD NPC using microarray and mass spectrometry technologies, and found that SCZD NPC showed aberrant expression levels of genes related to oxidative stress and cytoskeletal remodeling.[84] Consistently, the disease NPC showed abnormal migration and increased oxidative stress. These findings indicate that a significant fraction of SCZD originated from defects in neural progenitor cells.[84] Follow-up research revealed that the NPC derived from SCZD patient iPSC showed higher variability in the activation level of heat shock factor 1 (HSF1), which plays a dominant role in stress responses, in response to environmental stimulation compared to the control.[85] HSF1 regulates the transcription of heat shock proteins during the development of the brain to cope with the embryonic inflammatory response.[140–142] In HSF1 knockout mice, prenatal low-dose environmental stimulation remarkably increased brain structure abnormalities and seizure susceptibility after birth.[85] As in humans, perinatal stress, such as viral or bacterial infection to the mother, would increase the risk of SCZD in the offspring through the inflammatory response. HSF1 is probably key for the response of the brain to prenatal environmental stress, and its abnormalities induce the pathogenesis of neuropsychiatric disorders, such as SCZD.

MicroRNA in SCZD neurons

In addition to synaptic deficits and mitochondrial dysfunction, accumulating evidence suggests that microRNA may also play a role in the pathogenesis of SCZD. MicroRNA interact with mRNA to regulate the translation and degradation of target mRNA.[143] Zhao *et al.* and Toyoshima *et al.* independently studied the iPSC-differentiated neurons of patients with 22q11.2 microdeletion,[86,87] which is another most common SCZD-associated CNV,[144,145] and detected a lot of differentially expressed miRNA in the patient iPSC-derived cells. Differentially expressed miRNA are largely proposed to be involved in neurogenesis, axon guidance, and synaptic plasticity.[146–148] In Zhao *et al.*[86] several miRNA that were previously found to be differentially expressed in SCZD and ASD tissues, such as miR-34, miR-4449, miR-146b-3p, and miR-23a-5p, showed significantly increased expression in the patient iPSC-derived neurons.[149–155] In Toyoshima *et al.* downregulated expression of miRNA belonging to the miR-17/92 cluster and miR-106a/b promoted gliogenic differentiation of iPSC through enhancing the expression of p38α.[87] Recent research has suggested that microRNA are fundamental for neurodevelopmental deficits in SCZD. For instance, Han *et al.* recently found that miR-19 is highly expressed in adult NPC, but showed abnormality in the NPC derived from SCZD iPSC.[88] Importantly, miR-19 was found to play an essential role in the migration of newborn neurons in the brain. In parallel, Topol *et al.*[89] reported significant downregulation of miR-9 in the NPC of another cohort of SCZD patients, which also resulted in the deficits in neural migration that had been observed earlier,[84] which can be rescued by the overexpression of miR-9. These two studies indicate that the dysregulation of miR-9 and miR-19 may be risk factors for SCZD that contribute to the neurodevelopmental deficiencies in this disease.

Integrated understanding of impaired neural maturation

Combining the three major types of abnormalities revealed in SCZD patient iPSC-derived neurons, we summarize that deficits in the process of neuronal maturation, such as NPC migration and neuronal synapse formation, are essential features of the diseased neurons, and mitochondrial abnormalities and microRNA are largely involved through affecting neuronal maturation. For instance, increased oxidative stress and mitochondrial deficiencies contribute to the abnormal adherent junctions, apical polarity, and migration of NPC,[81,83,84] and insufficient expression of miR-19 in the SCZD NPC might also contribute to the deficient migration of new cells.[88,89]

Long interspersed element-1 retrotransposition in SCZD

In addition to dysfunctions in neuronal maturation, mitochondria, and microRNA, recent evidence suggests that long interspersed element-1 (LINE-1, L1) retrotransposition might also be involved in SCZD.

L1 is an autonomous retrotransposon that generates *de novo* insertions into new genomic locations, leading to somatic mosaicism (i.e., genetic variation within individual somatic cells) in the human brain,[156] which may cause neuronal disorders, such as Sturge–Weber syndrome and hemimegancephaly.[157,158] A recent study demonstrated that the L1 expression was increased in the neurons derived from iPSC of SCZD patients with 22q11 deletion.[90] Consistently, the authors observed enhanced L1 insertions into synapse- and SCZD-related genes in the SCZD patient brains. Hence, hyperactive L1 retrotransposition may play a role in the susceptibility and pathophysiology of SCZD.

iPSC MODELING OF BIPOLAR DISORDER

Pathophysiology of bipolar disorder

Compared to SCZD, modeling BD with iPSC technology started only recently (see Table 2).[159–164] BD is characterized by intermittent episodes of mania and depression.[165,166] More than 1% of the population worldwide suffer from BD.[167–169] Of the patients, approximately half show psychotic symptoms,[170,171] and 15% commit suicide when no appropriate treatments are given.[172] Hence, among all diseases, BD has been ranked by the World Health Organization as a top disorder of morbidity and lost productivity.[173]

Genetic epidemiological evidence from family, twin, and adoption studies has suggested that BD has an occurrence of 5–10% for first-degree relatives and the concordance rate of monozygotic twins is as high as 90%, thus being a highly heritable disorder.[174–176] Screening for susceptible genes based on genome-wide association studies has revealed single-nucleotide polymorphisms or unusual splicing variants in a series of genes, such as *ODZ4*, *CACNA1C*, *NCAN*, *DHH*, *TRPC4AP*, *SYNE1*, *ZNF804A*, *MAPK3*, *ITIH3/4*, *PBRM1*, and *ANK3*.[177–184] Owing to the complexity and heterogeneity of the genetics of BD, it has been difficult to

Table 2. Summary of hiPSC-based studies of bipolar disorder

Genetic mutation/ family	Patients	Types of investigated cells	Relevant phenotypes or major findings	References
Sporadic	Three BD-I patients and three controls	Induced neurons by pan-differentiation protocol	(i) The control and BD iPSC were very similar in the transcriptome; (ii) more features of ventral patterning	Chen *et al.*[159]
Familial BD	Four BD patients and four unaffected siblings	PAX6-positive NPC and MAP2-positive neurons	Higher expression of GAD1	Kim *et al.*[160]
Familial BD	Two BD brothers and their healthy parents	NPC and neurons	(i) Abnormalities in neurodevelopment and mRNA expression; (ii) WNT enhancer can restore the deficits	Madison *et al.*[161]
Sporadic	One male BD patient and one unaffected male control	NPC, NPC-derived neurons, and transdifferentiated neurons	(i) miR-34a showed strong expression in the BD NPC and neurons; (ii) enhanced miR-34a expression inhibited the neuronal differentiation and maturation of the NPC	Bavamian *et al.*[162]
Sporadic	Three LR BD-I patients, three NR patients, and four controls	Hippocampal DG granule cells	(i) Significant enhancement in genes related to the PKA/PKC-signaling pathway, action potential firing, and mitochondrial functions; (ii) hyperexcitability; (iii) mitochondria exhibited elevated functions; (iv) Li selectively rescued the LR patient neurons	Mertens *et al.*[163]
Sporadic	LR and NR patients	DG neurons	(i) Two different subdisorders of BD: LR and NR; (ii) larger fast after-hyperpolarization	Stern *et al.*[164]

BD, bipolar disorder; DG, dentate gyrus; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; LR, lithium-responsive; NPC, neural progenitor cell; NR, lithium-non-responsive.

develop gene-targeted or phenotypic animal models to recapitulate the spontaneous cycling of manic and depressive episodes,[185,186] which has resulted in slow progress in our understanding of the disease, especially at the cellular level.

Previous neuropathological studies have revealed a series of alterations in the animal models, the blood cells of living BD patients, and the post-mortem brains of deceased BD patients.[187] These changes include reduced neuronal and glial density in the prefrontal cortex and hippocampus,[188–190] upregulated activity of the PKA/PKC pathways,[191–193] and changes in neurotransmitter systems, including dopamine, 5-HT, and glutamate.[194–197] However, due to a lack of precise animal models,[185,186] whether these changes are primary for the disease is questionable, which has baffled the development of new drugs or therapies precisely targeting BD pathology. Current drugs for the treatment of BD include mood stabilizers (e.g., lithium, anticonvulsants valproate, carbamazepine), atypical antipsychotics, antiepileptic drugs, and antidepressants; however, antidepressants may cause unexpected mania or hypomania.[198,199] One of the most generalized drugs for BD is lithium (Li), a mood stabilizer that causes remarkable improvement in about one-third of the patients.[200,201] The proposed action of Li includes inhibition of the GSK-3β pathway;[202] the downregulation of protein kinase pathways, such as PKA and PKC;[203–206] and a neuroprotecting influence through increasing the expression of brain-derived growth factor and BCL2.[207,208] However, the exact mechanism by which Li stabilizes mood is still obscure, and the reason why a majority of patients are resistant to Li treatment has remained enigmatic. Therefore, developing accurate iPSC models and the downstream neuronal models is an urgent task for research on BD and drug development for BD.

Abnormalities in neurodevelopment and mRNA expression in BD iPSC-derived neurons

In 2014, Chen *et al.* established an iPSC model for BD based on the dual SMAD inhibition method and further differentiated the iPSC into neurons using a pan-differentiation protocol.[159] Although the iPSC were very similar in the transcriptome profile between the patients and the healthy controls, the neurons derived from the patient iPSC showed more features of ventral patterning while the neurons of healthy people were more shifted to the dorsal fate. This was later confirmed by a study in 2005 in which neurons differentiated from BD type I (BD-I) patients of an Amish pedigree showed higher expression of *GAD1*.[160] Compared to the control, the BD patient neurons showed higher expression of *NKX2.1* and *PAX2*, indicating that they were differentiated towards the fate of GABAergic interneurons originated from the MGE progenitor cells. In contrast, the neurons differentiated from healthy people showed high expression of dorsal telencephalic markers *PAX6*, *EMX2*, *TBR2*, *TCF3*, and *ZNF536*, as well as cortical marker *LHX2*.[209] However, the fate of both groups of cells can be regulated by the differentiation protocol. For instance, administration of purmorphamine to promote SHH signaling can increase the expression of *NKX2.1* in the differentiated cells in both groups, whereas enhancing the WNT-signaling pathway by Li treatment can promote *EMX2* expression.[159] These findings are consistent with the notion that BD probably involves embryonic developmental problems in the central nervous system.[210,211] Moreover, these observations provide new insight into the therapeutic effects of Li on BD. Microarray analysis revealed that the BD neurons showed greater expression of many ion channel subunits and membrane receptors, such as GABA and glutamate receptors, and potassium channel subunits. Particularly, the expression of the Ca^{2+} channel subunit was also significantly increased in BD neurons, which was consistent with previous clinical findings regarding the abnormality of calcium signaling in BD.[212,213] Consistent with the microarray analysis, Ca^{2+} imaging experiments revealed that the amplitude and dynamics of high potassium perfusion-stimulated Ca^{2+} transient events were different between the disease and control neurons.

Another study investigated the abnormalities in neurodevelopment and mRNA expression in neurons differentiated from a BD patient family, which consisted of two affected brothers and their healthy parents.[161] Surprisingly, although no significant differences were detected among the iPSC of the family, the stem cells of the patients could hardly differentiate into functional neurons, indicating that severe developmental deficits were involved in the pathogenesis of the two offspring patients. Importantly, application of CHIR-99021, which has been used to enhance the WNT-signaling pathway, can

restore the deficits of the BD iPSC to the normal level. This is consistent with the results of Chen *et al.* in that the progenitor cells of BD patients were reluctant to follow a dorsal fate due to the lack of WNT signaling.[159] Using NanoString digital mRNA expression profiling technology, Madison *et al.* investigated the transcriptional changes in the patient cells, including fibroblasts, iPSC, NPC, and the differentiated neurons with the help of CHIR-99021, to explore the mechanisms underlying the deficits that appeared during differentiation.[161] Although the fibroblasts and iPSC failed to show obvious difference, the patient NPC showed increased expression of ventral progenitor markers, such as *NKX2.2*, *NKX6.1*, and *IRX3*, while the expression of the dorsal telencephalic marker *PAX6* was attenuated. Notably, the patient NPC showed decreased expression of *DISC1*, a gene famous for its central role in neurodevelopment and in SCZD. Finally, the investigation into the neurons revealed that the expression of *BCL11B* (*CTIP2*) and *RELN* were obviously reduced in the BD patient neurons compared to the healthy control. *CTIP2* plays essential roles in nucleosome remodeling and deacetylation (NuRD) and thus is important for the epigenetic regulation of neurogenesis.[30,214,215] RELN is a glycoprotein involved in the formation of the cortex.[216] Using the iPSC model of this patient family, Bavamian *et al.* observed that miR-34a showed strong expression in the patient NPC and neurons compared to the parent control cells.[162] This finding was supported by further experiments based on fibroblast-transdifferentiated neurons and post-mortem brain analysis. Previously, it had been suggested that miR-34a plays important roles in neuronal development, synaptic plasticity, and learning and memory in mammals.[217–220] miR-34a is also one of the microRNA that can be suppressed by treatment with Li and valproic acid, which have often been used for the treatment of BD.[221,222] As the potential targets of miR-34a include *ANK3* and *CACNB3*, which are two established risk genes for BD, miR-34a might be involved in the therapeutic effects of mood stabilizers. Bavamian *et al.* demonstrated that the enhanced expression of miR-34a is the barrier for neuronal differentiation and maturation of the NPC derived from the patient iPSC. The discovery of miR34a in BD might explain why the iPSC with similar transcriptome patterns to those of the normal healthy controls can differentiate into NPC and neurons distinct from those of controls.

Hyperexcitability in BD iPSC-derived hippocampal neurons

In parallel, Mertens *et al.* established an iPSC model for six BD-I patients (of which half showed a positive clinical response to Li treatment while the other half were non-responsive) and systematically investigated the neurobiology of BD based on the Li selectivity.[163] As BD patients often show atrophy in the hippocampus,[189,190] Mertens *et al.* chose to investigate iPSC-derived hippocampal dentate gyrus (DG) granule cells.[163] Approximately 70% of the differentiated cells were DG granule cells indicated by the DG marker *PROX1*. As neurodevelopmental factors are probably involved in the pathogenesis of BD, RNA-seq analysis was carried out on immature neurons at the 3-week age. The results indicated significant enhancement in genes involved in the PKA/PKC-signaling pathways, the action potential (AP) firing-related sodium and potassium channel subunits, and the mitochondrial system. With the guidance of RNA-seq data, the authors carried out patch clamp recording and Ca^{2+} imaging investigation into the phenotype of the BD patient neurons, and demonstrated hyperexcitability in these neurons. In parallel, the mitochondria of the BD neurons exhibited elevated functions as indicated by higher membrane potential, which might provide energy necessary for the hyperexcitation of the neurons. In the meanwhile, the mitochondria showed a smaller size in the disease neurons, which might facilitate the transport of mitochondria to nerve terminals. To support that the BD iPSC model is suitable for the development of new clinical therapies and drugs, it is important to investigate the consistency of the hyperactivity phenotype shown in the BD patient-derived neurons in comparison to the clinical defects of the patients. In the study of Mertens *et al.* the recruited subjects included three Li responsive (LR) and three Li non-responsive (NR) patients. Hence, the authors compared the effects of Li on the hyperactivity phenotype of the two types of BD neurons. In the neurons derived from LR patients, Li significantly reduced the hyperactivity of the BD neurons and their mitochondria, whereas the NR neurons remained unaffected by Li. Therefore, the neuronal hyperactivity revealed by the BD iPSC can represent the clinical symptom of mania in the BD patients. To explore the mechanisms that may underlie the Li-caused reduction of neuronal activity in the LR neurons, the authors

again carried out RNA-seq analysis to search for the Li-affecting genes in the LR and NR neurons. The results indicated that Li can significantly affect the gene expression profiles of the LR patient neurons but not in the NR neurons. Further analysis revealed that Li rescued 84 genes in the LR neurons, to varying degrees, of which those involved in the PKA/PKC pathways and AP firing were included. Therefore, Li diminished the hyperactivity of the LR neurons, probably through reversing the activity of these pathways. Moreover, the PKA/PKC, AP-firing, and mitochondrial systems are likely involved in the pathogenesis of BD. Based on these findings, Stern *et al.* further investigated the hyperexcitability of DG-like neurons derived from B-lymphocyte-reprogrammed BD patient iPSC.[164] By comparing the LR and NR patient neurons, the authors found that the two groups of neurons showed different features in AP-firing profiles, which suggested two different subcategories of BD. Importantly, both the LR and NR neurons exhibited a larger fast after-hyperpolarization (AHP), which might play an important role in the sustained spiking abilities of the BD neurons. Therefore, the hyperexcitability in the patient neurons and its rescue by Li proved that the DG neuronal model is a valid tool for research into the pathology and pathogenesis of mania and for the development of new drugs or therapies aimed at BD.

CONCLUSION

The iPSC models generated from patients with SCZD and BD have been widely used for research into the progression and pathogenesis of these two neuropsychiatric disorders. Although iPSC may generate epigenetic features different from the original patient tissues, this type of cell is still the best to retain the primary genetic characteristics of the diseases. So far, much knowledge obtained from animal models and post-mortem tissues in past decades has been justified by iPSC-based research, and, importantly, a lot of new information about the diseases has been discovered with the help of iPSC. We believe that, with the differentiation of patient iPSC into divergent types of neurons or organoids and the participation of more patient cohorts in iPSC research, our understanding about these disorders will be much furthered.

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AUTHOR CONTRIBUTIONS

Y.N.L. contributed to the literature search and first drafting of the manuscript. S.Y.L. contributed to editing of the manuscript. J.Y. contributed to literature search, evidence review, writing, and editing of the manuscript.

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