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Modeling Hippocampal Neurogenesis Using Human Pluripotent Stem Cells

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SUMMARY

The availability of human pluripotent stem cells (hPSCs) offers the opportunity to generate lineage-specific cells to investigate mechanisms of human diseases specific to brain regions. Here, we report a differentiation paradigm for hPSCs that enriches for hippocampal dentate gyrus (DG) granule neurons. This differentiation paradigm recapitulates the expression patterns of key developmental genes during hippocampal neurogenesis, exhibits characteristics of neuronal network maturation, and produces PROX1+ neurons that functionally integrate into the DG. Because hippocampal neurogenesis has been implicated in schizophrenia (SCZD), we applied our protocol to SCZD patient-derived human induced pluripotent stem cells (hiPSCs). We found deficits in the generation of DG granule neurons from SCZD hiPSC-derived hippocampal NPCs with lowered levels of *NEUROD1*, *PROX1*, and *TBR1*, reduced neuronal activity, and reduced levels of spontaneous neurotransmitter release. Our approach offers important insights into the neurodevelopmental aspects of SCZD and may be a promising tool for drug screening and personalized medicine.

INTRODUCTION

The advent of stem cell biology has opened new avenues in the field of neuroscience research as well as therapeutic approaches for neurological diseases. Although it is clear that human pluripotent stem cells (hPSCs; i.e., both human embryonic stem cells [hESCs] and human induced pluripotent stem cells [hiPSCs]) can give rise to functional neurons, a current challenge is to develop differentiation strategies that can produce disease-relevant subtypes of neurons. To date, progress has been made to generate enriched populations of ventral midbrain dopaminergic neurons and spinal motor neurons to model Parkinson disease and amyotrophic lateral sclerosis, respectively (Perrier et al., 2004; Roy et al., 2006; Di Giorgio et al., 2008; Dimos et al., 2008; Marchetto et al., 2008; Kriks et al., 2011; Ma et al., 2011). In addition, cortical pyramidal neurons and forebrain interneurons have been generated from hiPSCs (Shi et al., 2012a,b; Vanderhaeghen, 2012; Maroof et al., 2013; Nicholas et al., 2013). Most of these methods induce differentiation that approximates the in vivo developmental program. Consequently, in addition to producing a neuronal subtype, hPSCs can potentially recapitulate the developmental stages of the neuron of interest (Hu et al., 2010; Shi et al., 2012b; Nicholas et al., 2013) and provide insights into the pathogenesis of neurodevelopmental diseases where deficits originate in developmental windows prior to the onset of clinical symptoms.

The dentate gyrus (DG) of the hippocampus is one of two areas of the brain where neurogenesis continues to occur throughout life. New neurons generated at the subgranular zone (SGZ) of the DG integrate play a fundamental role in learning and memory (Zhao et al., 2008). Aberrations in hippocampal neurogenesis have been implicated in epileptic seizures (Jessberger et al., 2007), Alzheimer disease (Tatebayashi et al., 2003), and cognitive defects characteristic of depression (Sahay and Hen, 2007; Mateus-Pinheiro et al., 2013) and schizophrenia (SCZD) (Reif et al., 2006; Tamminga et al., 2010; Walton et al., 2012; Hagihara et al., 2013). Unfortunately, it is difficult to investigate the early events of these central nervous system (CNS) disorders in the human system, and it is not clear whether findings from studies using rodent models will translate across species.

Here, we present a differentiation paradigm that produces an enriched population of hippocampal DG granule neurons using key developmental cues that specify the hippocampal DG identity. Using two differentiation approaches based on free-floating embryoid bodies (EBs) and neural progenitor cell (NPC) monolayers, we generated neurons expressing PROX1 and TBR1, markers found in mature DG granule neurons (Hodge et al., 2012; Iwano et al., 2012). The differentiation process recapitulates expression patterns of key developmental genes, including *NEUROD1* and *DCX*, that are critical for hippocampal neurogenesis (Miyata et al., 1999; Liu et al., 2000; Schwab et al., 2000; Gao et al., 2009). Furthermore, these neurons

formed functional networks characteristic of granule neuron maturation and are able to functionally integrate into the endogenous DG upon in vivo transplantation. To test the effectiveness of this protocol to uncover functional defects in human diseased neurons, we used this differentiation paradigm on SCZD patient hiPSCs and control hiPSCs as a proof-of-principle application. We found deficits in the generation of hippocampal granule neurons from SCZD hiPSC-derived hippocampal NPCs with reduced levels of *PROX1* and *TBR1*. Furthermore, SCZD granule neurons showed deficits in neuronal activity, as evidenced by the reduced frequency of spontaneous neurotransmitter release. Our strategy to generate a specific, disease-relevant subtype of neurons reduces the variability within the cellular population being examined and allows for the detection of early alterations in the developing SCZD hippocampal granule neurons. This approach may offer important insights into the neurodevelopmental aspects of SCZD and presents a promising tool for drug screening, diagnosis, and personalized medicine.

RESULTS

To develop a differentiation paradigm that enriches for hippocampal granule neurons, we first utilized free-floating EBs, which provide 3D spatial cues that have been shown to simulate aspects of pregastrulation development and early gastrulation (Kopper et al., 2010). The differentiation approach was optimized based on key morphological cues that specify the hippocampal DG identity during development. Mimicking the developmental signals that generate the anterior-posterior patterning of the forebrain, we first treated the EBs with a cocktail of anticaudalizing factors (DKK1, noggin, and SB431542) blocking the WNT, bone morphogenetic protein (BMP), and transforming growth factor-β (TGF-β) pathways to obtain telencephalic neural precursors (Watanabe et al., 2005). In addition, we applied cyclopamine, an antagonist of the Sonic Hedgehog pathway, to enrich for dorsal forebrain progenitors (Gaspard et al., 2008) (Figure 1A). After 20 days, the mature EBs were treated with Wnt3a, a WNT protein previously shown to be important for the maintenance of hippocampal progenitors and their differentiation into DG granule neurons (Lee et al., 2000; Machon et al., 2007; Kuwabara et al., 2009; Wexler et al., 2009; Karalay et al., 2011), as well as brain-derived growth factor (BDNF), a neurotrophic factor found to promote hippocampal neurogenesis throughout life (Scharfman et al., 2005; Erickson et al., 2010) (Figure 1A).

After 35 days of differentiation, we observed expression of TUJ1 and MAP2AB in the EBs, indicating the derivation of neurons using the differentiation paradigm (Figure 1B). Strikingly, we detected a significant expression of PROX1 in the EBs (~32%), a marker specific to DG granule neurons in the hippocampus (Hodge et al., 2012; Iwano et al., 2012), compared to the untreated group (~4.7%) (Figures 1B and 1C). We further confirmed this observation using quantitative RT-PCR (qRT-PCR), which showed a remarkable increase in *PROX1* expression (Figure 1E) and a significant reduction in *OCT4* expression in EBs treated with the cocktail of factors (Figure 1D).

To further authenticate the generation of DG granule neurons using this protocol, we assessed the EBs at various time points to track the development of these neurons during the differentiation process. Previous studies of both embryonic and adult neurogenesis in the DG have revealed a number of transcription factors, as well as their expression patterns, that are important for directing neuronal fate specification and lineage commitment during hippocampal neurogenesis (Hodge et al., 2012; Hsieh, 2012). We examined the expression dynamics of these key markers to determine the extent to which our protocol for in vitro differentiation recapitulated the in vivo neurogenesis process. Following 10 days of differentiation, we observed upregulated expression levels of *EMX2*, *FOXG1*, and *PAX6*, which are markers found in hippocampal NPCs (Pellegrini et al., 1996; Shinozaki et al., 2004; Shen et al., 2006; Osumi et al., 2008), together with *NEUROD1*, an indispensable transcriptional activator that regulates neuronal differentiation of granule neurons in the hippocampus (Miyata et al., 1999; Liu et al., 2000; Schwab et al., 2000; Gao et al., 2009) (Figure 1F). These hippocampal NPCs further matured into neuroblasts and postmitotic granule neurons, as indicated by the increasing levels of *DCX* and *TBR1*, together with *PROX1*, after 20 days of differentiation (Figures 1E and 1G). Taken together, these data indicate that we were able to obtain PROX1+ hippocampal granule neurons from hESCs and that these neurons were generated via a process that recapitulated important aspects of hippocampal neurogenesis in vivo. We then dissociated the EBs after 40 days of differentiation and coculture with human hippocampal astrocytes to allow formation of neural networks and facilitate further neuronal maturation. As a result, we observed neurons that had extensively branched dendritic arbors and were triple positive for PROX1, MAP2AB, and NEUN, indicative of postmitotic hippocampal granule neurons (Figures 1H and 1I).

hESC-Derived Hippocampal NPCs Can Give Rise to DG Granule Neurons

We further adapted our protocol to utilize NPC monolayers during differentiation. To this end, we plated EBs treated with anticaudalizing factors onto laminin/polyornithine-coated tissue culture surfaces at differentiation day 20 to