HHS Public Access

Author manuscript

Mol Psychiatry. Author manuscript; available in PMC 2015 November 01.

Published in final edited form as:

Mol Psychiatry. 2015 May ; 20(5): 549–550. doi:10.1038/mp.2014.194.

Dopaminergic differentiation of schizophrenia hiPSCs

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Dear Editor

Given the recent report that dopaminergic (DA) neurons are generated at extremely low efficiency from schizophrenia (SZ) patient-derived human induced pluripotent stem cells (hiPSCs)[1], it is important to communicate that we have successfully differentiated tyrosine hydroxylase (TH)-positive DA neurons from both SZ patients and controls at modest levels.

Robicsek *et al.*[1] adopted a protocol whereby neural induction occurs via dual SMAD inhibition in a monolayer culture (using the BMP inhibitor Noggin and the TGFβ inhibitor SB431542), followed by DA patterning through the addition of SHH for five days, and then SHH, FGF8, BDNF and ascorbic acid for four additional days (SI Table 1).[2] Using TH and DAT as markers of DA neurons[1], the authors demonstrated a significant defect in the ability of the SZ hiPSC lines to differentiate to DA neurons. Within the mammalian brain, however, the expression of TH[3] and DAT[4, 5] is widespread and thus not solely indicative of the DA neuronal subtypes most relevant to SZ (reviewed in[4]).

[34]We also used dual SMAD inhibition for neural induction (using the small molecules SB431542 and LDN193189), followed by patterning with SHH and FGF8, though via an embryoid body (EB)-intermediate (SI Table 1).[6] This yielded populations of neural progenitor cells (NPCs) that consistently, over a number of passages, differentiated to TH-positive neurons (Fig. 1B). Owing to concerns that this protocol may in fact generate hypothalamic precursor cells,[7] we attempted to increase the proportion of cells expressing the midbrain DA marker Forkhead box A2 (FOXA2), by culturing our low-passage NPCs with CHIR99021, a potent GSK3B inhibitor known to strongly activate WNT signaling,[8] in addition to SHH/FGF8 (Fig. 1C). This strategy led to the derivation of NPCs that consistently yielded increased numbers of TH (Fig. 1D,E) and FOXA2-positive (Fig. 1E) neurons. Though there was substantial variability in efficiency between individual hiPSC lines, we observed no meaningful differences consistent with SZ diagnosis (Fig 1D). There was limited overlap of FOXA2- and TH-positive cells (40-80% of TH-positive cells were FOXA2-positive, while 7-17% of FOXA2-positive cells were TH-positive, varying between

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Author Information: As per our agreement with Coriell Cell Repository, many of the hiPSC lines generated from control and SZ fibroblasts will be available from Coriell. Additionally, all control and SZ fibroblasts are in the process of being deposited with the NIMH RUCDR Repository.

The authors have declared that no competing interests exist.

individuals and experimental replicates), indicating that these TH-positive neurons do not represent midbrain DA fate (Fig. 1E); likely because CHIR99021 was added late in our differentiation paradigm and was not present not during the original patterning of our control and SZ neural rosettes.[9]

Hook *et al.*[10] recently described increased release of DA neurotransmitter, concomitant with increased numbers of TH-positive neurons, from a subset of SZ hiPSC lines. However, that report relied on default anterior neural patterning to generate NPCs and neurons[11] with a transcriptional profile most similar to fetal forebrain tissue,[12] whereas data presented here is from neurons derived from SHH/FGF8 treated EBs (SI Table 1). Though this report [10] (and ours) utilized the very same control and SZ hiPSC lines[11], direct comparisons are difficult given that the TH-positive neurons have different spatial patterning.

It is critical to note that the field still lacks a full electrophysiological characterization confirming that TH-positive neurons derived from SZ patients are in fact functionally mature DA neurons. Others have rigorously demonstrated DA-characteristic features, such as overshooting action potentials with prominent K+ currents,[13] after-spike hyperpolarizations,[13] tonical firing patterns[13, 14] and DA release,[7, 14] in control hiPSC-differentiated or fibroblast-induced DA neurons. Pharmacologically, the repetitive firing pattern of mature DA neurons is reversibly inhibited following the addition of DA (or a DA receptor agonist such as quinpirole).[13] Additionally, some, populations of DA neurons are susceptible to the toxin 1-methyl-4-phenylpyridinium (MPP+).[14] Moreover, because diverse neuronal populations express TH,[3, 15-17] these functional validations need to be accompanied by demonstration of markers associated with DA production and release, such as AADC and DAT.

So what could explain the different findings in these reports? One explanation may relate to the heterogeneity of SZ patients used to derive hiPSC lines, Robicsek *et al.*[1] derived lines from three patients with paranoid SZ whereas we, and Hook *et al.* [10] derived lines from three clinically heterogeneous SZ patients (SI Table 2). Additionally, the reprogramming technique and somatic cell source, as well as the demographic status and treatment history may also represent confounding variables (SI Table 2); however, as the particulars of the later are unknown, it is difficult to assess what contribution this may have had. [1819] Another possibility is that simple methodological differences, such as media composition, patterning protocols, neuronal density and/or length and extent of neuronal maturation, may account for the vastly different final compositions of the neuronal populations obtained in these reports. Ultimately, many of these methodological variables could lead to differences in oxidative stress, which has been increasingly linked to SZ in animal models[20-22] and human studies.[23] Moreover, increased reactive oxygen species and oxidative stress, impaired mitochondrial function and sensitivity to sub-threshold environmental stresses are among the phenotypes reported in a number of recent hiPSC-based[1, 12, 24, 25] and olfactory neural stem cell-based[26] studies of SZ.

In order to conclusively resolve whether SZ hiPSC derived DA neurons have specific defects in patterning, maturation or survival relative to controls, researchers need to not just utilize larger cohort sizes with known clinical and treatment history, but couple this to a

more rigorous phenotypic, biochemical and functional characterization of neuronal fate, particularly on neurons derived from protocols that generate midbrain DA neurons [7, 9], the DA subtype currently hypothesized relevant to SZ. Only in this way can we begin to identify neuronal subtype specific defects contributing to SZ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Kristen Brennand is a New York Stem Cell Foundation - Robertson Investigator. The Brennand Laboratory is supported by a Brain and Behavior Young Investigator Grant, National Institute of Health (NIH) grant R01 MH101454 and the New York Stem Cell Foundation.

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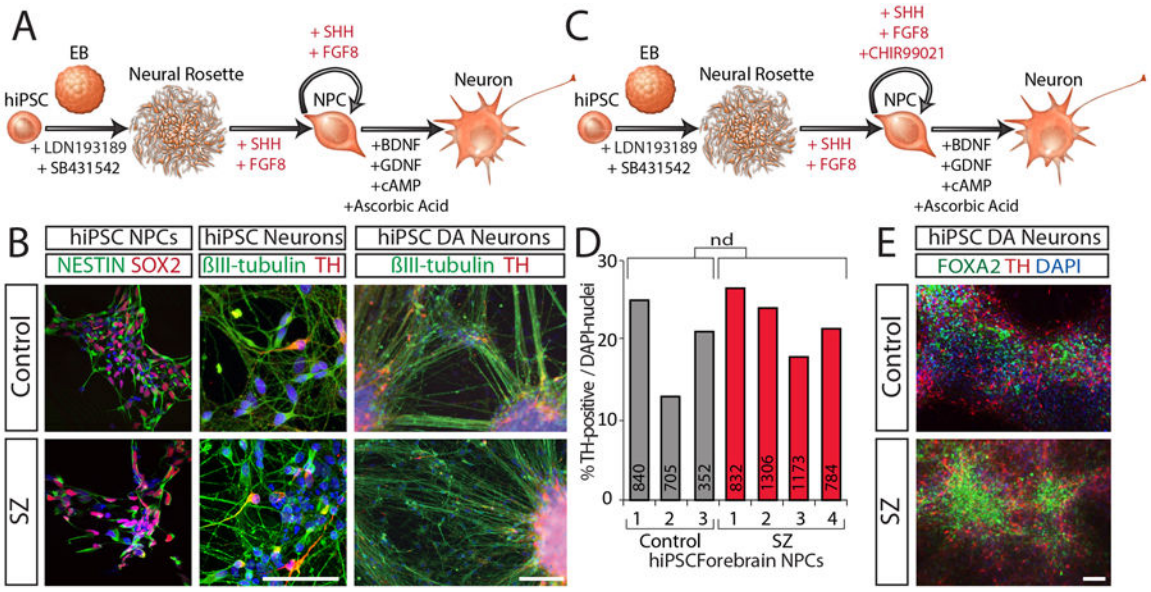

Fig. 1. Differentiation of control and SZ hiPSC DA NPCs

A. Schematic of SHH/FGF8 hiPSC DA neural differentiation. **B.** NPCs patterned with SHH and FGF8 differentiation to neurons expressing the DA marker tyrosine hydroxylase (TH) (red) and ßIII-TUBULIN (green). Scale bar 10μm. **C.** Schematic of SHH/FGF8/CHIR99021 hiPSC DA neural differentiation. **D.** No significant differences (nd) in the yield of TH-positive neurons after 4-weeks of neuronal differentiation between control and SZ DA NPCs when cultured with SHH, FGF8 and CHIR99021. Numbers within the bars indicate total number of DAPI-positive nuclei counted. **E.** Limited overlap in FOXA2-positive and TH-positive neuronal population. Scale bar 100μm.