Biological Psychiatry

Altered WNT Signaling in Human Induced Pluripotent Stem Cell Neural Progenitor Cells Derived from Four Schizophrenia Patients

To the Editor:

Schizophrenia (SZ) is a devastating psychiatric disorder hypothesized to be a neurodevelopmental condition (1,2) arising as a consequence of dysregulation of brain development (3,4). WNT signaling is important for neural patterning, proliferation and migration, and synapse formation (5); converging postmortem (6,7), rodent (8,9), and pharmacologic (10) evidence suggests that WNT signaling may contribute to SZ (11,12). We used human induced pluripotent stem cell (hiPSC) derived forebrain patterned neural progenitor cells (NPCs) (13,14) to investigate canonical WNT activity in a pilot cohort of four patients with SZ.

Because all research described herein was performed on deidentified human samples obtained for broadly consented scientific research by either American type culture collection or the Coriell Cell Repository, it was found to be exempt by the Internal Review Committee of the Icahn School of Medicine at Mount Sinai. This work was also reviewed by the Embryonic Stem Cell Research Oversight Committee at the Icahn School of Medicine at Mount Sinai.

We compared global transcription of forebrain hiPSC NPCs from six control subjects and four patients with SZ by RNA sequencing (GSE63738) (Table 1), cultured as described (13,14). As previously reported, hiPSC forebrain NPCs differentiate to a mixed neuronal population of glutamatergic and GABAergic neurons; there was no difference in the ability of control or SZ hiPSC NPCs to generate βIII-tubulin–positive neurons (14), and neither transcriptional nor immunohistochemical characterization revealed any diagnosis-dependent differences in the regional patterning of forebrain NPCs (13). Multidimensional scaling resolved most SZ and control hiPSC NPC samples (Figure 1A); 848 genes were significantly differentially expressed (false discovery rate $<$ .05) (Table S1 in Supplement 1), as illustrated by a heat map (Figure 1C) and a volcano plot (Figure 1D). The differentially expressed genes in SZ hiPSC NPCs were significantly 3.6-fold enriched compared with WNT target genes (p $<$ 10e-20) predicted by standard classification and regression tree methods (16). The differentially expressed genes (false discovery rate $<$.05) were submitted to Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov), which identified several significantly enriched pathways (Figure 1B and Tables 2 and 3), including the WNT signaling pathway: 17.3-fold enrichment (p $<$ 10e-13; false discovery rate $<$ 10e-11). The perturbed WNT genes are marked by red stars in the WNT signaling pathway diagram (Figure 1E and Table 2). Six of six differentially expressed WNT genes identified by RNA sequencing were confirmed when tested by quantitative polymerase chain reaction (Figure 1F and Table 2).

We investigated canonical WNT activity using the well-established TOPFlash assay, in which transcriptional activation of T-cell factor/lymphoid enhancer-binding factor binding sites drives expression of a luciferase reporter (17,18). The NPCs were infected 3–7 days before analysis with a Lentiviral (LV)-TOPFLASH luciferase reporter (generously provided by Karl Willert, University of California, San Diego) and a constitutive LV-Renilla reporter for normalization. The SZ hiPSC NPCs showed increased canonical WNT signaling relative to control samples (p $<$ 10e-5) (Figures 2A and 3), although increased WNT signaling was not present in every patient, and significant outliers often skewed results (Figure 3). Across six independent experimental replicates, the following fold changes in canonical WNT signaling were observed: 2.8, 4.2, 3.1, 2.7, 4.7, and 3.3 (Figure 3). The ultimate effector of canonical WNT signaling is β-catenin; Western blot analysis for β-catenin protein (1:10,000; Millipore, Billerica, Massachusetts), normalized to β-actin (1:10,000; Ambion, Foster City, California), revealed increased β-catenin protein levels in SZ hiPSC NPCs (Figure 2B).

WNT signaling has been implicated in neural migration (19). After 48 hours of culture with either canonical (20 ng/mL WNT3A) or noncanonical (5 ng/mL WNT7B) WNT signals, neither control nor SZ hiPSC forebrain NPCs showed significant changes in radial migration (98 total SZ neurospheres were analyzed relative to 56 total control neurospheres) (Figure 2C); increased canonical WNT signaling was not sufficient to recapitulate SZ aberrant migration in control hiPSC derived neurospheres (14).

Consistent with evidence suggesting that the WNT pathway could be aberrant in SZ (20), we demonstrate that SZ hiPSC forebrain NPCs derived from four patients have perturbations in WNT signaling, but we caution that 1) because of our small

Table 1. Known Clinical Information for Four Coriell Schizophrenia Patients

Coriell ID	Sex	Ethnicity	Age (Years)	Age of Onset	Phenotype	Hospitalizations?	Family History
GM02038	M	Caucasian	22	6 years	Suicide	?	Unknown
GM01792	M	Caucasian Jewish/ Scandinavian	26	Unknown	Episodes of agitation, delusions of persecution, and fear of assassination; at age 4 mild features of pervasive developmental disorder	?	Father and sister affected; brother autistic at age 4
GM01835	F	Caucasian Jewish	27	Unknown	Drug abuse; schizoaffective disorder	Yes	Father and brother affected
GM02497	M	Caucasian Jewish	23	15 years	Paralogical thinking, affective shielding, splitting of affect from content, and suspiciousness	Yes	Affected father, anorexic/schizoid sister

F, female; M, male.

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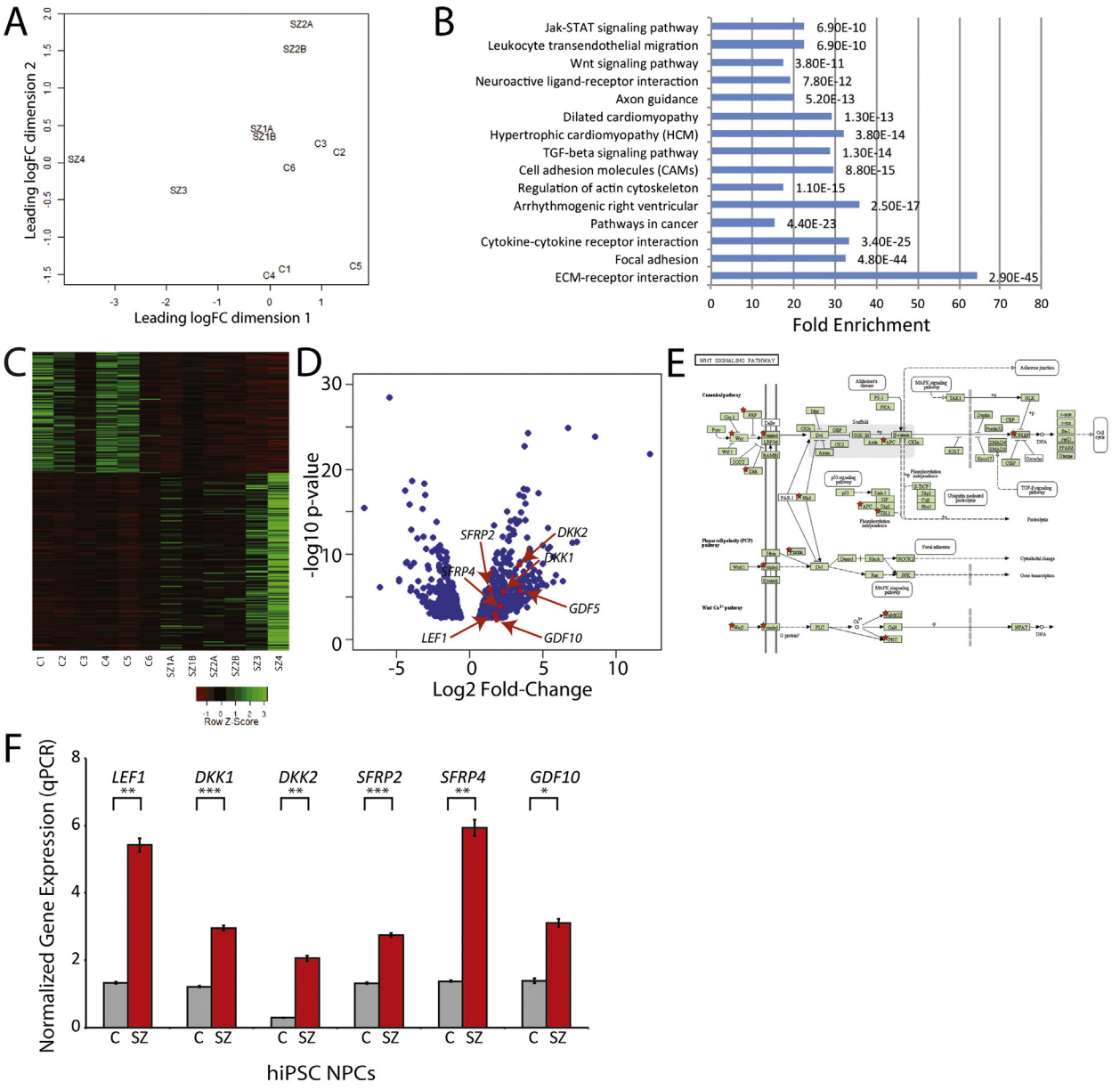

Figure 1. RNA sequencing comparisons of control and schizophrenia human induced pluripotent stem cell (hiPSC) neural progenitor cells (NPCs). **(A)** Multidimensional scaling of RNA sequencing gene expression of hiPSC NPCs from each of six control and four patients with schizophrenia segregates samples along the two leading fold change dimensions. Gene expression analysis was performed on passage-matched hiPSC forebrain NPCs cultured on Matrigel matrix (Corning Inc, Corning, New York). Cells were lysed in RNA-BEE (Tel-Test, Inc, Friendswood, Texas). RNA was chloroform extracted and treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin). RNA sequencing samples were prepared using the HiSeq 2500 RNA kit (Illumina, San Diego, California) for 100nt/paired end reads, and four samples were run per lane. Raw complementary DNA reads were aligned to the hg19 reference with the spliced gap aligner spliced transcripts alignment to a reference software, with count-based quantitation carried out via the Subread package featureCounts (http://bioconductor.org/packages/release/bioc/html/Rsubread.html) at the geneic and exonic levels for UCSC and ensemble annotation builds. **(B)** Pathway enrichment analysis based on Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov). The x-axis represents fold enrichment, and the y-axis denotes pathways. The false discovery rates are labeled on the right of the bar plot. **(C)** Heat map of control and schizophrenia hiPSC NPCs of 848 unique genes (false discovery rate $<$ .05). The count data were normalized and modeled as overdispersed Poisson data using a negative binomial model in the Bioconductor package edgeR (15). Fold changes, *p* values, and false discovery rates are obtained from the same package for integrative analysis. **(D)** Volcano plots of $-$log10 p value versus log2 fold-change messenger RNA levels for control and schizophrenia hiPSC NPCs. Key canonical WNT signaling genes, including lymphoid enhancer-binding factor 1 (*LEF1*), Dickkopf-1 (*DKK1*), *DKK2*, secreted frizzled-related protein-2 (*SFRP2*), *SFRP4*, growth differentiation factor 5 (*GDF5*), and *GDF10*, are indicated. **(E)** WNT signaling pathway. The differentially expressed genes by RNA sequencing are marked by red stars. **(F)** Quantitative polymerase chain reaction validation of perturbed WNT gene expression, normalized to the expression of the housekeeping genes *GAPDH* and *ACTIN*: *LEF1*, *DKK1*, *DKK2*, *SFRP2*, *SFRP4*, and *GDF10*. Error bars are SE. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. qPCR, quantitative polymerase chain reaction.

Table 2. Selected WNT Signaling Genes Differentially Expressed in Schizophrenia Human Induced Pluripotent Stem Cell Neural Progenitor Cells

		RNA Sequencing			qPCR	
Symbol	RefSeq ID	Fold Change	*p* Value	FDR	Fold Change	*p* Value
DKK1	NM_012242	2.32	2.23E-06	1.43E-04	2.43	7.78E-04
DKK2	NM_014421	3.35	1.45E-09	2.38E-07	7.11	1.08E-03
SFRP2	NM_003013	1.33	1.21E-06	8.61E-05	2.08	3.14E-04
SFRP4	NM_003014	2.05	1.22E-04	3.87E-03	4.31	3.37E-03
GDF5	NM_000557	3.42	1.75E-06	1.18E-04	—	—
GDF10	NM_004962	1.82	4.16E-03	6.58E-02	2.24	3.77E-02
LEF1	NM_016269	1.68	1.19E-03	2.51E-02	4.09	1.73E-03

FDR, false discovery rate; qPCR, quantitative polymerase chain reaction.

Table 3. Enrichment Analysis for Bone Morphogenetic Protein Signaling, Hedgehog Signaling, or G Protein–Coupled Receptor Signaling Pathways

Category	Term	Fold Enrichment	FDR
KEGG_PATHWAY	Hedgehog signaling pathway	18.7	.02
REACTOME_PATHWAY	Signaling by BMP	.7	1.00
REACTOME_PATHWAY	Signaling by GPCR	2.1	1.00

BMP, bone morphogenetic protein; GPCR, G protein–coupled receptor.

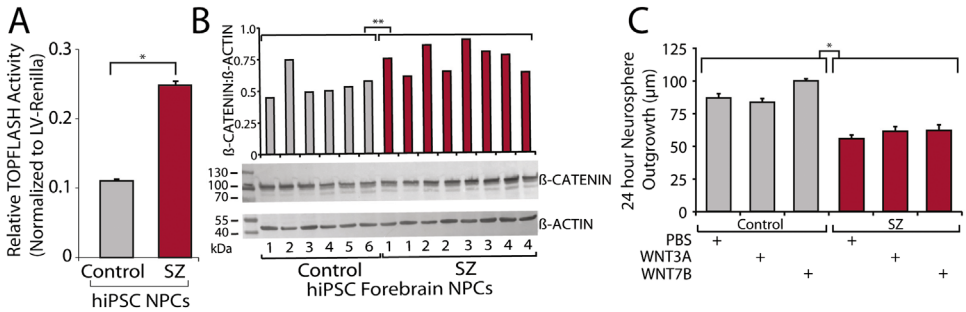

Figure 2. Perturbed WNT signaling in schizophrenia (SZ) human induced pluripotent stem cell (hiPSC) forebrain neural progenitor cells (NPCs). **(A)** Comparison of canonical WNT activity (assayed as LV-TOPFLASH reporter levels relative to LV-Renilla fluorescence) between control and SZ hiPSC forebrain NPCs, averaged by diagnosis. Luciferase levels were determined using the Dual-Glo Luciferase Assay System (Promega), measured on a FlexStation 3 (Molecular Devices, Sunnyvale, California) and then normalized to LV-Renilla florescence. **(B)** Increased β-catenin protein levels in SZ hiPSC forebrain NPCs. Western blot comparison of β-catenin and β-actin levels in control and SZ hiPSC NPCs. Western blots were repeated twice using independent protein lysates; Student *t* tests were used to test statistical differences between control and SZ Western blot β-catenin levels. β-actin was used as a loading control because we have found no evidence, by microarray or Nanostring nCounter gene expression assays (Nanostring Technologies, Inc, Seattle, Washington) or stable isotope labeling by amino acids in cell culture quantitative protein mass spectrometry, that it is differentially expressed in SZ hiPSC NPCs or neurons (13,14). **(C)** No effect of WNT on aberrant migration in SZ hiPSC forebrain NPCs. Control and SZ neurosphere outgrowth when cultured with phosphate-buffered saline (PBS), canonical WNT3A (20 ng/mL), and noncanonical WNT7B (5 ng/mL). Error bars are SE. $^{*}p < .05$, $^{**}p < .01$.

sample size, these phenotypes may not generalize across all patients with SZ, and 2) there was substantial variation in the specific SZ hiPSC NPC lines with increased WNT signaling between experimental replicates. The SZ hiPSC NPCs with elevated canonical WNT signaling showed significantly increased experimental variation, suggesting that this phenotype might more accurately reflect an increased variability in WNT signaling, perhaps because of increased susceptibility to an extrinsic factor, rather than implying a cell-autonomous difference in canonical WNT signaling.

A question of immediate interest is whether WNT signaling is also perturbed in SZ hiPSC neurons and, if so, in which neuronal cell types this is most evident. WNT signaling has been implicated in neural patterning, proliferation, differentiation, migration, and activity-dependent synaptic modulation (12,19,21–25). Given that WNT signaling is typically believed to increase neurogenesis (26) and that we and others have reported reduced neuronal connectivity in SZ hiPSC neurons (14,27), the (increased) direction of change in WNT signaling observed in our hiPSC NPCs is potentially surprising, although it may reflect an attempt at compensation for neural defects in other pathways. Perturbations in canonical WNT signaling in SZ hiPSC NPCs foretell a practical confounder for future hiPSC-based studies of SZ because aberrant canonical WNT signaling might affect the specification of SZ hiPSCs to certain neural fates. During neuronal differentiation, active WNT signaling is required for the specification of hippocampal (28) and midbrain dopaminergic fate (29,30), whereas repression of WNT signaling is required for cortical interneuron (31,32) and striatal (33,34) neuronal patterning; two more recent publications

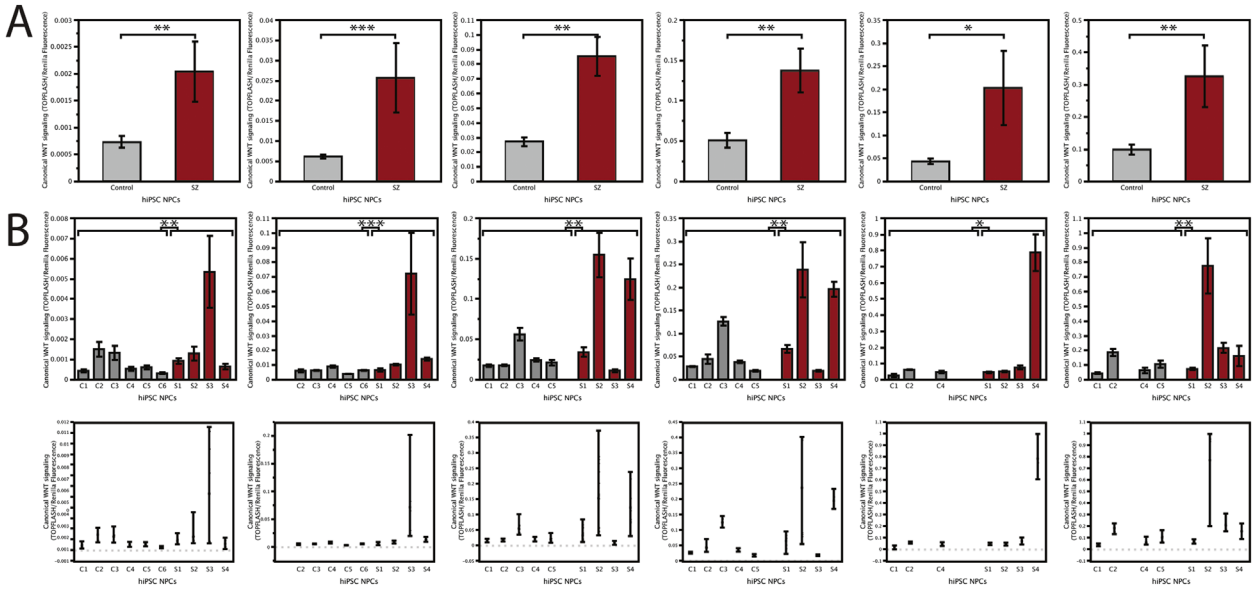

Figure 3. Experimental variability in assaying WNT signaling in schizophrenia (SZ) human induced pluripotent stem cell (hiPSC) forebrain neural progenitor cells (NPCs). **(A)** Six experimental replicates comparing canonical WNT activity (assayed as LV-TOPFLASH reporter levels relative to LV-Renilla fluorescence) between control and SZ hiPSC forebrain NPCs, averaged by diagnosis. With increasing passage, NPC lines can show reduced ability to differentiate to neurons or undergo spontaneous transformation to a highly proliferative cell with rounded morphology that cannot undergo neural differentiation at all; when either event occurred, that NPC line was dropped from subsequent experiments; for this reason, not all NPC lines were analyzed in independent experiments. **(B)** Six experimental replicates comparing canonical WNT activity (assayed as LV-TOPFLASH reporter levels relative to LV-Renilla fluorescence) between control and SZ hiPSC forebrain NPCs, averaged by individual. (Top row, mean ± SE. Bottom row, Variability chart showing individual data points.) For phenotypic analysis, statistical analysis was performed using JMP (SAS Institute, Carey, North Carolina). Box-Cox transformation of raw data was performed to correct nonnormal distribution of the data, and means were compared within diagnosis by one way analysis of variance using Student *t* test and Tukey-Kramer honest significant difference test. A nested analysis of values for individual patients was performed using standard least squares analysis comparing means for all pairs using Student *t* test for specific pairs and Tukey-Kramer honest significant difference test for multiple comparisons. Error bars are SE. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

reported differing abilities of SZ hiPSCs to differentiate into dopaminergic neurons (27,35).

Rodent-based (36), hiPSC-based (13,27,37,38), and olfactory neural stem cell–based (39) studies of SZ reported increased oxidative stress and reactive oxygen species. There is well-documented cross-talk between redox and WNT/β-catenin signaling (40–44); for example, treatment of cells with a low dose of hydrogen peroxide induces a rapid stabilization of β-catenin (43), whereas downregulation of canonical WNT signaling can decrease oxidative stress (45). If increased oxidative stress does contribute to perturbed canonical WNT signaling in SZ hiPSC NPCs, small variations in tissue culture–induced oxidative stress between experimental replicates may be one source of the large experimental variation observed among patients with SZ. Future studies comprising larger patient cohorts are necessary to determine whether aberrant canonical WNT signaling is a causal molecular factor contributing to aberrant neural patterning and neuronal maturation in SZ or simply a noncell autonomous consequence of increased oxidative stress (46).

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As per our agreement with Coriell Cell Repository, human induced pluripotent stem cell lines generated from control and schizophrenia fibroblasts will be available from Coriell.

The authors report no biomedical financial interests or potential conflicts of interest.

Authors AT, AS, and NT performed and analyzed the experiments. SZ and GF completed the RNA sequencing analysis. KJB designed the experiments and wrote the manuscript.

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