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Supplemental Information

Modeling Hippocampal Neurogenesis Using Human Pluripotent Stem Cells

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Supplementary Methods

Construction of PROX1-EGFP lentiviral reporter plasmid

The human prospero homeobox protein 1 (PROX1) gene is located on Chromosome 1 and spans approximately 53 kb of DNA. It consists of 5 exons and 4 introns. RefSeq lists the transcriptional variants 1 (NM_001270616.1) and 2 (NM_002763.4), which differ in transcription start site and first (non-coding) exon but encode the same protein (Fig. S1). To track PROX1-positive cells differentiating in culture, a lentiviral transcriptional reporter vector encoding EGFP was designed. PROX1 gene regulatory regions were identified with the aid of the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly. A composite regulatory sequence was assembled into the reporter vector from the following regions: (1) a 1001 bp sequence centered around an evolutionarily conserved TCF/LEF binding site (AAATGGCTTTGAAGTCTTCC) located at -49 kb from PROX1 ATG site (Petrova, Nykanen et al. 2008); and (2) a proximal promoter sequence, whose 3' end corresponds exactly to the 3' end of the first exon of the transcriptional variant 2 and whose 5' end is located 3205 bp upstream (Fig S1).

PCR amplification with Phusion High-Fidelity DNA Polymerase (NEB) was conducted using BAC clone RP11-783K13 (CHORI) as DNA template. The vector was constructed in two steps. First, the proximal promoter region was amplified using a forward primer (5'TATTATCGATTTACTCGAGAATGCGGGCTGCGAACCGCCG), which adds a ClaI site and an XhoI site, and a reverse primer (5'AACTGGATCCTCTTAGGAACTGGGAGGAGCGG), which adds a BamHI site. The PCR product was subjected to restriction digestion with ClaI and BamHI and introduced as a promoter in lentivector pCSC-EGFP. Next, the enhancer region was amplified using a forward primer (5'CGAAATCGATGCTGAAAATTGAGAGGTATAGAAC), which adds a ClaI site and a reverse primer (5'TAAACTCGAGCTGTCCCTAAACTGTGGCTTCA), which adds an XhoI site, and was introduced upstream of the proximal promoter region present in the above intermediate vector.

Electrophysiology

Whole-cell patch clamp recordings were performed from cells co-cultured with astrocytes after 6 weeks of differentiation. The bath was constantly perfused with artificial CSF (ACSF) (115 mM NaCl, 2 mM KCl, 3 mM CaCl2, 10 mM glucose and 1.5 mM MgCl2). The recording micropipettes (tip resistance 3–6 MΩ) were filled with internal solution (140 mM K-gluconate, 5 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 2.5 mM Na-ATP, 0.5 mM Na-GTP, 10 mM Na_2-phosphocreatine). Recordings were made using Axopatch 200B amplifier (Axon Instruments). Signals were filtered at 2 kHz and sampled at 5 kHz. The whole-cell capacitance was fully compensated. The series resistance was typically < 15 MΩ. For voltage clamp recordings, the membrane potential was held at -70 mV. To record the sodium and potassium currents, cells were depolarized in 5 mV increments. For current-clamp recordings, a hyperpolarized current was injected into the neuron to a membrane potential of around -50 mV. Step-depolarized currents were injected to elicit action potentials. All recordings were performed at room temperature and chemicals were purchased from Sigma. Frequency and amplitude of spontaneous postsynaptic currents were measured with the Mini Analysis Program software (Synaptosoft, Leonia, NJ). Statistical comparisons of WT and SZCD groups were made using the nonparametric Kolmogorov-Smirnov two-tailed test, with a significance criterion of $p = 0.05$.

For recording of transplanted neurons in brain slices, acutely dissected brains from 3 animals at 6 month post surgery were transferred to an icy cold cutting solution (110 mM choline-Cl^-, 2.5 mM KCl, 2.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.3 mM Na^+-ascorbate, 3.1 mM Na^+-pyruvate, 20 mM dextrose), bubbled with 95% O_2 and 5% CO_2. Three hundred microme-thick coronal brain slices were cut in a Vibratome (VT 1000S, Leica) and recovered for 30–60 min in ACSF (125 mM NaCl, 2.5 mM KCl, 2.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.3 mM Na^+-ascorbate, 3.1 mM Na^+-pyruvate, and 10 mM dextrose), then stored at room temperature. Pipettes for whole-cell recording (pipette resistance, 5–7 MΩ) were filled with internal solution (120 mM K-gluconate, 15 mM KCl, 4 mM $MgCl_2$, 0.1 mM EGTA, 10 mM Hepes, 4 mM MgATP, 0.3 mM Na_2GTP, and 7 mM phosphocreatine, pH, 7.2). Biocytin (2 mg/mL) was added to the internal solution for morphological analysis. Patch clamp recordings were performed at room temperature with an Axopach-200B amplifier (Molecular Devices). Signals were filtered at 2 kHz and sampled at 5 kHz using a Digidata 1320A analog-digital interface (Molecular Devices). A bipolar tungsten electrode was placed in the middle of the molecular layer (ML) at <200 µm from the recording cell for extracellular stimulation (0.5 Hz). All antagoniss were purchased from Tocris Bioscience.

Calcium imaging

Neuronal networks derived from hESCs and hiPSCs were previously infected with the lentiviral vector carrying the Synapsin-DsRed reporter construct. Cell cultures were washed twice with sterile Krebs HEPES Buffer (KHB) and incubated with 2–5 µM Fluo-4AM (Molecular Probes/Invitrogen, Carlsbad, CA) in KHB for 40 min at room temperature. Excess dye was removed by washing twice with KHB and additional 20-min incubation was done to equilibrate intracellular dye concentration and allow de-esterification. The threshold for activity was determined as the 95th percentile of the amplitude all the events detected in hESC and iPSC-derived neurons.Time-lapse image sequences (100× magnification) of 3,000 frames were acquired at 28 Hz with a region of 336 × 256 pixels using a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Japan) with a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Japan). To assess changes in calcium signaling in response to perturbation of neuronal activity, 1 μM tetrodotoxin, CNQX (10 μM), APV (20 μM), and Gaba (25 μM) were applied by bath application. Images were acquired with MetaMorph 7.7 (MDS Analytical Technologies, Sunnyvale, CA). Images were subsequently processed using ImageJ (http://rsbweb.nih.gov/ij/) and custom written routines in Matlab 7.2 (Mathworks, Natick, MA).

Immunocytochemistry and Quantification. Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.25% Triton-X100 in PBS. Cells were then blocked in PBS containing 0.25% Triton-X100 and 10% donkey serum before primary antibody incubation and overnight at 4°C. After 3 washes with PBS, cells were incubated with secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature. Fluorescent signals were detected using a Zeiss 710 LSM and images were processed with ZEN 2011. Primary antibodies used were Prox1 (1:500, ABcam); human Nestin (1:100, Chemicon); Tuj-1 (1:500, Covance); Map2 (1:200; Sigma); VGLUT1 (1:200, Synaptic Systems); GFP (1:250, gift from Aves lab); Sox2 (1:250, BD Biosciences); Gaba (1: 1000); Pax6 (1:200, Covance); Foxg1 (1:500, AbCam); NeuN (1:200, Millipore); and HuNu (1:200, Phosphosolution). Quantification of staining (Prox1, NeuN, Map2) was done using ImageJ software on fluorescent images acquired on Olympus

Ix51 inverted fluorescent microscope with 40x objective. Regions of Interest (ROI) were first selected on the Dapi image to obtain total cell count per image. The ROI list is transferred to corresponding images of nuclear stains (Prox1, NeuN) to identify co-localization with Dapi, as well as corresponding images of Map2 staining to identify positive cells.

References

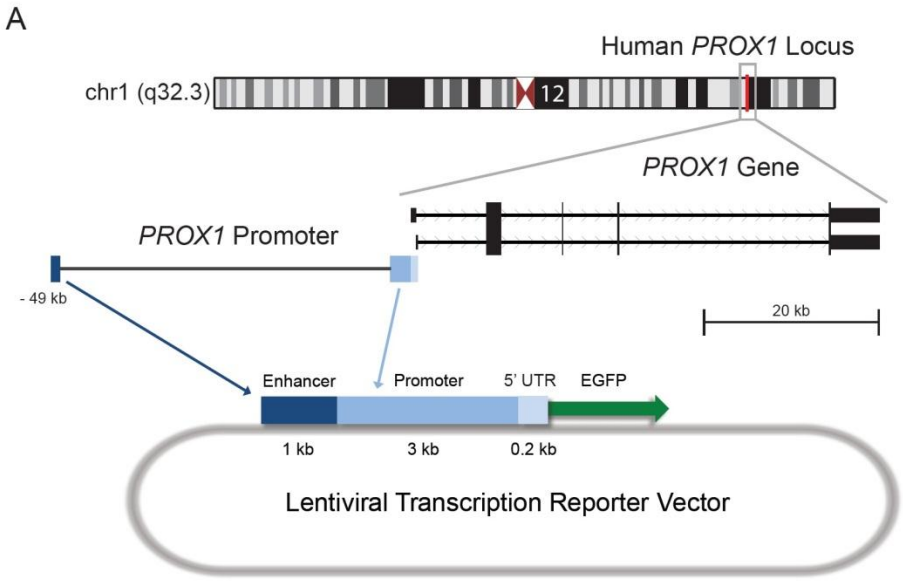
Petrova, T. V., A. Nykanen, et al. (2008). "Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype." Cancer Cell **13**(5): 407-419.

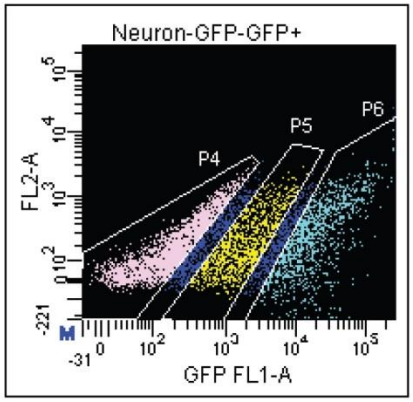
Figure Legends

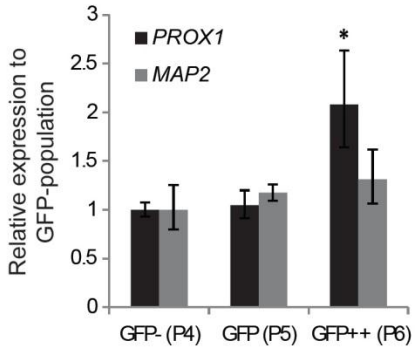
Fig. S1. Generation and assessment of lentiviral *PROX1-EGFP* construct. **A**, Schematic of lentiviral *PROX1-EGFP* construct. **B**, Fluorescence-activated cell sorting (FACS) of LV:*PROX1-EGFP*-infected, hESC-derived granule neurons at 8 weeks post differentiation to isolate populations of EGFP-negative (P4), low EGFP expressing (P5), and high EGFP expressing (P6) cells. **C**, Similar levels of *MAP2AB*, a neuronal marker, are detected by qPCR in all 3 populations. However, *PROX1* expression is enriched in the high EGFP-expressing neuronal population. **D**, Immunostaining for co-labeling of PROX1 and EGFP in lentiviral PROX1-EGFP-infected cultures. . Bar = 50 µm. **E**, Quantification of EGFP and PROX1 co-labeling showed that lentiviral PROX1-EGFP labeled ~50% of the PROX1+ cells and that ~95% of EGFP+ cells were stained positive by PROX1 antibody. (* $p<0.05$, **C**; n = 3 biological replicates, 2-tailed *t*-Test. **E**; n = 3 biological replicates).

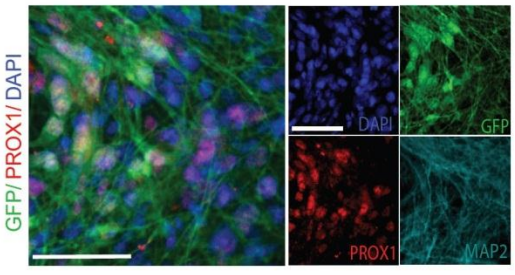
Fig. S2. Attenuated spontaneous neurotransmitter release in SCZD neurons. Quantification of the frequency and amplitude of postsynaptic currents in Control and SCZD neurons by individual lines. PROX1+ neurons are identified using the PROX1-EGFP lentiviral vector, 6-13 neurons recorded for each line.

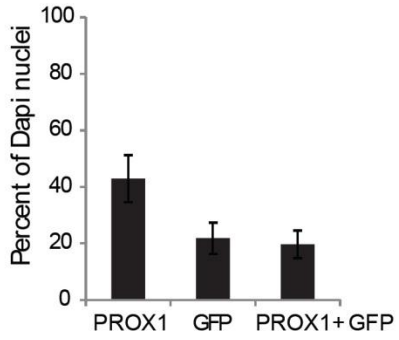
Supplementary Figure 1.





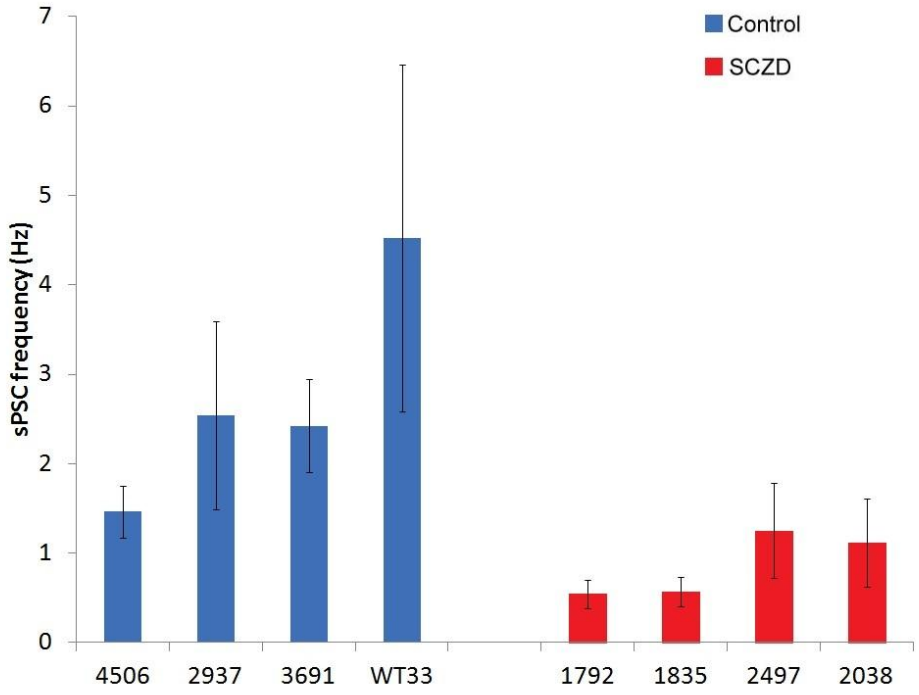




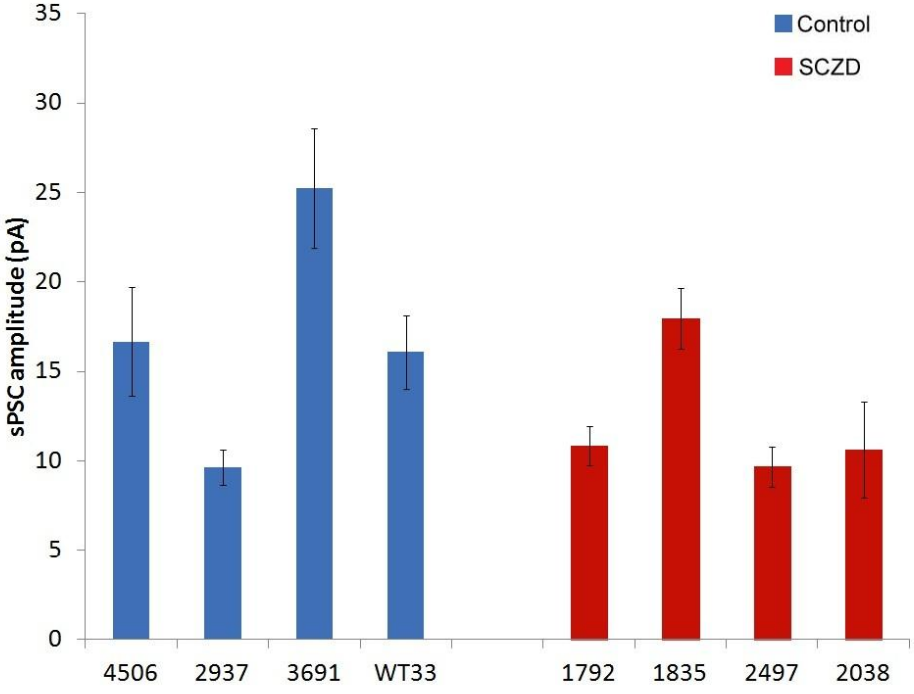


Supplementary Figure 2.

A



B



Supplementary Table 1: RT-qPCR primer sequences

hActin F	AAACTGGAACGGTGAAGGTG
hActin R	AGAGAAGTGGGGTGGCTTTT
hSox2 F	AGCTACAGCATGATGCAGGA
hSox2 R	GGTCATGGAGTTGTACTGCA
hOct4 F	GAGGAGTCCCAGGACATCAA
hOct4 R	TGGCTGAATACCTTCCCAAA
hEmx2 F	AGGGACGCACCATATTAACC
hEmx2 R	CACCTCTCCCTGTCTCTTTTG
hPax6 F	GCCCTCACAAACACCTACAG
hPax6 R	TCATAACTCCGCCCATTCAC
hNeuroD1 F	CCAGGGTTATGAGACTATCACTG
hNeuroD1 R	TCCTGAGAACTGAGACACTCG
hFoxg1 F	AGAAGAACGGCAAGTACGAGA
hFoxg1 R	TGTTGAGGGACAGATTGTGGC
hDCX F	TCAGGGAGTGCGTTACATTTAC
hDCX R	GTTGGGATTGACATTCTTGGTG
hProx1 F	GACTTTGAGGTTCCAGAGAGA
hProx1 R	TGTAGGCAGTTCGGGGATTTG
hTBR1 F	GGAGCTTCAAATAACAATGGGC
hTBR1 R	GAGTCTCAGGGAAAGTGAACG
hMap2 F	CAGGAGACAGAGATGAGAATTCC
hMap2 R	CAGGAGTGATGGCAGTAGAC