Assessing mechanisms of brain malformation in *SCN3A* encephalopathy using a stem cell-derived model

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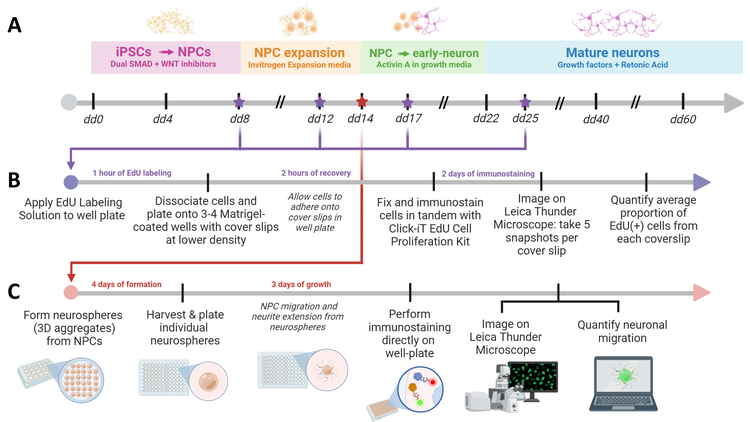
Introduction

- Pathogenic variants in *SCN3A*, encoding the voltage-gated Na+ channel α-subunit Nav1.3, are associated with *SCN3A*-related neurodevelopmental disorders (*SCN3A*-NDD), involving a clinical spectrum including: epilepsy, developmental delay/intellectual disability (DD/ID), and/or malformation of cortical development (MCD).
- MCD is atypical of a channelopathy yet is surprisingly a prominent phenotype in *SCN3A*-NDD patients. Although *SCN3A* is expressed highly in the brain during embryonic development, the mechanism(s) by which mutations in Nav1.3 result in MCD remain unknown.
- To address this question, we generated an induced pluripotent stem cell (iPSC) line from a patient harboring the recurrent *SCN3A*-I875T variant – which is associated with severe epilepsy, profound ID, and diffuse MCD – as well as a genetically corrected control line. To model human cortical development *in vitro*, we directed the differentiation of these isogenic iPSC lines through the NPC stage into cortical neurons.
- To specifically examine early-stage neurodevelopment, we developed assays to evaluate NPC proliferation and migration, hypothesizing that the *SCN3A*-I875T variant results in deficits in proliferation, migration, and neurite extension compared to corrected control NPCs.

Methodology

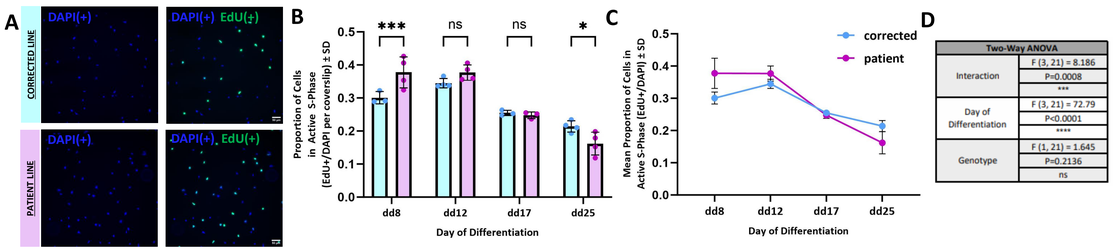
Figure 1. Directing the differentiation of iPSCs to NPCs to cortical neurons for use in functional assays



(A) Schematic of the 2D cortical differentiation directing iPSCs to NPCs to cortical neurons using a time-sensitive series of developmental signaling molecules. A patient-derived iPSC line expressing the *SCN3A*-I875T variant and an isogenic corrected control line were differentiated in parallel. **(B)** Schematic of S-phase entry assay to assess proliferation. At four different **d**ays of **d**ifferentiation (dd) – dd8, dd12, dd17, & dd25, ranging from the NPC to the early neuron stages – the EdU Click-iT Cell Proliferation Kit was used to determine the proportion of cells actively undergoing DNA replication. **(C)** Schematic of the NPC neurosphere migration assay. At dd14, NPCs were plated in a microwell culture plate to form 3D aggregates, or neurospheres, with uniform size and shape. Individual neurospheres were then plated to separate Matrigel-coated wells of a 96-well plate, and radial NPC migration and neurite extension was allowed to occur for 3 days until fixation and immunostaining. Figure generated with Biorender.com.

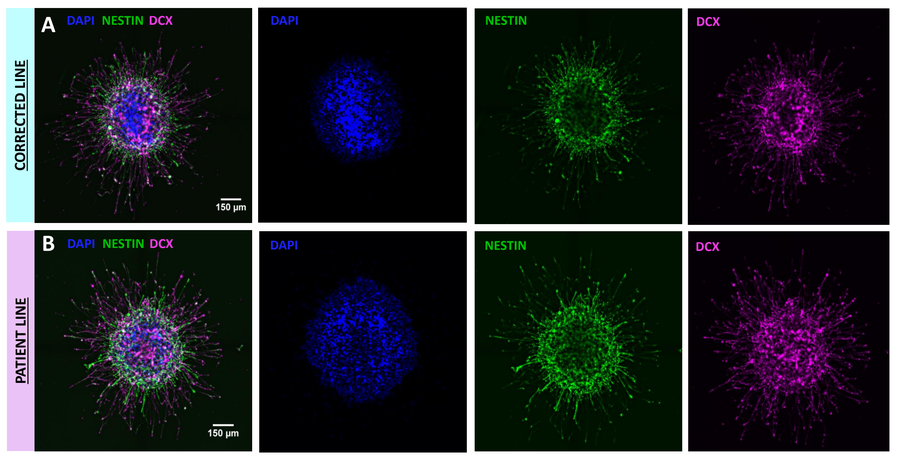
Results

Figure 2. S-phase entry analysis reveals altered proliferation time course in patient NPCs



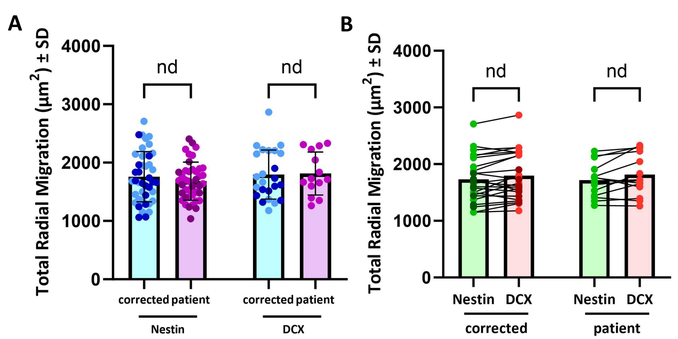
(**A**) Representative images at dd8 showing cells actively undergoing DNA replication (EdU+) out of the total cells in the frame (DAPI+). **(B-C)** The proportion of cells in active S-phase was determined at dd8, dd12, dd17, and dd25. Each point in (B) represents the average proportions of EdU(+) cells/total cells across 5 non-overlapping images per coverslip. *n=3-4* coverslips per line per timepoint, shown averaged in (C). The proportion of EdU(+) cells decreases over the course of the differentiation, with patient line showing more proliferation at dd8 and less at dd25 compared to corrected line. **(D)** A two-way ANOVA reveals a statistically significant interaction between genotype and day of differentiation, as well as a significant simple main effect of day of differentiation on the proportion of EdU(+) cells out of total cells. Results of post hoc multiple comparisons shown in (B).

Figure 3. NESTIN and DCX stain separate populations of cells in neurospheres



(**A-B**) NESTIN (a filament protein found in iPSCs and NPCs) and Doublecortin (DCX, a microtubule-associated phosphoprotein promoting neurite extension and cell migration) stain separate cell bodies and processes in neurospheres plated on Matrigel and allowed to radially migrate for 3 days until fixation and immunostaining.

Figure 4. Traces of patient vs. corrected neurospheres show no differences in radial migration



(**A-B**) Quantification of radial migration defined as *total radial migration = total neurosphere area – inner cell mass.* Symbol colors indicate neurospheres from plate replicates. **(A)** Total radial migration was determined using tracings from both NESTIN and DCX for each line. No statistically significant difference in radial migration was found between genotypes using either NESTIN (n(corrected)=43, n(patient)=41) or DCX (n(corrected)=24, n(patient)=15), by unpaired t-test. **(B)** Within-neurosphere analysis shows no differences in NESTIN versus DCX area for either corrected (n=24) or patient (n=14), by paired t-test.

Discussion

- We successfully implemented a novel 2D cortical differentiation protocol using a human iPSC line expressing the *SCN3A*-I875T variant derived from a patient with *SCN3A* encephalopathy as well as a CRISPR/Cas9-corrected control line, and developed two functional assays to evaluate neurodevelopmental phenotypes *in vitro*.
- Significant differences in proliferation between patient and corrected lines at dd8 and dd25 suggest that the timing of the transition period from iPSC to early NPC and from NPC to early neuron stages may be disrupted by the expression of the *SCN3A*-I875T variant, which may underlie abnormal corticogenesis leading to MCD in affected patients.
- NESTIN and DCX signals show distinct staining, suggesting that this neurosphere migration assay models the development of outer radial glia-like cells (NESTIN+) along which newborn neurons (DCX+) migrate and extend their processes during neurogenesis.
- The patient vs. corrected lines showed no differences in radial migration when using either antibody signal (NESTIN or DCX) to define total neurosphere area, suggesting that radial migration is not affected by the expression of the patient variant.

Implications & Future Directions

- The strongest genotype difference in cell proliferation at dd8 suggests the S-phase entry assay and neurosphere migration assay should be performed even earlier in the differentiation than dd8 and dd14, respectively, to better understand differences in developmental trajectories between lines.
- Future directions include performing these assays on a range of lines with different pathogenic variants in *SCN3A* and extending the study to patient-derived organoid models with a focus on investigating cortical lamination.

References

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