Neuronal impact of patient-specific aberrant NRXN1α splicing

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NRXN1 undergoes extensive alternative splicing, and non-recurrent heterozygous deletions in NRXN1 are strongly associated with neuropsychiatric disorders. We establish that human induced pluripotent stem cell (hiPSC)-derived neurons well represent the diversity of NRXN1α alternative splicing observed in the human brain, cataloguing 123 high-confidence in-frame human NRXN1α isoforms. Patient-derived NRXN1+/− hiPSC-neurons show a greater than twofold reduction in half of the wild-type NRXN1α isoforms and express dozens of novel isoforms from the mutant allele. Reduced neuronal activity in patient-derived NRXN1+/− hiPSC-neurons is ameliorated by overexpression of individual control isoforms in a genotype-dependent manner, whereas individual mutant isoforms decrease neuronal activity levels in control hiPSC-neurons. In a genotype-dependent manner, the phenotypic impact of patient-specific NRXN1+/− mutations can occur through a reduction in wild-type NRXN1α isoform levels as well as the presence of mutant NRXN1α isoforms.

Heterozygous exonic deletions in the neurexin-1 gene (NRXN1, 2p16.3) are associated with neuropsychiatric disorders1 (schizophrenia (odds ratio = 14.4)2, autism spectrum disorder (odds ratio = 14.9)3, epilepsy (odds ratio = 9.91)4 and developmental delay/intellectual disability (odds ratio = 7.47)5). Deletions occur non-recurrently (with different boundaries) between patients6 and the mechanisms underlying variable penetrance and diverse clinical presentations remain unknown. Homozygous Nrnx1 knockout mice display behavioral (for example, anxiety and social interaction) and electrophysiological (for example, decreased miniature excitatory postsynaptic currents)6–8 deficits, but only minor effects are observed in heterozygous mice. Engineered conditional heterozygous NRXN1+/− deletions in hiPSC-derived neurons impaired neurotransmitter release9. To better understand the clinical impact of NRXN1+/− mutations, it is critical to evaluate how distinct patient-specific deletions alter the NRXN1 isoform repertoire and impact synaptic function in a human context.

Mammals possess three neurexin genes (NRXN1, NRXN2 and NRXN3); each is highly alternatively spliced and can be expressed from three independent promoters to produce long alpha (NRXN1α) isoforms and shorter beta (NRXN1β) and gamma (NRXN1γ) isoforms. In humans, neurexins reach peak expression levels at birth10,11. Single-molecule long-read RNA sequencing (RNA-seq) of mouse prefrontal cortex (PFC) identified more than 200 Nrnx1α isoforms12,13; extensive Nrnx1 alternative splicing14,15 distinguishes neuronal cell types11,15 and is thought to recruit specific post-synaptic binding partners16.

We applied new methods17,18 to integrate targeted long- and short-read sequencing data, cataloguing and quantifying NRXN1α isoform repertoires in human post-mortem brain and across hiPSC-neuronal subtypes. hiPSC-neurons derived from cases of psychosis with heterozygous NRXN1 deletions exhibited significant perturbations in the NRXN1α isoform repertoire, including decreased levels of many wild-type isoforms and unexpected expression of patient-specific mutant NRXN1α isoforms. Single-cell (sc)RNA-seq revealed that NRXN1+/− hiPSC-neurons failed to reach full maturity. Reduced neuronal activity in NRXN1+/− hiPSC-neurons were ameliorated by overexpression of individual wild-type NRXN1α isoforms only in patient-derived hiPSC-neurons lacking expression of mutant isoforms, whereas neuronal activity was decreased in control hiPSC-neurons following expression of individual mutant NRXN1α isoforms, suggesting a possible novel dominant-negative mode of action for heterozygous NRXN1+/− mutations. In summary, we report and functionally evaluate psychiatric disease-associated changes in the NRXN1α isoform repertoire.

Results
We generated hiPSCs from four individuals with rare heterozygous intragenic deletions in NRXN1 (refs 16,19) and diagnosed with a...
psychosis disorder (two sharing ~136-kilobase (kb) deletions in the 3′ region of NRXN1 and two sharing ~115-kb deletions in the 5′ region of NRXN1; available clinical information provided in Table 1) as well as one related and three unrelated age-, sex- and ancestry-matched controls (Table 1, Fig. 1a and Extended Data Fig. 1a–f). The 3′-NRXN1+/− deletions encompassed three exons, impacting canonical splice site 4 (SS4), thought to impact post-synaptic binding affinities1, but none of the other five known canonical splice sites. Two to three hiPSC clones per individual were reprogrammed from fibroblasts using a non-integrating Sendai virus approach, followed by the differentiation of neural progenitor cells (NPCs) through dual SMAD inhibition22–24. NESTIN- and SOX2-positive hiPSC-NPC populations robustly differentiated into MAP2-positive hiPSC-neurons (Fig. 1b), a forebrain-like population consisting primarily of glutamatergic neurons, but also GABAergic neurons and astrocytes25, hiPSC-NPCs were also rapidly induced into glutamatergic and GABAergic neurons through NGN2 (ref. 26) and ASCL1/DLX2 induction27, respectively.

NRXN1+/− hiPSC-neurons display aberrant NRXN1 isoform expression. To survey the molecular impact of patient-specific NRXN1+/− deletions, we performed whole-transcriptome RNA-seq on hiPSC-NPCs and 6-week hiPSC-neurons from two to three hiPSC clones per individual (Supplementary Table 1). Following confirmation of sample identity28 and cell type composition (Extended Data Figs. 1 and 2), we analyzed 37 hiPSC-NPC and hiPSC-neuron samples. In the combined dataset, differential expression analysis between NRXN1+/− and controls identified 49 genes with a false discovery rate (FDR) < 10% and 148 genes with an FDR < 20%, when controlling for cell type as a covariate29 (Fig. 1c and Supplementary Tables 2–4). Differentially expressed genes were enriched for schizophrenia genome-wide association study (GWAS)-associated genes and transcriptional/epigenetic regulation gene sets (Fig. 1d and Supplementary Table 5). This could reflect perturbations downstream of NRXN1+/− and/or the independent contribution of other schizophrenia risk alleles. Although functions associated with common variants in autism spectrum disorder exhibit enhanced effects when found in combination with rare variant(s)30, and genetic risk underlying many neuropsychiatric disorders is thought to converge at pathways involved in synaptic function31–33 and epigenetic regulation34, our cohort is underpowered to examine this question in greater detail. We observed a significant correlation between the overall t-statistics from our case-control NRXN1+/− hiPSC-NPC/neuron comparison with post-mortem brain comparisons of schizophrenia, major depressive disorder, bipolar disorder and autism spectrum disorder (CommonMind34, National Institute of Mental Health Human Brain Collection Core (NIMH HBCC) and University of California, Los Angeles (UCLA) datasets35) (Fig. 1e and Supplementary Table 6). This enrichment for GWAS-linked genes and correlation with post-mortem datasets highlighted that patient-specific NRXN1+/− neurons captured some of the biological underpinnings associated more broadly with psychiatric disease36.

Total NRXN1 levels were significantly greater in hiPSC-neurons than hiPSC-NPCs (Fig. 1f; log2[fold change] (log2[FC]) = 3.03, Padj = 2.08 × 10−8), determined by linear regression model. Although total NRXN1 gene expression was not significantly decreased in NRXN1+/− hiPSC-NPCs or hiPSC-neurons compared to controls (Fig. 1f), we queried whether the expression of NRXN1α or NRXN1β isoforms shifts between control, 5′− and 3′−NRXN1+/− hiPSC-neurons. Consistent with differential promoter usage, 5′−NRXN1+/− hiPSC-neurons showed a decrease in NRXN1α isoforms (Fig. 1g; P = 0.001 by one-way ANOVA with Dunnett’s test), while NRXN1β isoforms were significantly increased (Fig. 1h; P = 0.001 by one-way ANOVA with Dunnett’s test). Alternatively, 3′−NRXN1+/− hiPSC-neurons demonstrated a more subtle increase in NRXN1α isoform usage (Fig. 1g; P = 0.01 by one-way ANOVA with Dunnett’s test), while usage of NRXN1β isoforms was decreased (Fig. 1h; P = 0.01 by one-way ANOVA with Dunnett’s test).

NRXN1 exon expression is highly correlated with its own gene expression; therefore, to identify genes associated with NRXN1 splicing, we calculated partial correlation coefficients between all genes and each NRXN1 exon (irrespective of genotype), adjusting for NRXN1 gene transcription. Genes with significant correlation coefficients (FDR < 0.01) to perturbations in NRXN1α splicing in hiPSC-neurons (regardless of NRXN1 gene expression) were enriched for synaptic gene sets (Fig. 1l). Similar results were observed in post-mortem adult brains35 (Extended Data Fig. 1o), in donors lacking rare NRXN1+/− deletions, suggesting a generalizable correlation between NRXN1 splicing patterns and global expression of synaptic genes.

As isoform quantification by short-read RNA-seq is limited36 and current annotations do not include all possible NRXN1 isoforms, such as potentially novel isoforms arising from the deletion allele, we developed a targeted hybrid sequencing approach to more carefully examine the impact of patient-specific deletions in NRXN1 on isoform expression.

Many NRXN1α isoforms are conserved between fetal PFC and hiPSC-neurons. Targeted single-molecule real-time (SMRT) isoform sequencing (Iso-seq) of mouse PFC previously identified 247 Nrxna1a isoforms37. Our hybrid approach integrated long-read Iso-seq (Pacific Biosciences) with short-read amplicon sequencing

| Patient | Family | Sex | Diagnosis | Age of onset (y) | IQ | CNV burden | Start (hg19) | Stop (hg19) | Size (kb) | Type | PRS | Ancestry |
|---------|--------|-----|-----------|-----------------|----|-------------|-------------|-------------|-----------|------|------|---------|
| 553     | N/A    | M   | Control   | N/A             | 127| -           | -           | -           | -         | -    | -    | -0.0003012 European |
| 2605    | N/A    | M   | Control   | N/A             | 126| -           | -           | -           | -         | -    | -    | -0.0003791 European |
| 3084    | N/A    | M   | Control   | N/A             | 8  | -           | -           | -           | -         | -    | -    | -0.0001327 Non-European |
| 642     | Mother 1 | F   | Paranoic PD | 17              | N/A| -           | -           | -           | -         | -    | -    | -0.000333 European |
| 973     | Proband 2 | M   | COBD + psychosis | 8          | 2p16.3 del | 51,190,677 | 51,306,133 | 115,456   | 5′-NRXN1+/− | -    | -0.0001820 Non-European |
| 972     | Mother 2 | F   | BD + psychosis | Unknown | MR | 2p16.3 del | 51,190,677 | 51,306,133 | 115,456   | 5′-NRXN1+/− | -    | -0.0001633 Non-European |
| 581     | Proband 1 | M   | COS      | 7               | 7  | 2p16.3 del | 50,162,758 | 50,298,963 | 136,205   | 3′-NRXN1+/− | -    | -0.000360 European |
| 641     | MZ twin 1 | M   | SA        | <14             | N/A| 2p16.3 del | 50,162,758 | 50,298,963 | 136,205   | 3′-NRXN1+/− | -    | -0.0003620 European |

1IQ, intelligence quotient; CNV, copy number variant; M, male; F, female; MZ, monzygotic; PD, personality disorder; COBD, childhood-onset bipolar disorder; BD, bipolar disorder; COS, childhood-onset schizophrenia; SA, schizoaffective disorder; del, deletion.

Table 1 | Clinical information for the hiPSC cohort used in this study

- Many NRXN1α isoforms are conserved between fetal PFC and hiPSC-neurons. Targeted single-molecule real-time (SMRT) isoform sequencing (Iso-seq) of mouse PFC previously identified 247 Nrxna1a isoforms. Our hybrid approach integrated long-read Iso-seq (Pacific Biosciences) with short-read amplicon sequencing.
**Fig. 1 | Cohort description and transcriptomic analysis.** **a.** A representation of individuals in the hiPSC cohort used for this study (5’−NRXN1−/− deletions in blue; 3’−NRXN1−/− deletions in red; controls in gray) with a schematic of the NRXN1 gene structure highlighting the exons encompassed by 5’− (blue) and 3’− (red) NRXN1−/− deletions. **b.** Validation of hiPSC-derived neural cells by immunostaining for SOX2 and NESTIN (NPCs) and MAP2 (6-week hiPSC-neurons) with DAPI-stained nuclei, three independent differentiations per line. **c.** A volcano plot showing log2[FC] between hiPSC-neurons (18 samples, 4 donors) and controls (19 samples, 4 donors) and the −log_{10}P value] determined by linear modeling for each gene with an FDR <20% in red, an FDR <10% in orange and NRXN1 in blue. **d.** Gene set enrichment analysis, with the −log_{10}P0.01 by one-way analysis of variance (ANOVA) with Dunnett’s test). **e,** Concordance computed using Spearman correlation of the t-statistics between two datasets. Comparisons were made between this study and post-mortem RNA-seq datasets of schizophrenia (SZ), major depressive disorder (MDD), bipolar disorder (BD) and autism spectrum disorder (ASD) from CommonMind™, NIMH HBCC and UCLA™. **f,** The sum of all NRXN1 transcript expression levels by cell type (19 samples, 8 donors for hiPSC-NPC; 18 samples, 8 donors for hiPSC-neurons) and genotype (19 samples, 4 donors for controls; 10 samples, 2 donors for 5’−NRXN1−/−; 8 samples, 3 donors for 3’−NRXN1−/−). **g,h,** Differential isoform usage in hiPSC-neurons across genotypes (9 samples, 3 donors for controls; 5 samples, 2 donors for 5’−NRXN1−/−; 5 samples, 2 donors for 3’−NRXN1−/−) when sub-setting for NRXN1ex or NRXN1Δ isoforms (h). The violin plots display the median and quartiles (‘P<0.05, **P<0.01 by one-way analysis of variance (ANOVA) with Dunnett’s test). **i,** Gene set enrichments for genes correlated with NRXN1 splicing in hiPSC-neurons.
(Illina), quantifying NRXN1α isoforms across three human fetal PFC, three adult dorsal lateral PFC (dIPFC) and one mouse PFC sample (Fig. 2a–d, Extended Data Figs. 3 and 4 and Supplementary Tables 7–10). A read-count threshold (≥7) filtered potentially spurious low-abundance isoforms (Extended Data Fig. 4 and Supplementary Note). The NRXN1α isoforms identified in human samples recapitated 86% of the isoforms detected in mouse (Fig. 2a). Levels of shared mouse–human NRXN1α isoforms were significantly correlated (Fig. 2b; r = 0.574, P = 3.09 x 10⁻⁴), with the most abundant isoforms best conserved between species (Fig. 2c,d). Mouse-specific Nrxi1α isoforms also revealed SS3 to SS6 splicing not seen in the human dataset (Fig. 2c,d).

We catalogued 123 human NRXN1α isoforms (predicted to be translated, ≥7 read-count threshold) across two control hiPSC-neuron, three adult dIPFC and three fetal PFC samples. Fetal PFC exhibited the highest NRXN1 expression (whole-transcriptome sequencing: Extended Data Fig. 1k) and the greatest NRXN1α isoform diversity (targeted hybrid analysis: fetal PFC (n = 83), adult dIPFC (n = 66) and hiPSC-neurons (n = 68); Fig. 2e,f and Supplementary Table 7). Overall, hiPSC-neurons modeled well the NRXN1α alternative splicing diversity found in vivo, particularly the high-abundance isoforms (Fig. 2g,h).

NRXN1α isoforms differ between glutamatergic and GABAergic neurons in mice[14,15]; therefore, we queried generated NGN2-glutamatergic neurons and ASCL1/DLX2-GABAergic neurons (Extended Data Fig. 5a–d). Whole-transcriptome RNA-seq revealed a significant decrease in total NRXN1 expression in NRXN1−/− NGN2-glutamatergic hiPSC-neurons[14,15] and ASCL1/DLX2-GABAergic hiPSC-neurons compared to controls (Extended Data Fig. 5e), a finding not seen in our forebrain hiPSC-neurons (Fig. 2a).

NRXN1α isoforms were significantly increased in 3′-NRXN1−/− hiPSC-neurons that resulted from splicing across the three deleted exons (Extended Data Fig. 1f), with the most abundant isoforms best conserved between species (Fig. 2c,d). Mouse-specific Nrxi1α isoforms also revealed SS3 to SS6 splicing not seen in the human dataset (Fig. 2c,d).

We catalogued 123 human NRXN1α isoforms (predicted to be translated, ≥7 read-count threshold) across two control hiPSC-neuron, three adult dIPFC and three fetal PFC samples. Fetal PFC exhibited the highest NRXN1 expression (whole-transcriptome sequencing: Extended Data Fig. 1k) and the greatest NRXN1α isoform diversity (targeted hybrid analysis: fetal PFC (n = 83), adult dIPFC (n = 66) and hiPSC-neurons (n = 68); Fig. 2e,f and Supplementary Table 7). Overall, hiPSC-neurons modeled well the NRXN1α alternative splicing diversity found in vivo, particularly the high-abundance isoforms (Fig. 2g,h).

NRXN1α isoforms are differentially expressed in NRXN1+/− hiPSC-neurons. We next quantified NRXN1α isoforms in patient-specific 3′-NRXN1+/− neurons (Extended Data Figs. 3 and 4); as this targeted strategy relied on the amplification of transcripts containing the first and last coding exons of NRXN1α (Supplementary Note and Extended Data Fig. 3a), it was unsuitable for the two 5′-NRXN1+/− cases, whose deletion encompassed the first coding exon. The correlation of NRXN1α isoform expression was higher between the two 3′-NRXN1+/− cases (r = 0.59) or between the two controls (r = 0.60) than across NRXN1 genotypes (r = 0.03–0.37) (Extended Data Fig. 6a).

Across two 3′-NRXN1+/− cases (relative to two controls), we observed reduced abundance of 49 of 99 (50%) wild-type isoforms (FC < 2; FDR < 0.01) (Fig. 4a–c and Supplementary Table 11). We further detected 31 mutant NRXN1α isoforms unique to 3′-NRXN1+/− hiPSC-neurons that resulted from splicing across the three deleted exons (Extended Data Fig. 1d), and never detected in control hiPSC-neurons or post-mortem samples (Fig. 4a–d). Mutant isoforms were confirmed in 3′-NRXN1+/− NGN2-glutamatergic and ASCL1/DLX2-GABAergic hiPSC-neurons (Fig. 4e,f). The novel junction site was validated through Sanger sequencing of TOPO-cloned isoforms (Extended Data Fig. 1f), PCR with reverse transcription (RT–PCR) across the 3′-NRXN1−/− deletion (Extended Data Fig. 1d) and quantitative PCR (qPCR; Extended Data Fig. 1e). All catalogued isoforms, including the mutant isoforms, were predicted to be translatable; translation of a 5xFLAG-tagged mutant NRXN1α isoform was confirmed by western blot (Extended Data Fig. 8g).

Across control and 3′-NRXN1+/− hiPSC-neurons, alternative splicing of NRXN1α occurred at the six previously annotated canonical splice sites, with almost no occurrences of non-canonical splicing (Extended Data Fig. 6b,c). In fact, there was very similar inclusion of all NRXN1α exons between control hiPSC-neurons and 3′-NRXN1+/− hiPSC-neurons, except for those exons encompassed by the 3′ deletion (Extended Data Fig. 6c). Overall, the 3′-NRXN1−/− deletion impacted the NRXN1α transcriptional repertoire by decreasing levels of wild-type isoforms and also by introducing novel isoforms not otherwise identified in control hiPSC-neurons or in post-mortem brain.

To consider any transcripts generated by alternative start or stop sites, we leveraged our whole-transcriptome RNA-seq data from hiPSC-neurons across NRXN1 genotypes (control, 5′- and 3′-NRXN1−/− deletions), to quantify the total levels of NRXN1α, NRXN1β and NRXN1γ primary isoforms (Extended Data Fig. 6j). This unbiased approach revealed significantly decreased NRXN1 expression in 3′-NRXN1+/− hiPSC-neurons and increased overall expression of NRXN1β in 5′-NRXN1+/− hiPSC-neurons (Extended Data Fig. 6k–m). The consistent change in messenger RNA expression levels of the NRXN1α, NRXN1β, and NRXN1γ isoforms in 5′- and 3′-NRXN1+/− hiPSC-neurons suggested that there was no compensation of mRNA expression across isoform groups (Extended Data Fig. 6n).

Perturbed neuronal maturation of NRXN1+/− hiPSC-neurons. We examined the extent to which aberrant isoform expression in NRXN1+/− neurons impacted neuronal differentiation. Although a small number of samples were considered, by focusing on the distribution across different cell types, rather than across case–control samples, integrated analysis of scRNA-seq from two control and
three NRXN1<sup>+/−</sup> hiPSC-forebrain neurons (Supplementary Table 12) revealed a dramatic shift in cell-type composition. NRXN1<sup>+/−</sup> cells were under-represented in the mature neuron clusters C4 (3′-NRXN1<sup>+/−</sup> \(P = 0.06\); 5′-NRXN1<sup>+/−</sup> \(P = 0.04\)), C8 (3′-NRXN1<sup>+/−</sup> \(P = 0.02\); 5′-NRXN1<sup>+/−</sup> \(P = 0.02\)) and C9 (3′-NRXN1<sup>+/−</sup> \(P = 0.01\); 5′-NRXN1<sup>+/−</sup> \(P = 0.01\)). These clusters exhibited the highest NRXN1
Fig. 3 | Identification of cell-type-specific NRXN1 isoforms from control isogenic hiPSC-neuronal subtypes. a, Overlap of NRXN1α isoforms identified in forebrain hiPSC-neurons (3 donors), NGN2-glutamatergic hiPSC-neurons (1 donor) and ASCL1/DLX2-GABAergic hiPSC-neurons (1 donor). b, A bar plot of the total read count for each NRXN1α exon along with the fraction in which each NRXN1α junction (row, 5′ end; column, 3′ end) is included in control NGN2-glutamatergic hiPSC-neurons (green, left) and control ASCL1/DLX2-GABAergic hiPSC-neurons (blue, right). c, A schematic of the NRXN1α isoform structures, with each row representing a unique NRXN1α isoform and each column representing a NRXN1 exon. The colored exons (purple, ASCL1/DLX2-GABAergic-specific, green, NGN2-glutamatergic-specific; orange, shared) are spliced into the transcript while the blank exons are spliced out. d, The abundance of each NRXN1α isoform across control isogenic hiPSC-neuronal subtypes. e, Validation of each isoform in control forebrain hiPSC-neurons (black, expressed).
Fig. 4  |  Identification of mutant NRXN1α isoforms.  

a. A schematic of the NRXN1α isoform structures, with each row representing a unique NRXN1α isoform and each column representing a NRX11 exon. The colored exons (red, 3′-NRXN1+/−-specific; gray, control-specific; orange, shared) are spliced into the transcript while the blank exons are spliced out.  
b. log2(FC) of each NRXN1α isoform in 3′-NRXN1+/− hiPSC-neurons (2 donors) compared to control hiPSC-neurons (2 donors).  
c. The abundance of each NRXN1α isoform across 3′-NRXN1+/− and control hiPSC-neurons.  
d. Validation of each isoform in post-mortem samples (black, expressed in post-mortem PFC).  

e. Pearson’s correlation of 47 NRXN1α isoforms between hiPSC-neurons (forebrain) and ASCL1/DLX2-GABAergic neurons from 3′-NRXN1+/− (1 donor) and control (1 donor) samples with a two-sided t-test.  
f. Pearson’s correlation of 57 NRXN1α isoforms between hiPSC-neurons (forebrain) and NGN2-glutamatergic neurons from 3′-NRXN1+/− (1 donor) and control (1 donor) samples with a two-sided t-test.
expression (Fig. 5a,e) and were distinguished by mature neuronal marker genes including MAPT, MAP2 and TUBB3 (Supplementary Tables 13 and 14). Interestingly, C14, a mature neuronal cluster, was also identified and contained almost exclusively 5′-NRXN1+− hiPSC-neurons (Fig. 5a,b,e). Conversely, the clusters (C0, C10) with the highest expression of astrocyte markers, including GFAP and s100B (Supplementary Tables 13 and 14), along with the more immature neuronal clusters (C1, C2, and C3), were equally comprised of control and NRXN1+− cells (Fig. 5d,e). We found no dramatic differences in the expression level and percentage of cells expressing common synaptic markers in the immature neuronal clusters (Extended Data Fig. 7) or impact on the relative patterning of glutamatergic, GABAergic or glial fates by scRNA-seq (Fig. 5a–e) or immunocytochemical analysis (Extended Data Fig. 2c,d). Within the largest and mostly mature neuron cluster (C4), patient NRXN1+− neurons showed significantly reduced expression of NRXN1 (P_e=3.07×10−24), as well as genes related to the development of the nervous system, development of axons and neuronal projection regulation, and regional patterning (Fig. 5f and Supplementary Table 15).

NRXN1+− hiPSC-neurons exhibit deficits in neuronal activity. The impact of NRXN1+− deletions on population-wide neuronal activity was evaluated across all four cases (both 5′- and 3′-NRXN1+− carriers), relative to two or three control donors in each experiment, using two independent methods of generating neurons. In more slowly maturing forebrain hiPSC-neurons, weekly recordings of spontaneous action potentials demonstrated a decrease in the number of spikes after 6 weeks of differentiation (Fig. 6a–c, 5′-NRXN1+− P=0.01; 3′-NRXN1+− P=0.0006 by one-way ANOVA with Dunnett’s test) with no change in the number of SYN1-positive puncta (Extended Data Fig. 2e,f). These findings were confirmed in NGN2-glutamatergic hiPSC-neurons, which also exhibited decreased spikes at 3 weeks and through 7 weeks of maturation into induced neurons from all four cases (Fig. 6d–f, 5′-NRXN1+− P=0.0002; 3′-NRXN1+− P=0.02 by one-way ANOVA with Dunnett’s test).

The effect of patient-specific NRXN1+− deletions on neuronal morphology was examined by comparing reciprocally labeled, low-density-plated tdTomato-positive control hiPSC-neurons and eGFP-positive NRXN1+− hiPSC-neurons, differentiated on a
control hiPSC-neuron lawn (Extended Data Fig. 8a–g), allowing us to focus on cell-autonomous NRXN1-related phenotypes, as all synaptic inputs of the traced neurons were expected to originate from control pre-synaptic neurons. Confocal imaging and semi-automated neuronal tracing after 6 weeks of differentiation revealed decreased neurite number (Extended Data Fig. 8b, 5′-NRXN1+/− P < 0.001; 3′-NRXN1+/− P = 0.002 by one-way ANOVA with Dunnett’s test) and total length (Extended Data Fig. 8e, 5′-NRXN1+/− P < 0.001; 3′-NRXN1+/− P < 0.001 by one-way ANOVA with Dunnett’s test) in NRXN1+− hiPSC-neurons compared to two controls.

The impact of NRXN1+− deletions on cellular signaling was assessed through interrogation of perturbed serine/threonine kinase activity in hiPSC-neurons (Extended Data Fig. 8h–m). The kinase activity of 5′- and 3′-NRXN1+− hiPSC-neurons was compared to controls individually. While there were unique kinases across the comparisons (3′-NRXN1+/−, Extended Data Fig. 8h; 5′-NRXN1+/−, Extended Data Fig. 8k and Supplementary Table 20), many of the kinases that showed differential kinase activity and differential expression by whole-transcriptome RNA-seq were shared across 5′- and 3′-NRXN1+− hiPSC-neurons, including TAOK1 and AKT1 (3′-NRXN1+−, Extended Data Fig. 8i); 5′-NRXN1+−, Extended Data Fig. 8l,m).

Expression of specific NRXN1a isoforms may impact neuronal activity in a genotype-dependent manner. We tested the extent to which decreased neuronal activity in NRXN1+− hiPSC-neurons reflected a decrease in wild-type NRXN1a isoforms and/or potential dominant-negative activity of mutant patient-specific NRXN1a isoforms. Individual NRXN1a isoforms were selected for overexpression studies: four wild-type isoforms shared between all hiPSC-neurons but significantly decreased in 3′-NRXN1+− hiPSC-neurons (Extended Data Fig. 8n), and four mutant isoforms unique to 3′-NRXN1− hiPSC-neurons and not detected in controls (Extended Data Fig. 8n). Lentiviral expression of the wild-type isoform resulted in a two- to sevenfold increase of NRXN1 expression after 48h (Extended Data Fig. 8o).

Expression of all four wild-type NRXN1a isoforms tested significantly increased population-wide neuronal activity after 2 weeks of expression in 5′-NRXN1+− deletion hiPSC-neurons (Fig. 6i; P < 0.05 by one-way ANOVA with Dunnett’s test to GFP alone). Reciprocally, expression of two of the four mutant NRXN1a isoforms evaluated significantly decreased neuronal activity after 2 weeks of expression in control hiPSC-neurons (Fig. 6h; P < 0.05 by one-way ANOVA with Dunnett’s test). Surprisingly, neither the wild-type nor mutant isoforms tested impacted 3′-NRXN1+− hiPSC-neuron activity (Fig. 6j) by one-way ANOVA with Dunnett’s test).

Overall, our data support a model whereby functional deficits in 5′-NRXN1+− neurons arise from NRXN1 haploinsufficiency and can therefore be rescued by overexpression of wild-type NRXN1a isoforms, but unexpectedly, haploinsufficiency in 3′-NRXN1− neurons is exacerbated by novel dominant-negative activity of mutant splice isoforms, and so cannot be rescued by simply increasing wild-type NRXN1a levels (Fig. 6k). Non-recurrent heterozygous deletions in NRXN1+− seem to impact neuronal function through different mechanisms, dependent on the precise patient-specific mutations involved.

Discussion

Our report links patient-specific, heterozygous intragenic deletions in NRXN1 to isoform dysregulation and impaired neuronal maturation and activity in a human and disease-relevant context. NRXN1a alternative splicing is disrupted in NRXN1− hiPSC-neurons; patient-specific perturbations include decreased expression of wild-type NRXN1a isoforms, and, in a subset of cases, the production of mutant NRXN1α isoforms. Mutant NRXN1α isoforms may be particularly biologically relevant as our experimental data demonstrated that overexpression of even a single mutant isoform was sufficient to perturb neuronal activity in control neurons and that overexpression of control isoforms could ameliorate activity deficits in patient hiPSC-neurons only in the absence of mutant isoform expression. Overall, our data, when combined with the published literature (reviewed in ref. 1), suggest that NRXN1α haploinsufficiency impedes neuronal maturation in addition to synaptic activity.

The consensus roles for neurexins are as organizers of neurotransmitter release by coupling calcium channels to the presynaptic machinery and trans-synaptic organizers of post-synaptic proteins resulting in stabilization of α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid receptors44–46. Although neurexins and their ligands have also been linked to synapse formation47–50, deletion of all three α-Nrxns or all three β-Nrxns impaired neurotransmitter release without altering glutamatergic synapse density in mice, and pan-Nrxn disruption of all α- and β-Nrxns dramatically reduced synapse stabilization and functional maturation. Our scRNA-seq, morphological and population-wide neuronal activity analyses fit a model whereby NRXN1+− hiPSC-neurons do not mature along the same trajectory as control hiPSC-neurons. Moreover, the lack of observed deficits in SYN1 puncta number and synaptic marker gene expression in NRXN1+− hiPSC-neurons suggests that synapses form but remain immature, consistent with the expression of synaptic-associated proteins in the immature neuronal clusters detected by scRNA-seq. While these data provide insights into the neuronal impact of patient-specific NRXN1+− deletions, the causal mechanisms remain unclear: does altered neuronal maturation lead to impaired synaptic function, or does aberrant synaptic activity limit neuronal maturation through an activity-dependent mechanism? Future experiments are needed to disentangle the extent to which perturbations in NRXN1 splicing independently perturb neuronal maturation, synaptic initiation, stability and activity across neurodevelopment and in a cell-type-specific manner. It is critical to disentangle the potentially additive effects of loss of wild-type NRXN1 isoforms and aberrant generation of mutant NRXN1 isoforms, particularly whether mutant isoforms are localized to the synapse, and the extent to which mutant isoforms impair the function of wild-type isoforms (perhaps similar to protocadherins44) or directly interfere with neuronal function.

Fig. 6 | Impact of specific NRXN1α isoforms on neuronal activity. a-c. The results of hiPSC-neuron (forebrain) MEA showing a representative raster plot for each genotype (a), a time course of spontaneous activity over two independent 6-week differentiations (b), and quantification of the number of spontaneous spikes at 6 weeks across control (30 wells, 2 donors), related non-carrier (3 wells, 1 donor), 5′-NRXN1+/− (30 wells, 2 donors) and 3′-NRXN1−/− (30 wells, 2 donors) samples (c). d-f. Results of NGN2-neuron MEA showing representative raster plots for each genotype (d), a time-course of spontaneous activity over 7 weeks of two independent differentiations (e) and quantification of number of spontaneous spikes at 6 weeks across control (22 wells, 2 donors), 5′-NRXN1−/− (22 wells, 2 donors) and 3′-NRXN1−/− (17 wells, 2 donors) samples (f). Two unique donors per genotype indicated by different shading within each plot. b,c,e,f. The plots display the mean with s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA with Dunnett’s test. g. A schematic of the time line used for overexpression of NRXN1α isoforms. h-j. Quantification of spontaneous spikes after overexpression of individual NRXN1α isoforms in control hiPSC-neurons (1 donor) (h), 5′-NRXN1+/− hiPSC-neurons (1 donor) (i) or 3′-NRXN1−/− hiPSC-neurons (1 donor) (j). k. A model for the contribution of both the NRXN1− genotype and NRXN1α isoform expression to changes in neuronal activity. g-j. One donor per genotype. The plots display the mean with s.e.m.; *P < 0.05 by one-way ANOVA after log-transformation with Dunnett’s test.
We demonstrated that hiPSC-neurons to a large extent recapitulate the in vivo splice repertoire. We observed significantly reduced expression of 49 (50%) wild-type NRXN1α isoforms in two 3′-NRXN1+/- cases and identified 31 mutant isoforms not detected in controls. Increasing even a single differentially expressed wild-type NRXN1α isoform ameliorated neuronal activity in NRXN1+/-
hiPSC-neurons that did not express mutant isoforms, whereas mutant isoforms decreased neuronal activity levels in control hiPSC-neurons. Protein models partially predicted protein structure of the top ten most abundant wild-type and mutant isoforms, and suggested that, whereas wild-type isoforms are largely conserved (Extended Data Fig. 9a,d), 3′-NRXN1/α− deletion isoforms may be more variable (Extended Data Fig. 9b,c,e), particularly near the carboxy terminus where the 3′-NRXN1/α− deletion is located (Extended Data Fig. 9 insets). Dominant-negative activity by patient-specific mutant NRXN1α may be a widespread phenomenon: up to 8/35 (23%) of identified NRXN1/α− exonic deletions in the PGC dataset may be capable of producing novel translated (mutant) isoforms. Moving forward, it will be important to empirically evaluate the extent to which these putative mutant isoforms can exacerbate NRXN1-related phenotypes.

The complexity of the NRXN1α transcriptional landscape varies by neuronal cell type1, is impacted by neuronal activity24,25, is epigenetically regulated26 and directly impacts synaptic plasticity27. We identified 54 NRXN1α isoforms shared across isogenic NGN2-glutamatergic and ASCL1/DLX2-GABAergic hiPSC-neurons and 25 subtype-specific isoforms. Comparison of NRXN1α isoforms across fetal and adult PFC samples revealed 55% of fetal isoforms to not be found in adult dPFC samples, suggesting that developmental regulation occurs (Extended Data Fig. 10), particularly decreased inclusion of exons 3α, 4, 5 (S1) and 17 (S6) in adult dPFC (Extended Data Fig. 10a,b). We similarly observed a developmental switching pattern in vitro, identifying a threefold increase in the exclusion of S4 (S4′) with neuronal differentiation from hiPSCs (Extended Data Fig. 6g–i). As the 3′-NRXN1/α− deletion encompassed S4, which undergoes activity-regulated exclusion25,27, we examined the impact of patient-specific mutations on the inclusion/exclusion of NRXN1α canonical splice sites following neuronal depolarization by KCl (4 h 50 μM KCl treatment, relative to a PBS vehicle). In control hiPSC-neurons, S4 exclusion (S4−/−) showed the greatest activity-dependent change (1.5-fold; P = 0.039 Wilcoxon test) (Extended Data Fig. 6d–f), whereas 3′-NRXN1/α− hiPSC-neurons did not (Extended Data Fig. 6d–f). Given that S4 regulates NRXN1 binding affinity to multiple post-synaptic partners (reviewed in ref.32), including NLGN1β/δ28, LRRTMα/β/δ29, and the CBLNs30, these initial findings warrant further investigation into how patient-specific NRXN1/α− deletions impact the NRXN1α repertoire throughout neurodevelopment, across more neuronal subtypes and in response to neuronal activity, to better understand how patient-specific isoform combinations impact protein–protein interactions and synaptic function.

Psychose risk reflects a complex interaction of rare2 and common variants31. While idiopathic post-mortem and hiPSC-based studies remain underpowered24,33, the effect sizes of NRXN1 deletions are dramatically larger2–5. As patient recruitment is limiting for this rare cohort size would be unsuitable as an idiopathic study, it is common to see overlapping loci49,51, we examined the impact of patient-specific mutations on neuronal fate, maturation and function in a cell-type-specific and activity-dependent manner represents a critical first step towards a more genetics-based form of precision medicine. Understanding how NRXN1/α− deletions perturb the splice repertoire and alter neuronal function could ultimately improve genetic diagnosis, prognosis and/or lead to new therapeutic targets.

Online content
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Reprogramming and differentiation. Skin biopsies were obtained from four cases, four unrelated controls and one non-carrier relative. Fibroblasts were re-genotyped using PsychChip and exome sequencing\(^{23}\). hiPSCs were reprogrammed via Sendai viral reprogramming (Life Technologies) and validated by karyotyping, gene expression and protein immunocytochemistry as described previously\(^{24}\). Human fibroblasts were grown in human fibroblast medium on 0.1% gelatin (in Milli-Q water) plates. Replicating but nearly confluent human fibroblasts were transfected with Cytotune Sendai virus (ThermoFisher Scientific), and then allowed to recover for at least 3 d before dissociation with TrypLE (ThermoFisher Scientific) to create a 10-cm dish of a million million dividing fibroblasts. Cells were switched to HUES medium (Dalbeck’s modified Eagle’s medium (DMEM/F12 (Invitrogen), 20% KO-Serum Replacement (Invitrogen), 1× glutathion (Invitrogen), 1× NEAA (Invitrogen), 1×2-mercaptoethanol (Sigma) and 20 ng ml\(^{-1}\) FGF2 (R&D Systems)) and fed every 2–3 d. hiPSC colonies were manually picked and clonally plated onto 24-well mouse embryonic fibroblast plates in HUES medium as described previously\(^{25}\). hiPSC-NPCs were seeded at low density in each well and transduced with lentivirus expressing TetO-ASCL1-GFP (R&D Systems) and tetO-NGN2-GABAergic neurons. hiPSC-NPCs were seeded at low density in HUES medium and fed every 2–3 d. hiPSC-neurons were cultured in neuronal differentiation medium and assayed for 3 weeks unless otherwise noted. A similar strategy was used to produce hiPSC-neurons transfected with Cytotune Sendai virus (ThermoFisher Scientific), and then allowed to incubate for 1–2 d after plating. hiPSC-neurons were cultured in neuronal differentiation medium with doxycycline four hours after transduction, doxycycline was added to initiate expression of TetO-NGN2-GFP-Puro or TetO-NGN2-ASCL1-GFP-Puro, and then the reaction was inactivated by incubation with stop buffer at 65 °C for 10 min. Human post-mortem samples were homogenized and lysed in 1 ml Trizol. RNA was chloroform-extracted and purified and DNAse-treated using the RNA Clean & Concentrator with DNAse Kit (Zymo Research).

Western blotting. Cells were isolated, suspended in 1× RIPA lyses buffer (Sigma) supplemented with Complete Protease Inhibitor tablets (1 per 50 ml) (Roche) and PhosSTOP phosphatase inhibitor tablets (1 per 10 ml), sonicated and then centrifuged at 14,000 g for 30 min at 4 °C. Total protein was separated on 4–20% SDS-polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride (Bio-Rad). The membrane was blocked with 4% bovine serum albumin (Sigma) in 1× TWEEN (Sigma) in PBS and probed with primary antibodies against FLAG (F7425, 2MG, Sigma) and NGN2, and then with HRP-conjugated secondary antibodies (Sigma) for 2 h at room temperature. The membrane was visualized using the Amersham ECL prime western blotting kit (GE, RPN2232). Imaging was performed using a Bio-Rad Gel Doc Imaging System; the membrane was cut during imaging.

Predicted NRXN1 isoform structures. The top ten most abundant wild-type and mutant splice isoform sequences were submitted to the ExPaSy translate tool (https://web.expasy.org/translate/), for translation into amino-acid sequences. The amino-acid sequences were submitted to Phyre 2 for predicting protein structure\(^{69}\). All models were predicted with 100% confidence.

Whole-transcriptome RNA-seq parameters. RNA-seq libraries were prepared using the Kapa Total RNA library prep kit with ribo-depletion and strand-specific complementary DNA library construction (Kappa Biosystems). Pair-end sequencing (125-base-pair reads) was performed using the Illumina HiSeq2500 platform (New York Genome Center). RNA-seq reads were aligned to GRCh37 with STAR\(^{70}\). Uniquely mapping reads overlapping genes were counted with featureCounts\(^{71}\) using annotations from ENSEMBL v70 while isoform expression was obtained using Kalliisto\(^{72}\).

Whole-transcriptome RNA-seq analysis. Samples were analyzed using publicly available code from Hoffman et al.\(^{73}\). Briefly, sample identity was checked by calling variants from the RNA-seq BAM files using GATK to produce gVCF files. These gVCF files were merged with variants from whole-exome sequencing data from the same donor and variant concordance was examined using bcftools. One sample with variant discordance was excluded from downstream analysis.

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that showed significant correlation coefficients (FDR < 0.01) were selected for further gene set enrichment analysis. This analysis was performed for hiPSC-neuron and CommonMind diPFC datasets.

Generation of NRXN1α cDNA for targeted sequencing. For targeted NRXN1α library preparation, first-strand cDNA synthesis was performed on 500 ng of RNA using 0.5 μM of a primer complementary to the last exon of NRXN1α (CAGAAAACGGGGAATGAGGCT). The cDNA was amplified in a 30-μl reaction with 4 μl of cDNA as a template, LA Tag, and primers complementary to the first (TGGTGGCCCTAAAGAGGCAAG) and last (same as above) coding exons of NRXN1α with 35 cycles (Supplementary Tables 16 and 17).

For targeted Illumina sequencing, the PCR product was then run on a 1% agarose gel and gel-extracted using the GeneJet gel extraction and DNA cleanup micro kit (ThermoFisher). The gel-extracted cDNA was library prepped using the Nextera XT DNA library preparation kit (Illumina). Libraries from four samples were pooled and then sequenced using Illumina MiSeq or HiSeq (2 × 150-base-pair reads).

For Pacific Biosciences targeted Iso-seq preparation, the NRXN1α PCR products were purified with Agencourt Ampure XP magnetic beads (Beckman Coulter) at a volume ratio of 0.5:1 (magnetic beads/PCR product). The cDNA products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) at a volume ratio of 0.5:1 (magnetic beads/PCR product). The cDNA was quantified using the Qubit HS dsDNA Kit and the quality was assessed using the Agilent 2100 bioanalyzer. SMRT bell sequencing libraries were prepared using the Pacific Biosciences DNA Template Prep Kit according to the 5-kb way ANOV A was performed to satisfy this assumption. Extracellular 15-min recordings of spontaneous action potentials were performed at 37 °C using a Maestro MEA system and AXIS software (Aionx Biosystems). Data were sampled at a rate of 12.5 kHz with a 200–2,950 Hz single-order Butterworth band-pass filter and a threshold for spike detection at 5.5 times the rolling standard deviation of the filtered field potential on each electrode. Spike time stamps were exported to Neuroexplorer (Aionx Biosystems) for creation of raster plots, and all other data were exported as a csv file for further analysis. If data stratified normality assumptions, one-way ANOVA with Dunnett’s test for multiple comparisons was performed on the spike counts. This multiple comparisons data violated this assumption of normality and therefore spike counts were log-transformed and one-way ANOVA was performed to satisfy this assumption.

Neuronal tracing. Unlabeled control hiPSC-NPCs were plated onto 24-well culture plates. Control hiPSC-NPCs were transfected with a scramble, gRNA-eGFP, and eGFP-expressing lentivirus while NRXN1−/− hiPSC-NPCs were transduced with a scramble gRNA-tdTomato-puro-expressing lentivirus while NRXN1−/− hiPSC-NPCs were transduced with a scramble gRNA-tdTomato-puro-expressing lentivirus. A small number of control tdTomato-labeled and NRXN1−/− eGFP-labeled hiPSC-NPCs were spiked into the unlabeled background of hiPSC-NPCs on plating. hiPSC-NPCs were then differentiated for 6 weeks into hiPSC-neurons and immunostained using mouse anti-α-β-III-tubulin (Aionx), rabbit anti-GFP (ThermoFisher), 1:1,000; rabbit anti-beta-amyloid RFP (ThermoFisher) 1:1,000; with Alexa donkey anti-mouse-488 and Alexa donkey anti-rabbit-568 secondary antibodies as described above. Individual neurons were imaged at x20 on the Zeiss 780 upright confocal microscope, followed by tracing and analysis using ImageJ Simple Neurite Tracer Plugin. One-way ANOVA with Dunnett’s test was used to test the impact of genotype on neuronal morphology. As only the eGFP 3′-NRXN1−/− samples were imaged on the same coverslip as tdTomato-labeled controls, we assess the impact of coverslip-to-coverslip variation between these lines using a two-way ANOVA.

qPCR validation. Total RNA was extracted using TriZol following the manufacturer’s instructions and qPCR reactions were performed on a QuantStudio 7 Flex Real-Time PCR System using the Power SYBR green RNA-to-Ct RT–qPCR kit and gene-specific primers (ThermoFisher). A 50 ng quantity of RNA template was added to the PCR mix (ThermoFisher). The reaction conditions were 48 °C for 30 min, and then 95 °C for 10 min followed by 40 cycles (95 °C for 15 s, 60 °C for 60 s). The primers used are listed in Supplementary Table 18.

Molecular cloning procedures. NRXN1α cDNA was generated as previously described for targeted short-read sequencing. Gel-extracted NRXN1α cDNA was ligated into the pCR4-TOPO backbone (ThermoFisher) following the manufacturer’s cloning guidelines. Individual colonies were picked and screened for full-length NRXN1α isoforms by Sanger sequencing (Genewiz). Two specific NRXN1α isoforms in the pCR4-TOPO backbone were chosen for further cloning into a lentiviral expression vector. A 4.5-kb fragment containing most of coding sequence of NRXN1α was amplified from the pCR4-TOPO backbone. An upstream region of the first coding exon and a downstream region of the last coding exon fused to a 2xFLAG tag were amplified from two synthetic plasmids containing these sequences (ThermoFisher). Fragment amplification was performed using Q5 polymerase following the manufacturer’s protocol using appropriate primers (Supplementary Table 19). Fragments were assembled using the NEBuilder HiFi DNA Assembly Master Mix following the manufacturer’s protocol and the cloning components into SbfI-hindIII sites in the pCFB shuttle plasmid (ThermoFisher), and positive clones were confirmed by restriction digest and Sanger sequencing (Genewiz). Lentiviruses were produced from these vectors as previously described21. Physical titration of lentivirus was performed by using a qPCR Lentivirus Titration Kit (ABM).

Single-cell sequencing. Six hiPSC-neuron (two control, two 3′-NRXN1−/− and two 5′-NRXN1−/−) samples were differentiated into hiPSC-neurons for 6 weeks as previously described. To collect the neurons, Accutase was added to the hiPSC-neurons and incubated at 37 °C for 10–15 min. The hiPSC-neurons were then gently pipetted 2–3 times, transferred into DMEM for washing and spun at 600 g for 4 min. The hiPSC-neuron-mixed pellets were re-suspended in 900 μl PBS-C 0.4% bovine serum albumin and passed through a 0.5-μm filter. The hiPSC-neurons were then counted and re-suspended to a concentration of 1,000 cells μl−1 in 25 μl scRNA-seq was performed on these samples using the Chromium platform (10x Genomics) with the 3′ gene expression (3′ GEX) V2 kit, using an input of ~10,000 cells. Libraries were prepared as directed by the manufacturer and sequenced.
on a HiSeq2500 (Illumina) targeting a minimum depth of 50,000 reads per cell. Sequencing reads were then aligned to hg38 using the CellRanger pipeline from 10x Genomics. One of the 3’-NRXN1+/− samples had a much lower number of mean reads per cell than the other samples and therefore was not considered in the final analysis. All downstream scRNA-seq analysis was performed using the Seurat package in R. Reads were filtered for mitochondria-, ribosome- and HLA-related genes, and for cells expressing a minimum of 200 genes. Each sample was then normalized and scaled, and variable genes were identified individually. The variable genes across samples were then used in the RunMultiCCA function considering 20 CCS. Clusters were identified on the integrated dataset using the FindClusters function and considering 12 dimensions and a resolution of 0.8. tSNE plots were generated using the same 12 dimensions. Marker gene as well as differential expression analysis using a non-parametric Wilcoxon rank sum test within each cluster was performed.

**Kinase assay.** Cell pellets generated from cultures of hiPSC-neurons from two control, the male 5’-NRXN1+/− and both 3’-NRXN1+/− samples were lysed on ice for 30 min using M-PER lysis buffer (ThermoFisher) containing 1:100 Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher). Samples were centrifuged (14,000 r.p.m., 10 min, 4 °C), and the supernatants were collected and assayed for total protein concentration (Pierce BCA Protein Assay Kit, ThermoFisher). Samples were run in triplicate on the kinome array, using one chip for control versus 3’-NRXN1+/− hiPSC-neurons and a second chip for control versus 5’-NRXN1+/− hiPSC-neurons. Identical protein amounts were loaded for each condition.

Probing of serine/threonine kinase activity was performed using the PamStation12 microarray (PamGene International) and STK (4-well) PamChips containing 144 consensus phosphopeptide sequences (3 of which are internal controls) per well, immobilized on porous ceramic membranes. Each PamChip well was blocked with 2% bovine serum albumin before 2 μg of protein in the manufacturer's kinase buffer (PamGene), 157 μM ATP and fluorescein isothiocyanate-labeled anti-phospho serine/threonine antibodies (PamGene) were added to each well. The homogenized samples containing the active kinases and assay mix were pumped through the wells to facilitate interaction between kinases in the sample and specific peptide substrates immobilized on the chip. The degree of phosphorylation per well was measured in real time using Evolve (PamGene) kinetic image capture software. The software captures fluorescein isothiocyanate-labeled anti-phospho antibodies binding to each phosphorylated peptide substrate every 6 s for 60 min. Peptide spot intensity was captured across multiple exposure times (10, 20, 50, 100 and 200 ms) during post-wash, and the linear regression slope was calculated and used as the signal (that is, peptide phosphorylation times (10, 20, 50, 100 and 200 ms) during post-wash, and the linear regression slope was calculated and used as the signal (that is, peptide phosphorylation per well was measured in real time using Evolve (PamGene) for control versus 3’-NRXN1+/− hiPSC-neurons and a second chip for control versus 5’-NRXN1+/− hiPSC-neurons. Identical protein amounts were loaded for each condition.

The threshold value was guided by previous reports suggesting that changes in phosphorylation per well was measured in real time using Evolve (PamGene) for control versus 3’-NRXN1+/− hiPSC-neurons and a second chip for control versus 5’-NRXN1+/− hiPSC-neurons. Identical protein amounts were loaded for each condition.

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**Statistical analysis.** Data from all phenotypes described above were first organized in a Microsoft Excel spreadsheet and analyzed using GraphPad Prism 8 software or Rstudio. For MEA, qPCR data analysis and imaging analysis, values are expressed as mean ± s.e.m. All boxplots and violin plots represent the median and quartiles of the data. Statistical significance was tested using either two-sided Student’s-t-test, one-way ANOVA with Dunnett’s post-hoc test or two-way ANOVA with Holm–Sidak's test as noted in the figure legends. Whole-transcriptome RNA-seq analysis was performed in R using DeSeq2 and single-cell sequencing analysis was performed in R using Seurat.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** To facilitate improved sharing between stem cell laboratories, all hiPSCs have been deposited at the Rutgers University Cell and DNA Repository (study 160; http://www.nimustencells.org/) and all sequencing data have been deposited to GEO (GSE137101, whole short-read RNA-seq and scRNA-seq; and GSE137127, targeted short-read RNA-seq) or SRA (PRJNA563972, Iso-seq).

**Code availability.** To facilitate improved reproducibility of our data analyses, all code has been deposited at https://github.com/zhushijia/STAR2bSMRT.

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**Author contributions.**

K.J.B., E.F., S.Z. and G.F. contributed to experimental design. P.G. and J.R. developed the cohort, consented the patients and obtained skin biopsies. K.J.B., E.F., L.L. and M.Fitzgerald generated all hiPSCs and NPCs for cell culture experiments. S.Z. designed and developed the hybrid sequencing-based isoform correction and identification method and completed all targeted sequencing analysis. G.F. and K.J.B. supervised all the computational data analysis. A.A., N.F, G.D. and R.S. completed Iso-seq library preparation and sequencing. N.B. differentiated hN22 and ASC1/LDLX2 hiPSC-neurons for targeted sequencing. E.C. conducted neuron-tracing experiments. M.P.D. and M.Fernando completed immunostaining and analysis for GABA. M.H. completed western blots. R.M. and K.A. performed kinase assay and analysis. E.F., G.E.H. and N.S completed whole-transcriptome RNA-seq analysis. H.S. completed variant calling on the transcriptome. N.T. provided fetal post-mortem tissue. K.J.B., E.F., G.F. and S.Z. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information.**

**Extended data** is available for this paper at https://doi.org/10.1038/s41588-019-0539-z. **Supplementary information** is available for this paper at https://doi.org/10.1038/s41588-019-0539-z. **Correspondence and requests for materials** should be addressed to G.F. or K.J.B. **Reprints and permissions information** is available at www.nature.com/reprints.
Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Validation of deletions in hiPSC cohort and whole transcriptome RNA-seq analysis.  

**a.** Schematic showing the structure of the *NRXN1* gene and location of 5’- (blue) and 3’-deletions (red). **b,c.** Mean and s.e. of Taqman CNV assay confirming 3’-CNV in fibroblasts from one 3’-*NRXN1*+/- case (b) and 5’-CNV in fibroblasts from both 5’-*NRXN1*+/- cases (c); two replicates per sample per probe. **d.** PCR of cDNA across exons encompassed by the 3’-deletion in controls and 3’-*NRXN1*+/- hiPSC-NPCs, two independent validations. **e.** Mean and s.e. of the log FoldChange by qPCR across the novel junction (exon 20-24) created by 3’-deletion across 2 controls and 2 3’-*NRXN1*+/- hiPSC-NPCs compared using a two-sided t-test. **f.** Sanger sequencing result from a TOPO cloned *NRXN1α* isoform from 3’-*NRXN1*+/- hiPSC-neurons (n = 1) confirming presence of novel exon junction (exon 20-24). **g.** Confirmation of the sex of each sample. **h.** PCA plot of combined hiPSC-NPC (19 samples, 8 donors) and hiPSC-neuron (18 samples, 8 donors) RNA-seq dataset showing separation by cell type on PC1. **i,j.** Volcano plot showing differentially expressed genes within hiPSC-NPCs (i) and hiPSC-neurons (j) individually. **k.** FPKM of *NRXN1* expression across fetal PFC (1), adult dIPFC (3) and control hiPSC-neurons (3). **l.** Circle plot showing hierarchical clustering of samples by cell type and by donor. **m,n.** Pearson correlation of gene expression within and between donors in hiPSC-NPCs (19 samples, 8 donors) (m) and hiPSC-neurons (18 samples, 8 donors) (n) by one-sided Wilcoxon test. **o.** Gene set enrichments for genes correlated with NRXN1 splicing in the Common Mind Consortium dIPFC dataset.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Cell type composition. **a**, Heatmap showing log2FPKM values of sub-type marker genes in hiPSC-neurons across genotypes. **b**, Cell type composition scores obtained using Cibersort in hiPSC-neurons (9 controls, 4 donors; 9 cases, 4 donors), by diagnosis. **c**, Images of GABA immunostaining overlaid with MAP2. **d**, Mean percent of GABA+ cells from control (8 images, 2 coverslips), 5’-NRXN1+/− (8 images, 2 coverslips) and 3’-NRXN1+/− (11 images, 3 coverslips). Error bars are s.e. **e**, Representative images of SYN1 immunostaining alone and overlaid with MAP2 immunostaining and DAPI. **f**, Mean intensity of SYN1+ puncta normalized to MAP2 intensity (3 images per coverslip, 8 coverslips per donor, 2 donors per genotype). Error bars are s.e.
Extended Data Fig. 3 | Pipeline Schematic and quality control of Iso-seq data. a, Schematic of the sample preparation for the hybrid sequencing approach. b, Schematic of the computational pipeline developed for the hybrid sequencing approach. c, UCSC genome browser view of whole transcriptome Iso-seq data across the NRXN1 locus from five hiPSC-neuron samples. d,e, Representative bioanalyzer traces from Iso-seq library prep passing QC (d) compared to library prep failed QC (e). f, Targeted short-read sequencing counts per million for 3'-NRXN1+/- specific junction site showing 3'-NRXN1+/- hiPSC-neurons passing QC in green and failing QC in red. g,h, Pearson’s correlation of junction site expression from targeted long read vs. targeted short read data showing one of the samples passing QC (g) and one sample failing QC (h).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Comparison of long and short read data for quantification and threshold testing. a, Correlation of NRXN1α junction expression from long read and short read sequencing across control (control 1 n = 39, control 2 n = 37) and 3′-NRXN1+/− (3′-NRXN1+/− 1 n = 36, 3′-NRXN1+/− 2 n = 45) hiPSC-neuron samples. Red triangles represent canonical junctions while black represent noncanonical. b, Correlation of NRXN1α isoform expression from long read quantification and short read quantification across control (Control 1 n = 90, Control 2 n = 88) and 3′-NRXN1+/− (3′-NRXN1+/− 1 n = 89, 3′-NRXN1+/− 2 n = 96) hiPSC-neuron samples. Colored triangles represent in-frame isoforms, predicted to be translated (red), untranslated (black) and TOPO cloned (green). c, Correlation of mouse and human NRXN1α isoform expression and corresponding Venn diagrams for the number of isoforms across expression thresholds (≥2 n = 112, ≥3 n = 88, ≥4 n = 75, ≥5 n = 63, ≥6 n = 60, ≥7 n = 57, ≥8 n = 54, ≥9 n = 52, ≥10 n = 50).
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Cell type specific NRXN1 expression. a, b, PCA of isogenic samples differentiated into three hiPSC-neuronal cell types colored by cell type (ASCL1/DLX2 (5), hiPSC-neuron (6), and NGN2 (6) (a) or by NRXN1 genotype (control (8), case (9) from 3 donors each) (b). c, Representative confocal images from 2 independent differentiations of control and NRXN1+/− ASCL1/DLX2-GABAergic hiPSC-neurons. d, Expression of glutamatergic, GABAergic and pan-neuronal marker genes across NRXN1+/− (9) and control (8) NGN2-glutamatergic and ASCL1/DLX2-GABAergic hiPSC-neurons. Boxplot shows median and IQR. e, Sum of all NRXN1 transcripts expressed across control (2 donors), 3′-NRXN1+/− (2 donors) and 5′-NRXN1+/− (2 donors) in NGN2-glutamatergic and ASCL1/DLX2-GABAergic hiPSC-neurons. f, g, NRXN1α (f) and NRXN1β (g) isoform usage expressed across control, 3′-NRXN1+/− and 5′-NRXN1+/− donors in NGN2-glutamatergic and ASCL1/DLX2-GABAergic hiPSC-neurons. Boxplot displays median and range with P < 0.01 indicated by ** from two way ANOVA with Holm-Sidak’s test.
Extended Data Fig. 6 | See next page for caption.
**Extended Data Fig. 6 | Examination of NRXN1α canonical splice sites and total NRXN1 isoform expression.**

**a.** Pearson’s correlation of NRXN1α isoform expression across control and 3’-NRXN1+/− hiPSC-neurons (n = 99 isoforms, r computed by t-statistics). **b,c.** Bar plot of the total read count for each NRXN1α exon along with the fraction that each NRXN1α junction is included in control hiPSC-neurons (b) and 3’-NRXN1+/− hiPSC-neurons. Red circle indicates the novel junction created by the 3’-NRXN1+/− deletion (c). **d.** Schematic of the experimental design to test activity induced regulation at NRXN1α canonical splice sites. **e.** Fold change of canonical splice site exclusion in controls (gray) and a 3’-NRXN1+/− hiPSC-neuron (red) plus KCl compared to PBS control (dotted line). **f.** Bar plot showing fold change of SS4 in KCl treated control and 3’-NRXN1+/− hiPSC-neurons (compared to PBS). **g.** Schematic of the experimental design to test developmental regulation at NRXN1α canonical splice sites. **h.** Fold change of canonical splice sites in control hiPSC-neurons at 2-weeks (light gray), 4-weeks (gray) and 6-weeks (dark gray) post-differentiation compared to NPCs (dotted line). **i.** Specific examination of developmental exclusion of SS4. Error bars are s.e. **j-m.** Expression of levels of all NRXN1 isoforms (j), NRXN1α (k), NRXN1β (l), NRXN1γ (m) across NRXN1 genotypes (8 control, 3 donors; 5 3’-NRXN1+/−, 2 donors; 5 5’-NRXN1+/−, 2 donors) in hiPSC-neurons. Violin plot displays density and range with P < 0.05 indicated by “*” from Wilcoxon Signed Rank Test. **n.** Pearson’s correlation of all NRXN1 isoforms (18 samples, 6 donors) with NRXN1α, NRXN1β and NRXN1γ (r values calculated using t-statistics).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Single cell expression of synaptic genes. a, Violin plot displaying density of the expression of NRXN and multiple synaptic marker genes in single cells across control (2 donors) and NRXN1+/− (3 donors) hiPSC-neurons. b, Violin plot displaying density and range of the expression of multiple synaptic genes identified as marker genes in immature neuronal clusters from scRNA-seq data across control (2 donors) and NRXN1+/− (3 donors) hiPSC-neurons.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Investigation of hiPSC-neuron morphology, cellular signaling, and NRXN1 overexpression. a, Strategy to label individual hiPSC-neurons. b–g, Mean neurite number across genotypes (control 71 neurons, 2 donors; 3’-NRXN1+/− 72 neurons, 2 donors; S’-NRXN1+/− 50 neurons, 2 donors) **** indicates $P < 0.00001$ and ***** indicates $P < 0.0001$ by one-way ANOVA with Holm-Sidak’s test; (b) or by coverslip (2 donors, 12 coverslips, 3 regions each) (c,d) mean neurite length across genotypes (control 71 neurons, 2 donors; 3’-NRXN1+/− 72 neurons, 2 donors; S’-NRXN1+/− 50 neurons, 2 donors); (e) or by coverslip (2 donors, 12 coverslips, 3 regions each) (f,g). Two donors per genotype indicated by different shading within each plot.
h, Differentially active kinases (3’-NRXN1+/− hiPSC-neurons: 6 samples, 2 donors; controls: 5 samples, 2 donors). i, Volcano plot of $-\log_{10}(P)$-value and log$_2$(FoldChange) from linear model of RNA-seq (3’-NRXN1+/− hiPSC-neurons: 5 samples, 2 donors; controls 6 samples, 2 donors); DE kinase associated genes labeled. j, Violin plot of median and quartiles of RPKM for kinase hits with largest fold-change in RNA-seq (3’-NRXN1+/− hiPSC-neurons: 5 samples, 2 donors; controls: 6 samples, 2 donors). k, Differentially active kinases in (S’-NRXN1+/− hiPSC-neurons: 3 samples, 1 donors; controls 5 samples, 2 donors). l, Volcano plot of $-\log_{10}(P)$-value and log$_2$(FoldChange) from linear model of RNA-seq (S’-NRXN1+/− hiPSC-neurons: 3 samples, 1 donors; controls 6 samples, 2 donors); DE kinase associated genes labeled. m, Violin plot of median and quartiles of RPKM for kinase hits with largest fold change values in RNA-seq (S’-NRXN1+/− hiPSC-neurons: 3 samples, 1 donors; controls 6 samples, 2 donors). n, Isoform constructs for overexpression with log$_2$(FoldChange) from hybrid sequencing dataset. o, Mean fold-change from qPCR of NRXN1 expression (3 replicates per condition. p, Representative western blot (2 replicates) for anti-FLAG (48hr expression of control-enriched NRXN1α-FLAG). q, Representative western blot (2 replicates) for anti-FLAG (48hr expression of 3’-NRXN1+/− specific NRXN1α-FLAG). All error bars are s.e.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Predicted protein models for wild-type and mutant NRXN1α isoforms. 

a, Superimposed image of the top ten most abundant wildtype NRXN1α isoforms in hiPSC-neurons. 
b, Superimposed predicted protein model of the top ten most abundant mutant isoforms. 
c, Superimposed predicted protein model of the top ten most abundant mutant isoforms compared to the most abundant wildtype isoform (grey). 
d, Individual predicted protein models of the top ten most abundant wild type isoforms. 
e, Individual predicted protein models of the top ten most abundant mutant isoforms. 
Insets in each panel highlight C-terminal region of NRXN1α isoforms where 3'−NRXN1+− deletion is located.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Investigation of NRXN1α isoform changes across development. a, b, Bar plot of the total read count for each NRXN1α exon along with the fraction that each NRXN1α junction is included in adult dIPFC samples (a) and fetal PFC samples (b); pink boxes represent potential developmentally regulated exons. c, Schematic of NRXN1α isoform structure, with each row representing a unique NRXN1α isoform and each column representing a NRXN1 exon. Colored exons (blue, fetal PFC specific; green, adult dIPFC specific; orange, shared) are spliced into the transcript while blank exons are spliced out. Abundance of each NRXN1α isoform across fetal PFC and adult dIPFC samples.
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  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | AxiS 2.4 |
|----------------|---------|
| Data analysis | Whole transcriptome RNAseq data was processed using publicly available code from Hoffman et al 2017 Nature Communications and other published R packages. Code will be made available on github.com/nadschro/SZvariant-synergy. |
| R version | 3.4.0 |
| R packages: | grid compiler stats4 parallel stats graphics grDevices |
| [8] utils datasets methods base | other attached packages: |
| [1] sets_1.0-17 VennDiagram_1.6.17 | [3] futile.logger_1.4.3 pheatmap_1.0.8 |
| [5] GSEABase_1.38.1 graph_1.54.0 | [7] annotate_1.54.0 XML_3.98-1.9 |
| [9] AnnotationDbi_1.38.2 HTsanalyzeR_2.28.0 | [11] igraph_1.1.2 gage_2.26.1 |
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| [17] xtable_1.8-2 colortools_0.1.5 | [19] preprocessCore_1.38.1 DESeq2_1.16.1 |
| [21] SummarizedExperiment_1.6.3 DelayedArray_0.2.7 | [23] matrixStats_0.52.2 GenomicRanges_1.28.4 |
| [25] GenomeInfoDb_1.12.2 iRanges_2.10.2 | [27] S4Vectors_0.14.3 readr_1.1.1 |
| [29] latex2exp_0.4.0 mixtools_1.1.0 | [31] ape_4.1 ggrepel_0.6.5 |
| [31] ape_4.1 ggrepel_0.6.5 | |
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**Data**

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

To facilitate improved sharing between stem cell laboratories, all hiPSCs have already been deposited at the Rutgers University Cell and DNA Repository (study 160; http://www.nimhstemcells.org/) and all sequencing data has been deposited to GEO (GSE137101 whole short-read RNA-seq and scRNA-seq and GSE137127 targeted short-read RNA-seq) and SRA (PRJNA563972 Isoseq). To facilitate improved reproducibility of our data analyses, all code has been deposited at github.com/zhushijia/STAR2bSMRT.

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**Life sciences study design**

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size was determined by availability of hiPCS lines from donors carrying 2p16.3 deletions. Sample size for RNA-seq was > 30, which allows an adequate observation to take the benefits of the Central Limit Theorem, i.e. normally distributed data. |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Three samples were excluded from whole-transcriptome RNA-seq analysis due incompatibility of verifyBamID results and check on sample gender with the sample ID. These criteria were pre-determined to establish proper interpretation of resulting RNAseq data. Iso-seq samples which failed QC (as discussed in Supplementary Text 1) were also not included in the final data and this criteria was empirically determined and validated within the manuscript. |
| Replication | hiPSC-NPCs and hiPSC-neurons underwent multiple differentiations and multiple wells for each phenotypic assay. Whole-transcriptome RNAseq results were compared to post-mortem brain samples to assess replication of findings. All attempts at replication were successful. |
| Randomization | Samples without 2p16.3 deletions were allocated to control groups while those with 2p16.3 deletions were allocated according to the position of the deletion. |
| Blinding | Neuronal tracing was done blinded to genotype. Other experiments were not performed blinded given that the measurements were obtained through automated software following cell culture. |

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**Reporting for specific materials, systems and methods**

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒  | Antibodies |
| ☒  | Eukaryotic cell lines |
| ☒  | Palaeontology |
| ☒  | Animals and other organisms |
| ☒  | Human research participants |
| ☒  | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒  | ChIP-seq |
| ☒  | Flow cytometry |
| ☒  | MRI-based neuroimaging |

### Antibodies

Antibodies used

- Rabbit anti-NESTIN, Millipore, ABD69, 1:500;
- Goat anti-SOX2, Santa Cruz, sc-17320, 1:500;
- Chicken anti-MAP2, ABcam, ab5392, 1:500;
- Mouse anti-SYN1 Millipore, 574778, 1:500;
Validation

All antibodies were previously validated (in Brennand et al 2011, Brennand et al 2015, Ho et al 2017, Yang et al 2017) for immunocytochemistry in human cells.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Skin biopsies were obtained from four cases, four unrelated controls and one non-carrier relative from an NIMH study into childhood-onset schizophrenia.

Authentication

Fibroblasts were re-genotyped using PsychChip and exome sequencing. hiPSCs were reprogrammed via sendal viral reprogramming (Life Technologies) and validated by karyotyping, gene expression and protein immunocytochemistry.

Mycoplasma contamination

Cells were tested for mycoplasma monthly with all test being negative.

Commonly misidentified lines

(See ICLAC register)

none

Human research participants

Policy information about studies involving human research participants

Population characteristics

No subjects were consented or recruited by the stem cell investigators. Only de-identified fibroblast samples, with limited clinical information (noted in Table 1) were provided by the team at NIMH. There were four controls, three males and one female as well as two male MZ twins with 3’NRXN1 deletion diagnosed with SA/SZ and a mother and son pair with 5’ NRXN1 deletion diagnosed with BD+psychosis. All NRXN1 deletion cases had onset before the age of 17.

Recruitment

Subjects were recruited as part of a longitudinal study of childhood onset schizophrenia, since closed, conducted at the NIMH and led by Judith Rapoport.

Ethics oversight

Informed consent was obtained from all fibroblast donors at the National Institute of Mental Health, under the review of the Internal Review Board of the NIMH. All hiPSC work was reviewed by the Internal Review Board 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.

Note that full information on the approval of the study protocol must also be provided in the manuscript.