Supplementary figure 1

a

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**Supplementary Figure 1.** Expression of αII spectrin in embryonic rat forebrain. Immunostaining of E18 rat brain sections shows that αII spectrin expression overlaps with MAP2 (a), βIV spectrin (b) and ankryin G (d). Immunoreactivity is enriched in the cortical plate but low in the intermediate zone and ventricular/subventricular zones. The lateral ventricle is in the lower right corner of each panel. Co-immunostaining of αII spectrin and βIV (c) or Ankyrin-G (e) on dissociated rat cortical neurons is shown at higher magnification to highlight co-labeling at the axon initial segment (arrows). The image in panel e is the same as that shown in Figure 2e but with the red channel (RFP) replaced with the green channel (Ankyrin-G). Scale bar: 100 μm for a, b and d; 50 μm for c and e.
Supplementary figure 2

(a) Diagram showing the comparison of Sptan1 CRISPR A (GGATCGGTACCACCAGCTTCA) and Sptan1 CRISPR B (GTGTGCCGAGGCTGACCGCC).

(b) Sequence analysis for Sptan1 CRISPR A:

- Wild type: 25,527 pairs of reads, 40.43% mutation rate.
- Mutation 1: 30,671 pairs of reads, 48.58% mutation rate.
- Mutation 2: 2,164 pairs of reads, 3.42% mutation rate.
- Mutation 3: 1,229 pairs of reads, 1.94% mutation rate.

Sptan1 CRISPR B: 76,000 reads

- Wild type: 39,725 pairs of reads, 52.27% mutation rate.
- Mutation 1: 20,736 pairs of reads, 27.28% mutation rate.
- Mutation 2: 6,137 pairs of reads, 8.07% mutation rate.
- Mutation 3: 1,879 pairs of reads, 2.47% mutation rate.
- Mutation 4: 1,509 pairs of reads, 1.98% mutation rate.
- Mutation 5: 1,211 pairs of reads, 1.59% mutation rate.
- Mutation 6: 766 pairs of reads, 1.98% mutation rate.
**Supplementary Figure 2.** a) Two short guide RNAs (sgRNAs) are used to target rat Sptan1 either at exon 2 or exon 8. b) Next generation sequencing (NGS) shows that 59.6% of CRISPR A and 47.8% of CRISPR B transfected cells exhibit out-of-frame indels (mutations occurring at a frequency of <1% are not shown). This is an underestimate of the actual Sptan1 knockout frequency for technical reasons (see text).
Supplementary figure 3

(a)

Ctrl CRISPR

Sptan1 CRISPR

(b)

Percent (%)/of GFP+ neurons being all spectrin+

(c)
Supplementary Figure 3. a) Representative confocal images of αII spectrin (red) and GFP expression (white) in rat cortical neurons dissociated from brains 4 days after IUE at E14-15 and cultured for 10 days. Upper row: in control CRISPR experiments, the GFP+ cell (top panels, arrow) is αII spectrin+, whereas after Sptan1 CRISPR knockout, the GFP+ CRISPR sgRNA-transfected cell lacks αII spectrin (bottom panels, arrowhead; outlined in the lower middle panel). Note that an adjacent non-transfected cell (bottom panels, arrow) is αII spectrin+. b) Nearly all (~96.6%) of control CRISPR and a small fraction (~16.5%) of Sptan1 CRISPR-transfected neurons display alpha II spectrin immunoreactivity. Percentage data are transformed to arcsin value for statistical analysis. n=107 cells for Sptan1 CRISPR A and n=118 cells for control CRISPR transfection from 3 experimental replicates. One tailed t test: ****, p<0.00001. c) Unlike αII spectrin, αI spectrin immunostaining (red) shows only slight, likely non-specific labeling of cultured rat cortical neurons, with no change in the RFP+ (white), Sptan1 CRISPR-transfected neurons (arrows). Bisbenzimide nuclear stain is blue in panels a and c. Scale bar: 10 μm in a, 50 μm in c.
Supplementary figure 4

(a) RFP, beta I Spectrin, beta III Spectrin, Merge

(b) RFP, beta IV Spectrin, beta II Spectrin, Merge

Scale bar
**Supplementary Figure 4.** a) Confocal image showing triple labeling for RFP (white), βI (green) and βIII (magenta) spectrins in cultured neurons after co-transfection with *Sptan1* CRISPR sgRNA and RFP. βI spectrin immunoreactivity is absent in all neurons, even the RFP-labeled neurons with *Sptan1* deletion (arrows), while βIII expression is strong in all neurons except those with Sptan1 deletion (arrows). b) Confocal image showing triple labeling for RFP, βIV (green) and βII (magenta) spectrins in cultured neurons after co-transfection with *Sptan1* CRISPR sgRNA and RFP. βIV and βII spectrin immunoreactivity is present in all cells except the RFP-labeled neuron (arrow) with *Sptan1* deletion. Note the expected intense labeling of βIV immunoreactivity at the axon initial segments except for the RFP+ cell (arrow). Bisbenzimide nuclear stain is blue. Scale bar: 50 μm.
Supplementary figure 5

(a) Confocal images showing GFP, SPTAN1, and MAP2 expression in control and Splotch1 KO neurons. (b) Bar graph showing the percent of neurons with SPTAN1 following transfection. (c) Additional confocal images depicting control and Splotch1 KO neurons. (d) Boxplot illustrating the average dendritic length per neuron. (e) Line graph showing the average number of dendrites per neuron.
Supplementary Figure 5. Deletion of Sptan1 in cultured mouse hippocampal neurons. a) Confocal image showing that control CRISPR-transfected mouse hippocampal neurons are positive for αII spectrin and MAP2, while Sptan1 CRISPR A-transfected neurons express MAP2 but not αII spectrin (the αII spectrin-negative cell is indicated by an arrow and outlined in light gray in the middle panel). b) Quantification shows ~75% of Sptan1 CRISPR A-transfected neurons lack αII spectrin immunoreactivity. c) Upper row: 2 representative hippocampal neurons transfected with control CRISPR plasmid; lower row: 2 representative hippocampal neurons transfected with Sptan1 CRISPR A that show fewer and much shorter processes. d) Average dendritic length and e) number of dendrites are significantly decreased in the Sptan1 CRISPR A group. In b) n=17 neurons in Sptan1 CRISPR A and n=21 neurons in control CRISPR groups. In d) n=17 neurons in sptan1 CRISPR A and n=22 in control CRISPR groups. N=3 replicates in both control and Sptan1 CRISPR transfection group. t test: *, p<0.05; ***, p<0.001.
Supplementary figure 6

(a) Imaging of axon initial segments (AIS) in control and CRISPR-treated neurons. Arrows indicate AIS in control neurons, and stars indicate AIS in CRISPR-treated neurons. Scale bars: 500 μm.

(b) Merged images showing GFP, α-spectrin, and Ankyrin-G expression in control and CRISPR-treated neurons. Scale bars: 50 μm.

(c) Splan1 CRISPR treatment showing increased AIS fluorescence intensity compared to control. Scale bars: 50 μm.

(d) e) Graphs showing axon initial segment length and AIS fluorescence intensity fraction to control (CTL). Statistical significance indicated by ***(p < 0.001).
**Supplementary Figure 6. Loss of αII spectrin alters axon outgrowth and AIS formation.**

a) Stitched confocal images from a control at P21 (previously transfected at E14) showing GFP-labeled axons originating from transfected cells (at right) running ventrally toward the white matter with extensive arborization. Some of the axons extend laterally, whereas others turn toward the midline and fasciculate to form the corpus callosum (arrows). b, c) Compared to control axons b), a representative *Sptan1* CRISPR-transfected brain c) shows decreased and disorganized callosal axons (boxed areas magnified at right in b, c) and arborization in the contralateral hemisphere (asterisks in both panels). d) Upper row: a representative mouse hippocampal neuron transfected with control CRISPR plasmid shows immunopositivity for αII spectrin and ankyrin-G (arrowheads); lower row: a representative neuron transfected with *Sptan1* CRISPR A plasmid is negative for αII spectrin staining and has very faint ankyrin-G staining. e) The length of the AIS labeled by ankyrin-G and ankyrin-G immunostaining intensity are significantly decreased by *Sptan1* CRISPR A. Scale bar: 500μm in a, 50 μm in b and c, 10μm in d. n=4 in the *Sptan1* CRISPR A group and n=6 in the control CRISPR group. *t* test: *** p<0.001.
Supplementary Figure 7. Overexpression of pathogenic SPTAN1 mutations in vitro. a, b) Confocal images after overexpression of wild type SPTAN1 (WT), in-frame SPTAN1 duplication (DUP) and in-frame SPTAN1 deletion (DEL) in mouse hippocampal neurons reveals that expression of pathogenic mutations significantly decreased dendritic complexity. Also note that the soma size is qualitatively decreased in cells with mutant overexpression (a). c, d) Overexpression of pathogenic mutations also shortened the AIS and impaired ankyrin-G clustering at the AIS. The SPTAN1 mutants are the same as shown in Fig. 6. Scale bar: 10μm. In b) n=7 for the WT SPTAN1 group, n=6 for the SPTAN1 Dup group and n=6 for the SPTAN1 Del group. In d) n=8 in for WT SPTAN1, n=6 for SPTAN1 Dup, and n=6 for the SPTAN1 Del group. ANOVA test: *, p<0.05; ***, p<0.001.
Supplementary figure 8

(a) CamKIIa-GFP
(b) CTIP2
(c) VGLUT1

GABA/BB
BB
Merge
Merge
Merge

Scale bars: 100 μm
Supplementary Figure 8. iPSC-derived neurons are excitatory glutamatergic neurons. a) iPSC-derived neurons infected with lentivirus expressing GFP under a CamKIIα promoter express GFP, indicating they are excitatory neurons. In the middle panel, only one GFP-negative cell is positive for the inhibitory neuronal marker, GABA. b) Most iPSC-derived neurons are CTIP2 positive, a cortical deep layer excitatory projection neuron maker. c) The neurons also VGLUT1, a vesicular glutamate transporter expressed by excitatory neurons. Bisbenzimide (BB) in blue. Scale bar: 50 μm.