Supplemental Methods

Synapse Induction Assay

Synapse induction assay was carried out as described (22). Dissociated rat hippocampal neurons were prepared from embryonic day 18 SD rat embryos, plated on poly D-lysine-coated 12 mm ϕ cover glasses in 24-well dishes at a density of 2.5 x 10^4 cells / well, and cultured in Neurobasal medium containing B27 supplement (Invitrogen). HEK293 cells were transfected with pCAG-ires-EGFP-based expression constructs at 11 days after starting the hippocampal neuron culture (11 DIV) using TransIT LT1 transfection reagent (Mirus Bio), trypsinized, suspended in Neurobasal/B27 medium, and layed onto the hippocampal neurons at DIV13 at a density of 1.5 x 10^4 cells together with astrocyte culture conditioned medium. The cocultured cells were maintained for 24 h, chilled on ice for 5 min, and fixed with 4% PFA, 4% Sucrose in phosphate buffered saline (PBS) at 4 °C for 20 min. After washing with PBS four times, the cells were immersed in a blocking buffer (5% normal goat serum, 3% bovine serum albumin (Sigma), 0.1% TritonX100 in PBS) and incubated with rabbit anti-Vglut1 (1/500, Synaptic Systems) and chicken anti-GFP (1/500, Invitrogen) diluted in the blocking buffer overnight at 4 °C. The bound antibodies were detected by Alexa633-conjugated anti-rabbit IgG and Alexa488-conjugated anti-chiken IgY (Jackson ImmunoResearch) and mounted on slideglasses with Vectastain mounting medium (Vector Lab). Fluorescent images were captured by confocal microscope (Olympus FV1000) with a 40x objective lens. The digitized images were quantitatively analyzed by ImageJ software using “Threshold”, “Create Mask”, “Image Calculator”, “Analyze Particle” and “Measure” functions. The Vglut1-immunopositive puncta number or puncta area overlapping

with GFP signals were measured for each image. 10-17 independent-images containing more than 60 GFP-cells in total were analyzed for each transfectant. For the detection of SLITRK6 protein we generated an antibody against the N-terminal region of SLITRK6 using the polypeptide SQTPVLSSRGSCDSLC. The peptide was conjugated to keyhole limpet hemocyanin and used for immunization of a rabbit by conventional methods. The antibody was purified by affinity chromatography with the immunized peptide. For cell surface immunolabeling, the antibody was added in the culture medium for live transfected cells, and incubated at 37 °C for 15 min. After rinsing with PBS twice, cells were fixed and the bound antibodies were detected by Alexa594-conjugated anti-rabbit IgG.

Neurite Assay

PC12 cell culture and alkaline phosphatase staining were carried out as described (2) otherwise noted below. Cells were plated onto collagen-coated 12-well dish at a density of 1.0×10^5 cells/cm^2 12h before transfection. The cells were transfected with pEF-ires-alkaline phosphatase-based expression constructs by using Lipofectamine 2000 (Invitrogen) for 12h and exposed to NGF by adding the medium supplemented with 50 ng/ml of mouse 7S NGF (Calbiochem) and kept for 48h. After the fixation and staining, digitized images of 30 randomly chosen independent fields were collected, and neurite number and neurite length of strongly stained cells with clear outlines (n = 105-216) were measured. Neurite number was defined as the number of major proximal neurites that crossed a circle with 43.5 μm radius centered on a cell nucleus. Neurite length was defined as the distance between the center of the cell nucleus and the distal end of the longest neurite in a neurite-bearing cell. All transfection experiments were carried out at least three times.

Table S1: Primers for *SLITRK6* analysis

Name	Sequence : (5' to 3')	Amplicon size (bp)
SLITRK6 Exon 1aF	CCATCAAAATTCACTTGCACT	450
SLITRK6 Exon 1aR	TTGTCAAGTCCCATAGCTTCA	
SLITRK6 Exon 1bF	AGCTGTGAAGTATGCCGTGA	550
SLITRK6 Exon 1bR	TACAGGCATCTGCTCCAGTG	
SLITRK6 Exon 2aF	ATCCGTCATTTGCTTTTTGG	550
SLITRK6 Exon 2aR	TTCCAGGTTTTCCAGTCCAT	
SLITRK6 Exon 2bF	GCAGATATTGAGATAGGTGCATTT	571
SLITRK6 Exon 2bR	TTGAAGATGTTGCTGCCAGA	
SLITRK6 Exon 2cF	GCAACAGCCCTCCATTTTT	566
SLITRK6 Exon 2cR	ATGGAGACCAAGGAACATGC	
SLITRK6 Exon 2dF	TTTGGAAATGCTTCACTTGG	566
SLITRK6 Exon 2dR	TTGGCATGGATGGGTTATTT	
SLITRK6 Exon 2eF	TTGGACTGCAGCAATGGATA	566
SLITRK6 Exon 2eR	TGCATCACTTCCTTCTTTCTCA	
SLITRK6 Exon 2fF	ATGAACAGCACATGGTGAGC	537
SLITRK6 Exon 2fR	AACAGAATCACAGCATTTCTGC	
SLITRK6 Exon 2gF	TGTACTCACGTCCAAGGAAGG	600
SLITRK6 Exon 2gR	TGCAAGAAAGTTAGGTATGTTTGA	
SLITRK6 Exon 2hF	GCTGAACATTTAAGTATCCCGAAT	550
SLITRK6 Exon 2hR	CCTATGAAATAAAGCAAACCCAAT	
SLITRK6 Exon 2iF	AATGTCAGTGCTGCTGTGAA	601
SLITRK6 Exon 2iR	AACCTCCTGCAGTGTGTGTG	
EXOME CONF-SLITRK6-670-F	TTGCCCTACTCCACCAGTGT	183
EXOME CONF-SLITRK6-670-R	GGTCCTGGAAGTTGAGTGGA	
NMD-SLITRK6_1-PACF	CGTTAATTAATGCAGAAAGAGGAGGAGTTGA	3509
NMD-SLITRK6_1-R	AGCTTGCTAAAGGCACTTGG	
NMD-SLITRK6_2-F	TGGCCTCCTGAAACAACTTC	3510
NMD-SLITRK6_2-NOTR	TAGCGGCCGCAACCTCCTGCAGTGTGTGTG	
NMD_SLIT-EXPF	TTGAGAAGAAAGTACGCAGTGG	238
NMD_SLIT-EXPR	CACAAGAGCCTCTGGATGAG	
rRNA-18S-F	CTGAGAAACGGCTACCACATC	
rRNA-18S-R	CGCTCCCAAGATCCAACTAC	

Figure S1: Audiograms of Affected Subjects

Pure tone audiograms for affected individuals showing bilateral sensorineural hearing loss sloping from mild – moderate to severe – profound across higher frequencies. Left ear shown by blue crosses and right ear shown by red circles. Horizontal axis is frequency in Hz. Vertical axis is hearing threshold in decibels.

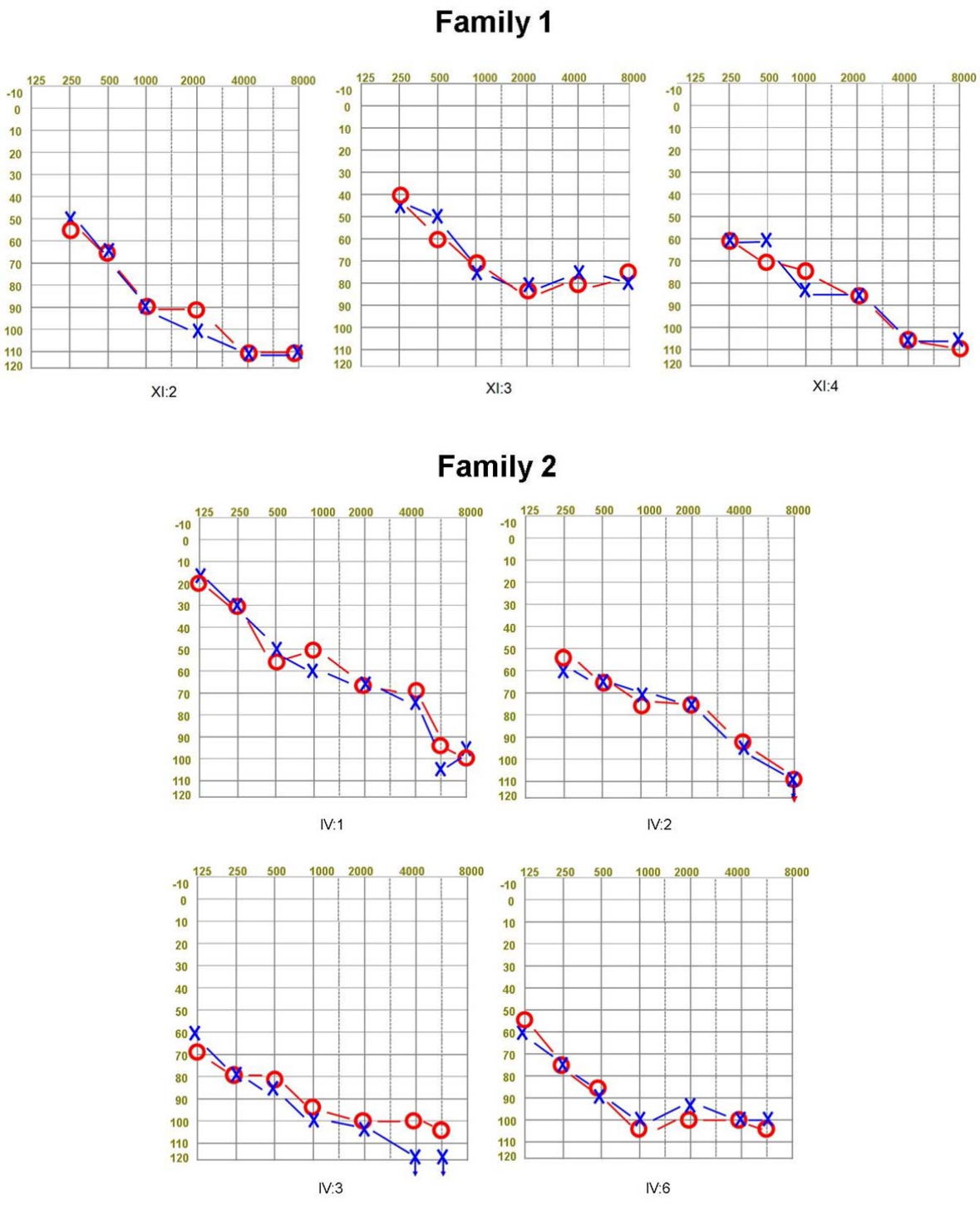


Figure S2: Microsatellite Analysis in Members of Family 1 for Markers Located Across the Chromosome 13q Critical Interval.

Family members indicated (X:1, X:2, XI:1, XI:2, XI:3 and XI:4) depicted in Figure 1A.

Marker	X:1		X:2		XI:1		XI:2		XI:3		XI:4	
D13S1255	1	2	1	2	2	2	1	2	1	2	1	1
rs1855259	-	-	-	-	-	-	A	G	A	G	A	G
rs7324849	-	-	-	-	-	-	A	C	A	C	A	C
rs1333404	-	-	-	-	-	-	A	G	A	G	A	G
D13S157	2	2	1	2	2	2	2	2	2	2	2	2
D13S790	1	2	1	2	1	2	2	2	2	2	2	2
D13S282	1	2	1	2	1	2	2	2	2	2	2	2
NM_032229.2:c.1240C>T	C	T	C	T	C	T	T	T	T	T	T	T
D13S1279	2	1	2	1	2	1	1	1	1	1	1	1
D13S1816	2	1	2	1	2	1	1	1	1	1	1	1
D13S886	1	2	1	2	1	2	2	2	2	2	2	2
chr13:93808431_17xCA	2	3	1	3	2	3	3	3	3	3	3	3
rs9584101	-	-	-	-	-	-	A	G	A	G	A	G
rs9523998	-	-	-	-	-	-	A	G	A	G	A	G
rs9524010	-	-	-	-	-	-	C	T	C	T	C	T

Figure S3: Genomic Organization of SLITRK6 (top panel) and Conserved Domain Architecture (lower panel).

The positions of the identified three variants are indicated (black arrow). Coding region: green bar with chevrons, 3' and 5' untranslated regions: red bar with chevrons.

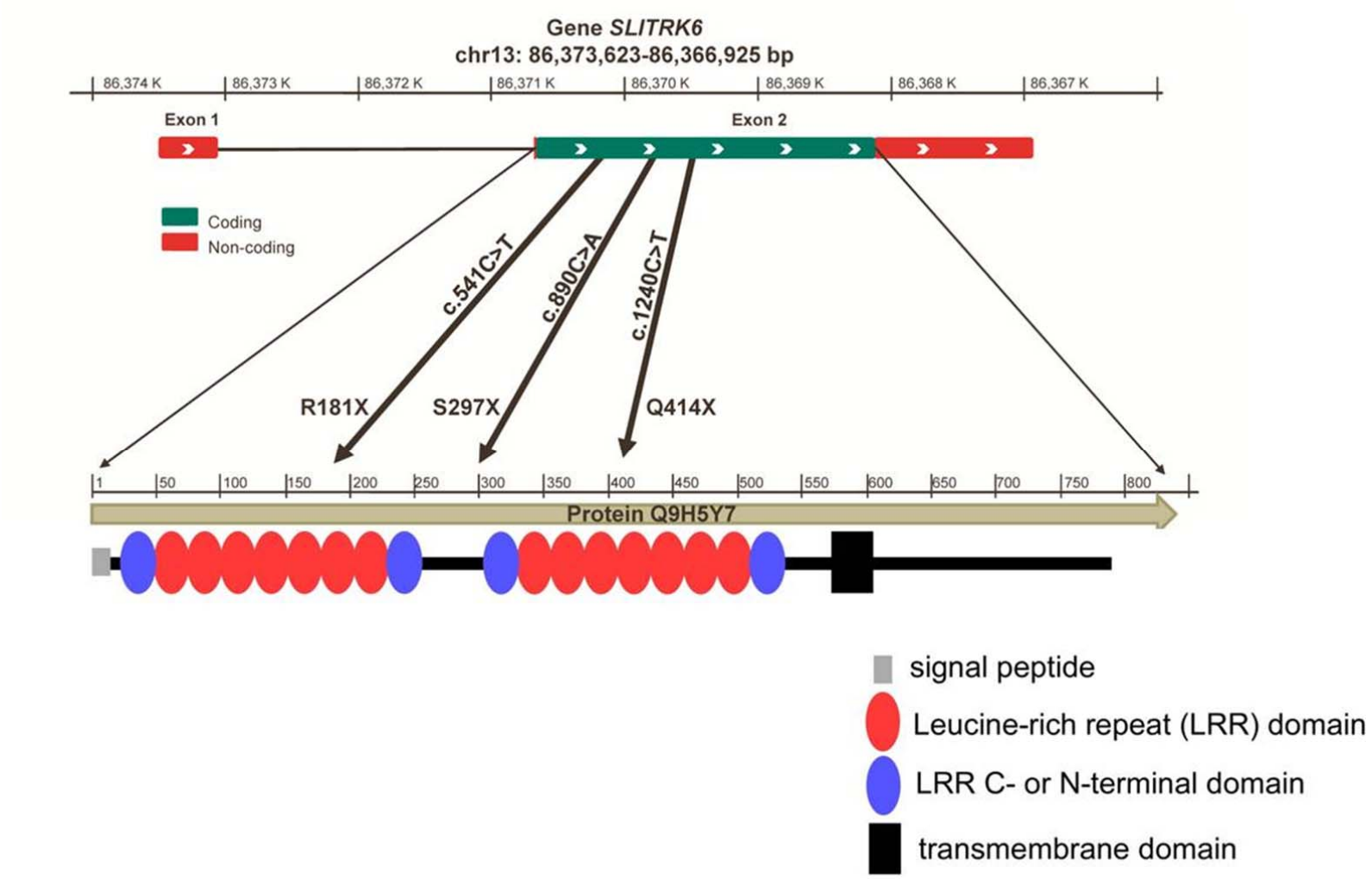


Figure S4: Results of Nonsense-Mediated mRNA Decay for the p.R181X, p.S297X), and p.Q414X mutation

SLITRK6 mRNA decay in HEK293 cells transfected with pA1 constructs. Twenty-four hours after transfection with *SLITRK6* minigenes, HEK293 cells were incubated with actinomycin-D, and the levels of WT or mutated SLITRK6 mRNA were measured by quantitative real-time PCR at different times.

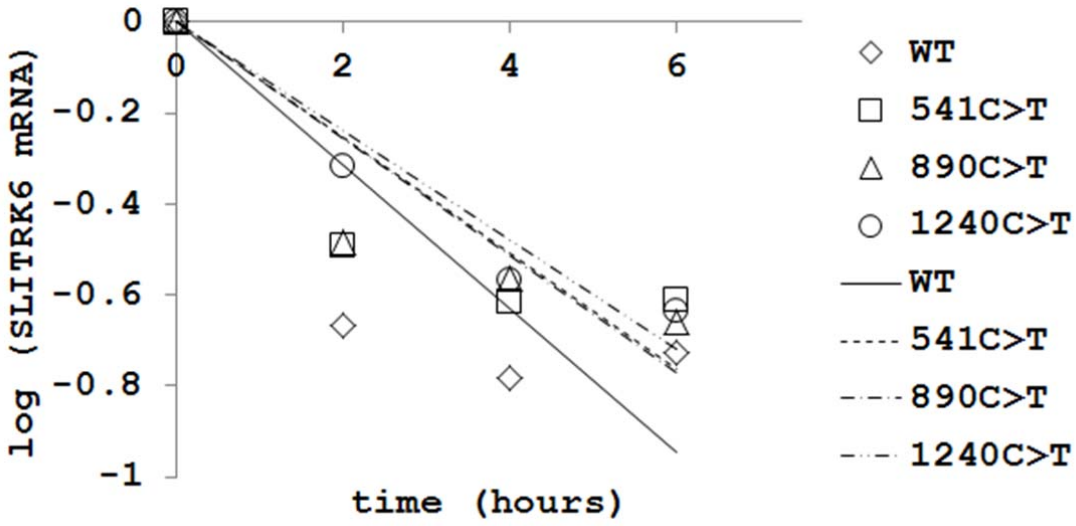


Figure S5. SLITRK6 Q414X, SLITRK6 S297X and SLITRK6 R181X are Functionally Impaired

(A) SLITRK6 ^{Q414X}, SLITRK6 ^{S297X} and SLITRK6 ^{R181X} were defective in synapse formation activities. HEK293 transfectants (Figure 2) were cocultured with rat hippocampal neurons (13 days in vitro). 1 day after, the cocultured cells were immunostained for an excitatory presynapse marker (Vglut1). *Left*, representative images. *Right,* quatitative analyses for Vglut1 positive overlapping with GFP derived signals. Puncta number or total puncta area was normalized for total GFP positive area for each image. n = 10-17 images. (B) SLITRK6 ^{Q414X}, SLITRK6 ^{S297X} and SLITRK6 ^{R181X} fail to inhibit the NGF-induced neurite outgrowth of PC12 cells. SLITRK6, SLITRK6 ^{Q414X},SLITRK6 ^{S297X}, SLITRK6 ^{R181X} expressing, or empty plasmid (pEF-ires-alkaline phosphatase) was transfected into PC12 cells. The mean neurite number and single neurite length are indicated for randomly selected more than 100 alkaline phosphatase-positive cells. Neurites equal to or longer than 43.5 μm (broken line) from a cell nucleus were subjected for the analysis. The expression of SLITRK6 resulted in the reduction of both neurite length and numbers in comparable degrees to that observed in mouse Slitrk6 expression (21). However, the three variants lacked these neurite-modulating activities. (A,B) *P < 0.05, **P < 0.01, ***P < 0.001 in the two tailed t-test, compared to the values of SLITRK6 WT transfectants. Scale bar, 10 μm.

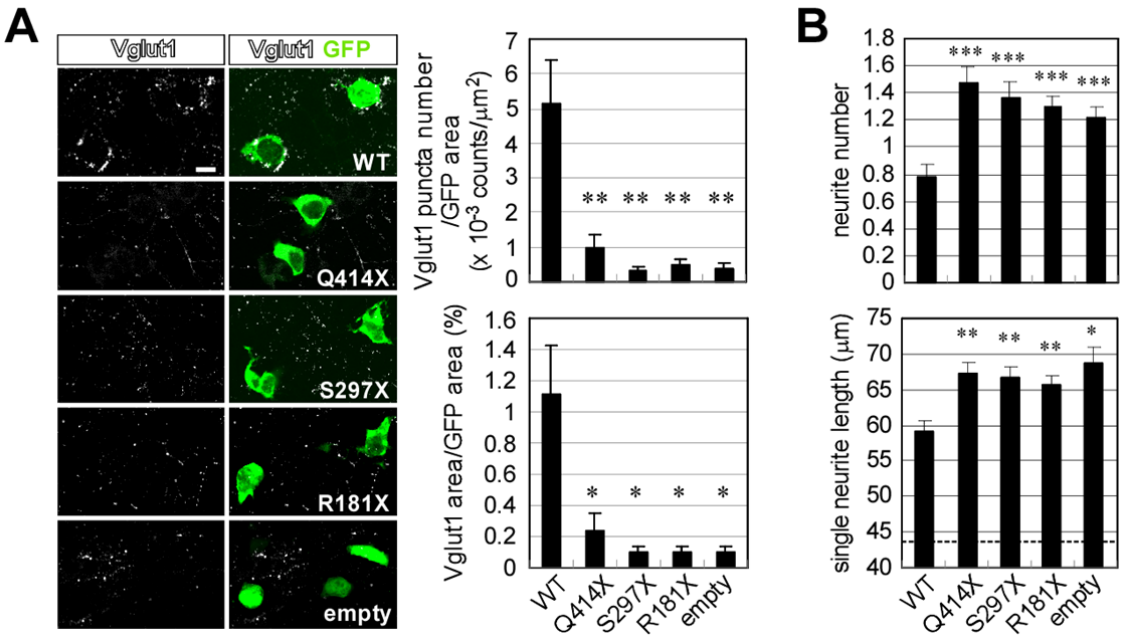
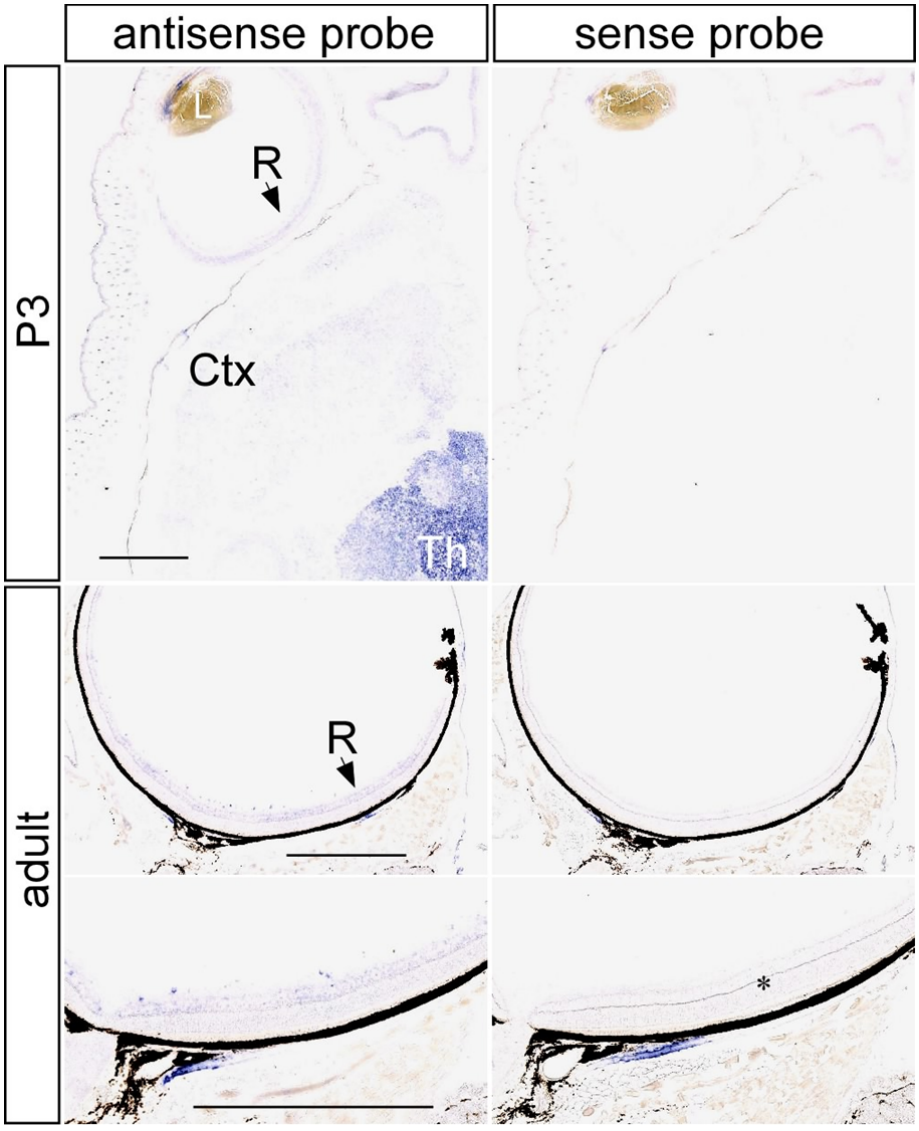


Figure S6. Supplemental Results for the *in situ* Hybridization Analysis

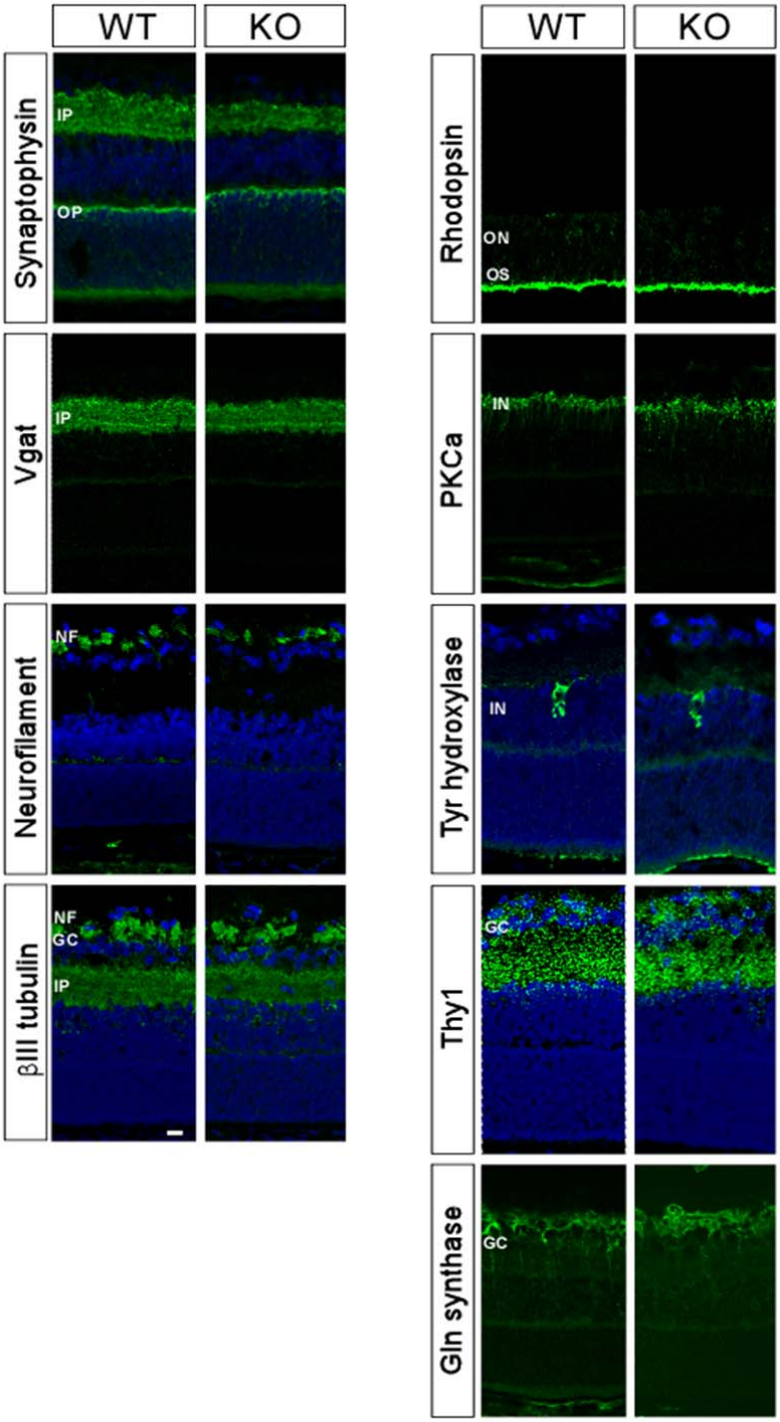
In situ hybridization was performed by using *Slitrk6* antisense and control sense strand probes. The experiments were undertaken for adjacent postnatal day 3 (P3) and adult (11 weeks-old) horizontal sections. In adult panels, the bottom pictures are the higher magnification of the retina in the top pictures.



Scale bar, 1 mm. R, retina; Th, Thalamus; Ctx, cerebral cortex. * The lining in outer plexiform layer is not an authentic *in situ* hybridization signal.

Figure S7. Representative Immunostaining Images for the Molecular Markers

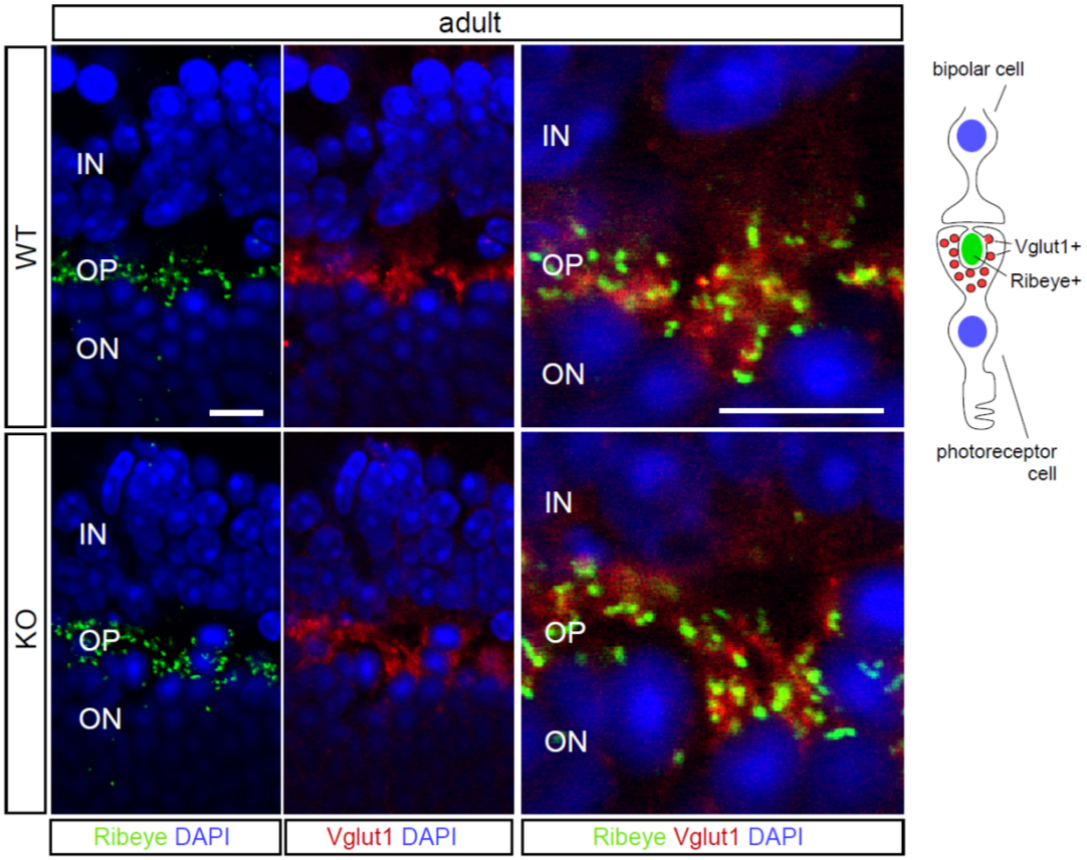
Immunostaining was performed by using antibodies for indicated molecular markers (green). In some images, DAPI nuclear staining (blue) was overlapped.



Scale bar, 10 μm. GC, ganglion cell layer; IN, inner nuclear layer; IP, inner plexiform layer; NF, nerve fiber layer; ON, outer nuclear layer; OP, outer plexiform layer; OS, outer segment of the photoreceptor layer.

Figure S8. Ribeye and Vglut1 Expression in Adult Mouse Eyes

Ribeye (ribbon synapse marker, *green*) and Vglut1 (synaptic vesicle marker, *red*) were examined in adult (14 months-old) mouse eyes by immunofluorescence staining. In the presynapse of the photoreceptor cells (located in OP), the Vglut1 signals lie on the synaptic vesicles and Ribeye signals lie in the clefts of the presynapstic membrane (synaptic ribbon). Ribeye signals are surrounded by the Vglut1 signals in these confocal images as illustrated bottom right. Both signals are equally detected at adult between WT and KO.



Scale bar, 10 μm. IN, inner nuclear layer; ON, outer nuclear layer; OP, outer plexiform layer. IP, inner plexiform layer; ON, outer nuclear layer.