Figure S1

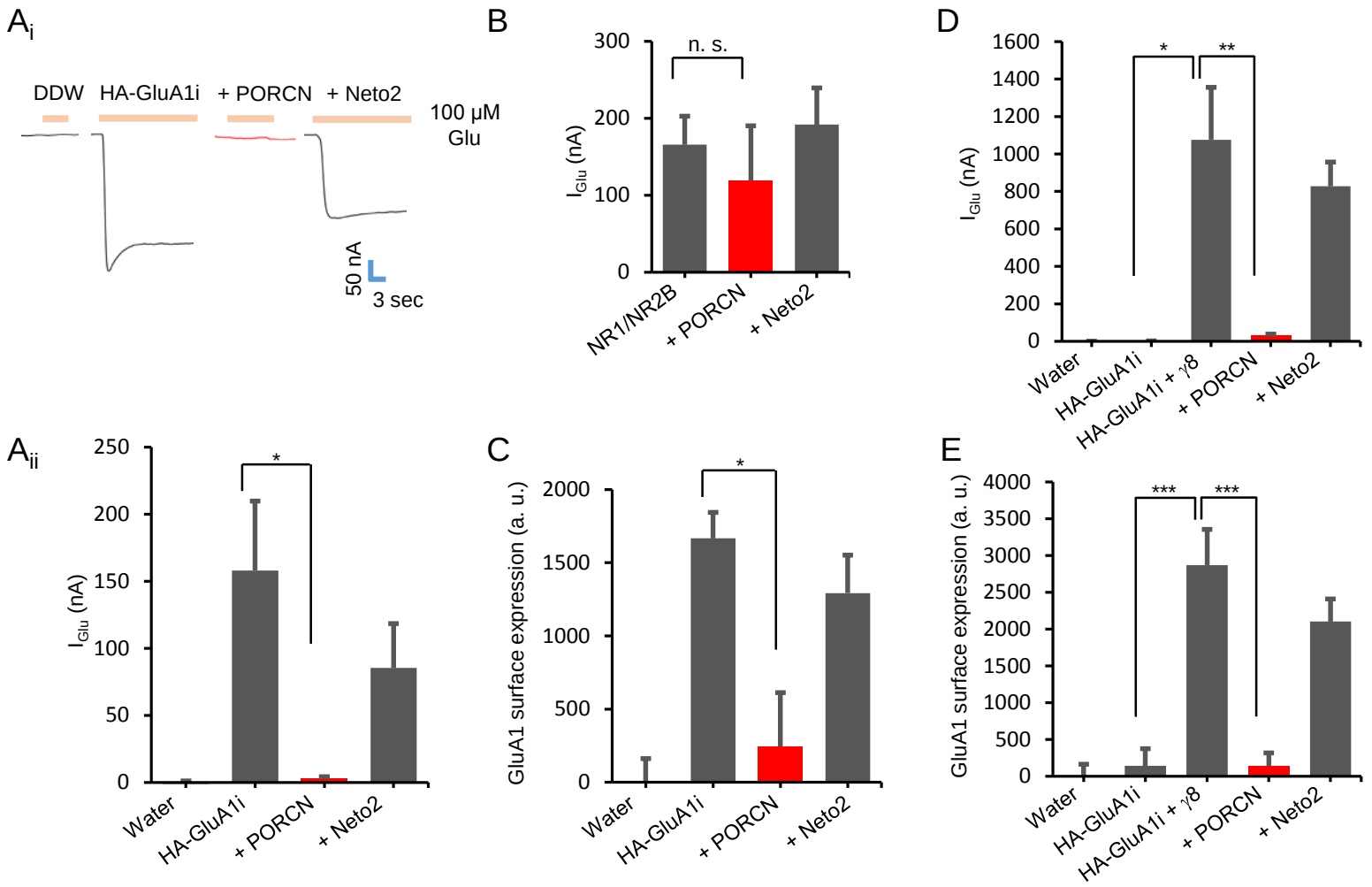

Figure S1, Related to Figure 2: A, B) Glutamate (100 µM)-evoked currents from oocytes injected with extracellular HA-epitope tagged GluA1 flip (HA-GluA1i) and PORCN cRNAs (1 ng each) were measured by two-electrode voltage clamp recording (Vh=-70 mV). PORCN reduced glutamate-evoked currents from HA-GluA1 (A), but not from NMDARs (NR1 and NR2B cRNA, 0.5 ng each) (B). The kainate receptor auxiliary subunit, Neto2, had no significant effect. C) Surface expression of HA-GluA1i was quantified by chemiluminescence assay with anti HA antibody. PORCN reduced surface expression of HA-GluA1i, whereas Neto2 did not. D, E). Glutamate-evoked currents were undetectable from oocytes injected with 0.1 ng HA-GluA1i cRNA alone. Upon co-injection with 0.016 ng γ-8 cRNA, robust currents were observed. PORCN (0.2 ng) reduced both glutamate-evoked currents (D) and surface expression of HA-GluA1i / γ-8 (E). The kainate receptor auxiliary subunit Neto2 had no significant effects. *p<0.05. **p<0.01. ***p<0.001. Data are shown as mean + SEM, n = 5-8 except n=3-5 for (B). DDW, distilled deionized water.

Figure S2

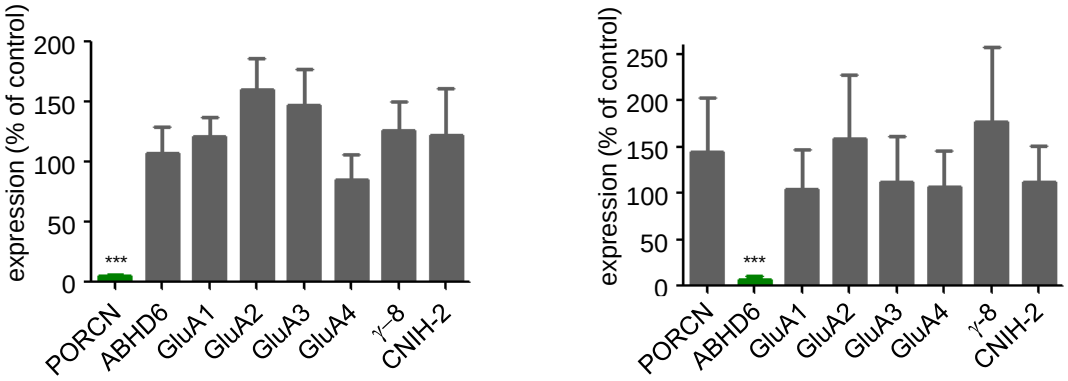

Figure S2, Related to Figure 3: qPCR following knockdown of PORCN (left) and ABHD6 (right) in hippocampal neurons six days after infection with lentivirus. Data are normalized to β-actin expression and control neurons. (n=3, mean + SD, ***p<0.001).

Figure S3

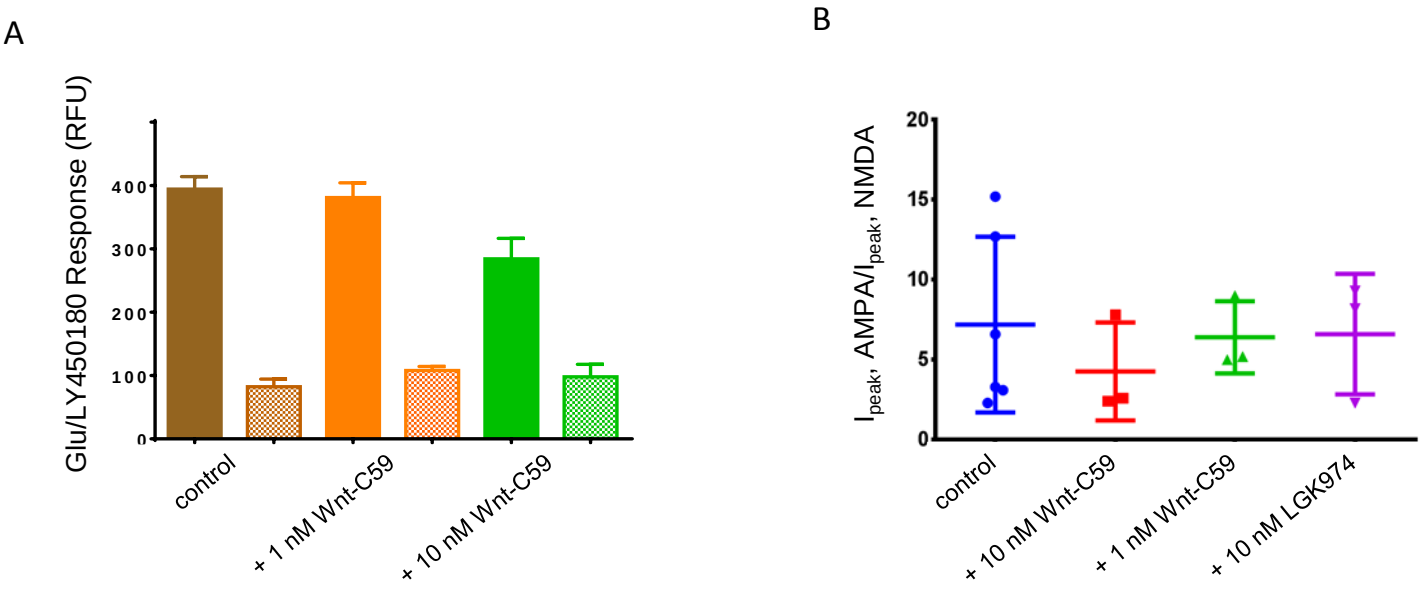

Figure S3, Related to Figure 2 and 3: The PORCN inhibitor Wnt-C59 does not abolish PORCN mediated effects on AMPARs. **A)** Quantification of glutamate-evoked calcium influx (10 μM LY450108 + 100 μM Glutamate) measured by FLIPR in HEK-293T cells transfected with $GluA1_o$ alone (solid bars) or together with PORCN (shaded bars) treated with 0, 1 or 10 nM Wnt-C59 for 28 hours. **B)** Ratio of peak currents evoked by 10 mM Glu in the presence of AP-5 and $MgCl_2$ (AMPAR mediated) and 100 μM Glu + 10 μM Gly in the presence of GYKI53655 (NMDAR mediated) from outside-out patches of rat hippocampal neurons at DIV 22-23 that have been treated with 0, 1, or 10 nM Wnt-C59, or 10 nM LGK974. Data are displayed as mean + SD.

Figure S4

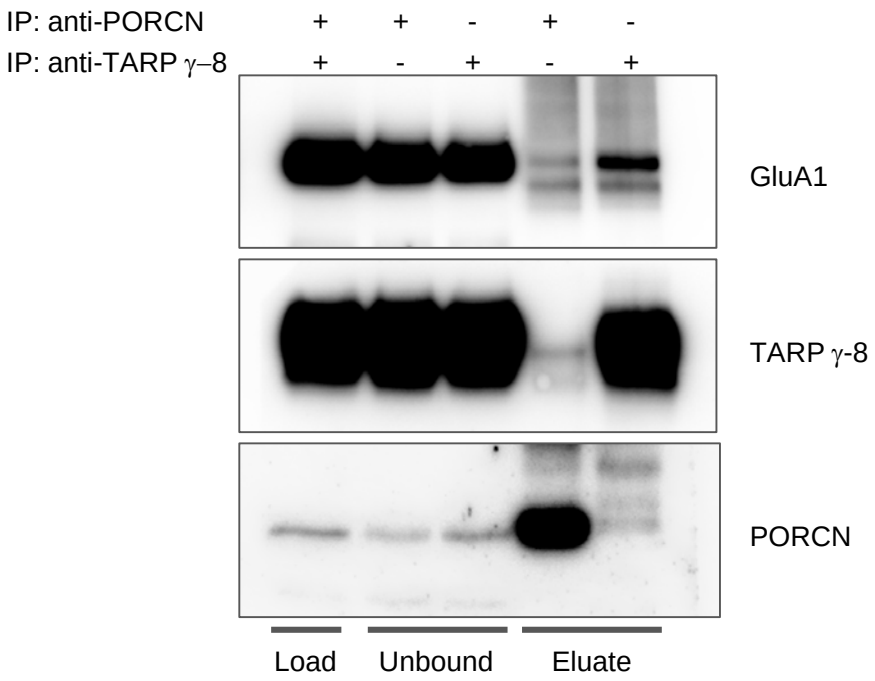

Figure S4, Related to Figure 5: Immunoprecipitation of PORCN- or TARP γ-8-containing AMPAR complexes from rat hippocampal membrane fractions using custom-made PORCN or γ-8 antibodies, respectively. Note that there is an overlapping AMPAR population that contains both, PORCN and γ-8. Eluate is concentrated 10-fold.

Figure S5

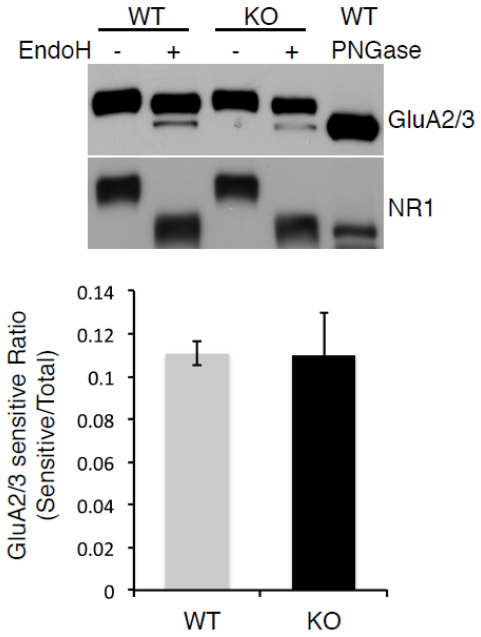

Figure S5, Related to Figure 6: Upper panel: Western Blot showing that in the hippocampus of PORCN KO mice the EndoH-sensitive and –resistant GluA2/3 populations are reduced proportionately. PNGAse F, a non-specific N-glycosidase, was used to remove all N-glycosylations. NR1 serves as a control. Lower panel: quantification (mean ± SEM).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Molecular biology

Gene Bank accession numbers of the clones are: GluA1flip (NM_001114183.1), GluA1flop (NM_000827), GluK2 (NM_021956), TARP γ-2 (NM_006078), TARP γ-8 (NM_031895.5), CNIH-2 (NM_182553), GSG1like (XM_574558.3), Brorin (NM_177033.3), C9orf4 (NM_014334.2), Neuritin 1 (NM_053346.1), Noelin 1 (NM_053573.1), PRRT1 (NM_001032285.1), LRRT4 (NM_178731.5), PORCN (NM_023638.3), ABHD6 (NM_025341.3), CPT1 (NM_153679.2). shRNA target sequences are CCTTCCACTTCAGCAACTATT (human PORCN) and GATGGATCTCGATGTGGTTAA (rat ABHD6).

Cell culture

HEK-293T cells (American Type Culture Collection) were cultured in high glucose DMEM (HyClone) supplemented with 10% fetal bovine serum, 1% sodium pyruvate and 1% penicillin/streptomycin at 37°C and 5% CO2. Cells were transiently transfected using Fugene HD Transfection Reagent (Promega) following the manufacturer´s protocols. cDNAs of receptors and auxiliary proteins were transfected in a 4:1 ratio unless otherwise stated. Experiments were performed 48 – 72 hours post transfection.

Primary cultures of rat hippocampal neurons were prepared as described previously (Kato et al., 2008). Briefly, whole hippocampi from E18 Sprague Dawley rat embryos were incubated with papain for 10 min at 37°C, triturated and washed with neurobasal medium supplemented with 2% B27, 1% penicillin/streptomycin, 0,5% fungizone and 0.5 mM glutamax. Cells were plated on either poly-D-lysine coated 6-well plates or 12 mm glass coverslips in 24-well plates at a density of 300,000 or 50,000 cells per well, respectively. Neurons were transiently transfected at DIV6-8 with Lipofectamine 2000 (Life Technologies) following the manufacturer´s protocol. Lentiviral transduction was performed at DIV14-16. At DIV20-23, neurons were used for experimental procedures.

Slice preparation

Transverse hippocampal slices were prepared from mice aged 17 – 30 days in accordance with protocols approved by the Institutional Animal Care and Use Committee of UAB. Animals were anesthetized by isoflurane inhalation and decapitated. The brain was quickly removed, and 300-350 µm slices were prepared using a slicer (Leica VT1200, Leica Instruments) in a solution containing (in mM) 135 NMDG, 1 KCl, 1.2 NaH_2PO_4, 1.5 $MgCl_2$, 0.5 $CaCl_2$, bubbled with 95% O2-5% CO_2 and incubated for 60 min in ACSF (125 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 2 $CaCl_2$, and 1 $MgCl_2$).

Preparation of Lentiviral Particles

shRNAs were cloned into pLKO_TRC_019 (The RNAi Consortium). Lentiviral particles were produced following standard procedures and were used at titers between 10^8 and 10^9.

Glycosidase Treatment

Mouse hippocampi were solubilized with 10 volumes of Glycoprotein Denaturing Buffer (New England Biolabs) containing Halt Protease Inhibitor (ThermoFisher Scientific). After brief sonication, samples were centrifuged at 20,000 x g for 10 min at 4 °C, and supernatants were collected. The solubilized samples were treated with EndoH or PNGaseF in G5 reaction buffer (New England Biolabs) for 3 hrs at 37 °C. The treated samples were then resolved on SDS-PAGE, which was followed by Western blot analysis with anti-GluA2/3 or anti-NR1 antibodies.

SDS-PAGE and Western Blotting

Subcellular fractionation of hippocampi was performed as described (Straub et al., 2011). Protein samples were separated on Bolt 4-12% Bis Tris Plus Gels (Life Technologies) and transferred to PVDF membranes using the iBlot Dry Blotting System (Life Technologies). Western Analysis was performed using the following primary antibodies: rabbit anti-GluA1, mouse anti-GluA2, rabbit anti-GluK2/3, rabbit anti-γ-2 (all Millipore), mouse anti-β-actin, mouse-anti-FLAG and rabbit anti-PORCN (for transfected cells) (all Sigma). Rabbit anti-PORCN (for native tissue) anti-CNIH-2 and rabbit anti-γ8 antibodies were generated using the synthetic peptides EKDHLEWDLTVSRP, DELRTDFKNPIDQGNPARARERLKNIERIC and CASGFLTLHNAFPKEA, respectively. After incubation with goat-anti-mouse or goat-anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz) blots were developed with ECL prime (GE Healthcare).

RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from cultured hippocampal neurons using the RNeasy Mini Kit (Qiagen) and was reverse transcribed using the GoScript Reverse Transcription Mix (Promega).

Quantitative real-time PCR was performed in an ABI Prism 7900HT Real-Time PCR System with 384-Well Block Module (ABI 7900 Sequence Detection System) using Perfecta Sybr Green Fast Mix ROX (Quanta Biosciences). Assays were run in a total volume of 20 µl containing 1 µl of the generated cDNAs, 400 nM of forward and reverse primers and 1x Sybr Green mix. The PCR parameters were: 50°C for 2 min, 95°C for 10 min for 40 cycles, 95°C for 15 sec, 60-65°C for 1 min, followed by a dissociation stage of 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. The following primers were used:

GluA1: F *GCCAGATCGTGAAGCTAGAA*, R *CCATCATAGGTGAGAGCAGAAG*

GluA2: F *ATGGGACAAGTTCGCATACC*, R *CCCTTTCACAGTCCAGGATTAC*

GluA3: F *GCTGTTGCTCCACTCACTAT*, R *GGGTCACGAGGTTCTTCATT*

GluA4: F *CCGAAACACAGACCAGGAATAC*, R *GCTTGGTGTGATGAGAGAGATG*

ABHD6: F *CTGGATGACCTGTCCATAGTTG*, R *GGATGGGATCAAAGGGATCTTC*

PORCN: F *TCCTTCCACAGCTACCTACA*, R *CTCAGACAGAAAGCCCACAA*

CNIH-2: F *CCTCCCTCATCTTCTTTGTCATC*, R *GTACCTCCAGAGGTGGTAGAA*

TARP γ-8: F *AGTGGCTCCTCAGAGAAGAA*, R *TTGGATTTGTAGACGCGAGAG*

Statistics

Data are represented as mean ± SEM from at least three independent experiments. Analyses involving three or more data sets were performed with a one way ANOVA with a Tukey Kramer post hoc analysis using Graphpad Prism software (Carlsbad, CA). Analyses involving two data sets were performed with an uncorrected student's t-test or with a student's t-test with a Welsh correction, only if the variances were statistically different. Significance was set as p-value <0.05.

SUPPLEMENTAL REFERENCES

Straub, C., Hunt, D.L., Yamasaki, M., Kim, K.S., Watanabe, M., Castillo, P.E., and Tomita, S. (2011). Distinct functions of kainate receptors in the brain are determined by the auxiliary subunit Neto1. Nature Neuroscience *14*, 866-873.