# Bidirectional Homeostatic Regulation of a Depression-Related Brain State by Gamma-Aminobutyric Acidergic Deficits and Ketamine Treatment

## *Supplement 1*

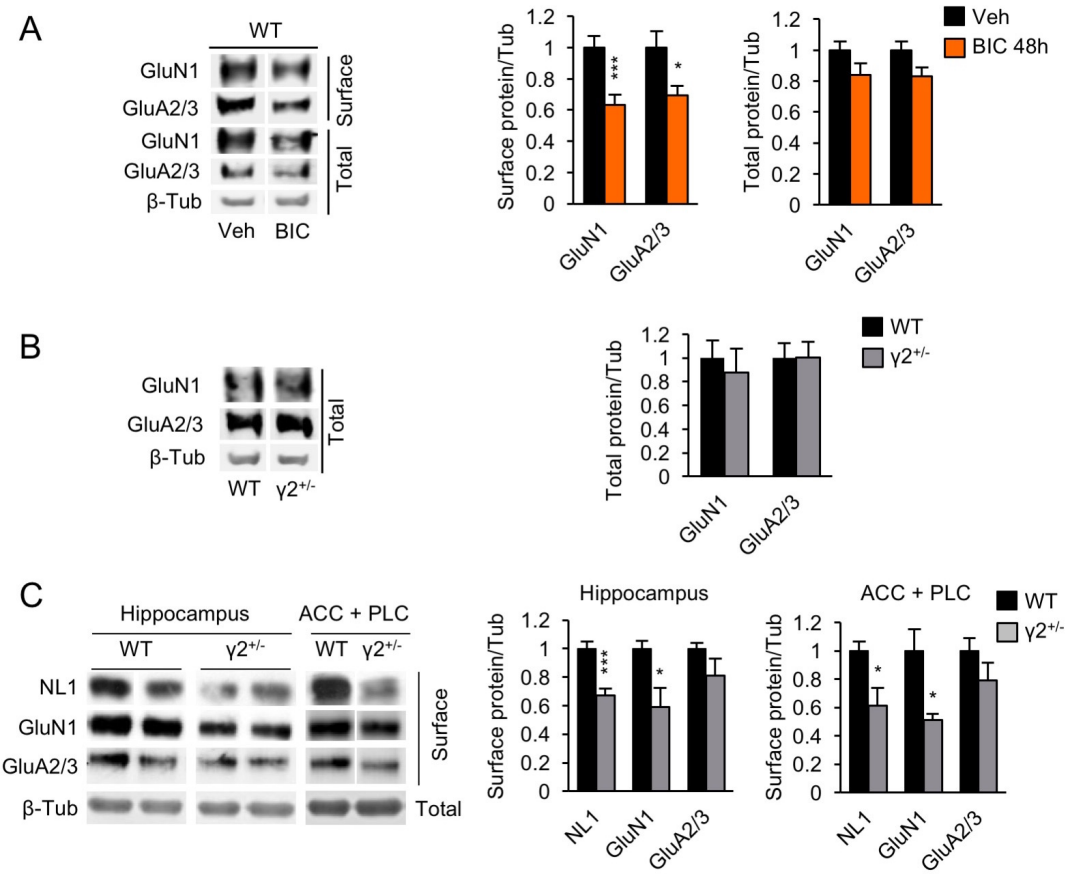


**Figure S1.** Total levels of GluRs are unaffected in $\gamma2^{+/-}$ vs. WT cortical cultures and BIC treated WT cultures. However, the cell surface expression of GluRs in BIC treated WT cultures is downregulated, similar to the effects seen in $\gamma2^{+/-}$ vs. WT cortical cultures. Lastly, downregulation of GluRs seen in C57BL/6J $\gamma2^{+/-}$ mice was reproduced in 129X1/SvJ $\gamma2^{+/-}$ mice. (**A**) Treatment of WT neurons with BIC (20 μM, 48 h) resulted in downregulation of cell surface GluRs in a manner comparable to effects seen in $\gamma2^{+/-}$ vs. WT neurons (GluN1: BIC 63.2 ± 6.5% of control, $P < 0.001$, $n$ = 14-15 cultures; GluA2/3: BIC 69.2 ± 6.4% of control, $P < 0.05$, $n$ = 11 per condition). Total protein levels remained unaffected by BIC treatment (GluN1: BIC, 84.0 ± 7.4% of control; GluA2/3: BIC, 83.2 ± 5.7% of control, n.s., $n$ = 10-11, for both comparisons). (**B**) Total GluR levels were unaltered in $\gamma2^{+/-}$ vs. WT cultures (GluN1: $\gamma2^{+/-}$, 88.0 ± 19.7% of WT; GluA2/3: $\gamma2^{+/-}$, 100.4 ± 12.5% of WT; n.s., $n$ = 8 per condition, for both comparisons). (**C**) The cell surface expression of NL1 and GluN1 in $\gamma2^{+/-}$ mice was reduced both in hippocampus (NL1: $\gamma2^{+/-}$, 67.1 ± 4.7% of WT; GluN1: $\gamma2^{+/-}$, 58.9 ± 13.6% of WT) and ACC/PLC (NL1: $\gamma2^{+/-}$, 61.1 ± 12.8% of WT; GluN1: $\gamma2^{+/-}$, 51.1 ± 4.4% of WT. $P < 0.05$, $n$ = 5-7, for all four comparisons), similar to data shown in Fig. 3A for C57BL/6J mice. The mean surface expression of GluA2/3

trended lower in $\gamma2^{+/-}$ vs. WT similar to data in Fig. 3A but the effect was not significant, most likely because the experiment was underpowered (hippocampus: $\gamma2^{+/-}$, 81.8 ± 15.9% of WT; ACC+PLC: $\gamma2^{+/-}$, 78.9 ± 12.5% of WT, n.s., $n$ = 3-4 for both comparisons). *$P$ < 0.05; ***$P$ < 0.001, $t$-tests.

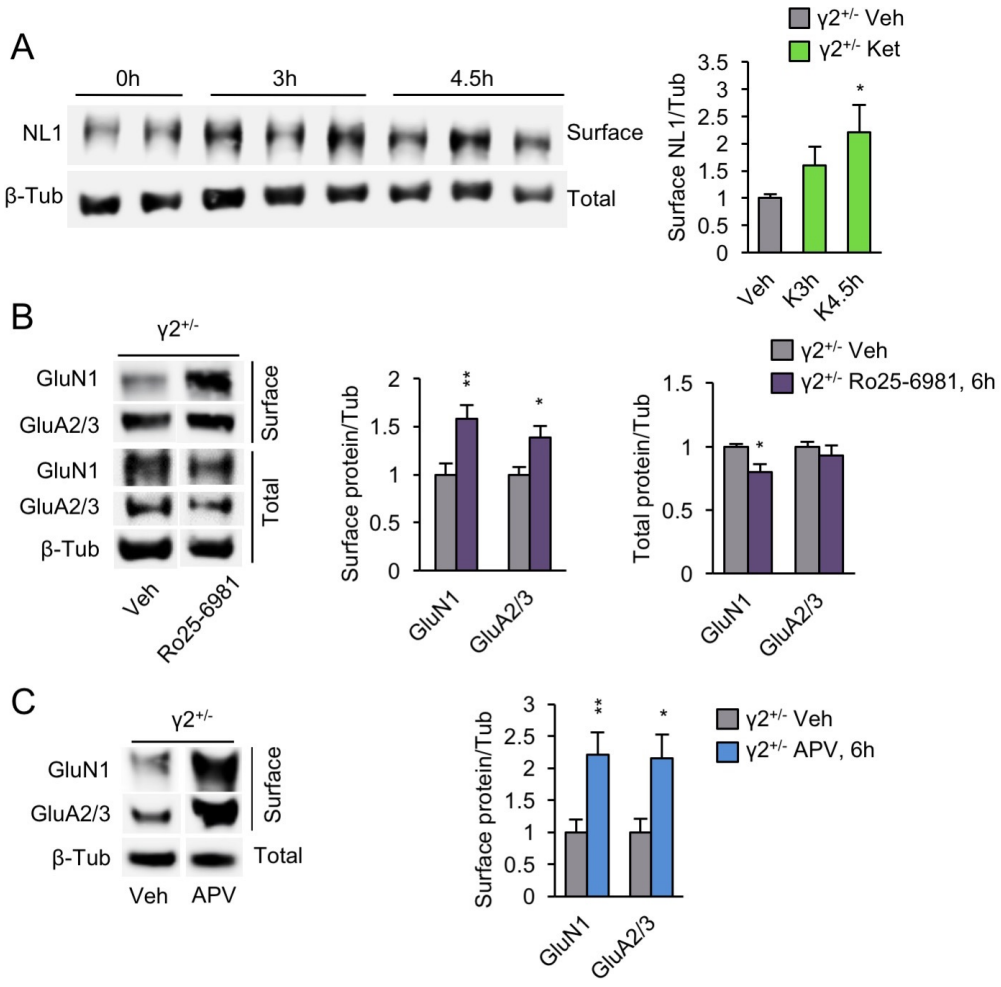


**Figure S2.** NMDAR antagonist induced cell surface expression of neuroligin 1 and glutamate receptors in $\gamma2^{+/-}$ cortical cultures. (**A**) Cell surface expression of NL1 in $\gamma2^{+/-}$ cultures was gradually increased by ketamine treatment, reaching significance at 4.5 h of treatment (Ket: 202.1 ± 35.6% of untreated controls, $P < 0.05$, $n = 10$ cultures, Dunnett's test). (**B**,**C**) Incubation of $\gamma2^{+/-}$ cultures with Ro25-6981 [(B) 10 μM, 6 h] or APV [(C)100 μM, 6 h] increased the cell surface levels of GluN1 and GluA2/3 (Ro25-6981: GluN1, 167.3 ± 15.2% of controls, $P < 0.01$, $n$ = 11-13; GluA2/3, 145.3 ± 12.9% of controls, $P < 0.05$, $n$ = 11-13; APV: GluN1, 221.0 ± 34.3% of controls, $P < 0.01$, $n$ = 7-8; GluA2/3, 215.8 ± 36.8% of controls, $P < 0.05$, $n$ = 10-11, *t*-tests), similar to treatment with ketamine (Fig. 1C). Total GluN1 and GluA2/3 were unaffected by Ro25-6981, except for a small but significant reduction in GluN1 expression (GluN1, 79.9 ± 6.4% of controls, $P < 0.05$, $n$ = 8-9; GluA2/3, 92.8 ± 8.1% of controls, n.s., $n$ = 8-9, *t*-tests).

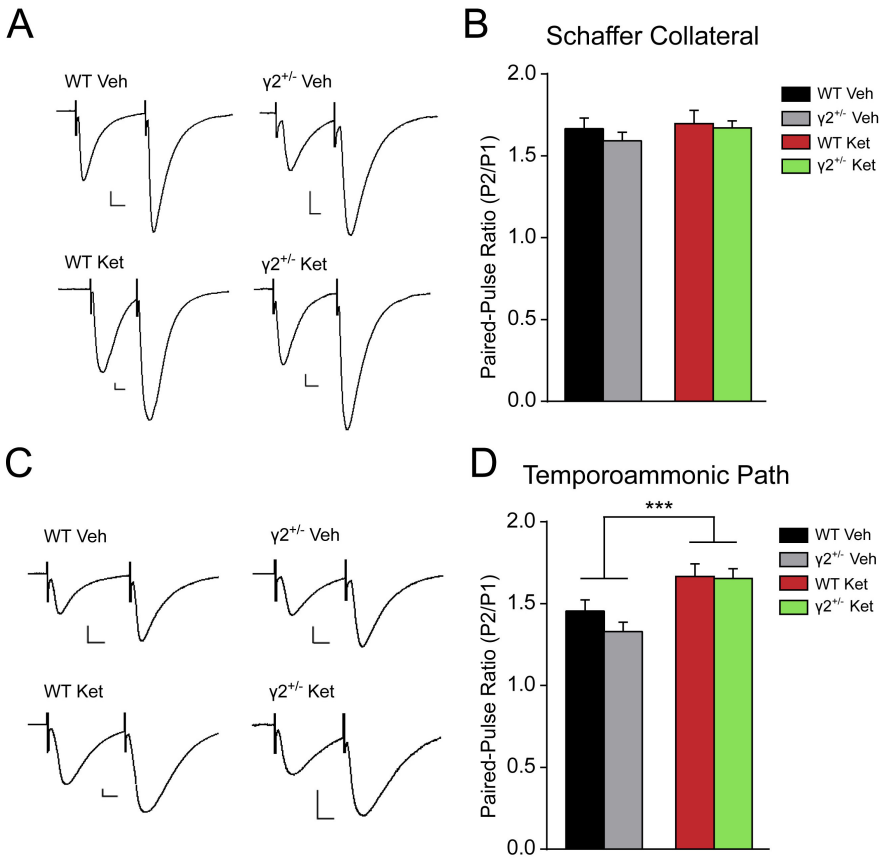


**Figure S3.** Paired-pulse ratios of evoked AMPAR responses at Schaffer collateral and temporoammonic synapses are unaltered in γ2$^{+/-}$ mice. Average traces of AMPAR EPSCs evoked by pairs of pulses 50 ms apart, at SC (**A**) and TA (**C**) synapses of vehicle or ketamine treated γ2$^{+/-}$ and WT mice, respectively. Corresponding quantifications in (**B**) and (**D**), respectively, show a ketamine-induced increase in the paired pulse ratio at TA synapses, regardless of genotype ($n$ = 15-21 cells; two-way ANOVA, TA path by treatment $F_{(1,65)}$ = 15.10, $P$ < 0.001). Scale bars: 50 pA, 10 ms.

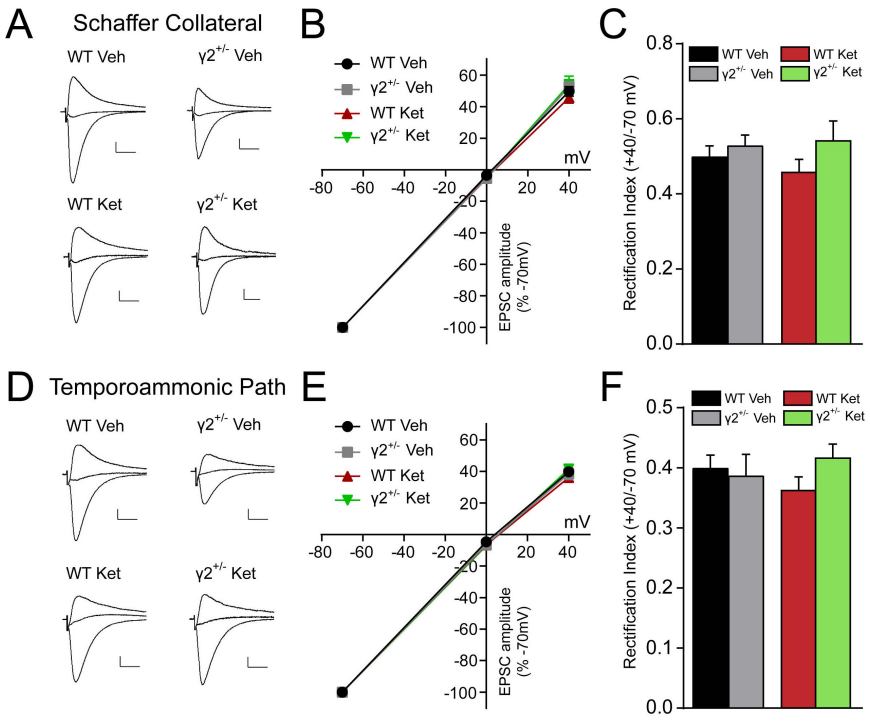


**Figure S4.** AMPAR rectification of hippocampal CA1 pyramidal neurons is unaltered by γ2 genotype or ketamine treatment. Average traces of AMPAR EPSCs evoked at SC (**A**) and TA (**D**) synapse, of saline (Veh) or ketamine (Ket) treated $\gamma2^{+/-}$ and WT mice, respectively. EPSCs were determined at -70, 0 and +40 mV holding potentials. Corresponding quantifications of rectification indexes are shown in (**B, C**) and (**E, F**) (*n* = 11-15 cells). Scale bars: 50 pA, 10 ms.

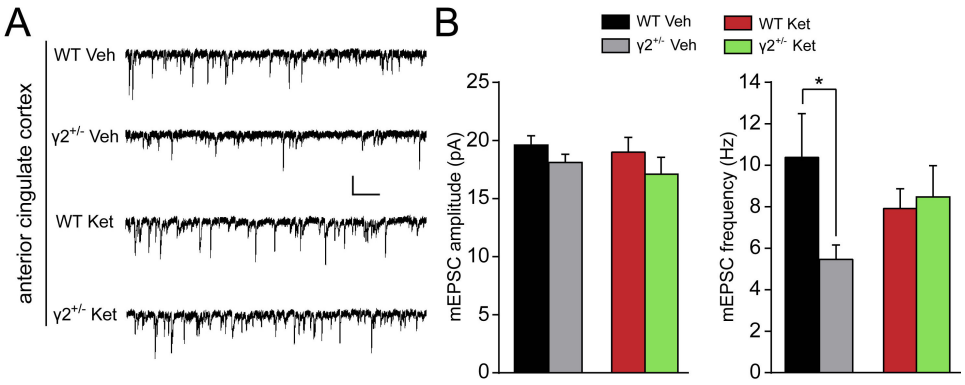


**Figure S5.** Reduction of mEPSC frequency in layer 2/3 anterior cingulate cortex pyramidal neurons of γ2$^{+/-}$ mice. (**A**) Representative traces of mEPSC recordings from ACC neurons of wild-type and γ2$^{+/-}$ mice injected with saline (Veh) or ketamine (Ket). (**B**) Quantification shows a significant decrease in mEPSC frequency in γ2$^{+/-}$ mice, which is reversed by ketamine treatment. (*n* = 7-8 cells; two-way ANOVA, mEPSC frequency by genotype $F_{(1,27)}$ = 2.353, *P* = 0.137, Bonferroni's test, **P* < 0.05). Scale bars: 20 pA, 250 ms.

## Supplemental Methods and Materials

### Production and Husbandry of Mice

Two different $GABA_AR$ $\gamma2^{+/-}$ mouse lines were used as part of this study, with virtually identical germline deletions of exon 8 of the *Gabrg2* locus. A first line of $\gamma2^{+/-}$ mice was maintained on a 129X1/SvJ background (> 40 generations of backcrossing) as previously described (1-3). A second line was generated on the C57BL/6J background by mating $\gamma2^{f/f}$ mice [containing a *Gabrg2* locus with exon 8 flanked by loxP sites (4)] with the oocyte-specific Cre-transgenic mouse line Tg(Zp3-cre)93Knw/J (stock number 003651, Jackson Laboratory, Bar Harbor, ME). Offspring with germline deletion of exon 8 were identified by PCR of tail DNA (4). The Zp3-Cre transgene was outcrossed and the resultant $\gamma2^{+/-}$ mice maintained on a C57BL/6J background (> 6 generations of backcrossing). The $\gamma2^{+/-}$ and WT mice used for experimentation were littermates produced by crossing $\gamma2^{+/-}$ mice and WT mice. The 129X1/SvJ line was used for preparation and analyses of cortical cultures, as well as biochemical and electrophysiological analyses of brain slices. The C57BL/6J line was used for biochemical and behavioral experimentation involving ketamine treatment. All mice were maintained on a standard 12h:12h light-dark cycle with food and water available ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University or by the IACUC of McGill University and performed in accordance with relevant guidelines and regulations of the National Institutes of Health.

### Drug Treatments

For treatment of cultures the drugs were diluted or dissolved in culture media to the following final concentrations: ketamine (10 μM, Ketaject, Phoenix Pharmaceutical, Inc., St. Joseph, MO); 2-amino-5-phosphonovaleric acid (APV, 100 μM, Sigma-Aldrich, St. Louis, MO); bicuculline (20 μM, R&D Systems, Minneapolis, MN); Ro25-6981 (10 μM, Sigma-Aldrich). For treatment of

mice (8-9 weeks old), ketamine (Ketaject diluted to 1 mg/ml in 0.9% saline) was administered at 10 mg/kg (biochemical and electrophysiological analyses) or 3 mg/kg (behavioral analyses) (i.p.) as previously described (5, 6). For biochemical and electrophysiological experiments, the mice were euthanized 24 or 72 h later by cervical dislocation and the brain tissue analyzed as described below.

**Cell Surface Biotinylation**

Cortical cultures from $\gamma 2^{+/-}$ and WT mice were generated from embryonic day 14-15 embryos (129X1/SvJ line) subject to cell surface biotinylation at DIV21 and purification using NeutrAvidin agarose beads (Thermo, Rockford, IL) as previously described (7). The biotinylated proteins were quantitated by SDS/PAGE/western blot using the primary antibodies mAb anti-NMDAR1 (1:500, BD Biosciences, San Jose, CA), mAb anti-neuroligin 1 (1:500, Synaptic Systems, Goettingen, Germany), rabbit anti-neuroligin2 antiserum (1:500, Synaptic Systems), rabbit anti-GluA2/3 (1:500, Millipore, Billerica, MA), and mAb anti-β-tubulin isotype I+II (1:1000, Sigma-Aldrich). The blots were developed using IRDye and VRDye secondary antibodies and an Odyssey CLx infrared imager (LI-COR, Lincoln, NE). Amounts of cell surface biotinylated proteins were normalized to amounts of β-tubulin in total extracts quantitated on parallel gels. For biotinylation of brain slices we adapted the protocol of Terunuma *et al.* (8). Briefly, 8-9-week-old mice were euthanized by cervical dislocation. Freshly isolated brains were rinsed with pre-chilled oxygenated artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $NaH_2PO_4$, 2 mM $MgSO_4$, and 10 mM D-glucose, equilibrated with 95% $O_2$ and 5% $CO_2$ on ice and quickly sliced into 1-mm thick coronal sections containing the mPFC or hippocampus, respectively, using a mouse brain matrix (acrylic brain matrix, Stoelting Co., Wood Dale, IL). The sections were incubated in 1 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in pre-chilled oxygenated ACSF and continuously equilibrated with 95% $O_2$/5% $CO_2$ on ice for 30 min. The tissue slices were washed with 50 mM glycine and 0.1%

bovine serum albumin in ACSF and the regions of interest isolated under a dissection microscope, triturated in lysis buffer and processed for western blots as described above for cultures.

**Immunofluorescent Staining of Cortical Cultures**

Immunofluorescent staining of neurons employed glia free cortical cultures prepared from embryonic day 14-15 embryos as previously described (129X1/SvJ line) (9). The cells were fixed, permeabilized and stained at 21 days *in vitro* as described (9) using double or triple staining with rabbit anti MAP2 (1:1000, Ab5622), guinea pig anti VGluT1 (1:500, LV1439669), mouse anti PSD95 (1:1500, catalog #28879, all from Millipore), mouse anti gephyrin (1:500, #147111), and rabbit anti VGAT (1:1000, #131002, both from Synaptic Systems) primary antibodies. The immunoreactivities were developed using AlexaFluor 488-conjugated donkey anti-mouse (1:500, Molecular Probes, Eugene, OR), Cy3 donkey anti-guinea pig (1:500, Jackson ImmunoResearch, West Grove, PA), and AlexaFluor 647 goat anti-rabbit secondary antibodies (1:500, Molecular Probes). Fluorescent images of pyramidal cell-like neurons were captured and digitized with a Zeiss Axiophot 2 microscope equipped with a 40 X 1.3 N.A. objective and an ORCA-100 video camera linked to an OpenLab imaging system (PerkinElmer). The density and size of immunoreactive puncta and the degree of colocalization of pre- and postsynaptic markers were quantified as described, with the investigator blinded to genotype and treatment (9).

**Electrophysiology**

Our rationale for analyses of altered synaptic transmission in $\gamma2^{+/-}$ vs. WT and vehicle vs. ketamine treated mice involved comparing defects in glutamatergic transmission at SC-CA1 vs. TA-CA1 synapses, as well as possible differential effects of genotype or drug treatment on AMPAR vs. NMDAR currents, and this required measurement of evoked EPSCs. In addition to

evoked EPSCs we also measured sEPSCs, which (unlike mEPSCs) can be recorded from the same cells because they require the same external solution. By contrast, characterization of IPSCs did not involve distinguishing different types of inputs or receptors. Therefore, recordings of mIPSCs were preferred over sIPSCs as mIPSCs can more reliably distinguish postsynaptic (mIPSC amplitude) from presynaptic changes (mIPSC frequency). Coronal slices (350 μm) containing the dorsal hippocampus or anterior cingulate cortex were prepared using a vibratome (Leica VT1200S), from 7- to 13-week-old 129X1/SvJ mice (of either sex), in a solution containing (in mM): 210 sucrose, 7 D-glucose, 25 $NaHCO_3$, 1.25 $NaH_2PO_4$, 2.5 KCl, 1.3 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2$, 7 $MgCl_2$, saturated with 95% $O_2$/5% $CO_2$. Slices were then allowed to recover at 30°C for 30 min, and subsequently at room temperature, in a solution containing (in mM): 118 NaCl, 11 D-glucose, 26 $NaHCO_3$, 15 HEPES, 1.25 $NaH_2PO_4$, 2.5 KCl, 0.4 Na-ascorbate, 3 myo-inositol, 2 Na-pyruvate, 2 $CaCl_2$, 1 $MgCl_2$, saturated with 95% $O_2$/5% $CO_2$. Whole-cell patch-clamp recordings were obtained from hippocampal CA1 pyramidal neurons or layer 2/3 anterior cingulate pyramidal neurons, under visual guidance by infrared differential interference microscopy (Nikon Eclipse FN1), between 1.5 h and 6 h after slicing. Perfusate (~1.5 ml/min at 30 ± 1°C) contained (in mM): 50 sucrose, 119 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 1 $NaH_2PO_4$, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, saturated with 95% $O_2$/5% $CO_2$. Cells were patched using borosilicate glass pipettes (2–5 MΩ) filled with (in mM): 127 CsCl, 8 NaCl, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 $Na_3$-GTP, and 2 Mg-ATP, pH 7.2 for mIPSCs, or 127 $CsMeSO_4$, 8 NaCl, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 $Na_3$-GTP, and 2 Mg-ATP, 0.1 spermine, 5 QX-314, pH 7.2 for EPSCs. Liquid junction potentials were left uncompensated. Signals were amplified and filtered (2 kHz) using an Axopatch 200B amplifier, sampled at 10 kHz using a Digidata 1440A, and recorded with Clampex 10.3 (Molecular Devices). To isolate mIPSCs, 0.5 μM TTX, 10 μM NBQX, and 25 μM D-APV were added to the perfusate. To isolate AMPAR-mediated EPSCs and sEPSCs, 50 μM PTX was added to the perfusate; 0.5 μM TTX was also added for mEPSCs. To isolate NMDAR-mediated EPSCs, 10 μM NBQX and 50 μM PTX were

added to perfusate lacking $MgCl_2$, and recordings were obtained following >30 min of pre-incubating the slice in the absence of $MgCl_2$. All recordings were obtained at $V_h$ = −70 mV, unless otherwise indicated. EPSCs were evoked using borosilicate glass pipette stimulators (~20-30 µm open tip diameter) filled with external solution. To stimulate the Schaffer collateral a stimulator was placed in *s. radiatum*, ~30-50 µm away from *s. pyramidale*. A second stimulator was placed in *s. lacunosum-moleculare* to activate the temporoammonic path. Both stimulators were positioned ~50-200 µm away from the primary apical dendrite of the recorded cell. Stimuli of 0.1 ms were delivered at 0.1 Hz, with a 5 s delay between the two paths. Paired-pulse ratios of AMPAR-mediated responses were acquired by applying a second stimulus of equal intensity at 50 ms after the first stimulus and calculating the ratio of EPSC2/EPSC1. Analyses were performed using Clampfit 10.3 (Molecular Devices). Passive membrane properties and access resistance were assessed between each sweep. Only cells with a stable access resistance throughout the recording period were included in the analysis. Average access resistance did not differ significantly across groups ($p$ > 0.10, Student's $t$-test).

**Behavior**

Behavioral analyses in the EPM and FST were performed essentially as described (10) except that the behavior was scored automatically using an EthoVision XT video tracking system (Noldus Information Technologies, Leesburg, VA). We used female mice (of the C57BL/6J $\gamma2^{+/-}$ line) at 10-14 weeks of age in accordance with previous behavioral characterizations of the $\gamma2^{+/-}$ model that relied exclusively on female mice (2, 3, 10).

**Statistics**

Statistical comparisons were performed using two-tailed student's $t$-tests or ANOVAs followed by posthoc analyses as detailed in the figure legends.

## Supplemental References


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