

SUPPLEMENTAL DATA

Generation of PIKE knockout mice (*PIKE* *-/-*)

In order to study the neuronal functions of PIKE-L on GluA2/GRIP1 association *in vivo*, we generated mice with targeted disruption in the *CENTG1* locus via homologous recombination using LoxP/Cre system. We first made a transgenic line with the loxP insertions in intron 2 and 7 of *CENTG1* gene (Fig S4A). *PIKE flox/+* mice were then bred with transgenic mice that express Cre recombinase in all tissues. Deletion of exons 3 to 6 resulted in removal of GTPase domain as well as introducing a frame shift mutation that created a new stop codon, leading to a truncated PIKE protein for all isoforms. Heterozygous mating generated newborn pups at expected Mendelian frequency that appeared indistinguishable from the wild-type littermates. Moreover, PIKE-deficient mice were viable, fertile, and of normal size and weight at birth (data not shown). We confirmed the ablation of PIKE-L protein by RT-PCR (Fig S4B) and Western blot analysis using anti-PIKE-L antibody (Fig S4C). Immunohistochemical staining using an antibody recognizing the C-terminus of PIKE-L revealed that no signal was detected in PIKE-null cortex, which further confirmed the successful removal of PIKE in the knockout lines (Fig S4D). PI3K activity was substantially reduced in brain extract from *PIKE -/-* mice (Fig S4E, top panel). In agreement with the reduced PI3K activity, Akt phosphorylation was also diminished (Fig S4E, middle panel).

MATERIALS AND METHODS

Antibodies and adenovirus

Antibodies against PIKE-L were raised against the N-terminus (PIKE-L (N)) or C-terminus (PIKE-L (C)) of rat PIKE-L (Liu et al, 2008; Tang et al, 2008). These antibodies were used at 1:1000 for Western blot (WB) and 1:200 for immunohistochemical (IHC) or immunofluorescent (IF) staining. Anti-phospho-AktS473 (1:1000 for WB), anti-active caspase 3 (1:500 for WB, 1: 100 for IHC) and anti-PARP (1:1000 for WB) were purchased from Cell signaling, USA. Anti-GRIP1 (1:1000 for WB, 1:200 for IF), anti-GluA1-N-terminal (1:500 for WB) and anti-myc (1:2000 for WB) were obtained from Calbiochem, USA. Anti-GluA2-N-terminal (1:1000 for WB, 1:500 for IF) was purchased from Millipore, USA; Anti-synaptotagmin (1:200 for IF), anti-GFP (1:1000 for WB) and anti-GST-HRP (1:2000) were purchased from Sigma-Aldrich, USA. Anti-HA (1:2000 for WB) and anti-Akt1 (1:1000 for WB) were purchased from Santa Cruz Biotechnology Inc, USA.

Adenovirus experiments were performed as previously described (Tang et al, 2008). Briefly, 1×10^6 pfu/ml control adenovirus, adenovirus carrying rat PIKE-L or adenovirus carrying shRNA against PIKE (5'-AGACTGGATTCCAGCTCAA-3') were applied to the cultured hippocampal neurons. After 48 h infection, the cells were used for various experiments as stated.

Yeast two-hybrid (Y2H) screening

Y2H screen was performed with the MATCH-MAKER Two-hybrid System 2 (Clontech, USA). The GTPase domain of PIKE was subcloned downstream of the Gal4 DNA-binding domain in pAS2-1 and was used as bait to screen a human fetal brain cDNA Library in pACT2. Clones that grew on plates lacking leucine, tryptophan and histidine with 50 mM 3-aminotriazole were selected and assayed for β -galactosidase activity.

Co-immunoprecipitation and *in vitro* binding assay

HEK293 cells, cultured rat neurons or mouse brain tissues cells were lysed to perform co-immunoprecipitation and *in vitro* binding assays as described (Ye et al, 1999). For PH domain interfering experiment, tissue lysates (1 mg) were incubated with the GST or GST-PIKE-L PH domain (50 μ g) for 4 h at 4 °C. Control IgG or anti-PIKE-L N-terminus antibody was then added to the lysates and the immunoprecipitation was performed at 4 °C for 16 h.

Immunofluorescent staining

For co-localization, cultured hippocampal neurons were fixed in 4% paraformaldehyde in PBS/4% sucrose and permeabilized in 0.1% TBST. Neurons were then blocked in 5% BSA and incubated overnight at 4 °C with primary antibodies as indicated. For cell surface receptor staining, glycine-treated cultured (Man et al, 2003) hippocampal neurons were fixed, blocked with 5% BSA and incubated with antibody against the N-terminal of GluA2 at 4 °C for overnight. After permeabilized in 0.1% TBST and block with 5% BSA, the neurons were then stained with anti-synaptotagmin at room temperature for 1 h. After PBS wash, the cells were incubated with FITC-conjugated anti-rabbit antibody and Cy3-conjugated anti-mouse antibody at room temperature for 2 h. Images were acquired using a 63X objective lens on Nikon C1 laser scanning confocal system. The 3-D stack series of images were taken on dendritic regions of a neuron and then projected to 2-D images.

***In vitro* PI3K assay**

GluA2 or p110 α were immunoprecipitated to determine total or AMAP-R-associated PI3K activity as described (Ye et al, 2000).

Cell surface biotinylation assay

Cell surface GluA2 expression was detected by total cell surface biotinylation followed by NeutrAvidin pull down (Pierce, USA) as reported (Man et al, 2007).

Generation of PIKE knockout mice

Heterozygous PIKE knockout C57BL/6 mice with a targeted deletion of exon 3 to 6 of CENTG1 were generated under contract by Ozgene (Australia). Mice were then bred to homozygosity by heterozygous mating. All animal experiments were performed according to the care of experimental animal guideline and approved by the Institutional Animal Care and Use Committee (IACUC) from Emory University.

Immunohistochemical staining

Brain tissues were fixed in 4% paraformaldehyde, paraffin embedded and sectioned (8 μ m) in standard procedures. After serial rehydration and permeabilization in 0.1% TBST, sections were immunostained using specific antibodies as indicated and counterstained with hematoxylin using Zymed Histostain SP kit (Invitrogen, USA).

Recording of hippocampal slices

Hippocampal slices were prepared as described (Chen et al, 2010). In brief, transverse hippocampal slices (400 μ m) from male mice (4-8 weeks old) were prepared using a microtome (Leica VT 1000S) in an ice-cold artificial cerebrospinal fluid (ACSF) contained (in mM): 220 sucrose, 2.5 KCl, 1.3 CaCl₂, 2.5 MgSO₄, 1 NaH₂PO₄, 26 NaHCO₃, and 10 glucose. For field

potential recording, the ACSF solution contained (in mM): 120 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.0 CaCl₂, 2.0 MgSO₄, 26 NaHCO₃, and 10 glucose. Hippocampal slices were transferred to a holding chamber recovery at 34 °C for 30 min, and then at room temperature (25 ± 1 °C) for additional 2–8 hr before recording. All solutions were saturated with 95% O₂/5% CO₂. A cut was made between CA1 and CA3 in hippocampal slices to prevent the propagation of epileptiform. Slices were placed in the recording chamber that was superfused (3 ml/min) with ACSF at 32–34°C. Field excitatory postsynaptic potentials (fEPSPs) were evoked in the CA1 stratum radiatum by stimulating Schaffer collaterals with a two-concentric bipolar stimulating electrode (FHC, ME) and recorded in current-clamp by the Axon MultiClamp 700B amplifier with ACSF-filled glass pipettes (1–5 MΩ). The strength of synaptic transmission was determined by measuring the initial (a 10–60% rising phase) slope of fEPSP.

LTP was induced using TBS (10 bursts of four pulses at 100 Hz, with each burst separated by 200 ms) or two trains of HFS (100 pulse delivered at 100 Hz stimulation, with intertrain interval of 10 sec). The stimulation intensity was adjusted to 25–30 % maximum response. The average of fEPSPs recorded between 55 and 60 min after TBS or HFS were used for statistical comparisons.

For whole-cell recording, the ACSF solution contained (in mM): 126 NaCl, 26 NaHCO₃, 3.0 KCl, 1.2 NaH₂PO₄, 2.0 CaCl₂, 1.0 MgSO₄, 10 glucose, 0.02 bicuculline or 0.1 picrotoxin. CA1 neurons were visualized with infrared optics using an upright microscope equipped with a ×40 water-immersion lens (Olympus, BX51WI) and infrared-sensitive CCD camera. NMDA-EPSC was determined by subtracting the traces obtained in 100 μM DL-AP5 at + 40 mV from those obtained before. The isolated AMPA-EPSC was measured at -60 mV in the presence of 100 μM DL-AP5. The pipettes (input resistance: 2–5 MΩ) were filled with the following internal

recording solution (in mM): 135 CsMeSO₄, 8 NaCl, 8 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 mM QX314 (pH 7.25, 290 mosM). The mEPSCs were recorded at – 80 mV in cells bathed a modified ACSF containing 5.0 mM CaCl₂ and 1 μM TTX was add to the ACSF. Data were collected when series resistance fluctuated within 20% of initial values, filtered at 1 kHz, and sampled at 10 kHz.

Glycine-induced LTP in hippocampal neurons.

E18 or P0 hippocampal neurons were cultured *in vitro* for 12-17 days. Neurons were recorded in whole-cell configuration at room temperature. The internal recording solution contained (in mM): 140 CsCl₂, 2.5 EGTA, 2 MgCl₂, 10 HEPES, 2 TEA, 4 KCl, 4 ATP (pH 7.3, 300–310 mosM) whereas the extracellular recording solution consisted of (in mM): 140 NaCl, 1.3 CaCl₂, 5.0 KCl, 25 HEPES, 33 glucose, 0.001 TTX, 0.001 strychnine, 0.02 bicuculline (pH 7.4, 325–335 mosM). The trigger level for event detection was set two times higher than baseline noise. Typically, neurons were recorded for at least 40 min, and those with resistance varied by more than 20% were rejected. In some experiments, GST or GST-PH (final concentration at 5μg/ml) was included in intracellular recording solution prior to glycine application.

Statistical analysis

Results were expressed as mean ± S.E.M. and were considered significant when $P < 0.05$. Statistic analysis of the data was performed using either Student's *t*-test or two-way ANOVA followed by Bonferroni post-tests by the computer program GraphPad Prism (GraphPad Software, USA).

REFERENCES

Chen YJ, Zhang M, Yin DM, Wen L, Ting A, Wang P, Lu YS, Zhu XH, Li SJ, Wu CY, Wang XM, Lai C, Xiong WC, Mei L, Gao TM (2010) ErbB4 in parvalbumin-positive interneurons is critical for neuregulin 1 regulation of long-term potentiation. *Proc Natl Acad Sci U S A* **107**: 21818-21823

Liu Z, Jang SW, Liu X, Cheng D, Peng J, Yepes M, Li XJ, Matthews S, Watts C, Asano M, Hara-Nishimura I, Luo HR, Ye K (2008) Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase. *Mol Cell* **29**: 665-678

Man HY, Sekine-Aizawa Y, Haganir RL (2007) Regulation of {alpha}-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking through PKA phosphorylation of the Glu receptor 1 subunit. *Proc Natl Acad Sci U S A* **104**: 3579-3584

Man HY, Wang Q, Lu WY, Ju W, Ahmadian G, Liu L, D'Souza S, Wong TP, Taghibiglou C, Lu J, Becker LE, Pei L, Liu F, Wymann MP, MacDonald JF, Wang YT (2003) Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. *Neuron* **38**: 611-624

Tang X, Jang SW, Okada M, Chan CB, Feng Y, Liu Y, Luo SW, Hong Y, Rama N, Xiong WC, Mehlen P, Ye K (2008) Netrin-1 mediates neuronal survival through PIKE-L interaction with the dependence receptor UNC5B. *Nat Cell Biol* **10**: 698-706

Ye K, Compton DA, Lai MM, Walensky LD, Snyder SH (1999) Protein 4.1N binding to nuclear mitotic apparatus protein in PC12 cells mediates the antiproliferative actions of nerve growth factor. *J Neurosci* **19**: 10747-10756

Ye K, Hurt KJ, Wu FY, Fang M, Luo HR, Hong JJ, Blackshaw S, Ferris CD, Snyder SH (2000) Pike. A nuclear gtpase that enhances PI3kinase activity and is regulated by protein 4.1N. *Cell* **103**: 919-930

SUPPLEMENTAL FIGURE LEGEND

Fig S1 Association of PIKE-L and GRIP1 PDZ4 domain

- A. Schematic representation of various GRIP1 deletion truncates used in the *in vitro* binding assay.
- B. Mapping of PIKE-L interaction domain in GRIP1. Various deletion truncates of GRIP1 tagged with bacterial GST (bGST) were purified and incubated with cell lysates from HEK293 cells expressing HA-PIKE-L. The GST proteins were pulled

down using glutathione beads and the associated PIKE-L was detected using anti-HA antibody (upper panel). The expression of GST-tagged GRIP1 truncates (asterisked) was also examined (lower panel).

Fig S2 PIKE-L interacts with GluR2

- A. HEK293 cells were co-transfected with GFP-PIKE-L, GluA2 or GluA1. Immunoprecipitation was performed using antibody as indicated and the associated PIKE-L was detected using anti-PIKE-L N-terminus antibody (1st panel). The expression of PIKE-L was detected using antibody against the N-terminus (N) of PIKE-L (2nd panel). The expression of GluA1 (3rd panel) and GluA2 (4th panel) was also examined.
- B. Schematic representation of various GluA2 deletion truncates used in the in vivo binding assay.
- C. Mapping of PIKE-L interaction domain in GluA2. HEK293 cells were transfected with GFP-PIKE-L and different mutants of GluA2 as shown in (B). Immunoprecipitated was performed using antibody as indicated and the associated myc-GluA2 truncates were detected using antibody against the N-terminus of GluA2 (top panel). The expression of the GluA2 (middle panel) and PIKE-L (bottom panel) was also examined.
- D. The association between PIKE-L and GluA2 is GRIP1 independent. HEK293 cells were transfected with GFP-PIKE-L, myc-GluA2 and various amount of myc-GRIP1. Interaction of PIKE-L and GluR2 was detected using immunoprecipitation (1st panel). The expression of PIKE-L (2nd panel), GluA2 (3rd panel) and GRIP1 (4th panel) was also examined (bottom panel).

Fig S3 PIKE-L facilitates glycine-induced cell surface expression of GluA2 in cultured neurons

Hippocampal neurons (21 DIV) were infected with control adenovirus (Ctr Ad), adenovirus expressing PIKE-L (Ad PIKE-L) or adenovirus carrying shRNA against PIKE (Ad shPIKE). The cells were then treated with PBS or glycine (200 μ M) as stated in the Experimental Procedures, stained with anti-GluA2 antibody under non-permeabilized condition and visualized by confocal microscope. Magnification of area indicated was shown at the bottom of each image. Scale bar represents 20 μ m.

Fig S4 Generation of *PIKE* $-/-$ mice

- A. Schematic representation of mouse *CENTG1* (top) and the targeted gene region (bottom). The location of loxP sites were marked as solid triangles and the FRT sites were marked as solid bars.
- B. The expression of PIKE mRNA in mouse brain. RT-PCR of cDNA isolated from progeny produced from heterozygote mating. The primers used in the reaction are indicated as arrows in (A).
- C. Western blot analysis of PIKE. Proteins extracts (50 μ g) of different tissues from wild-type (+/+), heterozygous (+/-) and knockout (-/-) were prepared and the expression of PIKE-L was detected using specific antibody against the N-terminal of human PIKE-L (top panel). The amount of tubulin in each sample was examined to demonstrate equal loading (bottom panel).

- D. Immunohistochemical staining of brains from age-matched (3-months-old) wild-type and *PIKE*^{-/-} mice using antibody specific to the C-terminal of PIKE. Scale bar represents 50 μm .
- E. Reduced PI3K/Akt activity in *PIKE*^{-/-} brain. Mouse brain tissues were isolated and subjected to immunoprecipitation with anti-p110 α antibody followed by in vitro PI3K assay (top panel). The phosphorylated Akt (middle panel) and total Akt (bottom panel) were determined by Western blot analysis using specific antibodies as indicated.

Fig S5 Cell surface expression of GluA2 under glycine treatment is impaired in *PIKE*^{-/-} neurons

Hippocampal neurons (21 DIV) were treated with PBS or glycine (200 μM) as stated in the Experimental Procedures, stained with anti-GluA2 antibody under non-permeabilized condition and visualized by confocal microscope. Magnification of area indicated was shown at the bottom of each image. Scale bar represents 20 μm .

Fig S6 Cell surface expression of GluA2 is rescued in *PIKE*^{-/-} neurons after PIKE-L overexpression.

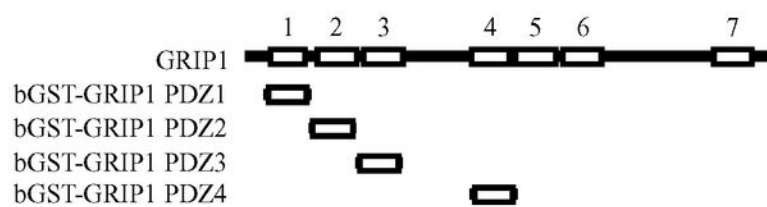
Hippocampal neurons (21 DIV) were infected with control adenovirus (Ctr Ad) or adenovirus overexpressing PIKE-L (Ad-PIKE-L) for 48 h. The cells were then stimulated with PBS or glycine (200 μM , 20 min) and stained with anti-GluA2 antibody under non-permeabilized condition.

Fig S7 Normal paired-pulse facilitation ratio of evoked EPSCs in *PIKE* ^{-/-} at SC-CA1 synapses.

- A. Sample Representative evoked EPSC traces by successive stimuli from control and *PIKE* ^{-/-} mice by holding membrane potentials at -70 mV in presence of 20 μ M bicuculline. Scale bars are 50 mV and 50 ms.
- B. Normal PPRs of evoked EPSCs at SC-CA1 in *PIKE* ^{-/-} hippocampus (n = 5 for both groups). Data are presented as the mean \pm SEM. No statistically significant differences were detected in all conditions.

Figure S1

A



B

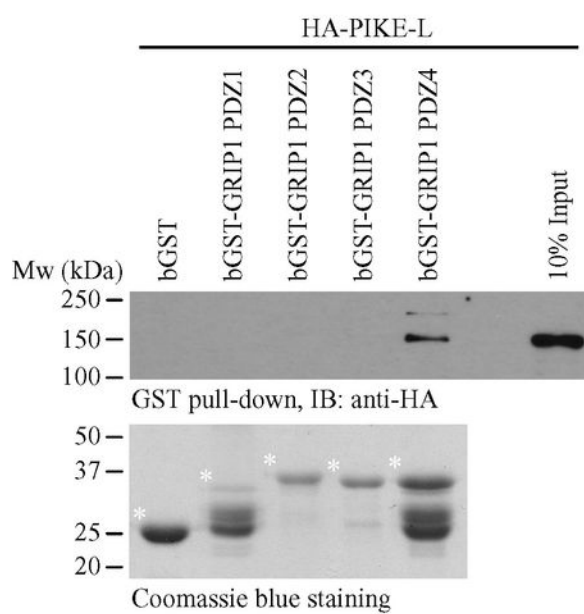
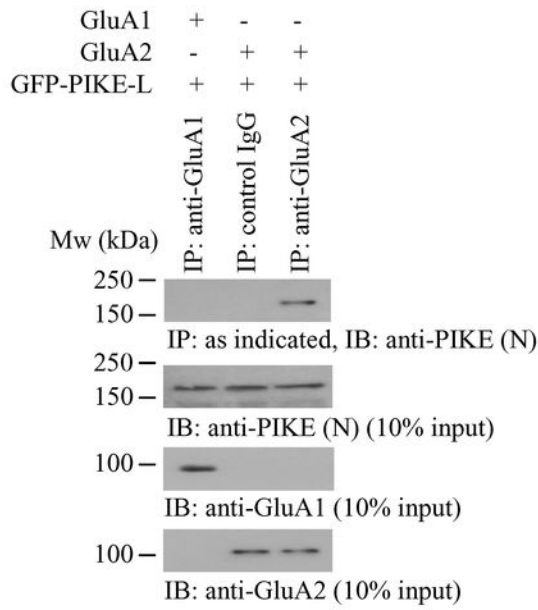
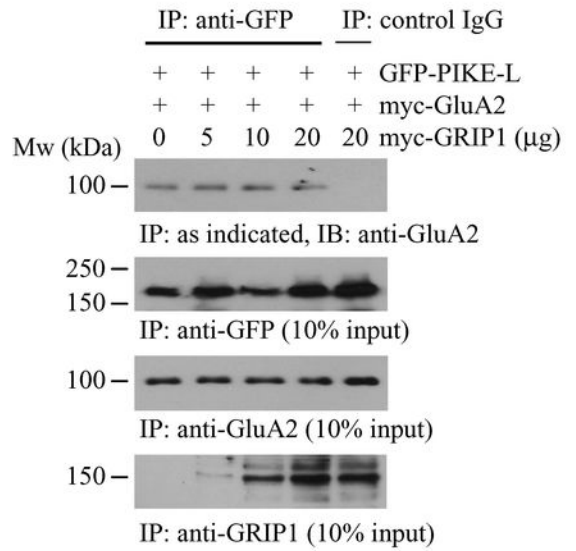


Figure S2

A



D



B

		<u>p85 binding</u>		<u>PDZ binding</u>	
myc-R2 WT	831	ALIEFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI			883
myc-R2 SVKE	831	ALIEFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKE			883
myc-R2 1-853	831	ALIEFCYKSRAEAKRMKVAKNPQ			853

C

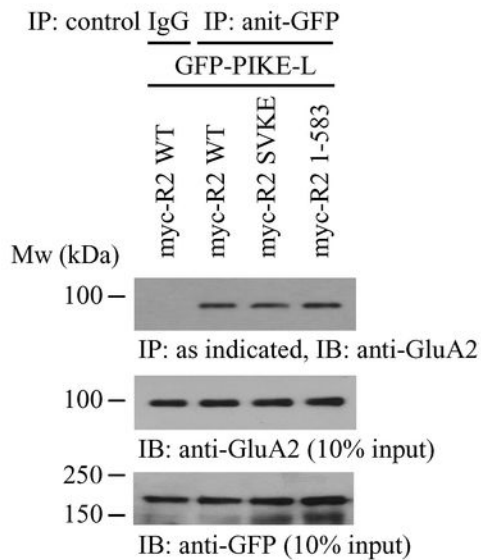


Figure S3

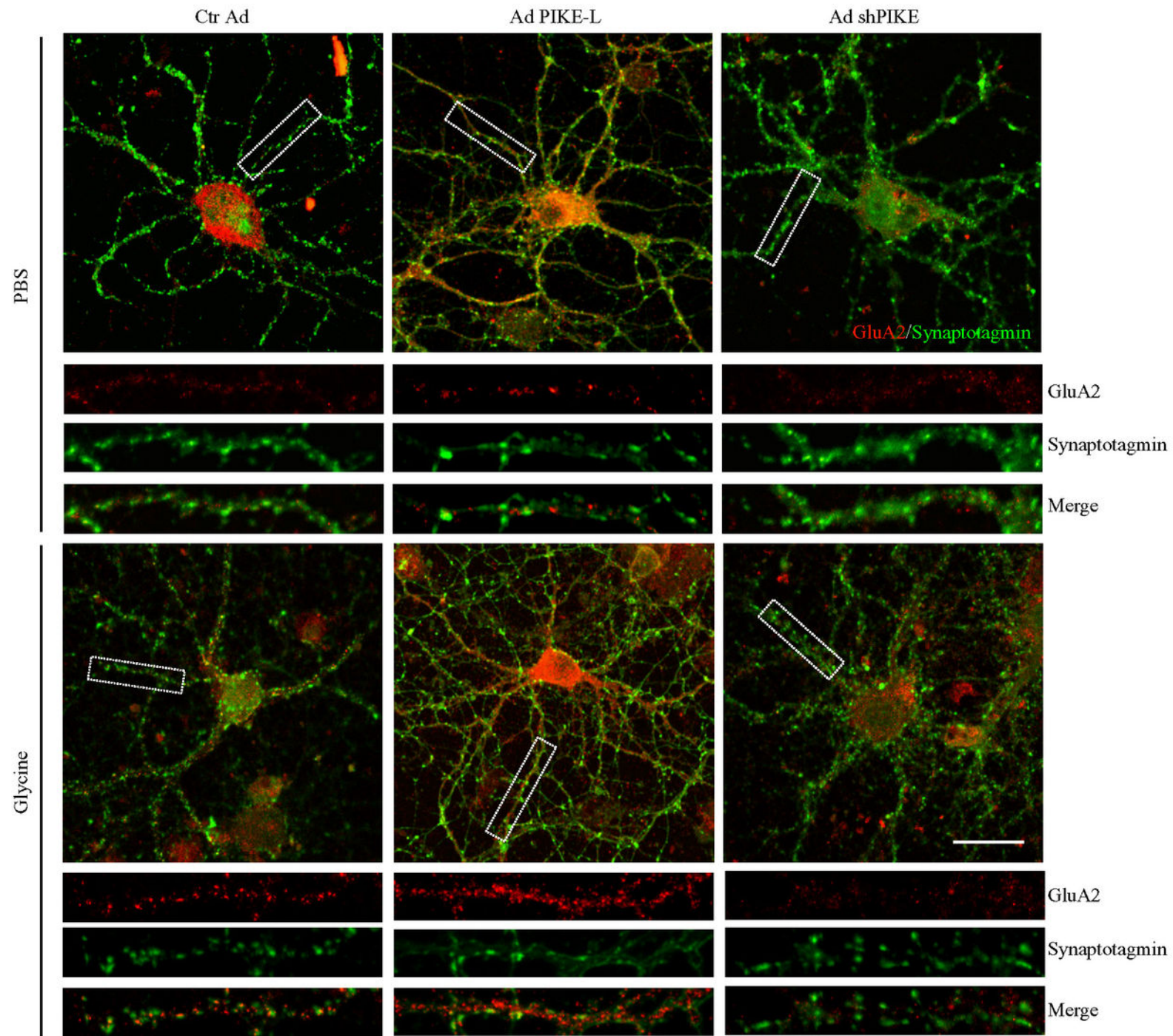
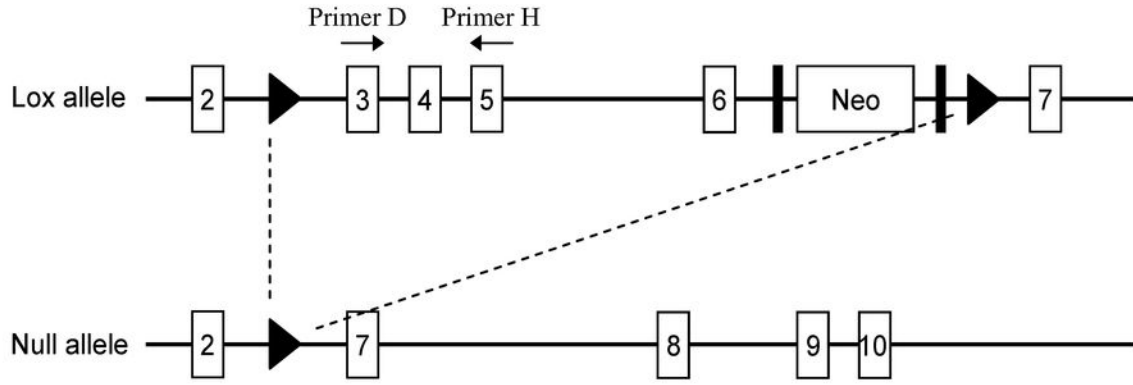
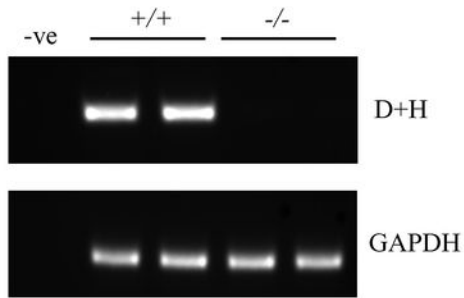


Figure S4

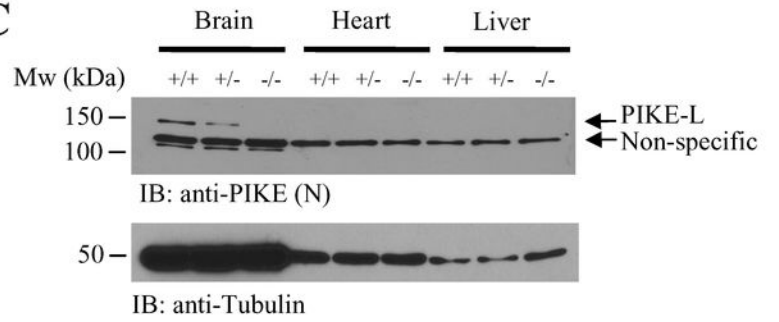
A



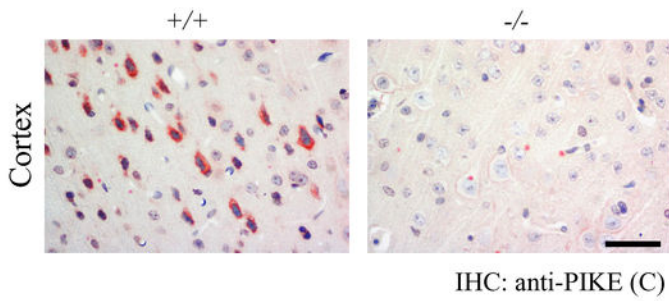
B



C



D



E

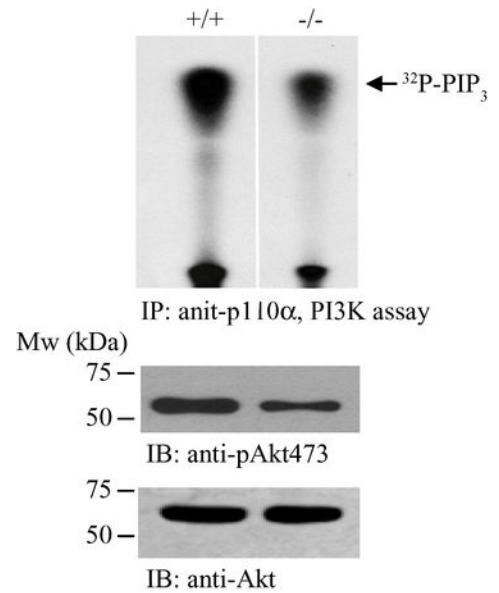


Figure S5

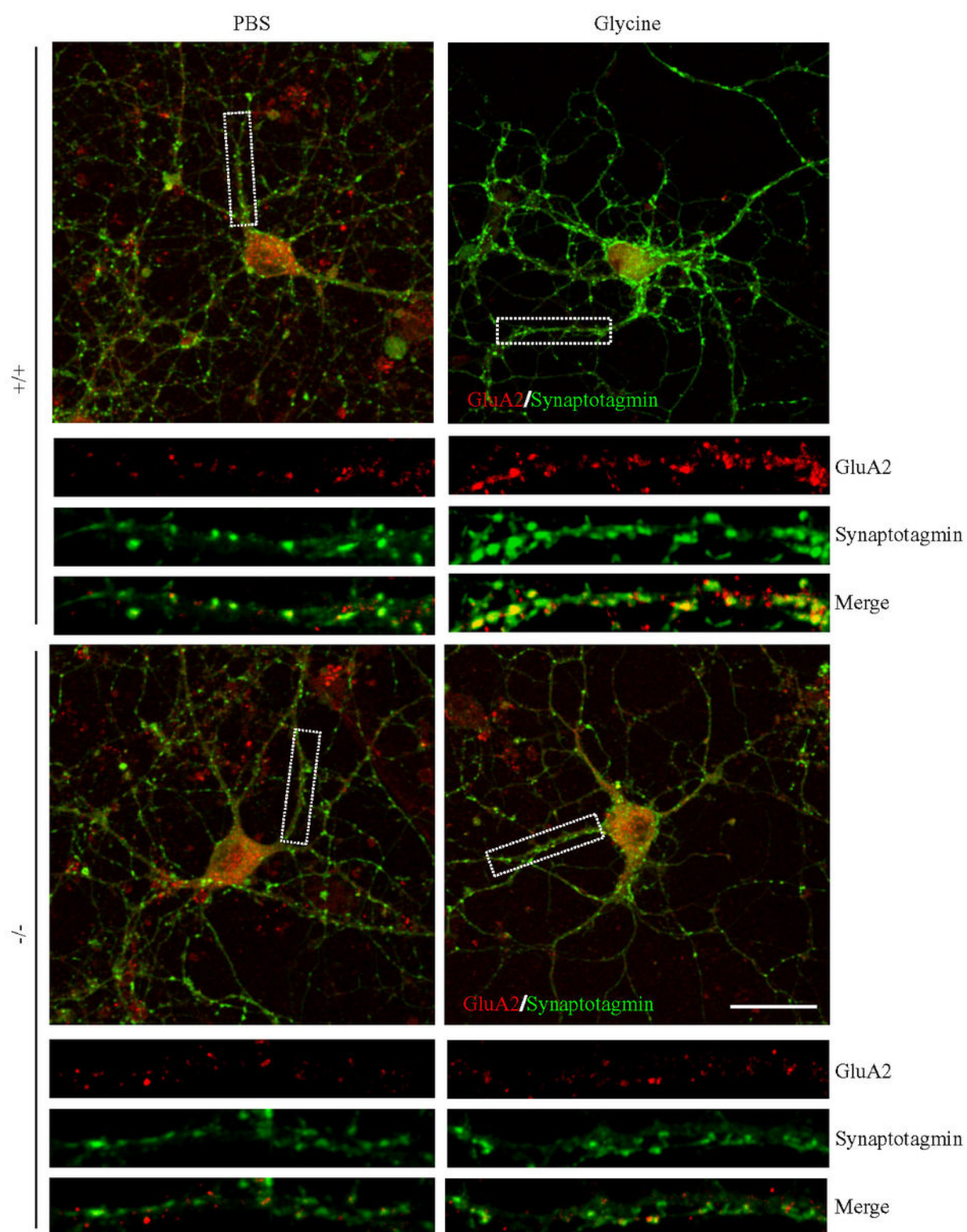


Figure S6

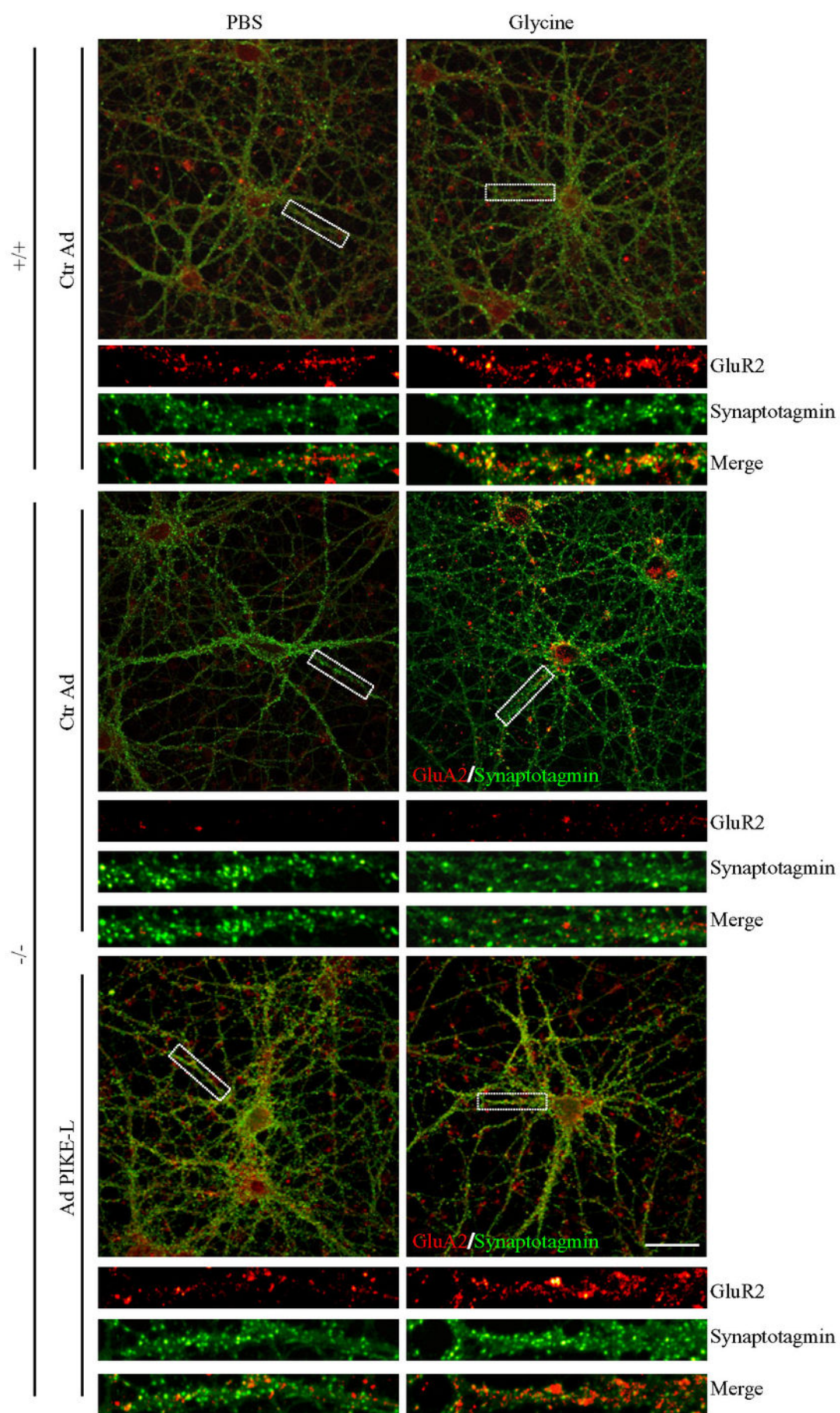


Figure S7

