Cell Reports

Supplemental Information

Bidirectional Control of Synaptic $GABA_AR$ Clustering by Glutamate and Calcium

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1. Supplemental Experimental Procedures

Animals

All experiments in this study were carried out in accordance with the guidelines issued by the Japanese Ministry of Education, Culture, Sports, Science and Technology and approved by the Animal Experiment Committee of the RIKEN and Nagoya Univ. IP_3R1 knock-out mice and Homozygous mRFP-gephyrin knock-in mice are described previously (Calamai et al., 2009; Matsumoto et al., 1996).

Primary cultures of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from E18–21 Wistar rat embryos as previously described (Goslin et al., 1998). Neurons were plated at a density of $1.3x10^4$ cells/cm^2 onto glass coverslips coated with 80 μg/ml poly-D, L-ornithine or 0.04% polyethyleneimine (Sigma). Cultures were maintained in Neurobasal medium supplemented with B27, 2 mM L-glutamine, and antibiotics (all from Life Technologies, CA, USA). Cells were cultured at 37°C in a 5% CO_2. At least three independent cultures were used for each experiment at 21–27 days *in vitro* (DIV) unless described.

Drug treatment

The following drugs were used: 2APB (100 μM; Daiichi Kagaku, Japan) to block IP_3R channel activity, U73122 (1 μM; Calbiochem, CA, USA) to inhibit IP_3 production and MCPG (250 μM; Tocris, MO, USA) to antagonize mGluR activity, TTX (1 μM; Tocris) to block voltage gated Na^+ channel, NMDA (50 μM; Tocris), glycine (5 μM) and TTX (1 μM) cocktail to induce the Ca^{2+} influx mediated dispersal of $GABA_AR$, DHPG (5 μM; Tocris) to enhance mGluR activity, dynasore (80 μM; Tocris) to prevent dynamin-dependent endocytosis, CysA (1 μM; Santa Cruz, CA, USA) and FK506 (1 μM; Tocris) to inhibit calcineurin activity, Gö6976 (500 nM; Calbiochem) to inhibit Ca^{2+} dependent PKC, PMA to activate PKC, and 4α-PMA as an inactive analogue of PMA (200 nM; Enzo Life Sciences inc. NY, USA). Stock solutions of 2APB, CysA, Gö6976, PMA and 4α-PMA were prepared in DMSO; other drugs were prepared in water. Neurons were acutely exposed to the different drugs for the indicated duration at 37°C in imaging medium comprising MEM without phenol red (Life Technologies), 20 mM HEPES, 33 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, and B27. To remove Ca^{2+} in the imaging medium (Fig. S1 C-D), MEM were pre-incubated with chelating resin Chelex (Bio Rad) for 60 min at room temperature before adding other components.

Immunocytochemistry and quantitative analysis

Immunochemical detection of $GABA_AR\gamma2$ subunit and gephyrin in cultured neurons was done as previously described (Bannai et al., 2009; Niwa et al., 2012). Endogenous $GABA_AR\gamma2$ subunits were labeled with a rabbit anti- $GABA_AR\gamma2$ subunits antibody (Niwa et al., 2012) by incubating live cells for 30 min at 37°C with 2.0 μg/ml of antibody diluted in imaging medium. Cells were then fixed for 15 min at room temperature (RT; 24–26°C) in paraformaldehyde (PFA; 4% w/v) solution prepared in PBS-0.02% NaN_3. Cells were permeabilized with triton X-100 (0.1% v/v) for 3 min at RT and the nonspecific staining was blocked with bovine serum albumin (BSA; 5% w/v; Sigma-Aldrich, MO, USA) for 30 min at RT. Neurons were then incubated with the mouse anti-synapsin I antibody (1:3000; Synaptic Systems, Goettingen, Germany) or guinea pig anti-PKCα antibody (1 μg/ml; Frontier institute,

Japan) or guinea pig anti-PKCβII antibody (1 μg/ml; Frontier institute) or guinea pig anti-PKCγ antibody (1 μg/ml; Frontier institute) in 2.5% BSA for 60 min at RT. After washes, the cells were incubated for 30 min at RT in Alexa Fluor®-conjugated secondary antibodies (5–10 μg/ml, Alexa Fluor 488 or Alexa Fluor 594; Life Technologies), washed, and mounted on slides with Vectashield (Vector Laboratories, CA, USA). For gephyrin labeling, cells were fixed immediately after the drug treatment and followed by permealization and blocking. Cells were incubated for 90 min at RT with the mouse anti-gephyrin antibody (0.33 μg/ml, clone mAb7a; Synaptic Systems) and the rabbit polyclonal anti-synapsin I antibody (1:400; Merck Millipore, MA, USA). The primary antibodies were visualized with secondary Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit antibodies (5–10 μg/ml; Life Technologies).

Immunofluorescent images from isolated neurons were acquired on an inverted microscope (IX-70; Olympus, Tokyo, Japan) equipped with a Plan Apo 60× oil immersion objective with a numerical aperture (NA) of 1.42 (Olympus), a cooled CCD camera (Orca-II-ER; Hamamatsu Photonics, Shizuoka, Japan), and appropriate filter sets for Alexa Fluor 488 (ex: 480 ± 10 nm, em: 530 ± 20 nm) and Alexa Fluor 594 (ex: 535 ± 15 nm, em: 580 nm long pass). All images from the same culture were acquired with the same sub-saturation exposure time.

Quantification of synaptic $GABA_AR$-, gephyrin-, synapsin and PKC-associated immunofluorescence was performed using "Integrated Morphometry Analysis" function of the MetaMorph software (Molecular Device Japan, Tokyo, Japan) as previously described (Bannai et al., 2009; Charrier et al., 2006; Levi et al., 2004; Levi et al., 2008; Niwa et al., 2012). $GABA_AR$- and gephyrin-immunoreactive clusters and synapsin-positive presynapses were defined by processing images with multidimensional image analysis (MIA) interface, i.e., a 2D object segmentation by wavelet transform (Racine et al., 2007) and "auto threshold for light object (isodata method)" function of MetaMorph. Synaptic $GABA_AR$ or gephyrin clusters were defined as clusters that overlapped at least 1 pixel with presynaptic terminals. The fluorescence intensity of $GABA_AR$ or gephyrin clusters was defined as the mean fluorescence intensity per cluster multiplied by the mean area per cluster. For PKC immunofluorescence showing diffuse labeling, we measured the fluorescence intensity per pixel of PKC overlapping with $GABA_AR$ clusters. All the data reported here showed same tendency in independent experiments using more than 3 independent culture sets. For population data, data values from 10 or more cells were divided by the average of control levels for that batch, and pooled for statistical analysis.

Time-lapse imaging of mRFP-gephyrin cluster

Time-lapse video microscopy was performed on hippocampal neuron cultures prepared from mRFP-gephyrin expressing knock-in mice (Machado et al., 2011) and as described before (Hanus et al., 2006). Experiments were performed on 19-20 DIV neurons and MEM recording medium (Phenol red-free MEM, 33 mM glucose, 20 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 1× B27) was used. Time-lapse imaging was carried out on an inverted Nikon Eclipse Ti microscope equipped with a 100× oil-immersion objective (NA 1.49) and excitation / emission filter (FF01-560/25 / FF01-607/36, Semrock) using an Andor iXon EMCCD camera (image pixel size, 160 nm, 300 ms exposure time) in a controlled environment maintained at 35ºC.

Electrophysiology

Whole cell patch clamp experiments on primary cultured neurons were carried out with following solutions. The internal solution contained (in mM): $CsCl_2$, 140; EGTA, 0.2; HEPES, 10; Mg-ATP, 2; GTP-Tris, 1; Na-phosphocreatine, 2.5 (pH 7.2-7.3, 280-290 mOsm). The extracellular recording solution contained (in mM): NaCl, 147; KCl, 2.1; HEPES, 8.8; D-glucose, 8.8; $CaCl_2$, 1.1; $MgCl_2$, 1.1; Pyruvic Acid, 0.026%(v/v) (pH 7.4, 310 mOsm).

Hippocampal brain slices were prepared from postnatal day 14 to 21 BL56/J IP3R1-/- mice and WT littermates. Mice were anesthetized with isoflurane and decapitated, and the brain was rapidly removed and transferred to ice cold cutting solution containing in mM: Choline-Cl, 120; KCl, 3; D-Glucose, 20; $MgCl_2$, 8; NaH_2PO_4, 1.25; $NaHCO_3$, 26; bubbled with 95% O_2/5% CO_2. Acute slices (300 µm thick) were cut on a VT1000S Vibratome (Leica), transferred at 34°C for 15-20min to artificial cerebrospinal fluid (aCSF) containing in mM: NaCl, 125; KCl, 2.5; D-Glucose, 25; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1.25; $NaHCO_3$, 25; 320 mOsm, bubbled with 95% O_2/5% CO_2, pH 7.35. Slices were allowed to recover, for at least one hour at RT before use. For experiments on acute slice, the internal solution contained (in mM): $CsCl_2$, 130; EGTA, 10; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 10; Mg-ATP, 2; GTP-Tris, 0.1; Na-phosphocreatine, 2.5 (pH 7.4, 290-300 mOsm). The extracellular recording solution was the aCSF solution.

Spontaneous mIPSCs were recorded in the whole-cell voltage-clamp configuration, in the presence of 2,3-Dihydroxy-6-nitrobenzo[f]quinoxaline-7-sulfonamide (NBQX, 10 µM), D-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 µM), and TTX (1 µM). When required the extracellular recording solution was supplemented with 250 µM MCPG or vehicle (NaOH). Whole-cell patch pipettes (3-5 MΩ) were pulled on a P-97 Micropipette Puller (Sutter Instruments), coated with sylgard-184/R6101 (Dow Corning), and filled with internal solution. All experiments were performed at room temperature.

Whole-cell recordings were obtained using AxopPatch 200B (Axon Instruments) amplifier and acquired with a Digidata1322A A/D converter (Axon Instruments) controlled by pClamp 9 (Axon Instruments). Signals were low-pass filtered at 5/10 kHz and acquired at 50/100 kHz. Series resistance and capacitance were corrected/compensated 70-80%. The membrane potential was held at −70 mV (junction potential left uncorrected). Under these recording conditions GABAergic chloride currents were recorded as inward currents. Detection of individual mIPSC was performed offline using the template-matching event detection algorithm in Clamp fit 9.2 (Axon Instruments). To avoid bias due to a different number of events from each recording, the same number of mIPSC events for each cell was taken from the beginning of the recording, for statistical analysis.

Production of a rabbit $GABA_AR$ β3 subunit antiserum

The rabbit anti-$GABA_AR$ β3 subunit antibody (anti-$GABA_AR$ β3) was raised as described previously (Todd et al., 1996). Purified fusion protein consisting of maltose-binding protein (MBP) and amino acids 345–408 of the mouse $GABA_AR$ β3, part of intracellular loop between transmembrane domains M3 and M4, were injected into rabbits to raise the antibody by the Support Unit for Animal Resources Development at the RIKEN BSI RRC. To obtain fusion proteins, the DNA sequence corresponding to amino acid 345-408 of the $GABA_AR$ β3 was amplified by PCR using FANTOM3 clone C630014N19 from RIKEN Genomic Sciences Research Complex as a template (Carninci et al., 2005), and subcloned into pMAL-C vector (New England Biolabs, MA, USA). Recombinant MBP fusion proteins were expressed in *E. coli* BL21 and purified with amylose resin (New England Biolabs).

The specificity of this antibody was confirmed by Western Blot using cell lysates from HeLa cells (RIKEN BioResource Center, Ibaraki, Japan) transfected with plasmids encoding the α1, β3, and γ2 $GABA_AR$ subunits (Niwa et al., 2012) or from extracts of rat hippocampal primary cultures (Fig. S5C). HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and transfected using TransIT-LT1 as described previously (Niwa et al. 2012). Confluent (70–80%) Hela cells cultured on 18 mm coverslips were lysed with 100 µl SDS-PAGE sampling buffer, while rat hippocampal primary cultures were lysed with RIPA buffer as described previously (Bannai et al., 2009) (see "**Surface biotinylation assay**"). The Western blot was done with 1 µg of proteins from 5 µl of cell lysates and using the rabbit $GABA_AR$ β3 serum at 1:2000.

Surface biotinylation assay

Surface biotinylation assay was carried out as previously described (Bannai et al., 2009; Saliba et al., 2007). Neurons were washed twice in PBS (1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and incubated for 30 min in 0.25 mg/ml sulfo-NHS-SS-biotin (Pierce) followed by 20 min washes in 100 mM glycine to quench excess biotin. Then neurons were lysed in radioimmunoprecipitation assay (RIPA) buffer: 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, protease inhibitor cocktail (Roche). Detergent-soluble extracts (150 µg) were incubated for 2 h with immobilized NeutrAvidin (Pierce, 60 µl 1:1 slurry) to purify biotinylated proteins. All steps were done at 4°C.

Western blot

Neurons were treated by SDS sample buffer: 62.5 mM TrisHCl, pH 6.8, 10 (w/v) % glycerol, 2% SDS, 5 (v/v) % β-ME, 0.05% BPB. The following primary antibodies were used: rabbit anti-$GABA_AR$ β3 antiserum (1:2000), rabbit anti-PKCα antiserum (1:100,000; Sigma-Aldrich), guinea pig anti-PKCβII antibody (200 ng/ml; Frontier institute) or guinea pig anti-PKCγ antibody (200 ng/ml concentration; Frontier institute). The primary antibodies were revealed using horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG (1:5000; GE Healthcare Japan, Tokyo, Japan) or goat anti-guinea pig IgG (1:5000; Cappel, CA, USA). Chemiluminescence from HRP reacted to Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) were detected by Imagequant LAS-4000 mini (GE healthcare) and quantified using Image J.

Ca^{2+} imaging

Ca^{2+} imaging was performed as described previously (Niwa et al., 2012). Neurons were incubated with 0.5 µM fluo-4 AM (Life Technologies) for 5 min at 37°C for loading. Fluo-4 signal was acquired at 0.2 Hz with a 200-ms exposure at room temperature (24–26°C), under an inverted microscope (IX-70; Olympus, JP) equipped with a 40× objective (NA 0.85, UPlanApo; Olympus), a cooled CCD camera (Orca-II-ER; Hamamatsu Photonics), and filters (ex, 480 ± 10 nm; em, 530 ± 20 nm). The ratio of the fluorescence intensities F/F0, where F is a fluorescence intensity and F0 is the intensity at t = 0, was obtained after subtraction of the background fluorescence.

QD-SPT experiments

QD labeling and SPT of $GABA_ARs$ and mGluR5s were performed as previously described (Bannai et al., 2006). Neurons were incubated with our rabbit anti-$GABA_AR\gamma2$ antibody (2.0 µg/ml) or anti-mGluR5 antibody (1.9–2.8 µg/ml) for 5 min, washed, and incubated with the biotinylated anti-rabbit Fab antibody (2.2 µg/ml; Jackson ImmunoResearch, PA, USA) for 5 min. Following washes, the coverslips were incubated with 1.0 nM streptavidin-coated QDs emitting at 605 nm or 625 nm (Life Technologies) in borate buffer for 1 min. After washes, functional presynaptic boutons were labeled with 2 µM FM4-64 (Life Technologies) in imaging medium containing 40 mM KCl for 15 s. Incubation with antibodies and washes were performed at 37°C in imaging medium.

Recording of $GABA_AR$-QD behavior and FM4-64 signals was performed at 37°C in the imaging medium using an inverted microscope (IX-70, -71 or -73 Olympus) equipped with an oil immersion objective (60×, NA > 1.42, Olympus) and a cooled-CCD camera (ORCA-II-ER, Hamamatsu Photonics) or an EM-CCD camera (Cascade, Roper Scientific; ImagEM, Hamamatsu Photonics). Fluorescent signals were detected using appropriate filter sets for QD (ex: 455 ± 70 nm, em: 605 ± 20 nm) and FM4-64 (ex: 535 ± 15 nm, em: 580 nm long pass). QD movies were recorded with an integration time of 76 ms with 512 consecutive frames

(38.9 s), or 200 frames for IP_3R1KO neurons. All recordings were finished within 30 min after labeling.

SPT data analysis

QD-SPD data were analyzed using TI workbench software written by Dr. T. Inoue (Waseda University) as described previously (Bannai et al., 2009; Niwa et al., 2012). $GABA_AR$-QD localization was determined by fitting QD images with a Gaussian model of the point spread function, and the trajectories were reconstructed. Only signals from single QDs with blinking were analyzed. The synaptic area was defined by processing FM4-64 images with wavelet decomposition (Racine et al., 2007). "synaptic" $GABA_AR$-QD trajectories were defined when they overlap with synaptic area + 2 pixels (284 nm). The $GABA_AR$-QD dwell time inside the synapse was defined as the duration of synaptic sub-trajectories.

Diffusion parameters, such as the diffusion coefficient and confinement size, were obtained from the mean square displacement (MSD) plot versus time (MSD-nτ *plot)* that were calculated for $GABA_AR$-QD trajectory by applying the following equation:

$$MSD(n\tau) = \frac{1}{N-n}\sum_{i=1}^{N-n}\left[\left(x\left((i+n)\tau\right)-x(i\tau)\right)^2+\left(y\left((i+n)\tau\right)-y(i\tau)\right)^2\right] \text{(Eq. 1)}$$

((Saxton and Jacobson, 1997)), where xi and yi are the positions of $GABA_AR$-QD in frame i, N is the total frame number, τ is the acquisition time in one frame (76 ms), and nτ is duration over which the displacement is calculated. Diffusion coefficients (*D*) were calculated by fitting first four points excluding the origin of the MSD-nτ plot with the following equation:

$MSD(n\tau) = 4Dn\tau + b$ (Eq. 2),

where *b* is a constant representing localization accuracy (Ehrensperger et al., 2007). The confinement domain size was obtained by fitting the MSD-nτ plot to the following equation:

$$MSD(n\tau) = \frac{L^2}{3}\left(1-\exp\left(-\frac{12Dn\tau}{L^2}\right)\right)+4D_{mac}n\tau \text{ (Eq. 3)}$$

(Kusumi et al., 1993), where L^2 is the estimated maximal area of diffusion when diffusion is confined, and D_{mac} is the diffusion coefficient on a long time scale. The diffusion of $GABA_AR$-QD with MSD-nτ plot that does not apply $|D\text{-}D_{mac}| < 0.1 \times D$ or $L < 0.001$ was defined as restricted motion, and only $GABA_AR$-QDs meeting this criteria were considered for calculations of confinement domain sizes (Ehrensperger et al., 2007). The sub-trajectories shorter than 29 frames were excluded for the calculation of D and L.

$GABA_AR$ γ2 mutant analysis

The $GABA_AR$ γ2L subunit was sub-cloned into pCDNA3.1zeo(+) as described previously (Niwa et al., 2012), and a *PstI* site, corresponding to amino acid LQ, were introduced between amino acids 4 and 5 of γ2L as previously described (Kittler et al., 2000). A Myc-epitope sequence (EQKLISEEDL) was then inserted into this *PstI* site to allow specific QD labeling of mutant γ2L through the myc tag. Point mutations on S327/343 (Fig.S7A) were introduced as described previously (Sawano and Miyawaki, 2000). Plasmid DNAs were transfected using Lipofectamine 2000 (Life technologies) at 3-4 DIV and QD-SPT was carried out 2 days after transfection. To label $GABA_AR$γ2 mutants, anti-Myc tag antibody, clone 4a6 (1:500, Merck Millipore) and biotinylated anti-mouse Fab antibody (2.6 μg/ml; Jackson ImmunoResearch, PA, USA) were used.

Statistical analysis and image preparation

Statistical analyses were performed using the Mann–Whitney *U* test, Welch's *t*-test, and Tukey's range test in ANOVA, with KaleidaGraph (Synergy Software, PA, USA). The numbers of QDs analyzed are shown in Table S1. Images were prepared for printing using

MetaMorph, TI Workbench, Microsoft Excel, Adobe Photoshop, and Adobe Illustrator.

2. Reference for Supplemental Experimental Procedures

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4. Supplemental Figures and Legends

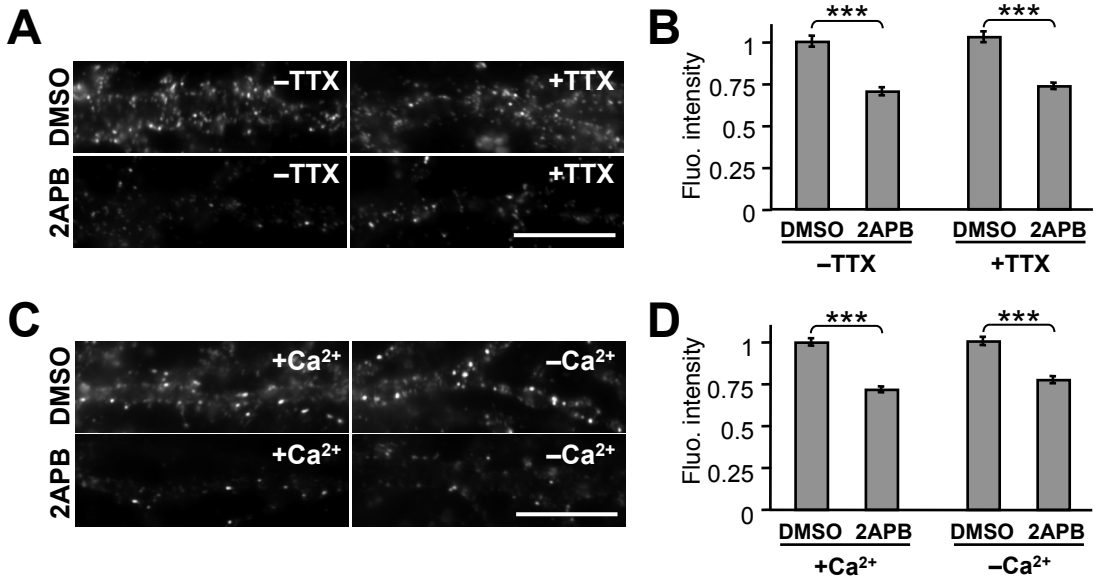

Figure S1. 2APB-induced synaptic $GABA_AR$ decrease is independent of synaptic activity and Ca^{2+} influx

A: Staining for $GABA_AR\gamma2$ of neurons treated with DMSO or 2APB for in the absence or the presence of TTX. **B**: Fluorescent intensities normalized to DMSO without TTX condition (average ± SEM) of synaptic $GABA_AR\gamma2$. **C**: $GABA_AR\gamma2$ cluster after 60 min DMSO or 2APB treatment with or without external Ca^{2+}. **D**: Normalized fluorescence (average ± SEM) of synaptic $GABA_AR\gamma2$. Average fluorescence DMSO with Ca^{2+} condition was assigned as 1. Scale bars: 10 μm. ***: $p < 0.005$, Welch's *t*-test, n = 29–30 cells/condition.

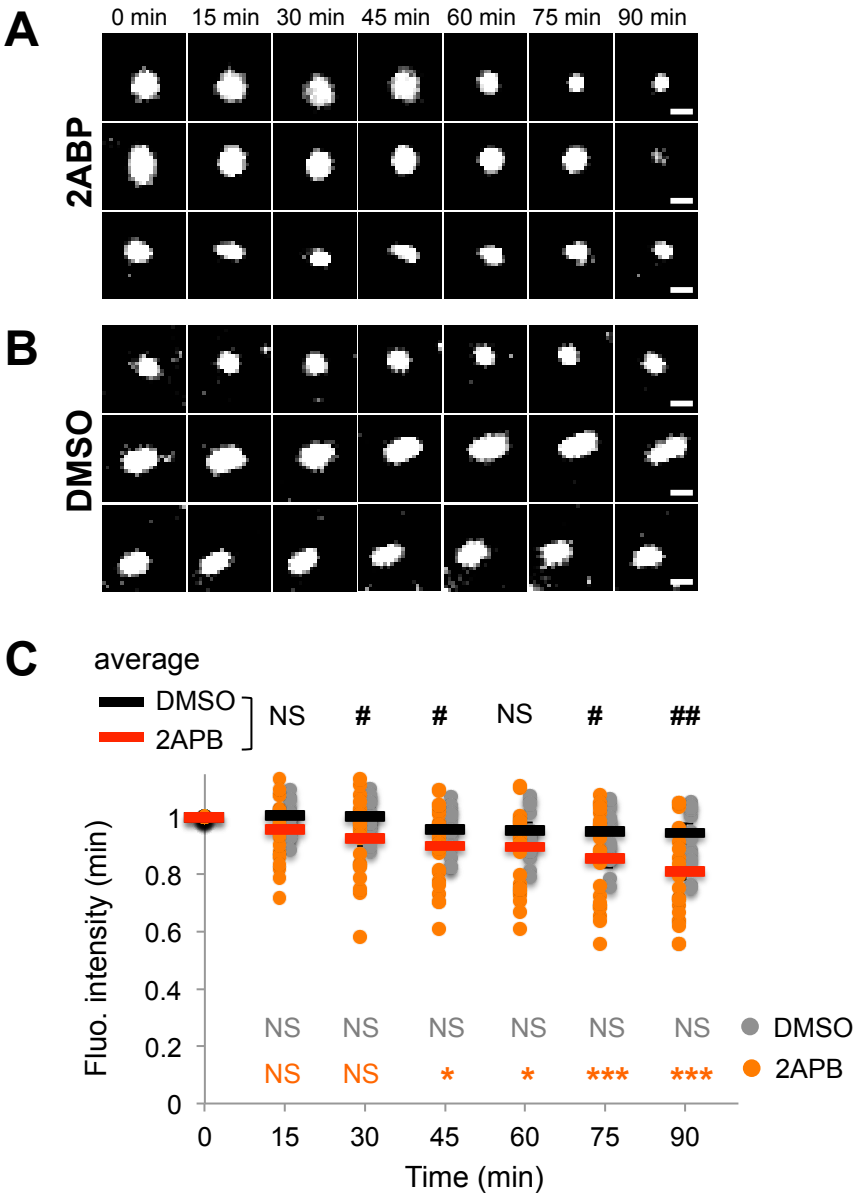

Figure S2. Time-lapse imaging of mRFP-gephyrin clusters

Cultured hippocampal neurons (19-20 DIV) from mRFP-gephyrin knock-in mice treated with 2ABP (100 µM, **A**) or DMSO (**B**). Three representative mRFP-gephyrin clusters shown for 2ABP (**A**) or DMSO (**B**). Scale: 1µm.

C: Time-dependent reduction in cluster fluorescence intensity following 2ABP (orange) but not DMSO (grey) treatment. Bars indicate average value (black: DMSO, red: 2APB).

Bottom: one-way ANOVA with Dunnett's post-hoc test to compare difference from 0 min (grey for DMSO, red for 2APB *: $p<0.05$ / ***: $p<0.001$, NS: non-significant. n= number of movies, DMSO: 15; 2ABP: 24). Top: Unpaired t-test with Welch's correction between DMSO and 2ABP for different time-points (#: $p<0.05$, ##: $p<0.01$, NS: non-significant).

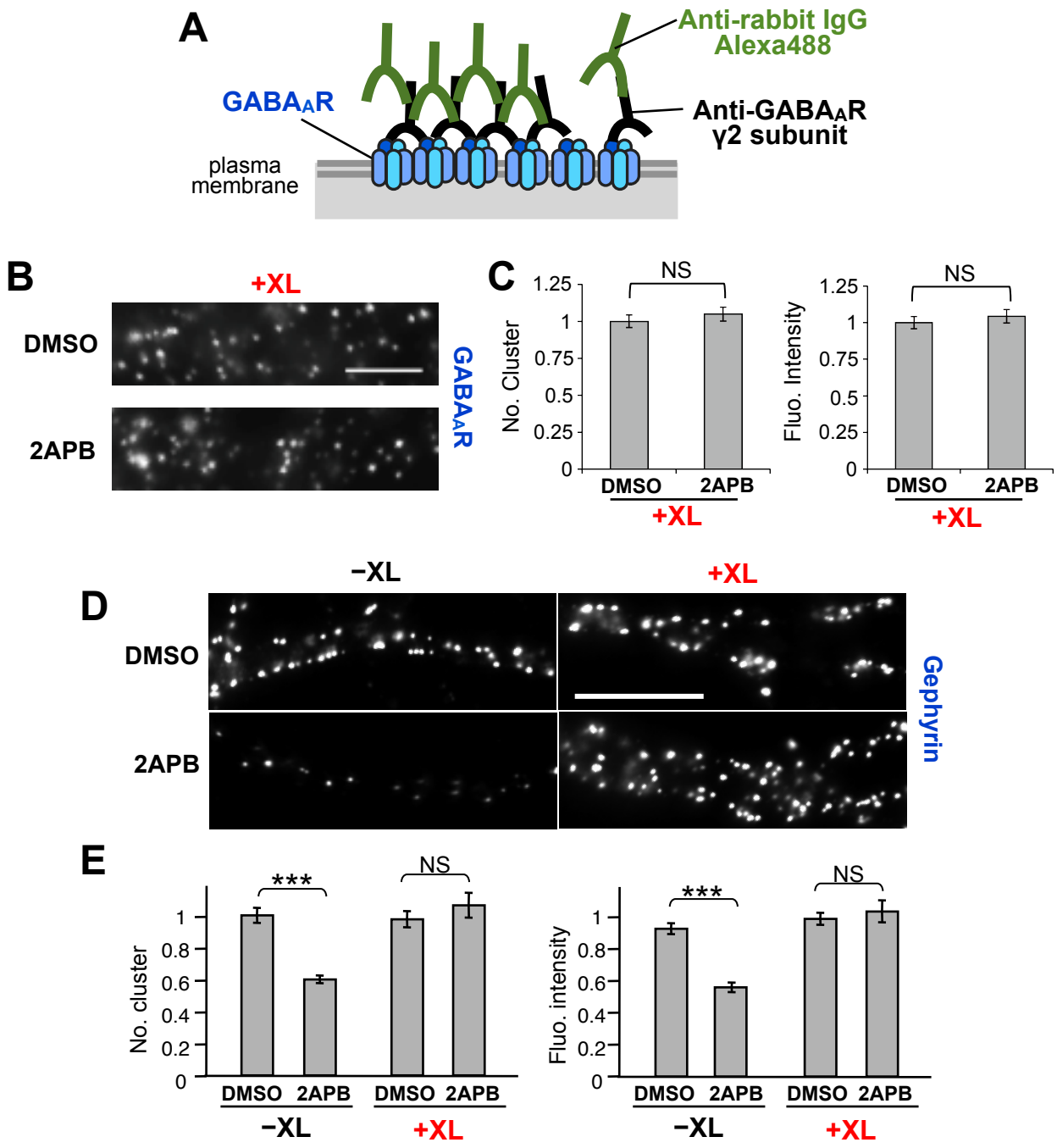

Figure S3. Suppression of 2APB-induced reduction in gephyrin immunofluorescence by $GABA_AR$ immobilization by $GABA_AR$ crosslinking (XL)

A: Diagram of crosslikning (XL). **B**: Examples of $GABA_AR$-immunoreactive clusters in dendrites with or without surface $GABA_AR$ cross-linking (+XL) treated with DMSO or 2APB for 90 min. Scale bar, 5 µm. **C**: Effects of $GABA_AR$ XL and 2APB treatment on the normalized number of clusters (left) and normalized fluorescence intensities (right) of gephyrin clusters (averages ± SEM) n = 30 cells/condition (3 cultures). XL of surface $GABA_AR$ completely inhibited 2APB-induced $GABA_AR$ declustering. **B**: Examples of gephyrin-immunoreactive clusters in dendrites with (+XL) or without (−XL) surface $GABA_AR$ cross-linking treated with DMSO or 2APB for 90 min. Note that 2APB-induced declustering of gephyrin was inhibited by surface $GABA_AR$ XL. Scale bar, 5 µm. **C**: Effects of $GABA_AR$ XL and 2APB treatment on the normalized number of clusters (left) and normalized fluorescence intensities (right) of gephyrin clusters (averages ± SEM). n = 30 cells/ condition (3 cultures). NS: $p > 0.05$; ***: $p < 0.005$, Welch's *t*-test, 2APB-induced reduction in gephyrin cluster size was completely suppressed by $GABA_AR$ XL.

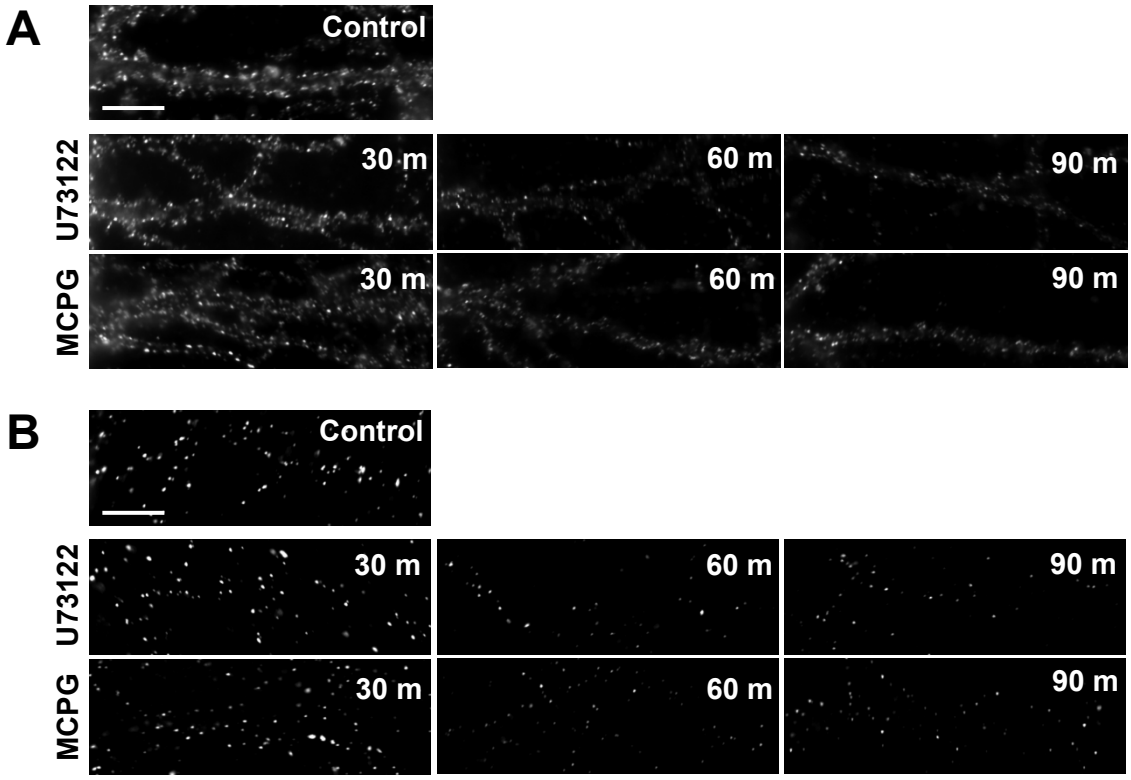

Figure S4. Prolonged blockade of IICR pathway results in declustering of $GABA_AR$ and gephyrin clusters.

Representative examples of immunoreactivity associated with $GABA_AR$ (**A**) and gephyrin (**B**) in the dendrite or rat hippocampal neurons (DIV21–27) of control and those treated 1 μM U73122 and 250 μM MCPG for 30, 60 and 90 min. Scale bar, 10 μm. For quantitative date, see Fig. 2B and D.

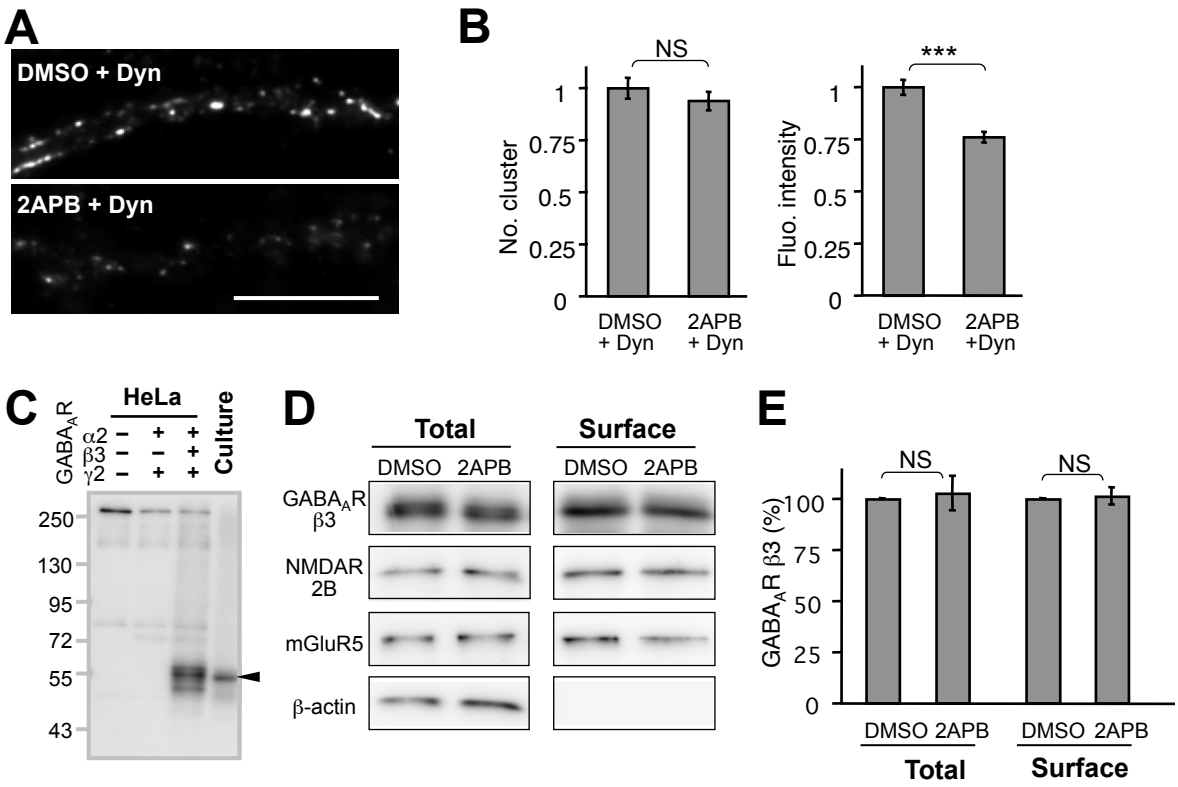

Figure S5. 2APB-induced synaptic $GABA_AR$ reduction is independent of receptor internalization.

A-B: 2APB-induced reduction in synaptic $GABA_AR$ cluster size was observed even in the absence of dynamin-dependent receptor endocytosis. Staining for $GABA_AR\gamma2$ of neurons treated with DMSO or 2APB for in the presence of 80 μM dynasore, a membrane-permeant dynamin inhibitor. **A**: Examples of $GABA_AR$ cluster. Scale bars: 10 μm. **B**: Normalized number of clusters and fluorescent intensity (average ± SEM) of synaptic $GABA_AR\gamma2$. NS: $p > 0.05$, ***: $p < 0.005$, Welch's t-test, n = 30−35 cells/condition. **C**: Westernblot analysis with HeLa cells and rat hippocampal culture lysate using our custom-made anti-$GABA_AR$ β3 antiserum. 55 kDa band (arrowhead) was detected in HeLa cells transfected with plasmids encoding $GABA_AR$ α2, β3, and γ2 subunits and hippocampal culture lysate, but not in non-transfected HeLa cells and those expressing only α2 and γ2 subunits. **D**: Amount of surface expressed biotinylated $GABA_AR$ β3 subunit, NMDAR2B subunit, and mGluR5 are not modified by 60 min 2APB treatment. Biotinylated membrane proteins (surface) isolated from detergent soluble fraction (total) with immobilized Neutroavidin, immunoblotted with antibodies against $GABA_AR\beta3$, NMDAR2B, and actin. **E:** Quantification of total and surface expressed $GABA_AR\beta3$ on DMSO and 2APB- treated cells. Protein level of DMSO condition was assigned as 100%. Both total and surface amount of β3 subunit was equivalent to that in control cells. NS: $p > 0.05$ Welch's *t*-test, n=9.

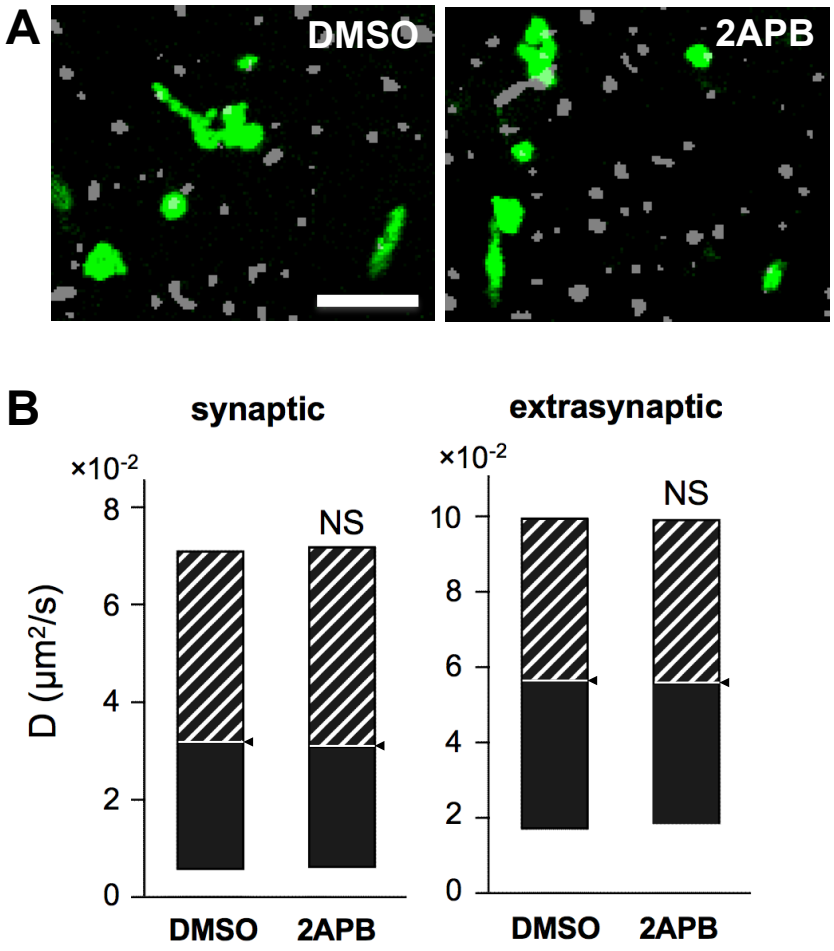

Figure S6. Minor effect of long-term 2APB treatment on lateral diffusion of neuronal mGluR5.

A: Examples of mGluR5-QD trajectories (green), reconstructed from recording sequences of 38.4 s overlaid with FM4-64 signals (gray) in order to identify synapses. Scale bar: 5 µm.
B: Diffusion coefficients (median ± IQR) of mGluR5-QDs inside (left) and outside (right) the synapse after 60-90 min 2APB treatment. NS: $p > 0.05$, Mann-Whitney *U* test. 2APB treatment did not significantly affect the diffusion coefficient of mGlu5s.

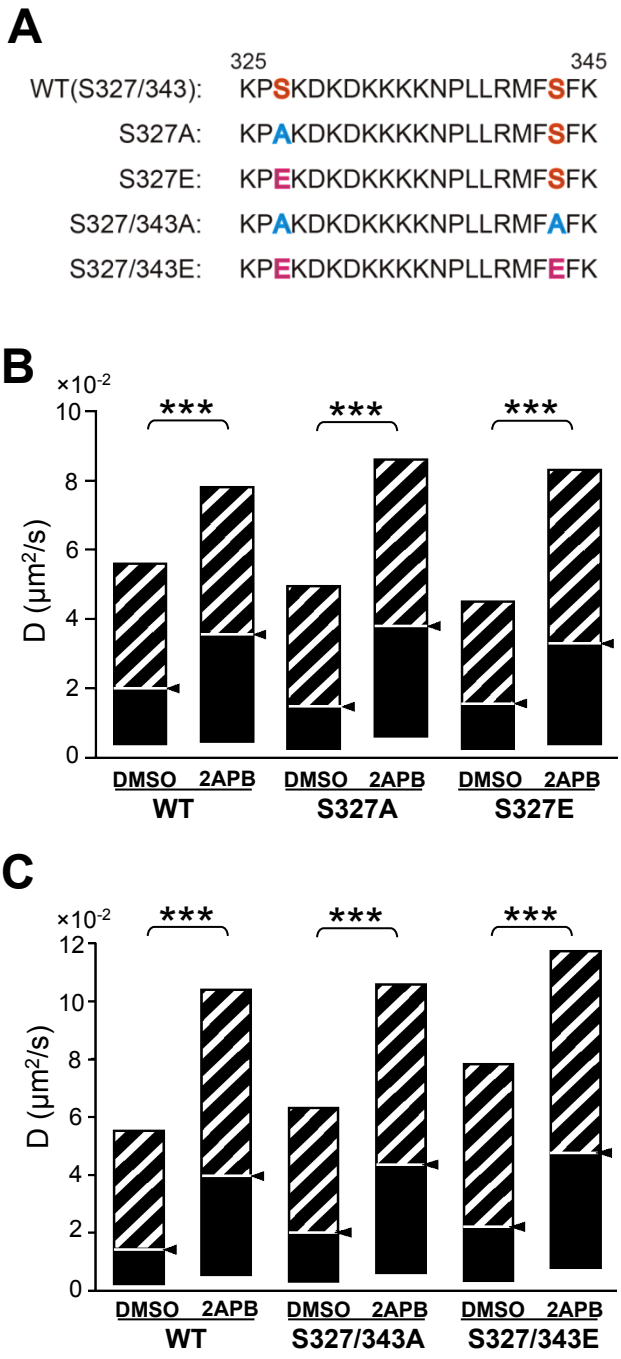

Figure S7. Phosphorylation of S327/343 in $GABA_AR$ γ2 subunit is insufficient to prevent the increase of $GABA_AR$ lateral diffusion caused by inhibition of IICR.

A: Amino acid sequences of a part of cytoplasmic loop region of $GABA_AR$ γ2 subunit. Serine residues shown in brown are S327 and S343 which were mutated into alanine (A, blue) or glutamate (E, magenta). **B**: Impact of 1 h 2APB treatment on diffusion coefficients (median±IQR) of $GABA_ARs$ with or without phospho-mimic mutation in S327. **C**: Diffusion coefficients (median±IQR) of $GABA_AR$ with WT, S327/343A, and S327/343E after 1 h treatment with DMSO or 2APB.
***: $p < 0.005$, Mann-Whitney *U* test. Cells were analyzed at 5-6 DIV.

5. Supplemental Table

Table S1. Number of analyzed $GABA_AR$-QD-trajectories.

Fig. No	Treatment	D (Synaptic)	D (Extra-synaptic)	Dwell time	Conf. size
Fig. 5A–D	**DMSO 0-30 min**	352	793	1337	189
	2APB 0-30 min	404	797	1623	219
Fig. 5E–H	**DMSO 60-90 min**	1031	1540	4091	593
	2APB 60-90 min	867	1253	3761	455
Fig. 5I–L	**WT**	268	550	839	137
	IP_3R1KO	260	637	679	122
Fig. 5M–P	**Control 60-90 min**	487	650	1640	294
	MCPG 60-90 min	672	900	2425	323
Fig. 5Q–T	**DMSO+CysA**	748	n. d.	3109	443
	2APB+CysA	791		2978	470
	DMSO+FK506	377		1383	209
	2APB+FK506	428		1494	248
Fig. 7B–E	**DMSO**	480	n. d.	2097	269
	Gö6976	688		3739	334
Fig. 7F–I	**4α-PMA+DMSO**	601	n. d.	2155	322
	4α-PMA+2APB	458		1947	219
	PMA+DMSO	546		1889	303
	PMA+2APB	546		1831	328
Fig.S6	**DMSO**	370	795	n. d.	n. d.
	2APB	421	853		

Fig. No	Treatment	D (Synaptic + Extra-synaptic)
Fig. S7B	**WT-DMSO**	896
	WT-2APB	646
	S327A-DMSO	751
	S327A-2APB	666
	S327E-DMSO	750
	S327E-2APB	739
Fig. S7C	**WT-DMSO**	1046
	WT-2APB	1167
	S327/343A-DMSO	1083
	S327/343A-2APB	1274
	S327/343E-DMSO	853
	S327/343E-2APB	1651