Supporting Information

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SI Materials and Methods

DNA Constructs. The N-terminally tagged α2-SEP DNA was a kind gift from S. Moss and has been described previously (1). To make γ2-SEP, SEP (kind gift from J. Henley, University of Bristol, Bristol, UK) was amplified by PCR using primers with flanking XhoI sites. SEP was introduced into the existing XhoI restriction sites of N-terminally tagged γ2-GFP (2), replacing the GFP between amino acids 4 and 5 of the mature protein. For improved eukaryotic expression, the ORF of γ2-SEP was amplified by PCR, introducing a Kozak sequence. Similarly, a Kozak sequence and SEP were introduced into $\gamma2^{S327A}$. For myc-tagged constructs the SEP tag was replaced with a 6× myc tag, using standard cloning techniques.

Immunoprecipitation. Cortical neurons at 12 days in vitro (DIV) were transfected with α2-SEP DNA by nucleofection before plating as previously described (3). Transfected cortical neurons were solubilized for 1 h in pull-down buffer [50 mM Hepes (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF with antipain, pepstatin, and leupeptin at 10 μg/mL] and centrifuged, and the supernatant was incubated with 2 μg of rabbit polyclonal anti-β3 antibody or rabbit IgG control antibody for 2 h. Protein A beads were added to the immunoprecipitation experiments for a subsequent 1-h incubation. Beads were washed four times with pull-down buffer and analyzed by SDS/PAGE and Western blotting with anti-GFP (SN 1:10; Neuromab).

Surface Biotinylation. For surface biotinylation, 11–13 DIV cortical neurons were incubated for 9 min at 37 °C in imaging media (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, 10 mM Hepes, adjusted to pH 7.4) or for 4 min in imaging media + 30 μM glutamate/1 μM glycine followed by return to imaging media for 5 min. Surface biotinylation and quantification have been previously described (4).

GluR-Mediated Dephosphorylation. For quantification of phosphorylated S327 in hippocampal slices, 14-d-old rats were killed by cervical dislocation in accordance with United Kingdom animal experimentation regulations. Sagittal hippocampal sections (150 μm) were cut in ice-cold ACSF (126 mM NaCl, 24 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM glucose, gassed with 95% O_2/5% CO_2) containing 1 mM kynurenic acid, using a vibrating slicer (Leica). Following a 1-h incubation at room temperature in ACSF + kynurenic acid, slices were washed in ACSF and incubated for 9 min at room temperature in ACSF or 4 min in ACSF supplemented with 30 μM glutamate/1 μM glycine followed by 5 min in ACSF. Following treatment, slices were immediately placed on ice. Slices were lysed in ice-cold RIPA buffer with phosphatase inhibitors [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 10 mM Na-Pyrophosphate, 10 nM Calyculin A, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS] containing protease inhibitors (Pierce Halt Protease inhibitor mixture) and solubilized by rotating for 1 h at 4 °C. Nuclear and cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4 °C. Equal amounts of protein (determined by BCA assay; Pierce) were immediately diluted with 3× sample buffer and resolved by SDS/PAGE electrophoresis. Transferred proteins were revealed by Western blot. For phosphorylation analysis at S327, membranes containing transferred proteins were blocked in 0.2% BSA in TBS-T for 1 h and incubated for 1 h at room temperature with rabbit anti-$\gamma2pSer^{327}$ (Abcam), followed by a 1-h incubation with an HRP-conjugated secondary antibody. For normalization to total γ2 levels, membranes transferred in parallel were blocked in 4% milk in PBS-T for 1 h and incubated for 1 h at room temperature with rabbit anti-γ2 (Alomone) followed by 1 h incubation with an HRP-conjugated secondary antibody. All bands were detected with an LAS4000 imager (GE Healthcare) and quantified using ImageJ software.

Neuronal Culture, Transfection, and Live Cell Imaging. Cultures of hippocampal neurons were prepared as described previously (5) and plated on poly-L-lysine coated coverslips (0.5 mg/mL). Neurons were transfected by Amaxa nucleofection with 3–4 μg of plasmid DNA before plating. For γ2 subunit expression, neurons were cotransfected with untagged α1 and β3 subunit cDNAs at a ratio of 2:1:1 (2). For analysis of α2-SEP cluster overlap with excitatory synapses, cotransfection with homer1c-dsRed (a kind gift from L. Groc, Centre National de la Recherche Scientifique, Bordeaux, France) was performed as above.

Cells were used at 12–16 DIV. Fluorescence was captured using an Olympus microscope (BX51WI) with a 60× Olympus objective coupled to an EM-CCD camera (Ixon; Andor). Excitation was provided by a monochromator (Cairn) or from a mercury-spiked xenon arc lamp (Cairn). Appropriate filters were chosen for SEP-tagged constructs, QDs, FM 4-64, and homer-dsRed. Imaging media used for all experiments contained 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, and 10 mM Hepes and was adjusted to pH 7.4 with NaOH before use. Cells were imaged under perfusion (4 mL/min) and heating (35–37 °C). Glutamate, glycine (both Sigma), APV, and bicuculline (both Tocris) were applied in the perfusate. APV (100 μM) and bicuculline (100 μM) were applied 2 min before and during treatment with glutamate and glycine. Addition of drugs did not change the pH of the imaging solution. Incubation with calcineurin autoinhibitory peptide (6) (Calbiochem) was made in imaging media for 30 min at 37 °C. Incubation with cyclosporin A (Calbiochem, 10 μM, 10 min) (7) was made in imaging media (0.01% DMSO) and media containing cyclosporin A at 10 μM was used in the perfusate. Incubation with dynasore (80 μM, 0.27% DMSO) (8) (Sigma) was made in imaging media for 10 min at room temperature. For this experiment, vehicle (0.27% DMSO) was used as control.

$\alpha2^{SEP}$-$GABA_AR$ images were acquired every 5 s. For the recovery experiment, single images (averages of 16 successive acquisitions) were taken at 0, 10, and 40 min to reduce photobleaching.

Labeling of $\alpha2^{SEP}$-$GABA_ARs$ with quantum dots was performed using a mouse anti-GFP antibody (NeuroMAB, clone N38) and an anti-mouse 605-nm QD (Invitrogen). Labeling of endogenous $GABA_ARs$ was made with antibodies to an extracellular region on the $GABA_AR$ α2 subunit (Synaptic Systems; followed by anti-rabbit 605nm QD) or β2/β3 subunit (MAB 314; Millipore; followed by anti-mouse 605nm QD). Labeling was performed at RT in 100 μL of solution spotted on parafilm (coverslips were inverted and placed onto solution droplet); incubation with primary antibody (10 μg/mL) was for 5 min in imaging media, and QD incubation (0.5 nM) was for 1 min in PBS containing 10% HRS (for anti-α2 labeling) or 1% Casein (all other labeling) to block nonspecific binding (9). Coverslips were washed six to eight times in imaging media after each incubation. QD movies were recorded at 6.5 Hz.

Labeling of active presynaptic terminals with FM 4-64 (Invitrogen) was performed by four incubations (10) in 100 μL imaging media with the coverslip inverted on parafilm for 1 min at RT,

with the following: 10 μM FM 4-64 and 60 mM KCl; 2 μM FM 4-64; imaging media only; and 1 mM advasep-7 (Biotium).

Fixed Cell Imaging. For stimulation of endogenous $GABA_AR$ cluster dispersal with glutamate in untransfected neurons, coverslips were incubated in a Hepes-buffered primary antibody solution (1:100) for 10 min with GABA receptor γ2 antibody. Coverslips were then washed three times in PBS and immersed in either imaging media or imaging media with 30 μM glutamate, 1 μM glycine (4 × 1 min). Coverslips were fixed with 4% paraformaldehyde/sucrose solution in PBS for 5 min before being blocked without permeabilizing in block solution (PBS containing 10% horse serum and 0.5% BSA) for 10 min. Secondary antibody dilutions were performed in block solution and washes in PBS.

For staining of myc-tagged γ2 $GABA_ARs$, coverslips were fixed with 4% paraformaldehyde/sucrose solution in PBS for 5 min before being blocked without permeabilizing in block solution (PBS containing 10% horse serum and 0.5% BSA) for 10 min. 9E10 myc antibody was then diluted in block solution and coverslips were incubated in antibody solution for 1 h before washing and incubation with Alexa Fluor fluorescent secondary antibody for 1 h. Antibodies were used at the following dilutions: mouse anti-myc 9E10, 1:100; and rabbit anti-$GABA_AR$ γ2 subunit, 1:200 (Alomone). Alexa Fluor 594 conjugated anti-mouse and anti-rabbit secondary antibodies were from Molecular Probes and were used at 1:1,000. Coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and sealed with nail varnish. Cells were visualized using a Zeiss Pascal upright confocal microscope and LSM software with a Plan-apochromat 63× oil-immersion lens with 1.4 numerical aperture. Excitation was via a HeNe laser at λ = 543 nm and an Argon laser at λ = 488 nm. To assay the percentage of cells transfected with α2-SEP also expressing γ2-myc constructs, we found multiple α2-SEP cells and counted whether these were positive for myc staining.

GAD-6 staining was performed using standard immunofluorescence techniques (using permeabilization with 0.2% Triton). Mouse monoclonal anti-GAD was obtained from GAD6 hybridoma cells (supernatant 1:100); Alexa Fluor 594 (anti-mouse) was used at 1/500 as secondary as above.

Analysis of $GABA_AR$ Expression at the Cell Surface Using COS-7 Cells. COS-7 cells were cultured and transfected as previously described (4) either with 3 μg of α2-SEP cDNA alone or cotransfected with 1 μg β3-myc and 1 μg γ2-myc cDNAs. Anti-GFP and anti-myc surface stainings were performed using standard immunofluorescence techniques.

Image Analysis. For intensity measurements in clusters or processes, analysis was performed manually in ImageJ. The StackReg macro (11) was used to correct for minor coverslip drift in (x, y). Regions were drawn around either clusters or processes (depending on which one was being analyzed) that captured their positions across the image stack. Somatic clusters were ignored, and all dendritic clusters or processes were analyzed. After background subtraction, measurements were normalized to the average intensity in the first 10 frames (50 s) of the movie, during which time cells were under control conditions. Averaging across regions gave a fluorescence profile over time for each cell. Images were acquired every 5 s. The average intensity from 5 consecutive frames (25 s) ending at $t = 9$ min was used for analysis and comparison.

For analysis of single $GABA_AR$ and $GABA_AR$ cluster dynamics, trajectories were created automatically from movies of either QD-tagged receptors or $\alpha2^{SEP}$-$GABA_AR$ clusters (for movies of 10 min duration, we used the StackReg plug-in to correct for minor x–y drift as above). For each frame, an image segmentation algorithm was run that classifies image pixels on the basis of their local symmetry at a specified scale. Pixels with maximal rotational symmetry (corresponding to local maxima in intensity) were classified as being due to fluorescent objects of interest, and their locations were given to subpixel accuracy by taking the weighted average of connected target pixels. The pointing accuracy of the system is ∼50 nm. Tracks were generated by linking particle positions between frames, using homemade software written in Mathematica (Wolfram Research). Briefly, the Euclidian distance between frames was minimized using a function that allowed for particle blinking such that tracks could be connected across blinking events. Instantaneous diffusivities (D_{inst}) were calculated from interframe displacements along track segments according to the 2D diffusion law ($x^2 = 4D_{inst}t$) and fitting to the first 5 points of the displacement curve was used to estimate the diffusion coefficient. Diffusion coefficients for whole tracks (used for α2-SEP cluster tracks) were estimated using the mean-squared displacement (MSD) obtained from track segments of length 30 frames. The gradient from the first 10 points of the MSD curve was used to give an estimate of D. QD track segments were classified as being inside $GABA_AR$ clusters if their midpoint was within 0.75 μm of a cluster centroid. Cluster positions were found automatically from images taken immediately before the corresponding QD movie. For comparisons before and after drug treatments, QD-tagged $GABA_AR$ mobilities were derived from movies of duration 250 frames = 38.5 s.

Kymograph representations (a line scan through a process of interest, projected over time) of $GABA_AR$ cluster movements and single $GABA_AR$ dynamics were made in ImageJ, using the MultipleKymograph macro. In cases where the line segment of interest was curved, the Straighten macro was used. $\alpha2^{SEP}$-$GABA_ARs$ were classed as synaptic if their centroids were within 1 μm of the nearest puncta of the relevant synaptic marker used (both sets of positions were obtained automatically, as above). For analysis of $\alpha2^{SEP}$-$GABA_AR$ cluster intensity, images were thresholded at a fixed threshold of 20 and clusters were found using the "Analyze Particles" macro in ImageJ, with allowed sizes lying between 4 and 80 square pixels to eliminate noise and larger bright areas (i.e., soma). For endogenous $GABA_AR$ staining, pixels above a fixed threshold of 20 were analyzed for mean intensity and variance, using ImageJ.

Local Stimulation. Local electrical stimulation of axons was made using a stimulating electrode via a patch pipette (resistance ∼1 MΩ) filled with imaging media, as detailed above (3, 10). The stimulation protocol (20 Hz, 1 min) was verified by recording whole-cell responses in voltage-clamp mode (not shown). For these experiments, images of $\alpha2^{SEP}$-$GABA_AR$ fluorescence were acquired every 2 s. $\alpha2^{SEP}$-$GABA_AR$ clusters within 15 μm of apparent stimulated synapses were defined as being stimulated. Intensity analysis was based on the average intensity in five consecutive frames ending at $t = 240$ s. In Fig. S4, imaging media containing TTX (100 μM), APV (50 μM), and CNQX (100 μM) (all Tocris) were used both to fill the patch pipette and as a perfusate.

Statistical Analysis. All experiments were performed on neurons from at least three individual preparations. Unless otherwise stated, P values given are from two-tailed t tests (type 2). Values are given as mean ± SEM; error bars represent SEM.

1. Tretter V, et al. (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. *J Neurosci* 28:1356–1365.

2. Kittler JT, et al. (2000) Analysis of GABAA receptor assembly in mammalian cell lines and hippocampal neurons using gamma 2 subunit green fluorescent protein chimeras. *Mol Cell Neurosci* 16:440–452.

3. Macaskill AF, et al. (2009) Miro1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses. *Neuron* 61:541–555.
4. Smith KR, et al. (2010) Identification and characterisation of a Maf1/Macoco protein complex that interacts with GABAA receptors in neurons. *Mol Cell Neurosci* 44:330–341.
5. Banker G, Goslin K (1991) *Culturing Nerve Cells* (MIT Press, Cambridge, MA), 2nd Ed.
6. Terada H, et al. (2003) Inhibition of excitatory neuronal cell death by cell-permeable calcineurin autoinhibitory peptide. *J Neurochem* 87:1145–1151.
7. Misonou H, et al. (2004) Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat Neurosci* 7:711–718.
8. Newton AJ, Kirchhausen T, Murthy VN (2006) Inhibition of dynamin completely blocks compensatory synaptic vesicle endocytosis. *Proc Natl Acad Sci USA* 103:17955–17960.
9. Groc L, Choquet D, Chaouloff F (2008) The stress hormone corticosterone conditions AMPAR surface trafficking and synaptic potentiation. *Nat Neurosci* 11:868–870.
10. Branco T, Staras K, Darcy KJ, Goda Y (2008) Local dendritic activity sets release probability at hippocampal synapses. *Neuron* 59:475–485.
11. Thévenaz P, Ruttimann UE, Unser M (1998) A pyramid approach to subpixel registration based on intensity. *IEEE Trans Image Process* 7:27–41.

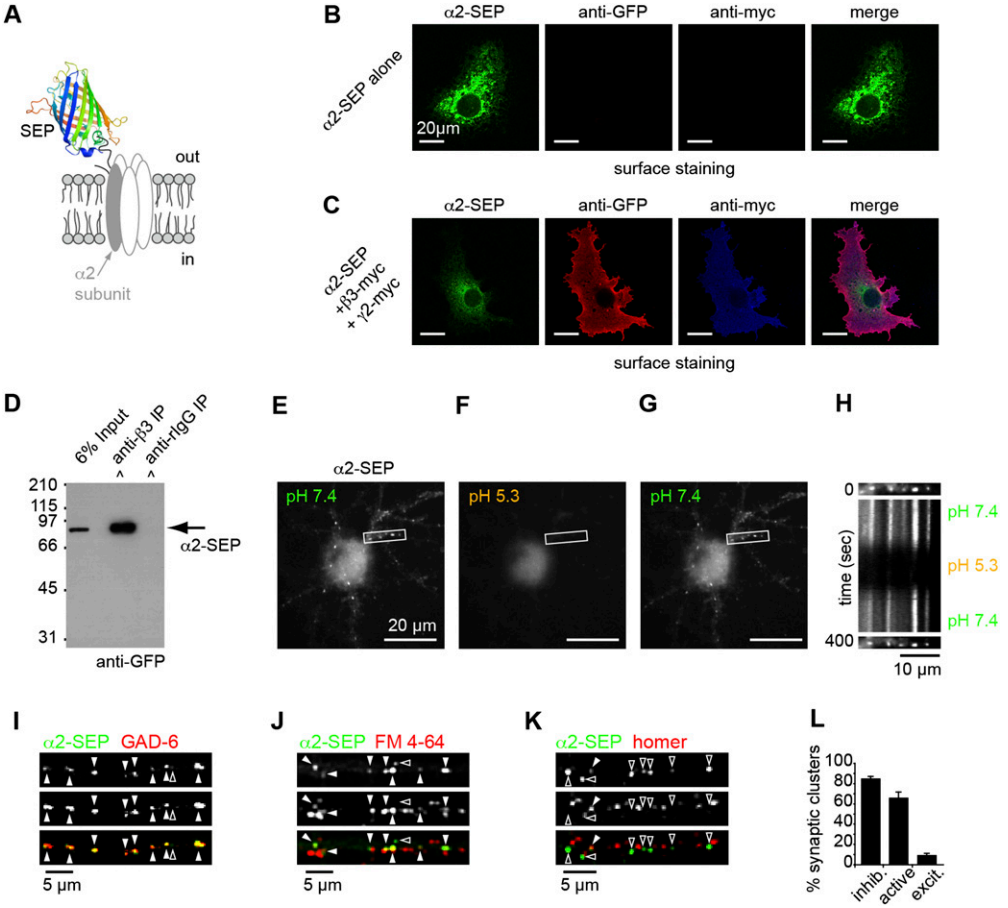


Fig. S1. $\alpha2^{SEP}$-$GABA_AR$ clusters are at the cell surface and found apposed to active presynaptic terminals. (*A*) Schematic showing the $GABA_AR$ α2 subunit tagged at the N terminus with SEP. (*B*) Expression of α2-SEP alone in COS cells results in no surface expression, as shown by the lack of GFP surface staining due to retention in the endoplasmic reticulum. (*C*) Coexpression of α2-SEP with myc-tagged β3 and γ2 subunits allows surface $\alpha2^{SEP}$-$GABA_AR$ expression, as shown by positive GFP surface staining in cotransfected cell. (*D*) Immunoprecipitated $GABA_AR$ β3 subunit-containing complexes from α2-SEP transfected cortical neurons were analyzed by SDS/PAGE and Western blotting with an anti-GFP antibody. Western blot showing that α2-SEP coimmunoprecipitates with native $GABA_A$ receptors. The α2-SEP construct has the expected molecular weight of ≈85 kDa, and there are no α2-SEP degradation products. (*E–G*) $\alpha2^{SEP}$-$GABA_AR$ fluorescence signal is from surface receptors. Fluorescence from dendrites is eclipsed on a transient switch to pH 5.3 solution (*F*) and returns on switch back to pH 7.4 (*G*). (*H*) Kymograph showing pH-dependent $\alpha2^{SEP}$-$GABA_AR$ fluorescence signal in dendrite boxed in *E–G*. Fluorescence from synaptic (clustered) and extrasynaptic (diffuse) receptors is transiently lost in low pH, indicating that dendritic fluorescence signal is from surface $GABA_ARs$. (*I–K*) Overlap of $\alpha2^{SEP}$-$GABA_AR$ clusters with inhibitory synapses (GAD-6 staining, *I*), active presynaptic terminals (FM 4–64 staining, *J*), and excitatory synapses (transfected homer-dsRed, *K*). Channels are shown merged (*Bottom*). Solid arrowheads indicate synaptic clusters, and open arrowheads indicate clusters not apposed to active presynaptic terminals. (*L*) Bar graph showing fraction of $\alpha2^{SEP}$-$GABA_AR$ clusters apposed to inhibitory synapses (GAD-6, 84.3 ± 1.8%, 21 cells), active presynaptic terminals (FM 4-64, 65.3 ± 5.2%, 19 cells), and excitatory synapses (dsRed homer, 9.1 ± 1.3%, 32 cells).

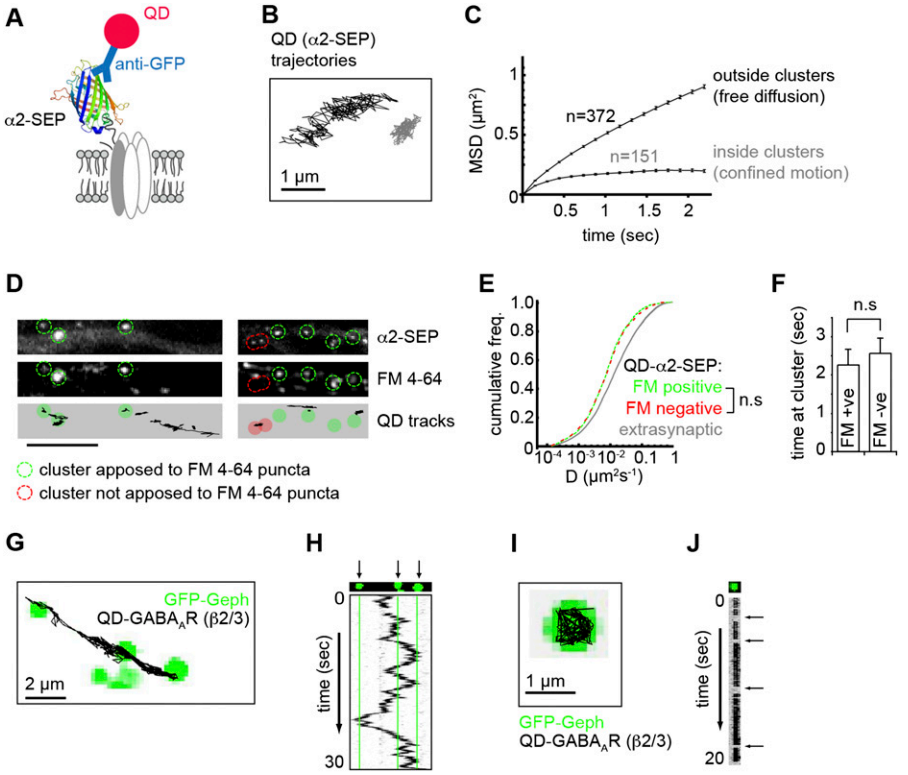


Fig. S2. $\alpha2^{SEP}$-GABA$_A$Rs undergo free diffusion and can exhibit confined motion within $\alpha2^{SEP}$-GABA$_A$R clusters, and native QD-labeled GABA$_A$Rs can exchange between inhibitory synapses and can be confined to single synapses. (*A*) Schematic showing labeling of $\alpha2^{SEP}$-GABA$_A$Rs with a QD, coupled via a GFP antibody. (*B*) Example trajectories showing QD-labeled $\alpha2^{SEP}$-GABA$_A$R motion outside of and within an $\alpha2^{SEP}$-GABA$_A$R cluster (black and gray, respectively). Time elapsed = 30 s. (*C*) Plots of mean squared displacement (MSD) for GABA$_A$R trajectories inside (gray) and outside (black) $\alpha2^{SEP}$-GABA$_A$R clusters. MSD plot is nearly linear for GABA$_A$Rs outside clusters, suggesting "free" diffusion, whereas the sublinear plot for trajectories inside clusters suggests confined motion within a GABA$_A$R cluster. (*D*) FM 4-64 labeling was used to distinguish between $\alpha2^{SEP}$-GABA$_A$R clusters either apposed to FM-labeled terminals (termed FM positive) or not apposed to FM-labeled terminals (termed FM negative). Zones corresponding to FM positive (green), FM negative (red), and extrasynaptic (gray) regions are shown, as well as maximum projection of a QD–$\alpha2^{SEP}$-GABA$_A$R movie from this dendrite. (Scale bar, 10 μm.) (*E*) Instantaneous diffusion coefficients for QD-tagged $\alpha2^{SEP}$-GABA$_A$Rs inside FM-positive (green) and FM-negative (red) clusters and in extrasynaptic regions. Single GABA$_A$Rs are less mobile when within GABA$_A$R clusters [$P < 2 \times 10^{-16}$, Kolmogorov–Smirnov (K-S) test, n_{in} = 3,048, n_{out} = 10,946), but mobilities inside clusters that are apposed or not apposed to FM-positive terminals are not significantly different (P = 0.7, K-S test, n_{act} = 1,547, n_{nonact} = 1,501). Analysis is from seven cells. (*F*) Mean residency times of single GABA$_A$Rs at FM-positive (+ve) and FM-negative (−ve) clusters are not significantly different (n = 7 cells, $P > 0.05$). (*G*) Trajectory of native QD-labeled GABA$_A$R (black) diffusing in a dendrite expressing a GFP-tagged form of the inhibitory synaptic scaffold protein gephyrin (green). Time elapsed = 30 s. Antibody: GABA$_A$R β2/β3 subunit. (*H*) Kymograph showing lateral motion of QD-GABA$_A$R in *G*. Vertical green lines show positions of gephyrin clusters. Native GABA$_A$R can diffuse into and out of inhibitory postsynaptic domains. (*I*) Trajectory of native QD-labeled GABA$_A$R (black) exhibiting motion confined to a single gephyrin cluster. (*J*) Kymograph showing restricted motion of QD-GABA$_A$R in *I*. Arrows indicate periods of QD blinking.

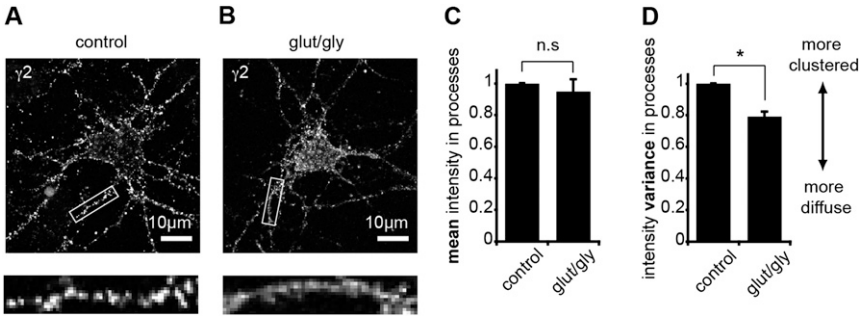


Fig. S3. Endogenous GABA$_A$R clustering is dispersed by GluR activation. (*A*) Example cell showing γ2 staining under control conditions. (*Lower*) Zoom in of boxed region. (*B*) Example cell showing γ2 staining after GluR activation (glut/gly, 4 min). (*Lower*) Zoom in of boxed region. (*C*) Bar graph showing mean fluorescence intensity in dendritic processes, normalized to control (n = 3 preps). Fluorescence intensity is not significantly different on GluR activation (0.95 ± 0.08 normalized to control, $P > 0.05$, paired t test). (*D*) Bar graph showing fluorescence intensity variance in processes, normalized to control. Variance in fluorescence intensity decreases on GluR activation (0.79 ± 0.05 normalized to control, P = 0.02, paired t test), suggesting dispersal of endogenous GABA$_A$R clusters.

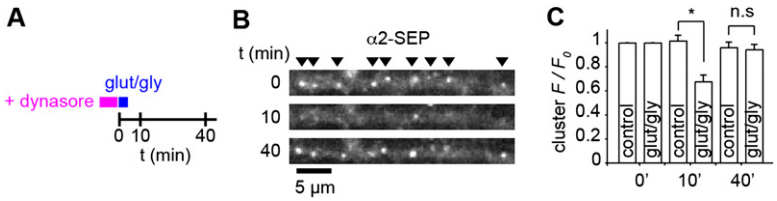


Fig. S4. $GABA_AR$ cluster recovery is not inhibited by blocking endocytosis. (*A*) Schematic of experiment. Images were taken at 0, 10, and 40 min after a 10-min dynasore treatment to block $GABA_AR$ internalization. (*B*) $\alpha2^{SEP}$-$GABA_AR$ clustering can recover after dispersal induced by GluR activation. (*C*) Cluster F/F_0 at t = 0, 10, and 40 min. Loss of $\alpha2^{SEP}$-$GABA_AR$ cluster fluorescence is significant at t = 10 min (P = 0.02) but is not significantly different at t = 40 min ($P > 0.05$); $n_{control}$ = 5 cells, $n_{glut/gly}$ = 6 cells.

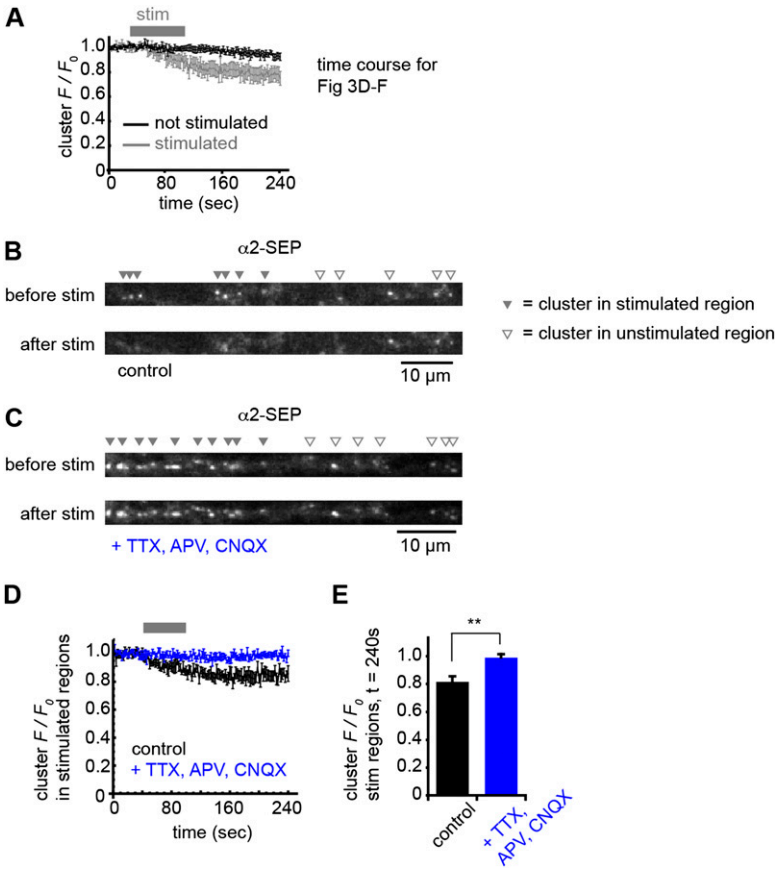


Fig. S5. Local $GABA_AR$ cluster dispersal is not a result of direct electrical stimulation of the dendrite. (*A*) Time course of $\alpha2^{SEP}$-$GABA_AR$ cluster fluorescence in stimulated (gray) and nonstimulated (black) regions (cf. Fig. 3 *D–F*). Electrical stimulus is indicated by a gray bar. (*B*) $\alpha2^{SEP}$-$GABA_AR$ fluorescence in dendrite before and after electrical stimulus (20 Hz, 1 min) in control solution. (*C*) $\alpha2^{SEP}$-$GABA_AR$ fluorescence in dendrite before and after electrical stimulus in solution containing TTX, APV, and CNQX to block action potentials and excitatory synaptic transmission. (*D*) Time course of fluorescence in $\alpha2^{SEP}$-$GABA_AR$ clusters in stimulated regions in control (black) and drug cells (blue). Electrical stimulus is indicated by a gray bar. (*E*) F/F_0 at t = 240 s is significantly reduced in stimulated regions in control solution compared with drug solution (P = 0.002, $n_{control}$ = 5 cells, n_{drug} = 5 cells).

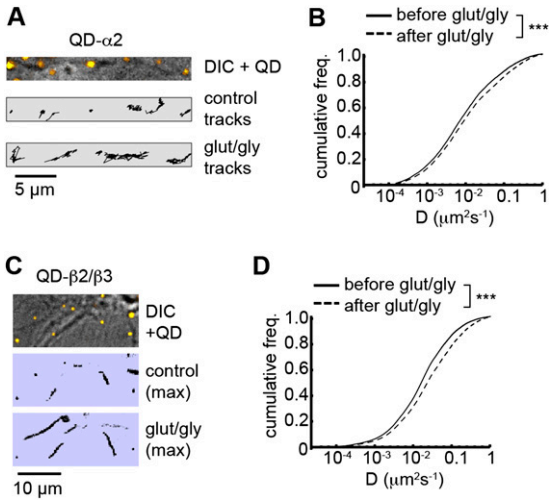


Fig. S6. GluR activation increases lateral mobility of endogenous $GABA_A$Rs. (*A*) (*Top*) DIC image of dendrite containing $GABA_A$Rs labeled with QDs (yellow) via α2 subunit antibody. (*Bottom*) $GABA_A$R trajectories in this dendrite before and after GluR activation with glut/gly. (*B*) Instantaneous diffusion coefficients for endogenous $GABA_A$Rs from before (solid line) and after GluR activation (dashed line). Receptor mobility increased significantly by 1.20-fold (n_{before} = 7,450, n_{after} = 5,798, $P = 1 \times 10^{-12}$, K-S test). (*C*) (*Top*) DIC image with endogenous β2/β3 subunits labeled with quantum dots overlaid in yellow. (*Middle* and *Bottom*) Maximum projection of QD movie taken before and after GluR activation. (*D*) Instantaneous diffusion coefficients for endogenous $GABA_A$Rs from before (solid line) and after GluR activation (dashed line). Receptor mobility increased significantly by 1.27-fold (n_{before} = 4,212, n_{after} = 3,328, $P = 1 \times 10^{-12}$, K-S test).

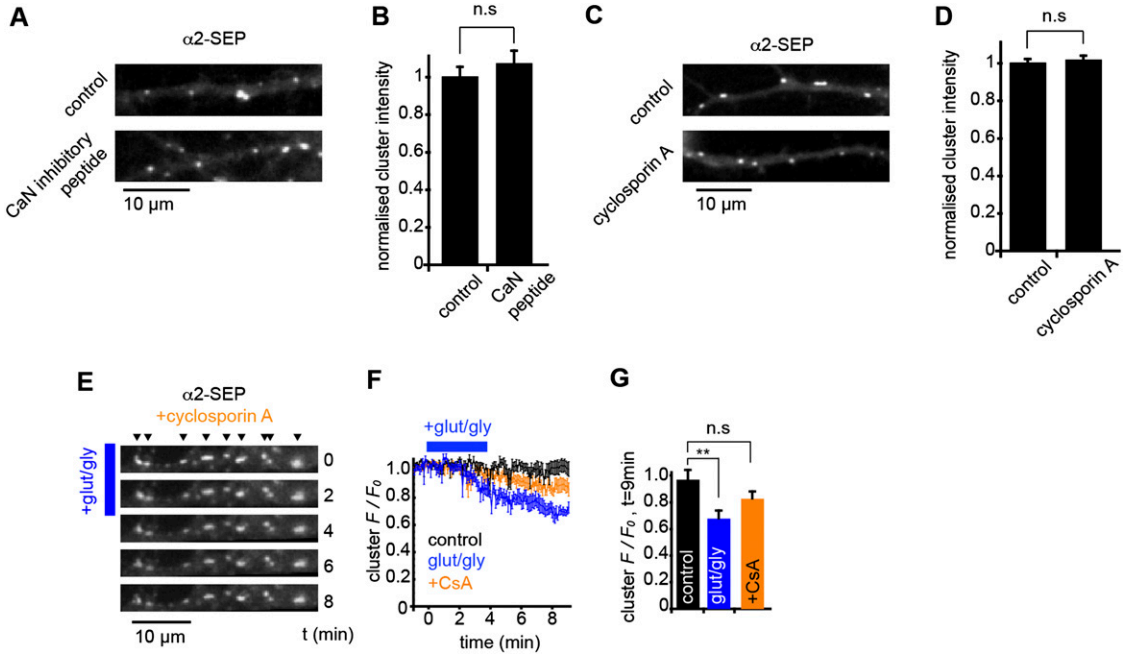


Fig. S7. Treatment with calcineurin autoinhibitory peptide or cyclosporin A does not affect $\alpha2^{SEP}$-$GABA_A$R cluster intensity, and cyclosporin A treatment prevents $GABA_A$R cluster dispersal. (*A*) Example dendrites containing $\alpha2^{SEP}$-$GABA_A$Rs: control (*Upper*) and after treatment with CaN autoinhibitory peptide (*Lower*). (*B*) Bar graph showing mean cluster intensity, normalized to control. Cluster intensities are not significantly different after treatment with peptide (P = 0.61, $n_{control}$ = 41 cells, $n_{peptide}$ = 41 cells). (*C*) Example dendrites containing $\alpha2^{SEP}$-$GABA_A$Rs: control (*Upper*) and after treatment with cyclosporin A (*Lower*). (*D*) Bar graph showing mean cluster intensity, normalized to control. Cluster intensities are not significantly different after treatment with cyclosporin A (P = 0.55, $n_{control}$ = 27 cells, n_{CsA} = 24 cells). (*E*) $\alpha2^{SEP}$-$GABA_A$R fluorescence on GluR activation after pretreatment with cyclosporin A (CsA). (*F*) Time course of $\alpha2^{SEP}$-$GABA_A$R cluster fluorescence from this experiment: vehicle, black, n = 4 cells; vehicle + glut/gly, blue, n = 4; glut/gly + CsA, orange, n = 5. (*G*) F/F_0 in $\alpha2^{SEP}$-$GABA_A$R clusters at t = 9 min. $GABA_A$R dispersal seen under vehicle + glut/gly (P = 0.01) was blocked by CsA ($P > 0.05$).

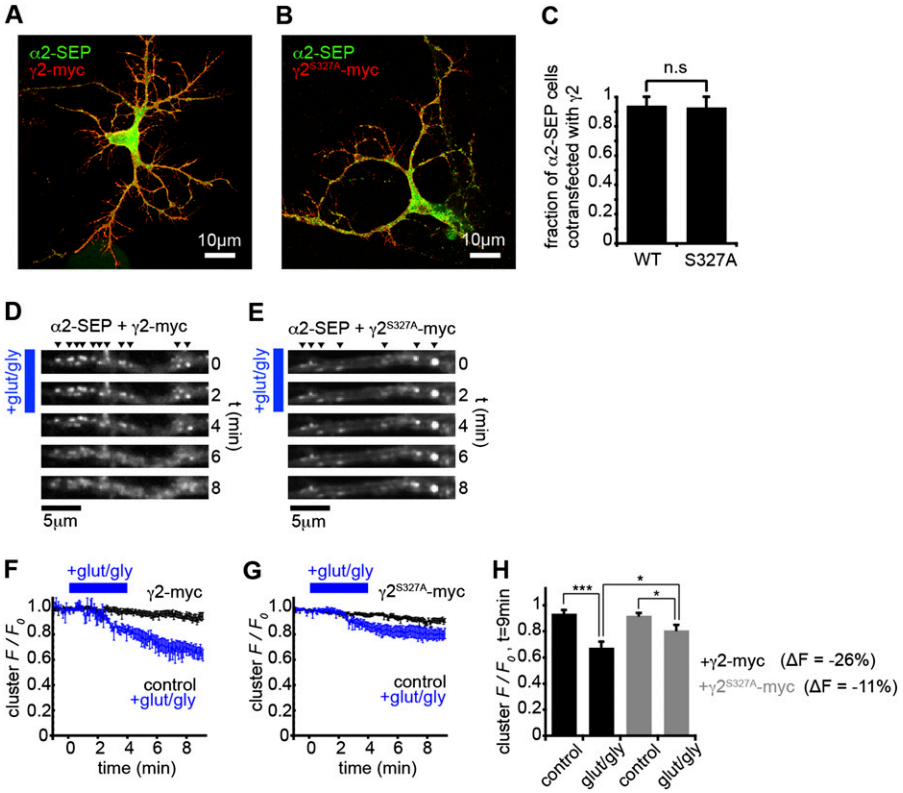


Fig. S8. Transfection of $\gamma2^{S327A}$-myc construct partially blocks GluR-dependent $\alpha2^{SEP}$-$GABA_AR$ cluster dispersal. (*A*) Example image of a cell coexpressing α2-SEP (green) and γ2-myc (red). (*B*) Example image of a cell coexpressing α2-SEP (green) and $\gamma2^{S327A}$-myc (red). (*C*) Bar graph showing fraction of α2-SEP cells also positive for γ2-myc staining. WT, 0.917 (11/12 cells); S327A, 0.923 (12/13 cells). Cotransfection fractions were not significantly different ($P > 0.05$). (*D* and *E*) $\alpha2^{SEP}$-$GABA_AR$ fluorescence on GluR activation (blue bar) in cells expressing γ2-myc (*D*) or $\gamma2^{S327A}$-myc (*E*). (*F*) Time course of $\alpha2^{SEP}$-$GABA_AR$ cluster F/F_0 on GluR activation in cells cotransfected with γ2-myc. Control, black, $n = 7$ cells; glut/gly, blue, $n = 7$. (*G*) Time course of $\alpha2^{SEP}$-$GABA_AR$ cluster F/F_0 on GluR activation in cells cotransfected with $\gamma2^{S327A}$-myc. Control, black, $n = 7$ cells; glut/gly, blue, $n = 7$. (*H*) $\alpha2^{SEP}$-$GABA_AR$ dispersal is decreased significantly by S327A mutation. Cluster F/F_0 at $t = 9$ min: WT control, 0.93 ± 0.03; WT glut/gly, 0.67 ± 0.04; S327A control, 0.92 ± 0.02; S327A glut/gly, 0.81 ± 0.04. $GABA_AR$ dispersal is reduced in cells expressing $\gamma2^{S327A}$-myc (decrease in cluster F/F_0 for glut/gly-treated cells is significantly greater for WT control-expressing cells than for cells expressing the S327A mutation; $P = 0.04$).

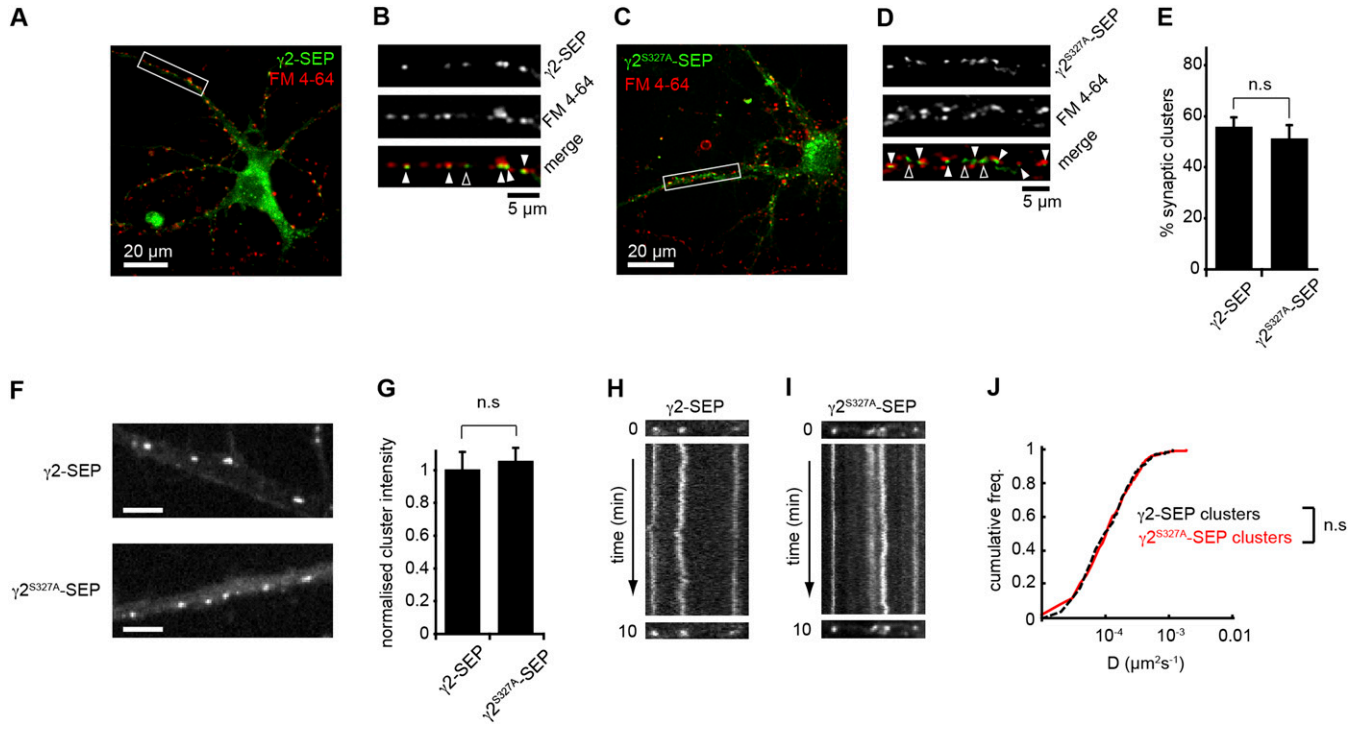
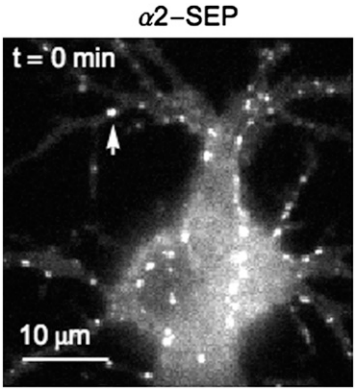
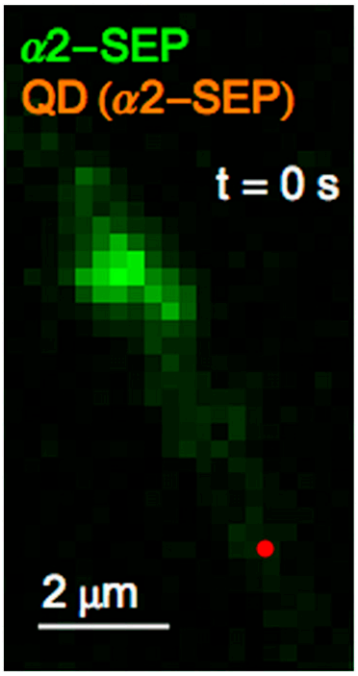


Fig. S9. Mutation of serine 327 to alanine in the γ2-SEP-$GABA_AR$ subunit does not affect $\gamma2^{SEP}$-$GABA_AR$ synaptic targeting, cluster intensity, or cluster mobility. (*A*) Neuron expressing γ2-SEP (green) with presynaptic terminals FM 4-64 stained (red). (*B*) Section of process boxed in *A*: γ2-SEP clusters (*Top*) and FM 4-64 puncta (*Middle*) are shown merged (*Bottom*). Solid arrowheads indicate synaptic clusters, and open arrowheads indicate clusters not apposed to active presynaptic terminals. (*C* and *D*) As in *A* and *B*, for neuron expressing $\gamma2^{S327A}$-SEP. (*E*) Bar graph representing percentage of synaptic clusters. γ2-SEP, 55.8 ± 3.3% (n = 22 cells); $\gamma2^{S327A}$-SEP, 50.9 ± 3.3% (n = 16 cells), not significantly different (P = 0.31). (*F*) Example dendrites containing γ2-SEP (*Upper*) and $\gamma2^{S327A}$-SEP (*Lower*). (*G*) Bar graph showing mean cluster intensity, normalized to wild-type control. $\gamma2^{S327A}$-SEP cluster intensities are not significantly different (P = 0.84, n_{WT} = 14 cells, n_{S327A} = 14). (*H*) Kymograph showing dynamics of γ2-SEP clusters over 10 min. (*I*) Kymograph showing dynamics of $\gamma2^{S327A}$-SEP clusters over



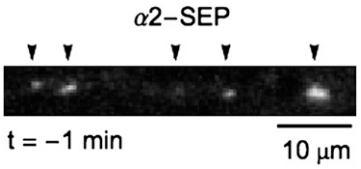
Movie S1. $GABA_AR$ clusters are stable under resting conditions. Movie of neuron expressing $\alpha2^{SEP}$-$GABA_ARs$: Imaging period is 10 min under resting conditions. Intensities and positions of $\alpha2^{SEP}$-$GABA_AR$ clusters (an example is indicated by an arrow) are stable over time, with some $GABA_AR$ clusters undergoing small lateral displacements.

Movie S1



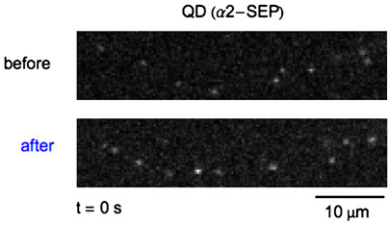
Movie S2. Single QD-labeled $\alpha2^{SEP}$-$GABA_AR$ can rapidly move into and out of $\alpha2^{SEP}$-$GABA_AR$ clusters. Movie shows position of QD-labeled $\alpha2^{SEP}$-$GABA_AR$ (red) in relation to SEP-$GABA_AR$ cluster (green) over 55 s under resting conditions. Receptor trajectory (orange) showing previous motion is built up as the receptor moves into and out of the cluster.

Movie S2



Movie S3. $GABA_AR$ clusters rapidly disperse on GluR activation. Movie shows $\alpha2^{SEP}$-$GABA_AR$ clusters before, during, and after GluR activation (glut/gly application indicated by blue text).

Movie S3



Movie S4. $GABA_AR$ mobility increases on GluR activation. Movie shows QD-labeled $\alpha2^{SEP}$-$GABA_AR$s diffusing in a dendrite before and after GluR activation.

Movie S4