Cell Reports
Inventory of supplemental information for:
Glutamate receptor modulation is restricted to synaptic microdomains
Gyorgy Lur and Michael J. Higley

Figure S1: Calibration of 2-photon glutamate uncaging. This figure explains the primary experimental method used throughout the paper.

Figure S2: Characteristics of NMDAR-mediated currents and Ca2+ transients. This figure provides evidence that the Ca2+ influx measured in Figure 1, Figure 2, Figure 4, Figure 5 and Figure 7 is primarily due to NMDAR activation.

Figure S3: Modulation of Ca2+ transients evoked by back-propagating action potentials. This figure provides evidence (in addition to Figure 4 and Figure 5) that α2Rs and $GABA_B$Rs are located in the same subcellular compartment.

Figure S4: Dialysis with RGS4 antibody enables neuromodulatory cross-talk. This figure utilizes an alternative method to inhibit the actions of RGS4, strengthening the results in Figure 7.

Figure S5: Schematic drawing aiding the Discussion of the results.

Supplemental Experimental procedures: This section provides extensive description of the experimental methods and materials used in the paper, including the source and catalog numbers of the antibodies used throughout the study.

Supplemental references: This section cites work establishing the methodology used in the paper.

Supplemental Figures

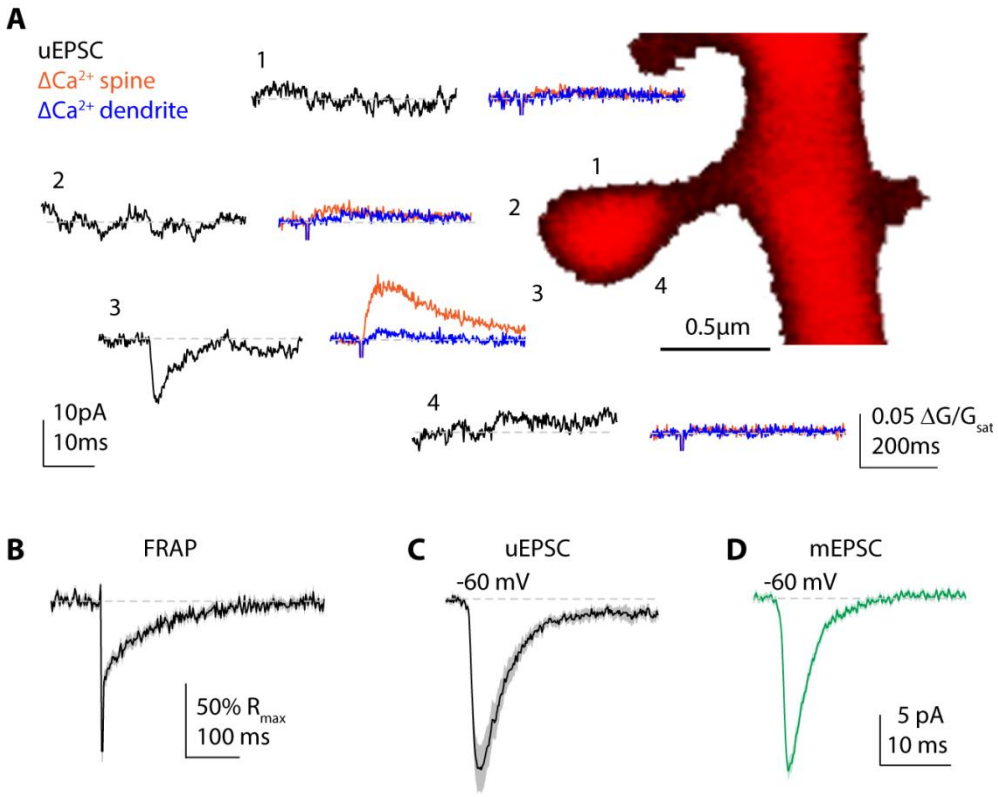

Figure S1. Calibration of 2PLU at a single dendritic spine. (**A**) Probing multiple uncaging sites (numbers) around a single spine head. Black traces show somatic excitatory post synaptic currents (uEPSCs) while colored traces represent Ca^{2+} transients in the spine head (red) and the neighboring dendritic shaft (blue) evoked at the positions indicated by their respective numbers. (**B**) The power output of the uncaging Ti-Sapphire laser is adjusted so that, when positioned over the spine head, it produces 50% photobleaching of the Alexa594 dye that rapidly recovers after the stimulus (fluorescent recovery after photobleaching – FRAP). (**C**) The size and kinetics of uEPSCs evoked by 2PLU at the established 50% FRAP laser power (n=18 spines) are comparable to (**D**) miniature EPSCs (n=161 events) measured in the same cells (n=4 cells). Traces show mean ± SEM (solid line and shaded area, respectively).

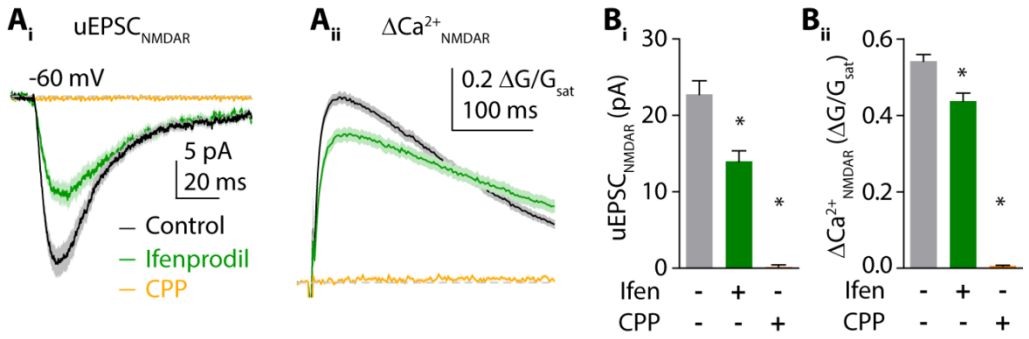

Figure S2. Characteristics of NMDAR-mediated currents and Ca^{2+} transients. (**Ai**) Mean ± SEM traces (solid lines and shaded areas) of 2PLU-evoked NMDAR-mediated uEPSCs and (**Aii**) Ca^{2+} transients from single spines in control (black, n=46 spines), ifenprodil (green, n=33 spines), and CPP (orange, n=12 spines). (**Bi**) Bar graphs represent mean ± SEM amplitude of uEPSCs and (**Bii**) Ca^{2+} transients in control (gray) versus ifenprodil (green) or CPP (orange). *: $p<0.05$, two tailed, unpaired t-test.

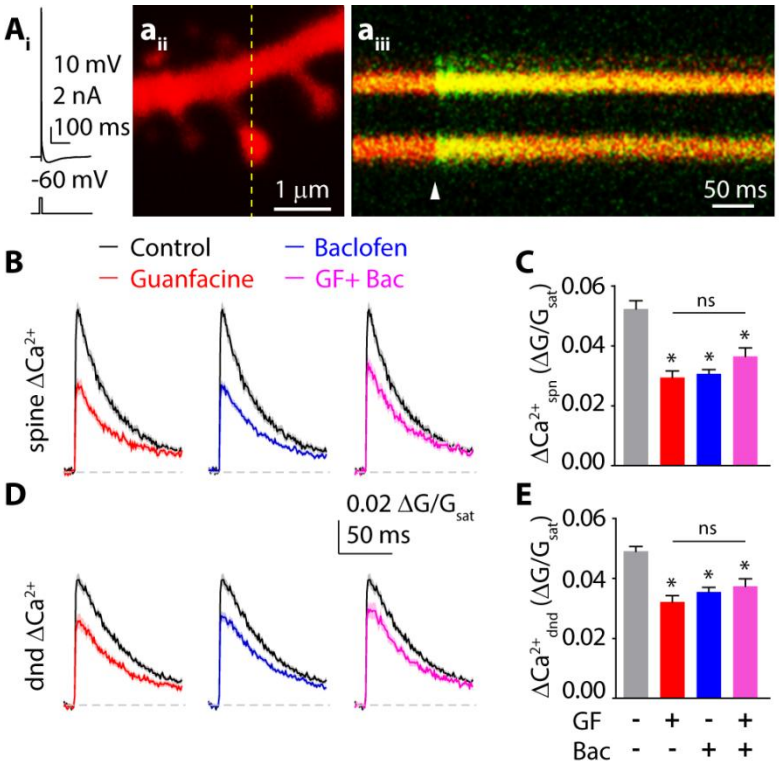

Figure S3. α2Rs and $GABA_B$Rs modulate Ca^{2+} transients evoked by back-propagating action potentials. (**Ai**) Example action potential (top) evoked by somatic current injection (bottom). (**Aii**) 2-photon image of a dendritic spine and (**Aiii**) fluorescence trace acquired in the line scan indicated by dashed line in (**Aii**). White arrowhead indicates the timing of the AP. (**B**) Mean ± SEM (solid lines and shaded areas, respectively) traces of bAP-evoked Ca^{2+} transients in control (black), guanfacine (red), baclofen (blue), or guanfacine combined with baclofen (magenta) in the spine head. (**C**) Bar graphs show the mean Ca^{2+} transient amplitude ± SEM. (**D**-**E**) Same as (**B**-**C**) for Ca^{2+} transients in the dendritic shaft. *: $p<0.05$, Tukey`s multiple comparison test.

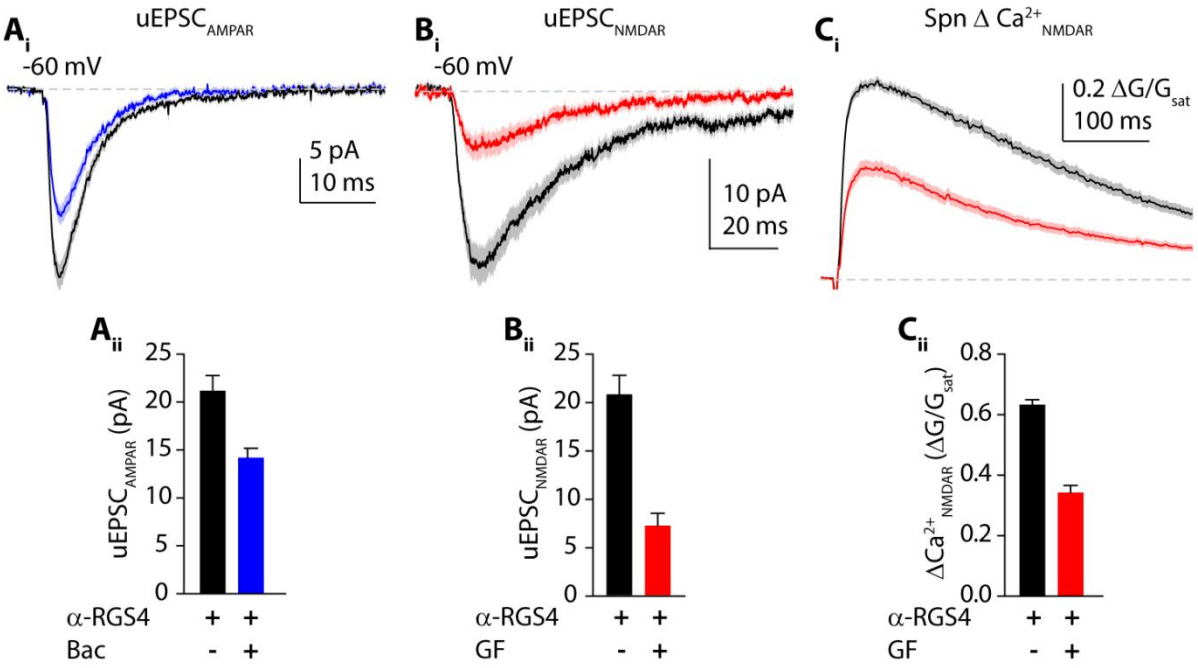

Figure S4. Dialysis with anti-RGS4 antibody enables cross-talk between α2R and $GABA_BR$ signaling. The internal solution contains an antibody specifically binding RGS4. (**Ai**) Mean (solid lines) ± SEM traces (shaded areas) of AMPAR-mediated currents in control (n=31 spines, black) and in baclofen (n=34 spines, blue). (**Bi**) Mean ± SEM traces (solid lines and shaded areas, respectively) of 2PLU-evoked, NMDAR-mediated uEPSCs and (**Ci**) Ca^{2+} transients in control (n=30 spines, black) and in guanfacine (n=32 spines, red). (**Aii**) Bars show mean ± SEM of AMPAR currents in control (black and baclofen (blue). (**Bii**) Bars represent mean ± SEM of NMDAR-mediated currents and (**Cii**) ΔCa2+ in control (black) and in guanfacine (red). *: p<0.05, unpaired t-test.

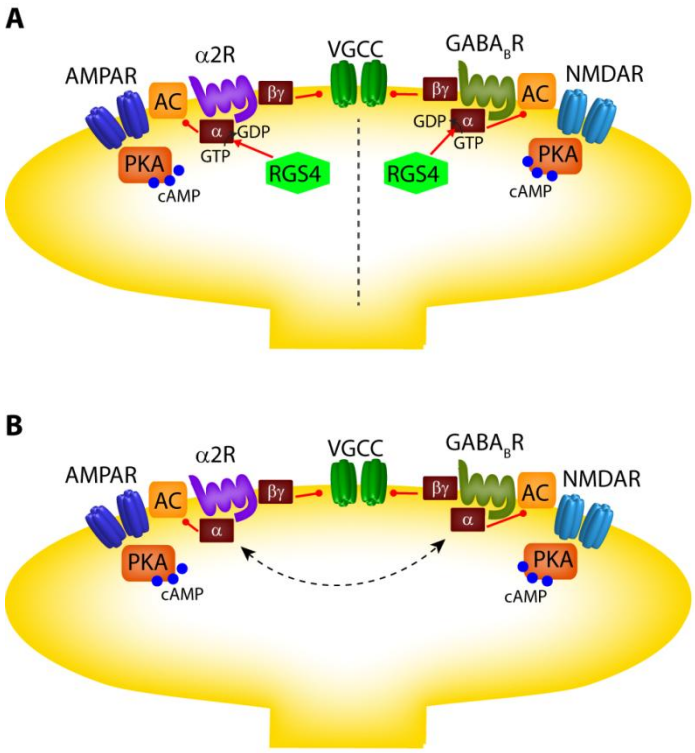

Figure S5. RGS4 restricts Gα_i mobility promoting segregated PKA-dependent regulation of glutamate receptors. Schematic shows α2R and GABA$_B$R signaling pathways targeting AMPARs and NMDARs, respectively. Both cascades utilize Gα_i subunits to block cAMP production by inhibiting adenylate cyclase (AC) activity. This in turn reduces PKA-dependent phosphorylation of the target glutamate receptor. VGCCs are modulated by the membrane delimited G$\beta\gamma$ subunits in a PKA-independent manner. (**A**) Cross-talk between modulatory pathways targeting AMPARs and NMDARs is restricted by RGS4 activity promoting signaling microdomains within the spine head. (**B**) Blocking RGS4 removes the selectivity of α2Rs and GABA$_B$Rs towards their respective glutamate receptors.

Supplemental Experimental procedures:

Slice Preparation

All animal handling was performed in accordance with guidelines approved by the Yale Institutional Animal Care and Use Committee and federal guidelines. Glutamate uncaging experiments were conducted using acute prefrontal cortical (PFC) slices from wild-type C57/Bl6 mice (P22-36). Under isoflurane anesthesia, mice were decapitated and coronal slices (300 μm) were cut in ice-cold external solution containing (in mM): 110 choline, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 3 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, 11.6 sodium ascorbate and 3.1 sodium pyruvate, bubbled with 95% O_2 and 5% CO_2. Slices containing the prelimbic-infralimbic regions of the PFC were then transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 0.4 sodium ascorbate, 2 sodium pyruvate and 3 myo-inositol, bubbled with 95% O_2 and 5% CO_2. After an incubation period of 15 min at 34 °C, the slices were maintained at 22–24 °C for at least 20 min before use.

Electrophysiology and imaging

All experiments were conducted at near physiological temperature (32-34°C) in a submersion-type recording chamber. Whole-cell patch-clamp recordings were obtained from layer 5 pyramidal cells (400-500 μm from the pial surface) identified with video-infrared/differential interference contrast. For voltage-clamp recordings, glass electrodes (1.8-3.0 MΩ) were filled with internal solution containing (in mM): 135 $CsMeSO_3$, 10 HEPES, 4 $MgCl_2$, 4 Na_2ATP, 0.4 NaGTP, 10 sodium creatine phosphate and 0.2% Neurobiotin (Vector Laboratories) adjusted to pH 7.3 with CsOH. For current-clamp recordings (Fig. S3, potassium was substituted for cesium. Red-fluorescent Alexa Fluor-594 (10 μM, Invitrogen) and the green-fluorescent calcium (Ca2+)-sensitive Fluo-5F (300 μM, Invitrogen) were included in the pipette solution to visualize cell morphology and changes of intracellular Ca2+ concentration, respectively. Neurons were filled via the patch electrode for 10 min before imaging. In experiments for Fig. S4, we added an RGS4 antibody (Millipore, RBT17) at 1:100 dilution to the internal solution and substituted Cs-gluconate for $CsMeSO_3$ to improve giga-seal formation. Cells were dialyzed with the anti-RGS4 antibody for 10 minutes before imaging. For whole-cell voltage-clamp recordings, series resistance was 10-22 MΩ and uncompensated. Electrophysiological recordings were made using a Multiclamp 700B amplifier (Molecular Devices), filtered at 4 kHz, and digitized at 10 kHz.

2-photon imaging was accomplished with a custom-modified Olympus BX51-WI microscope (Olympus, Japan), including components manufactured by Mike's Machine Company (Higley and Sabatini, 2010). Fluorophores were excited using 840 nm light from a pulsed titanium-sapphire laser (Ultra2, Coherent). Emitted green and red photons were separated with appropriate optics (Chroma, Semrock) and collected by photomultiplier tubes (Hamamatsu).

For Ca2+ imaging, signals were collected during 500 Hz line scans across a spine and the neighboring dendritic shaft. Reference frame scans were taken between each acquisition to correct for small spatial drift over time. Ca2+ signals were first quantified as increases in green fluorescence from baseline normalized to the average red fluorescence ($\Delta G/R$). We then expressed fluorescence changes as the fraction of the G/R ratio measured in saturating Ca2+ ($\Delta G/G_{sat}$). To calculate G_{sat}, we imaged a 1:1 mixture of internal solution and 1 M $CaCl_2$ in a sealed recording pipette in the specimen plane under conditions identical to those used during recordings. Normalizing G/R to the saturated Ca2+ signal compensates for variations in fluorophore concentration and optical collection efficiency across experiments and laboratories.

2-Photon Glutamate uncaging and bAP activation

For focal stimulation of single dendritic spines, we used 2-photon laser uncaging of glutamate (2PLU). To photorelease glutamate, a second Ti-Sapphire laser tuned to 720 nm was introduced into the light path using polarization optics. Laser power was calibrated for each spine by directing the uncaging spot to the middle of the spine head. We adjusted uncaging power to achieve 50% photobleaching of the Alexa 594 dye filling the spine (Fig. S1). The power used for 2PLU ranged from 8 to 25 mW. For synaptic stimulation, we typically uncaged glutamate at 3-4 separate locations around a single spine head to find a "hot spot", the place of the largest response (Fig. S1). Back propagating action potentials (bAPs) were evoked by injecting brief current pulses (2 nA, 2 ms) into the cell through the recording pipette.

Data acquisition and analysis

Imaging and physiology data were acquired using National Instruments data acquisition boards and custom software written in MATLAB (Mathworks, (Pologruto et al., 2003)). Off-line analysis was performed using custom routines written in MATLAB and IgorPro (Wavemetrics). AMPAR-mediated EPSC amplitudes were calculated by finding the peak of the current traces and averaging the values within a 0.3 ms window. NMDAR-mediated currents were measured in a 3 ms window around the peak for isolated responses and 140 ms after the stimulus for non-isolated responses collected at +40 mV. 2PLU or AP-evoked ΔCa2+ was calculated as the average $\Delta G/G_{sat}$ over a 100 ms window, starting 5 ms after the uncaging or the AP was triggered. Statistical comparisons were conducted in GraphPad Prism 5. Unless otherwise stated, all data were analyzed using two-tailed, unpaired T-tests.

Pharmacology and reagents

2PLU experiments were performed in normal ACSF supplemented with MNI-glutamate (2.5 mM) and D-serine (10 μM). To isolate AMPAR-mediated currents in voltage clamp experiments, we added TTX (1 μM), picrotoxin (50 μM), CGP55845 (3 μM), and CPP (10 μM) to the ACSF. To isolate NMDAR-mediated currents, we modified our original ACSF to contain 0 mM Mg and 3 mM Ca2+ and included TTX (1 μM), picrotoxin (50 μM), CGP55845 (3 μM), and NBQX 10 μM. To selectively block GluN2B containing NMDARs in some experiments we included ifenprodil (3 μM) in the ACSF. In experiments investigating the effects of baclofen, CGP55845 was omitted from the solutions. For PKA pharmacology, we applied H89 (10 μM) or N6-benzo-cAMP (100 μM, Millipore). In some experiments, we included PKI(6-22) (20 μM) in the recording pipette. To block the actions of RGS4 we added CCG50014 (5 μM). All compounds were from Tocris except where noted.

Western blot analysis

For RGS4, phospho-GluA1 (S845) and phospho-GluN2B (S1166) western blot analysis, we prepared 300 μm thick brain slices containing the PFC from p22-42 C57/bl6 mice as described above. Following the recovery period, slices were distributed between 5 holding chambers containing normal ACSF for control or ACSF supplemented with either guanfacine 40 μM, baclofen 5 μM, H89 10 μM or forskolin 50 μM. Holding chambers were then incubated for an additional 10 minutes at 32-34°C before the prefrontal cortex was dissected out of the slices on ice. Tissue samples were homogenized and sonicated in ice cold lysis buffer containing 20 mM Tris, 1 mM EDTA and 1x Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and 0.5% SDS, pH 8.0. After a 10 minute centrifugation at 14000 rpm, the supernatant was collected and protein content was determined using Pierce BCA Protein Assay (Thermo Scientific). Samples containing equal amounts of protein were separated on a 6% poly-acrylamide gel and transferred to PVDF membranes. After blocking for 1h at room temperature with 3% non-fat milk and 0.02% Na-azide in Tris buffered salt solution with 0.05% Tween 20 (TBST), membranes were immunoreacted with primary antibody against phosphorylated

GluA1 S845 (Millipore, 04-1073), phosphorylated GluN2B S1166 (a generous gift from Suzanne Zukin, Albert Einstein College of Medicine) or RGS4 (Millipore, RBT17) in 1% milk and 0.02% Na-azide in TBST, 1:1000, overnight. After washing off excess primary antibody and incubation with the appropriate HRP conjugated secondary antibody (GE Healthcare, UK) for 2 hours at room temperature in TBST, bands were visualized using HyGlo Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific Inc.) and exposed onto autoradiography film (Denville Scientific Inc.). Membranes were then stripped from antibodies using Restore Plus Western Blot Stripping Buffer (15 minutes at room temperature, Thermo Scientific), re-blocked and immunoreacted with non-phospho specific anti-GluA1 (generously provided by Susumu Tomita, Yale University), anti-GluN2B (Millipore, MAB5778) or anti-β-tubulin (SIGMA) primary antibody followed by the appropriate HRP-secondary antibody to establish total amount of GluA1, GluN2B or β-tubulin in the samples. Autoradiography films were developed in a Kodak automatic developer, then scanned and analyzed with ImageJ. Phosphorylation was quantified as phoshorylated / total protein and normalized to the control values of each experiment.

Immunofluorescence and proximity ligation assay (PLA)

C57/bl6 mice (p22-40) were transcardially perfused with ice cold phosphate buffer (PB) followed by ice cold 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PB. After dissection, brains were post-fixed in 4% PFA for 3.5 hours at 4 °C. Sections (70 μm) containing the prefrontal cortex were cut on a vibrotome (Leica) and washed in PB. To expose synaptic proteins tissue sections were permeabilised with 0.1% Triton X-100 (SIGMA) in PB then treated with 0.25 mg/ml pepsin for 10 minutes at 37 °C in 0.2 N HCl. Nonspecific antibody binding was blocked in 10% normal goat serum (NGS, SIGMA) and 1% bovine serum albumin (BSA, SIGMA) in PB for 1 hour. To co-label GPCRs with the postsynaptic marker PSD95 or the presynaptic marker Bassoon, primary antibodies against PSD95 (UC Davis/NIH NeuroMab Facility, 75-028, 1:400 dilution) or Bassoon (Synaptic Systems, 141-021, 1:200), α2R (Neuromics, RA14110, 1:400) and $GABA_bR$ (Millipore, AB2255, 1:400) in 5% NGS, 1% BSA, 0.1% Triton X-100, 0.02% NaN3 in PB were applied overnight at 4 °C. Appropriate secondary antibodies labelled with Alexa 488 (1:1000), Alexa 555 (1:500) and Alexa 647 (1:500) (Invitrogen) were applied for 2 hours. Then sections were mounted on glass microscope slides with ProlongGold (Invitrogen). Images were randomly collected from the prefrontal cortical region of the sections (up to 20 images per mouse for both PSD95 and Bassoon, 4 mice total) using a Leica SP2 inverted confocal microscope (Leica Microsystems) with a 63x oil immersion objective at 10x optical zoom with the pinhole set to 1 Airy unit. Images were then analysed in Cell Profiler (Broad Institute) utilizing object detection to calculate the co-localization of GPCRs with pre- and postsynaptic markers. Images were then shifted by 15 pixels diagonally in ImageJ and re-analyzed with the same pipeline in Cell Profiler. Co-localization is given as the percentage of PSD95 or Bassoon puncta overlapping with both α2R and $GABA_BR$ staining.

To perform proximity ligation assay (PLA), 70 μm sections containing the prefrontal cortex were obtained from 3 mice and pepsin treated as described above. After blocking non-specific antibody binding with 10% donkey serum and 1% BSA in PB for 1 hour, glutamate receptors were co-labelled with GPCRs using primary antibodies against GluA1 (Synaptic Systems, 182 011, 1:2000) or GluN1 (BD Biosciences, 556308, 1:1000) and α2R (Neuromics RA14110, 1:2000) or $GABA_bR$ (Alomone Labs, AGB-001, 1:2000) overnight at 4 °C. Tissue sections were then mounted on microscope slides and PLA was performed using a Duolink In Situ kit (SIGMA) in accordance with the manufacturer's instructions. Briefly, primary antibodies were washed off in wash buffer A (Tris buffered saline with Tween 20, pH 7.4) and sections were incubated with PLA probes, diluted 1:5 in the supplied antibody diluent at 37 °C for 2 hours. PLA probes were then washed off with buffer A and sections were incubated with the Ligase, diluted 1:40 in Ligation buffer, for 1 hour at 37 °C. The tissue was then washed in buffer A and the signal was amplified for 30 minutes at 37 °C using the Polymerase diluted 1:80 in Amplification buffer. Sections were then washed in buffer B (Tris buffer saline, pH 7.5) and a coverslip was mounted

on the slide with Duolink In Situ Mounting Medium. Images were randomly collected from the prefrontal cortical region of the sections (10-11 images for each antibody pair from each mouse) on a Leica SP2 confocal microscope with a 63x oil immersion objective at 3x optical zoom with the pinhole set to 1 Airy unit. Fluorescent puncta were counted using object recognition in Cell Profiler.

Supplemental References

Higley, M.J., and Sabatini, B.L. (2010). Competitive regulation of synaptic Ca2+ influx by D2 dopamine and A2A adenosine receptors. Nature neuroscience *13*, 958-966.

Pologruto, T.A., Sabatini, B.L., and Svoboda, K. (2003). ScanImage: flexible software for operating laser scanning microscopes. Biomedical engineering online *2*, 13.