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Authors
Shen, Ao
Nieves-Cintron, Madeline
Deng, Yawen
et al.

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Functionally distinct and selectively phosphorylated GPCR subpopulations co-exist in a single cell

Ao Shen¹, Madeline Nieves-Cintron¹, Yawen Deng¹², Qian Shi¹, Dhrubajyoti Chowdhury¹, Jinyi Qi³, Johannes W. Hell¹, Manuel F. Navedo¹ & Yang K. Xiang¹,⁴

G protein-coupled receptors (GPCRs) transduce pleiotropic intracellular signals in a broad range of physiological responses and disease states. Activated GPCRs can undergo agonist-induced phosphorylation by G protein receptor kinases (GRKs) and second messenger-dependent protein kinases such as protein kinase A (PKA). Here, we characterize spatially segregated subpopulations of β₂-adrenergic receptor (β₂AR) undergoing selective phosphorylation by GRKs or PKA in a single cell. GRKs primarily label monomeric β₂ARs that undergo endocytosis, whereas PKA modifies dimeric β₂ARs that remain at the cell surface. In hippocampal neurons, PKA-phosphorylated β₂ARs are enriched in dendrites, whereas GRK-phosphorylated β₂ARs accumulate in soma, being excluded from dendrites in a neuron maturation-dependent manner. Moreover, we show that PKA-phosphorylated β₂ARs are necessary to augment the activity of L-type calcium channel. Collectively, these findings provide evidence that functionally distinct subpopulations of this prototypical GPCR exist in a single cell.
Activation of G protein-coupled receptors (GPCRs) transduces the canonical G protein-dependent signal as well as noncanonical G protein-independent signals, frequently via β-arrestins. In the past decades, it has been appreciated that some ligands can differentially activate a GPCR via a phenomenon known as functional selectivity or biased signaling. Depending on the receptor, different mechanisms have been proposed for biased GPCR signaling, which include ligand efficacy bias, receptor conformational bias, cell type and/or expression level-caused cellular bias. One of the universal features of GPCRs is that they undergo agonist-induced phosphorylation by a variety of kinases, which may also allow distinct structural features that favors receptor binding to different signaling partners. Molecular and structural details underlying biased agonism need to be further elucidated, especially how a single ligand–receptor pair can selectively transduce different signals in space and time in a single cell.
β₂AR, a prototypical GPCR, is involved in memory and learning in the central nervous system, and cardiovascular and metabolism regulation in peripheral systems. Stimulation of β₂AR promotes phosphorylation of serine 355 and 356 at the receptor C-terminal domain by GRKs, contributing to receptor desensitization and endocytosis. β₂AR also undergoes phosphorylation by PKA at serine 261 and 262 in the third loop and serine 345 and 346 in the C-terminal domain. Here we apply super-resolution imaging together with single molecular analysis to probe β₂AR subpopulations that undergo phosphorylation by GRKs and PKA after agonist stimulation. Our results show that GRKs and PKA selectively label two distinct subpopulations of β₂AR that are spatially segregated on the plasma membrane and undergo distinct membrane trafficking in both fibroblasts and neurons. Moreover, these two subpopulations exert distinct functions in modulating L-type calcium channel (LTCC) activity and neuron excitability.

Results

PKA and GRKs target spatially segregated β₂AR subpopulations. In this study, we characterized the subcellular distribution of β₂ARs upon agonist-induced phosphorylation by PKA and GRKs. We used two sets of well-characterized phospho-specific antibodies: anti-pS261/262 (monoclonal 2G3 and 2E1) and anti-pS355/356 (monoclonal 10A5, polyclonal 22191R, and 16719R) antibodies, and here with mutant β₂AR lacking either the PKA (PKAmut) or GRK (GRKmut) sites (Supplementary Fig. 1). β₂ARs localize on cell membrane at resting state (Fig. 1a). Using super-resolution structured illumination microscopy (SIM), we found that after acute stimulation with the β₂AR agonist isoproterenol (ISO, 30 s or 1 min), both PKA- and GRK-phosphorylated β₂ARs are primarily segregated at the plasma membrane (PM) of HEK293 cells (Fig. 1b, top panel; Fig. 1c, d). Pearson’s coefficient 0.078 ± 0.016 for ISO 30 s and 0.058 ± 0.015 for ISO 1 min, mean ± s.e.m, three independent experiments). Comparably, PKA- and GRK-phosphorylated β₂ARs highly co-localize with total β₂AR (Fig. 1b, bottom two panels; Fig. 1c, d). Pearson’s coefficient 0.671 ± 0.035 and 0.510 ± 0.039 for ISO 30 s, 0.601 ± 0.039 and 0.507 ± 0.033 for ISO 1 min, respectively, mean ± s.e.m, three independent experiments). After prolonging stimulation with ISO for 5 to 10 min, PKA- and GRK-phosphorylated β₂ARs display further spatiotemporal segregation: GRK-phosphorylated β₂ARs undergo internalization and form puncta inside the cells, whereas PKA-phosphorylated β₂ARs stay on the PM (Fig. 2a, b; Supplementary Fig. 2).

The segregation between PKA- and GRK-phosphorylated β₂AR was validated biochemically with immuno-isolation of GRK-phosphorylated FLAG-β₂AR with anti-pS355/356 antibody. The remaining β₂AR was subsequently immuno-isolated with anti-FLAG antibody, GRK-phosphorylated β₂ARs and PKA-phosphorylated β₂ARs were enriched in first and second immuno-isolations, respectively (Figs. 1e and 2c). We also applied surface biotinylation-based fractionation to separate PM from intracellular endosome after stimulation with ISO for 10 min. At a minimal dose of 1 nM ISO, β₂AR displayed phosphorylation only at the PKA sites, and the phosphorylated receptors remained at the PM. At a saturated dose of 1 μM ISO, GRK-phosphorylated β₂ARs were partitioned in the endosomal fraction, whereas PKA-phosphorylated β₂ARs remained in the PM fraction (Fig. 2d). These data demonstrate two subpopulations of β₂AR undergoing phosphorylation by PKA and GRKs and displaying distinct spatial distribution in a single cell.

PKA- and GRK-pβ₂ARs display distinct oligomeric states. We applied our recently developed single-molecule pulldown (SiMPull) assay to gain structural insight into β₂AR subpopulations that are modified by PKA or GRKs (Fig. 3a) (Fig. 3b, c). We co-expressed FLAG-mYFP-β₂AR and FLAG-mCherry-β₂AR at a 1:1 ratio in HEK293 cells, treated the cells with ISO, and pulled down the receptors with either anti-FLAG antibody or phospho-specific antibodies in SiMPull (Fig. 3a; Supplementary Fig. 3a). A reference construct with mYFP and mCherry fused into a single protein displayed 58.8% ± 1.6% (mean ± s.d., three independent experiments) overlap between the two proteins in SiMPull (Fig. 3b, c). The incomplete co-localization arises primarily from immature/inactive chromophores as both mYFP and mCherry display about a 75% fluorescent maturation ratio. PKA-phosphorylated mCherry-β₂AR had more than 20.4% ± 1.6% (mean ± s.d., three independent experiments) overlap with mYFP-β₂AR. In contrast, GRK-phosphorylated β₂AR had less than 3.2% ± 0.5% (mean ± s.d., three independent experiments) overlap between the mCherry and mYFP versions of β₂AR (Fig. 3b, c). As control, the total β₂AR pulled down with anti-FLAG antibody displayed 13.2% ± 0.8% (mean ± s.d., three independent experiments) of overlap between mYFP and mCherry (Fig. 3b, c).

SiMPull can reveal stoichiometry of protein complexes via single-molecule chromophore photobleaching step analysis when proteins are fluorescently labeled at a one-to-one ratio. For example, the photobleaching of a single monomeric YFP (mYFP) is a discrete process; thus the fluorescence intensity of a protein complex with one or several mYFP molecules drops in a stepwise fashion, and the number of steps reveals the number of mYFP-tagged proteins in the complex (Fig. 4a; Supplementary Fig. 3e–g). This approach was validated with reference membrane receptors including mYFP-fused monomeric CD86 and mYFP fused dimeric CD28 (Supplementary Fig. 3b). We then used this method to directly determine the number of β₂ARs in a complex by counting discrete steps of photobleaching of mYFP molecules. Consistent with our previous report, SiMPull photobleaching analysis revealed that β₂ARs can be found as a mixture of monomers and dimers (42.7% ± 1.5% dimers, mean ± s.d., 36 independent experiments). The composition of β₂AR was not affected by the expression levels of the receptor.
(Supplementary Fig. 3c) or concentrations of ISO (Fig. 4b). In the same cell lysates treated with ISO, PKA-phosphorylated β2ARs pulled down with anti-pS261/262 antibody were mainly dimers (68.8% ± 2.1% dimers, mean ± s.d., 26 independent experiments, Fig. 4c and Supplementary Fig. 3d). In comparison, GRK-phosphorylated β2AR pulled down with anti-pS355/356 antibody displayed a predominant monomeric composition (86.5% ± 2.1% monomers, mean ± s.d., 33 independent experiments, Fig. 4c).

Together, these results suggest that PKA- and GRK-phosphorylated β2ARs have different oligomeric assembly states.

β2AR subpopulations are spatially segregated in neurons. Activation of βARs promotes PKA-phosphorylation of ion channels including LTCC and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) to modulate

Fig. 2 PKA- and GRK-pβ2AR undergo distinct membrane trafficking. a: FLAG-β2ARs expressed in HEK293 cells were stimulated with ISO for indicated times. Confocal imaging shows PKA- and GRK-phosphorylated β2ARs, which were stained with anti-pS261/262 (PKA-pβ2AR) and anti-pS355/356 (GRK-pβ2AR) antibodies, respectively. Scale bar, 5 μm. Representative of n = 16 and 15 cells, respectively, three independent experiments. b: Numbers of fluorescent objects in each cell in a were quantified with ImageJ (n = 19, 16, and 15 cells, respectively, three independent experiments). Error bars denote s.e.m.; multiplicity adjusted P values are computed by one-way ANOVA followed by Tukey’s test between indicated groups. c: Immuno-isolation of PKA- and GRK-phosphorylated β2ARs in HEK293 cells using same procedure as Fig. 1d after stimulation with 1 μM ISO for 10 min. The β2AR in total IP and sequential IPs were resolved in SDS-PAGE, and probed with anti-FLAG, anti-pS261/262 (PKA-pβ2AR), and anti-pS355/356 (GRK-pβ2AR) antibodies, respectively. Representative of three independent experiments. d: FLAG-β2ARs expressed in HEK293 cells underwent cell surface biotin labeling and then were stimulated with ISO (1 nM or 1 μM) for 10 min. The biotin-labeled proteins on the plasma membrane were pulled with streptavidin beads, and the leftover biotin-labeled proteins in endosome were isolated by a second precipitation with streptavidin beads. Membrane (M) and endosome (E) fractions were resolved in Western blot with antibodies against FLAG, pS261/262 (PKA-pβ2AR), and pS355/356 (GRK-pβ2AR), showing separation of GRK- and PKA-phosphorylated subpopulations of β2AR. Representative of three independent experiments. Molecular weight markers (in kDa) are indicated on the left.
**Fig. 3** PKA- and GRK-pβ2AR display minimal overlap at the single-molecule level. **a** Schematic of single-molecule pulldown (SiMPull) assay. **b** Representative SiMPull images of a fusion mCherry-mYFP protein, total β2AR, PKA-phosphorylated β2AR, and GRK-phosphorylated β2AR pulled down from cell lysates expressing mCherry-β2AR and mYFP-β2AR at 1:1 ratio. Scale bar, 5 μm. Representative of n = 8, 10, 7, and 9 images, respectively, three independent experiments. **c** Quantification of overlap percentage between mCherry and mYFP from images in **b** (n = 8, 10, 7, and 9, respectively). Error bars denote s.d.; multiplicity adjusted P values are computed by one-way ANOVA followed by Tukey’s test between indicated groups.
membrane potential and synaptic activity\(^{23-25}\). In hippocampal immature neurons, while PKA- and GRK-phosphorylated \(\beta_2\)ARs were found in both soma and dendrites, similar to what had been observed in HEK293 cells, super-resolution images showed lack of co-localization between PKA- and GRK-phosphorylated \(\beta_2\)ARs (Supplementary Fig. 4). Interestingly, as neurons mature, more GRK-phosphorylated \(\beta_2\)ARs existed in cell bodies and less is found in distal dendrites (Fig. 5). In contrast, PKA-phosphorylated \(\beta_2\)ARs were enriched in both proximal and distal dendrites relative to soma in both mature and immature dendrites (Fig. 5; Supplementary Fig. 5). These data indicate PKA- and GRK-phosphorylated subpopulations of \(\beta_2\)AR are in different subcellular compartments in mature neurons and support specific

![Image](https://example.com/image.png)
roles of PKA-phosphorylated β2ARs in dendritic regions for regulation of ion channels during synaptic transmission.

PKA-pβ2ARs control activation of LTCC in hippocampal neurons. We have previously shown that the LTCC Cavα1.2 subunit forms a membrane complex with β2AR in the brain, and recently we have reported that ISO induces a β2AR-dependent activation of LTCC in neurons, and PKA-phosphorylation of serine 1928 of α1.2 displaces the β2AR from α1.2 and promotes channel activation. Deletion of β2AR genes (β2AR KO) abolished ISO-induced increases in overall channel activity (nPo) of LTCC Ca2+ as measured by single-channel recordings in hippocampal neurons (Fig. 6). In contrast, deletion of β1AR gene (β1AR KO) did not affect the ISO-induced LTCC responses (Fig. 6). The results suggest that β2AR but not β1AR play a specific role in modulation of LTCC Ca2+ activity in neurons.

We then introduced WT or mutant β2ARs lacking either PKA-phosphorylation sites (PKAmut) or GRK-phosphorylation sites (GRKmut) in hippocampal neurons with deficiency of both β1AR and β2AR genes (DKO) to examine the function of PKA-phosphorylation sites on β2AR in activation of LTCC. While WT and β2AR-GRKmut promoted PKA-phosphorylation of S1928 on α1.2 after ISO stimulation, deletion of PKA-phosphorylation sites on β2AR abolished ISO-induced PKA-phosphorylation of α1.2 S1928 (Fig. 7a). Unexpectedly, stimulation of WT and mutant β2ARs promoted similar increases in phosphorylation of serine 1700 on α1.2 in DKO neurons (Fig. 7a). S1700 is another PKA-phosphorylation site on α1.2 important for upregulation of LTCC activity in heart, but is not relevant in neurons. Moreover, reintroduction of WT and β2AR-GRKmut promoted ISO-induced dissociation of the receptor from α1.2 in both DKO hippocampal neurons and HEK293 cells (Fig. 7b; Supplementary Fig. 6), and increases in nPo of LTCC Ca2+ in neurons, whereas activation of β2AR-PKAmut failed to do so (Fig. 7c, d). Together, these data indicate that ISO-induced PKA-phosphorylation of β2AR is necessary to transduce signal to promote PKA-phosphorylation and activation of LTCC Ca2+ in hippocampal neurons.

Discussion

This study reveals that a GPCR (β2AR) can be present in functionally distinct subpopulations that are distributed at different subcellular locations in a single cell. These β2AR subpopulations undergo agonist-induced phosphorylation by GRKs and second messenger-dependent PKA, respectively, in which GRK- and PKA-phosphorylated subpopulations are segregated into distinct microdomains on the plasma membrane. Moreover, GRK- and PKA-phosphorylated β2AR subpopulations display distinct membrane trafficking in both fibroblasts and hippocampal neurons. While GRK-phosphorylated β2ARs undergo endocytosis, PKA-phosphorylated β2ARs remain on the cell surface. This is consistent with the literature that GRKs play a dominant and necessary role in agonist-induced β2AR endocytosis.

In hippocampal neurons, there is a further segregation of GRK- and PKA-phosphorylated β2AR subpopulations in a neuron maturation-dependent manner. In immature neurons with 6–8 days of culture in vitro, there is a small but appreciable enrichment of PKA-phosphorylated β2ARs in dendrites whereas GRK-phosphorylated β2ARs are relatively enriched in soma. In mature neurons with 18–21 days of culture in vitro, GRK-phosphorylated β2ARs are almost entirely enriched in soma and excluded from dendrites. In contrast, PKA-phosphorylated β2ARs are further enriched in proximal and distal dendrites. It is known that many proteins including sodium and potassium ion channels develop specific functions in neurons due to their selective targeting and distributions on axon, dendrites, and soma in a single neuron. To our knowledge, our data show that a GPCR, based on the subcellular distribution, can exist in different functional subpopulations in a fully mature neuron, which offers a distinct mechanism to develop functional heterogeneity of a protein in highly differentiated neurons. The molecular mechanisms underlying specific locations of individual subpopulations remain to be examined. Some known β2AR binding partners, such as G proteins, arrestins, A-kinase anchoring proteins (AKAPs), and caveolins, co-localize with one or both subpopulations in particular, AKAP79 is involved in agonist-induced PKA-phosphorylation and is essential for β2AR-induced activation of LTCC. It is also expected that both lipids and proteins that are in association with the receptor can play a role. On the basis of their subcellular distribution, PKA-phosphorylated β2ARs may be more relevant to synapse transmission. Indeed, deletion of agonist-induced PKA-mediated phosphorylation of β2AR completely abolishes the receptor-induced activation of LTCC in hippocampal neurons, supporting the critical role of PKA-phosphorylated β2AR subpopulation in synaptic regulation. In contrary, due to the important role of GRKs for endocytosis of β2AR, the GRK-phosphorylated subpopulation may be more likely involved in delivery of signal to the nucleus for gene expression.

Using single molecular approach, we gain further insight on β2AR subpopulations phosphorylated by GRKs and PKA. Our data show that GRKs primarily phosphorylate monomeric β2ARs whereas PKA mainly targets dimeric receptors. These data show biochemical evidence that GPCR composition may dictate post-translational modifications of individual receptors after agonist stimulation. It reveals that the dimeric β2AR subpopulations are not only specifically modified by PKA, but also retained at the cell surface after agonist stimulation. This together with the necessary role of PKA-phosphorylated β2ARs in activation of LTCC strongly argues the presence of functional dimeric β2AR subpopulations in neurons. It should be noted that the single molecular analysis in this study was done using cells with over-expressing β2AR. Although our data indicated that the composition of β2AR was not affected by the expression levels, we could not exclude the possibility that over-expression conditions can change the stoichiometry of the receptor. Endogenously targeting of β2AR with split fluorescent protein would be able to address this question.
There are many questions remaining to be addressed. For example, while our data show that ISO does not induce dynamic changes in ratio of β2AR monomer and dimers, others have reported that some ligands such as ICI-118551 and Carvedilol are capable to induce dynamic changes in monomer and dimer ratio of the receptor. In the same vein, we speculate that certain ligands may be able to induce dynamic changes in GRK and PKA-phosphorylated populations. Other interesting questions include why monomeric receptors are more sensitive to GRKs than PKA and vice versa and whether phosphorylation leads to change in ratio of β2AR monomer and dimers. Potential explanations may include: the pre-coupling of different kinase (and scaffold proteins) with receptors; different accessibility of two kinases (receptor intracellular loops vs. C-terminal); and different locations at lipid rafts or non-rafts microdomains. Moreover, it remains to be determined whether neurons maturation affects the monomer and dimer ratio of the receptor. These are the questions that we look forward to pursuing in the near future.

Together, these data provide molecular mechanisms on the existence and functional relevance of distinct subpopulations of β2AR. It is conceivable that PKA- and GRK-phosphorylated

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**Fig. 5** Subpopulations of β2AR are spatially segregated between dendrites and soma in hippocampal neurons. Primary hippocampal neurons expressing FLG-β2AR at 18-21 days in vitro (DIV, mature, a) or 6-8 DIV (immature, b) were treated with 1 μM ISO for 5 min (a) or 10 min (b). Neurons were stained with phospho-specific antibodies against S261/262 (PKA-pβ2AR) and S355/356 (GRK-pβ2AR) as indicated to show phosphorylated β2ARs in soma, and proximal and distal dendrites (Scale bar, 2 μm. n = 25 and 20 cells, respectively, three independent experiments). Representative SIM images in a and b were from one single neuron. c Numbers of fluorescent objects per 20 μm² regions were quantified with ImageJ in 18-21 DIV mature neurons (PKA-pβ2AR, soma 29.6 ± 1.7, proximal dendrites 42.6 ± 2.2, and distal dendrites 38.6 ± 1.8; GRK-pβ2AR, soma 50.4 ± 2.7, proximal dendrites 14.9 ± 0.7, and distal dendrites 5.1 ± 0.6). d Numbers of fluorescent objects per 20 μm² regions were quantified with ImageJ in 6-8 DIV immature neurons (PKA-pβ2AR, soma 31.4 ± 1.7 and dendrites 42.6 ± 1.8; GRK-pβ2AR, soma 45.1 ± 2.0, and dendrites 36.8 ± 1.5). Error bars denote s.e.m., multiplicity adjusted P values are computed by one-way ANOVA followed by Tukey’s test between indicated groups.
subpopulations can lead to divergent downstream intracellular signals in space and time, offering a molecular basis on biased signaling transduced from activation of β2AR in a single cell. This study unveils a paradigm that can be extended to study other GPCRs in general. It also offers a structural platform to discover and study biased drugs that selectively modulate individual subpopulations of a GPCR.

Methods

Cell culture and transfection. Human embryonic kidney HEK293 cells were from American Type Culture Collection (ATCC) and were maintained in Dulbecco’s modified Eagle medium (Mediatech, VA) supplemented with 10% fetal bovine serum (Sigma, MO). Primary mouse hippocampal neurons were isolated and cultured from early postnatal (P0-P1) wild type, β2AR knockout (KO), β2AR KO and β2AR/β2AR double knockout (DKO) mouse pups, and primary rat hippocampal neuronal cultures were prepared from E17–E19 embryonic rats. Briefly, dissected hippocampi were dissociated by 0.25% trypsin treatment and triturated. Neurons were plated on poly-l-lysine-coated (Sigma, MO) glass coverslips for imaging and 6-well plate for biochemistry at a density of 7500 cells/cm² and 10,000 cells/cm², respectively. Neurons were cultured in Neurobasal medium supplemented with GlutaMax and B-27 (Thermo Scientific, MA).

Antibodies and chemicals. Mouse monoclonal antibodies against β2AR at serine 261/262 (clone 2G3 and 2E1) and at serine 355/356 (clone 10A5) were kindly provided by Dr. Richard Clark (UT Houston). Polyclonal antibodies against α11.2 residues 754–901 for total α11.2 (PFI), α11.2 residues 1923–1935 for phosphorylated serine 1928 site (LGRAPSFHLECKL, pS1928) and α11.2 residues 1694–1709 for phosphorylated serine 1700 site (EIRRAPSGDLTAEEEL, pS1700) were made in house. Other antibodies used in the experiments include: anti-FLAG-M1 and biotinylated anti-FLAG-M2 (F3040 and F9291, Sigma, MO), biotinylated goat anti-mouse IgG, and goat anti-rabbit IgG (111065R) were purchased from Santa Cruz Biotechnology (SCBT, CA). Polyclonal antibodies against α2a (m20 and sc-570) and phosphorylated serine 11.2 residues 1754–1926 (clone 2G3 and 2E1) and at serine 355/356 (clone 10A5) were kindly provided by Dr. Moritz Bunemann (Philipps University of Marburg, Germany), in which CFP was replaced by FLAG-mYFP. FLAG and FLAG-mYFP-tagged β2AR and its phosphorylation-deficient mutants were inserted into lentiviral vector pLenti-H1-CAG (kindly provided by Dr. Sergi Simo, UC Davis), and were used to produce neuron-preferential lentivirus according to a method described elsewhere. Neurons at 10 DIV were infected by same titer of lentiviruses as indicated, and were used in single-molecule pulldown analysis at 14 DIV.

SIM and confocal microscopy imaging. HEK293 cells growing on poly-d-lysine-coated coverslips were transfected with FLAG-β2AR or one of its mutants. Rat hippocampal neurons Growing on poly-d-lysine-coated coverslips were transfected with FLAG-β2AR at either 6–8 DIV or 18–21 DIV.

Cells were serum-starved for 2 h and stimulated with 1 μM ISO at indicated times. Cells were then fixed, permeabilized, and co-stained with indicated antibodies with a final concentration of 1 μg/ml for each antibody, which were revealed with a 1:1000 dilution of Alexa fluor 488 or Alexa fluor 594 conjugated goat anti-mouse or anti-rabbit IgG antibodies, respectively (A-11032, A-11037, A-11029, A-11034. Life technologies, CA). Fluorescence images were taken by Zeiss LSM 700 confocal microscope with a ×63/1.4 numerical aperture oil-immersion objective lens or Nikon 3D structured illumination (N-SIM) super-resolution microscope with a ×100/1.49 numerical aperture TIRF oil-immersion objective lens (MIB Imaging Facility, UC Davis). For SIM data, each sample was imaged from three angles and each angle was phase-shifted 5 times, 15 raw data images were then convolved to a reconstructed super-resolution image. Quantitative image analysis was carried out on unprocessed images using ImageJ software (http://rsb.info.nih.gov/ij). Co-localization analysis was assessed by calculating the Pearson’s correlation coefficient between two-color channels in the indicated images using the co-localization plug-in for ImageJ. Numbers of fluorescent objects within each 20 μm² regions in images of neurons were quantified using the Squash plug-in for ImageJ.

Plasmids and viruses. Plasmids containing FLAG-tagged mouse β2AR (FLAG-β2AR) and its phosphorylation-deficient mutants lacking pS261/262 or pS355/356 (FLAG-β2AR-PKAmut or FLAG-β2AR-GRKmut) were described before. DNA constructs containing HA-tagged rat L-type calcium channel (LTCC) α1.2, β2a and α4,8 subunits were described elsewhere. Plasmids containing monomeric CFP-CRD8 and constitutively dimeric CFP-CD28 were kindly provided by Dr. Moritz Bunemann (Philips University of Marburg, Germany), in which CFP was replaced by FLAG-mYFP. FLAG and FLAG-mYFP-tagged β2AR and its phosphorylation-deficient mutants were inserted into lentiviral vector pLenti-H1-CAG (kindly provided by Dr. Sergi Simo, UC Davis), and were used to produce neuron-preferential lentivirus according to a method described elsewhere. Neurons at 10 DIV were infected by same titer of lentiviruses as indicated, and were used in single-molecule pulldown analysis at 14 DIV.

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Fig. 6 β2AR is necessary for ISO-induced upregulation of LTCC Ca2.1,2 activity in hippocampal neurons. a Representative single-channel recordings of LTCC Ca2.1,2 currents in hippocampal neurons from wild type (WT), β2AR knockout (KO) and β2AR KO mice at 7–14 DIV after depolarization from −80 to 0 mV without (black traces) and with stimulation of 1 μM ISO (red traces) in the patch pipette. b Data in a were quantified and plotted. The ISO-induced increases in LTCC overall channel activity (nPo) were detected in WT and β2AR KO neurons, but absent in β2AR KO neurons (n = 8, 11, 4, 6, 20, and 11 cells, respectively). Error bars denote s.e.m., exact P values are computed by Mann-Whitney test.
**Fig. 7** PKA-β₂AR is necessary for activation of β₂-AR-α₁.2 complex in hippocampal neurons. WT or mutant β₂AR lacking either PKA sites (PKAmut) or GRK sites (GRKmut) was expressed in hippocampal neurons lacking both β₁AR and β₂AR genes (DKO). a Neurons were either not stimulated (ND) or stimulated with 1 μM ISO for 5 min. The total α₁.2 was immunoprecipitated; the phosphorylation of α₁.2 at S1928 and S1700 was probed with phospho-specific antibodies and normalized to total α₁.2. Representative of three independent experiments. Molecular weight markers (in kDa) are indicated on the left. b The endogenous α₁.2 was pulled down in SiMPull as depicted; representative images show that mYFP-β₂AR pulled down together with α₁.2. Scale bar, 5 μm. Quantification of the numbers of mYFP-β₂AR bound to α₁.2 shows that PKA-phosphorylation of β₂AR is required for dissociation of the β₂AR-α₁.2 complex. Representative of n = 13/12, 13/15, and 12/12 images for WT, PKAmut, and GRKmut β₂AR groups, four independent experiments. c DKO hippocampal neurons at 7–14 DIV expressing WT and mutant β₂AR were subjected to single-channel recording of LTCC currents using the same method as Fig. 6. d The overall channel activity (nPo) of LTCC Ca₁.2 was quantified from c (n = 11, 10, 14, 10, 12, and 10 cells, respectively). Error bars denote s.e.m., exact P values are computed by Student’s t-test in a and b, and by Mann–Whitney test in d.
SiMPull co-localization and photobleaching analysis. Single-molecule co-localization between mYFP and mCherry was performed using scripts written in Matlab (MathWorks). Briefly, images of the two regions were taken using mYFP and mCherry excitation. The fluorescent spots in both images were fit with Gaussian profiles to determine the center positions of mYFP and mCherry molecules to half-pixel accuracy. The mCherry and mYFP molecules within a 1-pixel distance (~150 nm) were considered as co-localized. The overlap percentage was calculated as the number of co-localized mYFP molecules divided by the total number of mYFP molecules.

Single-molecule fluorescence time traces of immobilized mYFP-tagged proteins were manually scored for the number of bleaching steps by a well-established method. To avoid false co-localization, samples were imaged (fluorescence frames) at an optimal diffusion coefficient (100–400 molecules in a 2 μm2 imaging area). The number of photobleaching steps (single frame intensity drops of equal size) in each trace was manually determined. The fluorescence trace of each molecule was classified as having 1–4 bleaching steps or was discarded if no clean bleaching steps could be identified (Supplementary Fig. 3). At least 1000 molecules were analyzed for each condition. The distribution of observed bleaching events and discarded traces is reported in Supplementary Table 1.

Determination of stoichiometry of fluorescent proteins. mYFP molecules are about 75% fluorescent active (visible) and 25% fluorescent inactive (invisible) in cells. This means that a constitutively dimeric mYFP-CD28 will statistically yield 56.25% 2-steps photobleaching events when both mYFP are active in the dimers; and 37.5% 1-step photobleaching events when only one copy of mYFP are fluorescently active in the dimers; and 6.25% dark events when both mYFP are fluorescently inactive in the dimers, which are undetectable in SiMPull. Accordingly, the percentage of monomer and dimer of a target protein was calculated by fitting bleaching data with the equation:

\[
P_{\text{dimer}} = \frac{7500 \times Q_{\text{2-steps}}}{56.25 - 18.75 \times Q_{\text{2-steps}}}, \quad P_{\text{monomer}} = 100 - P_{\text{dimer}}.
\]

Here, \(P_{\text{dimer}}\) is the percentage of dimer of a target protein, \(P_{\text{monomer}}\) is the percentage of monomer of a target protein, and \(Q_{\text{2-steps}}\) is the quotient of the numbers of 2-steps bleaching events divided by numbers of overall bleaching events.

Immunooisolation and co-immunoprecipitation. For immunooisolation tests, HEK293 cells stably expressing Flag-βAR were serum-starved for 2 h and stimulated with 1 μM ISO for 30 s or 10 min, then harvested by lysis buffer (10 mM Tris pH 7.4, 1% NP40, 150 mM NaCl, 2 mM EDTA) with protease and phosphatase inhibitor cocktail. Clarified lysates were incubated with anti-p5355/356 βAR (sc-16719R) and protein-A agarose (Thermo Scientific, MA) overnight at 4 °C. The beads were collected as the first immuno-isolation. Subsequently, the supernatants were further incubated with anti-FLAG-M2 agarose (Sigma, MO) for 2 h at 4 °C. These beads were collected as the second immuno-isolation. A control group was incubated with 1 μM mYFP antigen (MCVCIc), which was used as a control to block N and P0-1 type Ca2+ channels, respectively, and (S)–(–) BayK-8644 (500 nM) was included in the pipette solution to promote longer open times and resolve channel openings. Indeed, BayK-8644 is routinely used to augment detection of L-type channels in single-channel recordings. To examine the effects of β-adrenergic stimulation on the L-type Ca2+1.2 single-channel activity, 1 μM isoproterenol was added to the pipette solution in independent experiments. Note that we have previously used the L-type Ca2+1.2 channel blocker nifedipine (1 μM) to confirm the recording of L-type Ca2+1.2 currents under control conditions and in the presence of isoproterenol. Single-channel activity was recorded during a single pulse protocol (2 s) from holding potential of −80 mV to 0 mV every 5 s. An average of 50 sweeps were collected with each recording file under all experimental conditions. The half-amplitude event-detection algorithm of pClamp 10 was used to measure overall single-channel L-type Ca2+1.2 activity as nP0, where n is the number of channels in the patch and P0 is the open probability. Note that the number of channels in each patch recording (n) was not estimated and that all data are presented as “nPo” (product of n and channel open probability). nP0 values were pooled for each condition and analyzed with GraphPad Prism software.

Statistical analysis. Data were analyzed using GraphPad Prism software and expressed as mean ± s.d. or mean ± s.e.m. as indicated in figure legends. Differences between two groups were assessed by appropriate two-tailed unpaired Student’s t-test or nonparametric Mann–Whitney test. Differences among three or more groups were assessed by one-way ANOVA with Tukey’s post hoc test. P < 0.05 was considered statistically significant.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
A.S. and Y.K.X. conceived and designed experiments. A.S., M.F.N., M.N.-C. and Q.S.
performed experiments. Y.D. helped in data analysis. D.C. provided hippocampal neuron
culture. J.Q. provided analytic tools. A.S. and Y.K.X. analyzed data and wrote the
manuscript with inputs from M.F.N. and J.W.H. Y.K.X. provided overall project supervision.

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