β-blockers augment L-type Ca\(^{2+}\) channel activity by targeting spatially restricted β\(_2\)AR signaling in neurons

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Abstract

G protein-coupled receptors (GPCRs) transduce pleiotropic intracellular signals in mammalian cells. Here, we report neuronal excitability of β-blockers carvedilol and alprenolol at clinically relevant nanomolar concentrations. Carvedilol and alprenolol activate β2AR, which promote G protein signaling and cAMP/PKA activities without action of G protein receptor kinases (GRKs). The cAMP/PKA activities are restricted within the immediate vicinity of activated β2AR, leading to selectively enhance PKA-dependent phosphorylation and stimulation of endogenous L-type calcium channel (LTCC) but not AMPA receptor in rat hippocampal neurons. Moreover, we have engineered a mutant β2AR that lacks the catecholamine binding pocket. This mutant is preferentially activated by carvedilol but not the orthosteric agonist isoproterenol. Carvedilol activates the mutant β2AR in mouse hippocampal neurons augmenting LTCC activity through cAMP/PKA signaling. Together, our study identifies a mechanism by which β-blocker-dependent activation of GPCRs promotes spatially restricted cAMP/PKA signaling to selectively target membrane downstream effectors such as LTCC in neurons.

Introduction

GPCRs often signal not only through canonical G proteins but also through noncanonical G protein-independent signaling, frequently via G protein receptor kinases (GRKs) and β-arrestins (1, 2). One of the universal features of GPCRs is that they undergo ligand-induced phosphorylation at different sites by either GRKs or second messenger dependent protein kinases such as protein kinase A (PKA). The phosphorylated GPCRs thus may present distinct structural features that favor receptor binding to different signaling partners, engaging distinct downstream signaling cascades (3-5). Some ligands can differentially activate a GPCR via a phenomenon known as functional selectivity or biased signaling (6, 7). For example, stimulation of β2-adrenergic receptor (β2AR), a prototypical GPCR involved in memory and learning in the central nervous system (CNS) and regulation of metabolism and cardiovascular function, promotes phosphorylation by both GRKs and PKA (8-11). We
have recently identified spatially segregated subpopulations of \( \beta_2 \)AR undergoing exclusive phosphorylation by GRKs or PKA in a single cell. These findings indicate specific GPCR subpopulation-based signaling branches can co-exist in a single cell (12). GRK-mediated phosphorylation promotes pro-survival and cell growth signaling via \( \beta \)-arrestin-dependent mitogen-activated protein kinase (MAPK/ERK) pathways, prompting the search for biased ligands that preferentially activate \( \beta \)-arrestin pathways (13-18). On the other hand, our recent studies show that the cAMP/PKA-dependent phosphorylation of \( \beta_2 \)AR controls ion channel activity at the plasma membrane in primary hippocampal neurons (12).

\( \beta \)-blockers are thought to reduce cAMP signaling because they either reduce basal activity of \( \beta \)ARs or block agonist-induced receptor activation. While \( \beta \)-blockers are successful in clinical therapies of a broad range of diseases, their utility is limited by side effects in both the CNS and peripheral tissues (19, 20). Indeed, studies have revealed that some \( \beta \)-blockers display partial agonism and can promote receptor-Gs coupling at high concentrations \textit{in vitro} (21-23). Accordingly, some \( \beta \)-blockers display intrinsic properties mimicking sympathetic activation (sympathomimetic \( \beta \)-blockers) (24-26). The mechanism remains poorly understood because classic cAMP assay do not show even minimal cAMP signal induced by these \( \beta \)-blockers (24, 25).

In this study, we show that the \( \beta \)-blockers carvedilol and alprenolol can promote Gs protein coupling to \( \beta_2 \)AR and cAMP/PKA but not GRK activity at nanomolar concentrations. Thus, these \( \beta \)-blockers are emerging as partial agonists even at low concentrations rather than strict antagonists in mammalian cells. This cAMP/PKA signaling is spatially restricted, selectively promoting phosphorylation of \( \beta_2 \)AR and \( \text{Cav}_1.2 \) by PKA which augments LTCC activity in primary hippocampal neurons. Furthermore, we have engineered a mutant \( \beta_2 \)AR that can be selectively activated by carvedilol but not by the orthosteric agonist isoproterenol (ISO) to stimulate PKA but not GRK. Together, these studies identify a unique mechanism by which \( \beta \)-blockers activate \( \beta_2 \)AR at low concentrations, which promotes Gs/cAMP/PKA signaling branch
and selectively targets downstream LTCC channels in neurons. This observation may also explain sympathomimetic effects of β-blockers in the CNS.

Results

Carvedilol and alprenolol selectively promote β2AR-mediated PKA-phosphorylation of β2AR.

In this study, we applied two sets of well-characterized phospho-specific antibodies, anti-pS261/262 and anti-pS355/356 to examine a series of β-blockers for their effects on the phosphorylation of β2AR at its PKA and GRKs sites, respectively (12, 27, 28). We found that various β-blockers including alprenolol (ALP), carvedilol (CAR), propranolol (PRO) and CGP12177 (177) were able to stimulate phosphorylation of β2AR at PKA sites expressed in HEK293 cells, similar to the βAR agonist isoproterenol (ISO) (Figure 1A and Figure 1-figure supplement 1A). In contrast, other β-blockers, i.e., ICI118551 (ICI), timolol (TIM) and metoprolol (MET), were not able to do so (Figure 1A). The ligand-induced phosphorylation of β2AR was blocked by β2AR-specific antagonist ICI but not β1AR-specific antagonist CGP20712A (CGP) (Figure 1B and C, and Figure 1-figure supplement 1B and C). We chose CAR and ALP for further study. We found that CAR and ALP promoted phosphorylation of β2AR by PKA even at nanomolar concentrations (Figure 2A and B, and Figure 2-figure supplement 1A and B), which was paralleled by concentration-dependent increases in phosphorylation of ERK (Figure 2-figure supplement 2). The roles of β2AR and PKA in this phenomenon were confirmed by inhibition of β2AR with ICI and inhibition of PKA with H89, respectively (Figure 2C and D, and Figure 2-figure supplement 1C and D). In contrast, those β-blockers induced at best minimal increases in phosphorylation of β2AR at GRK sites and only at high concentrations, consistent with a previous report (29) (Figure 1A and Figure 2-figure supplement 3). As positive control, the βAR agonist ISO promoted robust increases in both PKA and GRK phosphorylation of the receptors at different concentrations ranging from nanomolar to micromolar (Figure 1 and 2, and Figure 2-figure supplement 2 and 3). In the CNS, β2AR emerges as a prevalent postsynaptic norepinephrine effector at glutamatergic
synapses (30-33). Consistent with the data from HEK293 cells, we found β-blockers
CAR and ALP activated β2AR and promoted phosphorylation of the receptor by PKA
in hippocampal neurons (Figure 2E). Together, these data suggest that certain
β-blockers selectively promote PKA phosphorylation of β2AR in HEK293 and primary
hippocampal neurons.

Carvedilol and alprenolol promote Gsα recruitment to β2AR and increase
spatially restricted cAMP signal.
The western blot data on PKA phosphorylation of β2AR indicates a stimulation of the
receptor-mediated Gs/AC/cAMP pathway by these β-blockers. We measured
ligand-induced Gsα recruitment to β2AR with an in situ proximity ligation assay (PLA),
which allows direct visualization and quantification of protein-protein interactions. We
showed that ISO, CAR and ALP were able to increase the PLA signals between β2AR
and Gsα, indicating recruitment of Gsα to β2AR (Figure 3A). As control, TIM had no
effect on the recruitment of Gsα to β2ARs. The role of Gs/AC in CAR-induced PKA
phosphorylation of β2AR was further validated by AC-specific inhibition with
2′,5′-dideoxyadenosine (ddA, Figure 3-figure supplement 1). These data indicate
that CAR and ALP are able to stimulate β2AR-Gs signal to increase PKA
phosphorylation of the receptor.

β-blockers have been thought to generally block β2AR-induced cAMP signal. We
hypothesized that the cAMP signal induced by β-blockers is restricted to local plasma
membrane domains containing activated receptor, which is not detectable with
traditional cAMP assays likely due to limited sensitivity. We applied the highly
sensitive FRET-based biosensor ICUE3 to detect the dynamics of cAMP signal in
living cells (34, 35). The full agonist ISO promoted cAMP signal in HEK293 cells while
all β-blockers failed to do so (Figure 3B), in agreement with the classic definition of
β-blockers. However, when cells were treated with non-selective phosphodiesterase
(PDE) inhibitor IBMX, CAR, ALP and CGP12177 were able to induce small but
significant cAMP signal in HEK293 cells (Figure 3C), indicating a role of PDE in
suppressing and restricting the distribution of cAMP in the cells. When \( \beta_2 \)AR was exogenously expressed in HEK293 cells, CAR and ALP were able to induce cAMP signal in HEK293 cells even without PDE inhibition (Figure 3-figure supplement 2), probably due to insufficient cAMP-hydrolytic activity of endogenous PDEs to counter cAMP production induced from overexpressed \( \beta_2 \)AR. We then engineered a targeted cAMP biosensor by fusing the biosensor ICUE3 to the C-terminus of \( \beta_2 \)AR (\( \beta_2 \)AR-ICUE3), aiming to detect increases of cAMP within the local domain of the receptor. CAR and ALP promoted cAMP signals within the immediate vicinity of activated \( \beta_2 \)AR even at nanomolar concentrations (Figure 3D and E). The local increases of cAMP were abolished by inhibition of \( \beta_2 \)AR with ICI or inhibition of ACs with ddA (Figure 3E). We also used two generic plasma membrane (PM) targeted ICUE3 sensors to further characterize how the CAR and ALP generated cAMP signals are localized when compared to the full agonist ISO. Interestingly, neither CAAX-ICUE3 targeting to the non-rafts regions of PM nor LYN-ICUE3 targeting to the rafts regions of PM could sense cAMP induced by CAR and ALP, while ISO induced cAMP were readily detectable on PM (Figure 3-figure supplement 3), this further demonstrates that CAR and ALP only promote cAMP within the immediate vicinity of \( \beta_2 \)AR. These data confirm that CAR and ALP promote cAMP/PKA activity within the immediate vicinity of activated \( \beta_2 \)AR, in contrast to the broad distribution of cAMP/PKA activities induced by ISO in the cells.

Carvedilol augments the endogenous \( \beta_2 \)AR-dependent PKA phosphorylation of \( \text{Ca}_V_1.2 \) and its channel activity in hippocampal neurons.

Local cAMP signals possess the potential to selectively regulate downstream effectors in receptor complexes or within the vicinity of activated receptors. In the CNS, \( \beta_2 \)AR emerges as a prevalent postsynaptic norepinephrine effector at glutamatergic synapses, where \( \beta_2 \)AR functionally interacts with AMPA receptor (AMPAR) and L-type \( \text{Ca}^{2+} \) channel (LTCC) \( \text{Ca}_V_1.2 \), and regulates neuronal excitability and synaptic plasticity (30-33). CAR and ALP, but not TIM significantly increased PKA phosphorylation of S1928 and S1700 of central \( \alpha_1.2 \) subunit of \( \text{Ca}_V_1.2 \) in
hippocampal neurons when both β2AR and LTCC were endogenously expressed (Figure 4A, and Figure 4-figure supplement 1A). However, CAR and ALP failed to promote phosphorylation of the AMPAR subunit GluA1 on its PKA site serine 845 (Figure 4B, and Figure 4-figure supplement 1B). Like Cav1.2, AMPARs are associated with β2AR, Gs, AC and PKA (30-33). These results indicate high selectivity in targeting downstream substrates by this β-blocker-induced signaling in hippocampal neurons. Meanwhile, the CAR and ALP-induced PKA phosphorylation of LTCC were blocked by β2AR inhibitor ICI, AC inhibitor ddA, and PKA inhibitor H89, but not CaMKII inhibitor KN93, validating the activation of β2AR-cAMP-PKA pathway (Figure 4C, and Figure 4-figure supplement 1C). We then examined the effects of CAR on PKA-dependent activation of LTCC Cav1.2 channels using cell-attached single channel recordings in hippocampal neurons. As shown before, ISO stimulates LTCC activity (Figure 5) (12, 30). Consistent with the phosphorylation data, CAR but not TIM significantly increased the open probability, channel availability and mean ensemble average of endogenous LTCC in rat hippocampal neurons (Figure 5 and Figure 5-figure supplement 1). CAR stimulated channel activity when present in the patch pipette solution but not when applied outside the patch via bath perfusion (Figure 5 and Figure 5-figure supplement 1D). Moreover, backfilling experiments with CAR found that L-type channels activity was relatively low at the beginning of the recording but then it significantly increased as the drug diffused to the pipette tip (Figure 5F-G and Figure 5-figure supplement 1C). Consistent with our hypothesis and prior studies (30), ISO applied outside the patch via bath perfusion was still able to stimulate LTCC activity (Figure 5-figure supplement 1B). These data indicate that CAR promotes spatially restricted cAMP/PKA activities for selective augmentation of LTCC activities in neurons. We further found that the activation of LTCC by CAR promoted cell death in cortical neuron cultures, and inhibition of β2AR or LTCC counteracted carvedilol-induced neuronal toxicities (Figure 5-figure supplement 2).

Carvedilol but not isoproterenol selectively activates a mutant β2AR to augment LTCC activity in neurons.
Structure-functional analyses of β2AR have previously revealed distinct residues important for binding to catecholamines and β-blockers (36-39). We hypothesized that mutation of Ser204 and Ser207 sites within β2AR binding pocket would abolish receptor hydrogen bonds with the catecholamine phenoxy moieties, thus reducing binding affinity to agonist ISO while having no effect on β-blocker binding (Figure 6A). Such a mutant β2AR could thus be selectively activated by CAR. We co-expressed the cAMP biosensor ICUE3 together with either wild-type (WT) β2AR or mutant S204A/S207A β2AR in MEF cells lacking endogenous β1AR and β2AR (DKO) to detect receptor signaling induced by different ligands. The mutant S204A/S207A β2AR induced a moderate cAMP signal at high but not low concentrations of ISO (Figure 6B). In contrast, after stimulation with CAR, the β2AR mutant S204A/S207A promoted significant cAMP signals at nanomolar concentrations; the overall concentration response curve was similar to those induced by WT β2AR (Figure 6B). Accordingly, the ISO-induced PKA phosphorylation of β2AR S204A/S207A mutant was selectively abolished at nanomolar concentrations. At higher concentrations, ISO was able to induce reduced PKA phosphorylation of the β2AR S204A/S207A mutant when compared to WT β2AR, consistent with the data of cAMP signals (Figure 6C, and Figure 6-figure supplement 1A). Meanwhile, ISO failed to induce GRK phosphorylation of β2AR S204A/S207A mutant at different concentrations (Figure 6C). In comparison, CAR induced equivalent PKA phosphorylation of β2AR WT and S204A/S207A mutant at different concentrations (Figure 6D, and Figure 6-figure supplement 1B). These data suggest that CAR, but not ISO selectively activates the S204A/S207A mutant β2AR at nanomolar concentrations. We then tested the effects of β2AR S204A/S207A mutant on LTCC channel activity after treatment with CAR in hippocampal neurons. In DKO neurons expressing the mutant S204A/S207A β2AR, CAR, but not ISO (30 nM) promoted PKA phosphorylation of LTCC α1.2 (Figure 7A and B and Figure 7-figure supplement 1). In agreement, CAR, but not ISO significantly increased the open probability, channel availability and mean ensemble average of LTCC (Figure 7C-7G). Together, CAR but not ISO selectively activates the S204A/S207A mutant β2AR at low concentrations and increases channel opening
probabilities.

Discussion

In a classic view, agonist stimulation promotes both PKA and GRK phosphorylation of activated GPCRs. Although previous studies have reported that some β-blockers promote βAR-Gs coupling and thus might display partial agonism, this phenomenon is only observed at high concentrations and *in vitro* with reconstituted systems (21-23).

In this study, using a combination of highly sensitive tools such as engineered FRET-based cAMP sensors and single channel recording together with detection by phospho-specific antibodies, we show for the first time that β-blockers such as CAR and ALP can promote receptor-Gs coupling at nanomolar concentrations in living cells, which is clinically relevant in contrast to superphysiological concentrations in previous studies. In detail, as low as 1 nM alprenolol as well as 1 nM carvedilol induce 20-40% of maximal effects (as obtained with 1 μM isoproterenol) with respect to phosphorylation of β2AR by PKA and to cAMP production detected by the ICUE3 sensor coupled to β2AR. Unlike agonists, activation of β2AR by β-blockers selectively transduce G protein/cAMP/PKA signaling but not GRK signaling. More importantly, the β2AR-induced cAMP signal is highly spatially restricted to the local domain of activated β2AR, which selectively promotes activation of receptor-associated LTCC but not receptor-associated AMPAR, two downstream ion channels essential for adrenergic regulation of neuronal excitability in hippocampal neurons. The differential signaling by carvedilol with respect to LTCC and AMPAR is especially remarkable because both channels form complexes with β2AR that are localized within dendritic spines. Moreover, we have engineered a mutant β2AR that is selectively activated by β-blockers but not by catecholamines at low concentration. Our study defines CAR and ALP as Gs-biased partial agonists of βAR for highly spatially restricted cAMP/PKA signaling to CaV1.2 in neurons. The study exemplifies a unique mechanism by which β-blockers shape the compartmentalization of βAR signaling and a highly restrictive distribution of ligand-induced activation of GPCR targeting a specific downstream effector.
PKA-mediated phosphorylation is thought to play critical roles in heterologous desensitization of GPCRs and in receptor switching from Gs to Gi coupling (40, 41), whereas GRK-mediated phosphorylation is implicated in β-arrestin recruitment and β-arrestin-dependent ERK activation (13-18). We have recently characterized that PKA and GRKs phosphorylate distinct subpopulations of β2AR in a single fibroblast or neuron (12). While GRK phosphorylation of β2AR is only observed at high concentrations of agonists, PKA phosphorylation can be induced with minimal doses of agonist (12, 27, 28, 42). Here, our data show CAR does not promote GRK phosphorylation at low concentrations and induces a slow and minimal GRK effect at high concentrations when compared to those induced by ISO. The CAR-induced GRK effects are minimally related to the PKA effects. Previously, CAR has been recognized as a biased β-blocker that preferentially activates β-arrestin/ERK pathways (29, 43). Despite the prominent role of GRK phosphorylation in full agonist ISO-induced β2AR-β-arrestin/ERK signaling, our data clearly indicate that GRK phosphorylation of β2AR is not necessary for CAR-induced activation of ERK, consistent with a recent study showing a distinct general mechanism of β-arrestin activation that does not require the GRK-phosphorylated tail of different GPCRs (44). Meanwhile, other studies show that in the absence of all G proteins, GPCRs fail to transduce β-arrestin/ERK signaling (45). These data indicate the necessity of G proteins in GPCR-induced arrestin activation. In our study, we observed a concentration-dependent correlation between PKA phosphorylation of β2AR with ERK activity induced by β-blockers, suggesting the potential role of Gs and PKA in CAR-induced β2AR-β-arrestin/ERK signaling are overlooked. In comparison, Gi is not required for CAR-induced β2AR/β-arrestin signaling even though CAR induces Gi recruitment to β1AR for transducing β1AR/β-arrestin signaling (46). Moreover, our results are also in line with a recent report that activation of β2AR with as low as femtomolar concentrations of ligands causes sustained ERK signaling (47), further support a PKA but GRK-dependent mechanism in GPCR-induced ERK activation. Future studies will help us understand how ligand-induced GPCRs utilize distinct
mechanisms in activating β-arrestin/ERK pathway.

Engineered GPCRs have been widely applied in investigating structural and biological processes and behaviors by precisely controlling specific GPCR signaling branches (48). Previous mutagenesis studies have shown that β2AR with S204/207A mutation loses binding to adrenaline but still binds with several β-blockers including ALP (37). Based on this and recent advances in βAR structures with agonists and β-blockers (38, 39), we have generated a S204/207A mutant that bestow β2AR with the ability to be selectively activated by β-blockers such as CAR and to transduce cAMP/PKA signaling. At nanomolar concentrations, while ISO fails to stimulate PKA phosphorylation of the S204/207A mutant β2AR, the mutant receptor still retains CAR-induced stimulation of PKA-phosphorylation of the receptor. The CAR-induced activation of mutant β2AR triggers the β2AR/Gs/cAMP/PKA signaling pathway and selectively targets downstream effectors in primary hippocampal neurons. Interestingly, the S204/207A β2AR mutant is not only refractory to its agonists but also completely lost both ISO- and CAR-induced GRK-phosphorylation of β2AR. Further studies comparing this mutant with previous reported β2AR-TYY and Y219A mutants that lack Gs and GRKs coupling, respectively (18, 49), will facilitate the analysis of the physiological relevance of Gs/cAMP/PKA-dependent and GRK-dependent signaling pathways and enable researchers to explore β-arrestin/ERK pathway devoid of individual signaling branches.

β-blockers are a standard clinical treatment in a broad range of diseases. Many β-blockers possess intrinsic sympathomimetic activities (19, 20), which are problematic due to the side effects through stimulation of βARs (19, 20), a feature that limits the clinical utility of the drugs. Here, we show that β-blockers promote activation of β2AR by recruiting Gs that selectively transduces cAMP/PKA signal but not GRK signal. Meanwhile, binding of β-blockers to β1AR has been shown to enhance cAMP levels locally by dissociating a β1AR-PDE4 complex, thereby reducing the local cAMP-hydrolytic activity (50), β1AR and β2AR thus could utilize different mechanisms
for β-blocker-induced signaling. Another interesting observation is that the β-blocker-induced β2AR-cAMP signal is sufficient to promote PKA phosphorylation of both β2AR and the receptor-associated CaV1.2 of LTCC, but not another substrate, the AMPAR GluA1 subunit. Both LTCC and AMPAR are shown to associate with the β2AR in hippocampal neurons (30-33). Therefore, the preference of one local membrane target over another local target indicates a highly restricted nature of the cAMP-PKA activities, potentially dependent on the recently identified distinct subpopulations of β2AR and associated signaling molecules in the neurons (12). Nevertheless, the PKA phosphorylation leads to augmentation of LTCC activity, potentially contributing to the neuronal toxicities. Therefore, activation of GPCR at low ligand concentrations should be taken into consideration when designing and screening new therapeutic drugs.

Materials and methods

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HEK293 cells stably expressing FLAG-β2AR

HEK293 cells stably expressing FLAG-β2AR-S207A
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**Animals**

β1AR/β2AR double knockout (DKO) mouse were obtained from Jackson Laboratories to produce P0-P1 postnatal DKO pups, SD pregnant rats were obtained from Charles River Laboratories to provide E17-E19 embryonic rats. All of the animals were
handled according to approved institutional animal care and use committee (IACUC) protocols (#20234 and #20673) of the University of California at Davis and in accordance with the NIH guidelines.

**Plasmids**

DNA constructs expressing FLAG-tagged human β2AR (FLAG-β2AR) and HA-tagged rat L-type calcium channel (LTCC) α1.2 were described before (12). FLAG-tagged human β2AR with S204/207A double mutations (FLAG-mutant) was generated by Gibson assembly method (Thermo Fisher) using FLAG-β2AR and synthetic gBlocks with the double mutations as templates (Integrated DNA Technologies). FRET biosensor ICUE3, CAAX-ICUE3 and LYN-ICUE3 were described before (34). To make the β2AR-ICUE3 fusion biosensor, ICUE3 was fused to the C-terminal of FLAG-β2AR with Gly-Ser linker. HA-Gsα was made by replacing CFP with HA tag, using Gsα-CFP as template (a gift from Dr. Catherine Berlot, Addgene plasmid # 55793).

**Antibodies and Chemicals**

Mouse monoclonal antibodies against β2AR at serine 261/262 (clone 2G3) and at serine 355/356 (clone 10A5) were kindly provided by Dr. Richard Clark (UT Huston). Polyclonal antibodies against β2AR (sc-570) and phosphorylated β2AR at serine 355/356 (sc-16719R) were purchased from Santa Cruz Biotechnology. Polyclonal antibodies against α1.2 residues 754-901 for total α1.2 (FP1), residues 1923-1935 for phosphorylated serine 1928 site (LGRRApSFHLECLK, pS1928) and residues 1694-1709 for phosphorylated serine 1700 site (EIRRAIpSGDLTAEEEL, pS1700) were described before (51). Polyclonal antibodies against GluA1 residues 894-907 for total GluA1, residues 826-837 for phosphorylated serine 831 site (LIPQQpSINEAIK, pS831) and residues 840-851 for phosphorylated serine 845 site (TLPRNpSGAGASK, pS845) were described before (51). Other antibodies used in the experiments include: anti-FLAG (F3040, Sigma), anti-HA (MMS-101R, Covance), Alexa fluor 488 conjugated goat anti-rabbit IgG and Alexa fluor 594 conjugated goat anti-mouse IgG.
(A-11034 and A-11032, Thermo Fisher), DyLight 680 conjugated goat anti-mouse IgG and anti-rabbit IgG (35518 and 35568, Thermo Fisher), IRDye 800CW conjugated goat anti-mouse IgG and anti-rabbit IgG (926-32210 and 926-32211, Li-cor).

Isoproterenol (I2760), timolol (T6394), alprenolol (A8676), propranolol (P0884), metoprolol (M5391), CGP12177A (C125), CGP20712A (C231), ICI118551 (I127), 3-isobutyl-1-methylxanthine (I5879) and 2',5'-dideoxyadenosine (D7408) were purchased from Sigma. Carvedilol (15418) was from Cayman Chemical, H89 (H-5239) was from LC Labs, pertussis toxin (179B) was from List Labs.

**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 (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Sigma). HEK293 cells stably expressing FLAG-β2AR was from previous study (35). HEK293 cells stably expressing FLAG-mutant β2AR was generated in this study. Briefly, cells transfected with β2AR-mutant were selected by G418 resistance (Corning) and cell clones were obtained by limiting serial dilution, monoclonal cells were analyzed by western blots and the one with comparable β2AR expression to FLAG-β2AR stable cells was chosen.

Mouse embryonic fibroblasts (MEFs) from β1AR/β2AR double knockout (DKO) mouse was described in previous study (52) and were maintained in DMEM supplemented with 10% FBS. Primary mouse hippocampal neurons were isolated and cultured from P0-P1 early postnatal DKO mouse pups, and primary rat hippocampal neurons were prepared from E17-E19 embryonic rats using methods described previously (53, 54). Briefly, dissected hippocampi were dissociated by 0.25% trypsin (Corning) and trituration. Neurons were plated on poly-D-lysine-coated (Sigma) glass coverslips in 24-well plate for imaging and in 6-well plate for biochemistry at a cell density of 50,000/well and 1 million/well, respectively. Neurons were cultured in Neurobasal medium supplemented with GlutaMax and B-27 (Thermo Fisher).
HEK293 cells were transfected with plasmids using polyethylenimine according to manufacturer’s instructions (Sigma). Neurons were transfected by the Ca\textsuperscript{2+}-phosphate method (55). Briefly, cultured neurons on 6-10 DIV were switched to pre-warmed Eagle’s minimum essential medium (EMEM, Thermo Fisher) supplemented with GlutaMax 1 hour before transfection, conditioned media were saved. DNA precipitates were prepared by 2x HBS (pH 6.96) and 2 M CaCl\textsubscript{2}. After incubation with DNA precipitates for 1 hour, neurons were incubated in 10% CO\textsubscript{2} pre-equilibrium EMEM for 20 minutes, then replaced with conditioned medium and cultured in 5% CO\textsubscript{2} incubator until use.

**Confocal Microscopy Imaging**

Rat hippocampal neurons were transfected with FLAG-β\textsubscript{2}AR on 10 DIV, treated for 5 minutes with 10 nM or 1 μM indicated drugs on 12 DIV. Mouse DKO hippocampal neurons were transfected with FLAG-β\textsubscript{2}AR or FLAG-mutant and HA-α\textsubscript{1.2} at 1:1 ratio on 6-8 DIV, and stimulated with indicated drugs and times 24 hours after transfection. Treated cells were fixed, permeabilized, and co-stained with indicated antibodies with a final concentration of 1 μg/ml for each antibody, which were revealed by a 1:1000 dilution of Alexa flour 488 conjugated goat anti-rabbit IgG or Alexa fluor 594 conjugated goat anti-mouse IgG, respectively. Fluorescence images were taken by Zeiss LSM 700 confocal microscope with a 63×/1.4 numerical aperture oil-immersion lens.

**Proximity ligation assay**

HEK293 cells growing on poly-D-lysine coated coverslips were transfected with FLAG-β\textsubscript{2}AR or FLAG-mutant, HA-G\textsubscript{sa} and pEYFP-N1 at 8:1:1 ratio. 24 hours after transfection, cells were serum-starved 2 hours, treated 100 nM indicated drugs for 5 minutes. Following stimulation, cells were fixed, permeabilized, and co-stained with anti-β\textsubscript{2}AR antibody (1:100 dilution) from rabbit in conjunction with anti-HA antibody (1:1000 dilution) from mouse. The proximity ligation reaction was performed according to the manufacturer’s protocol using the Duolink in situ detection orange
reagents (Sigma). Images were recorded with Zeiss LSM 700 confocal microscope with a 63×/1.4 numerical aperture oil-immersion lens. To quantify the PLA signals, the number of red fluorescent objects in each image was quantified using the Squassh plug-in for ImageJ software (56), and divided by the number of transfected cells.

Fluorescence resonance energy transfer (FRET) measurement

FRET measurement was performed as previously described (35). Briefly, HEK 293 cells were transfected with ICUE3 or β2AR-ICUE3, DKO MEFs were co-transfected with ICUE3 and FLAG-β2AR or FLAG-mutant. Cells were imaged on a Zeiss Axiovert 200M microscope with a 40×/1.3 numerical aperture oil-immersion lens and a cooled CCD camera. Dual emission ratio imaging was acquired with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters (475DF40 for cyan and 535DF25 for yellow). The acquisition was set with 0.2 second exposure in both channels and 20 second elapses. Images in both channels were subjected to background subtraction, and ratios of yellow-to-cyan were calculated at different time points.

Western blot

HEK293 cells stably expressing FLAG-β2AR or FLAG-mutant were serum-starved for 2 hours and treated with indicated drugs and times, 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. Rat hippocampal neurons on 10-14 DIV were treated with indicated drugs and times, then harvested by lysis buffer (10 mM Tris pH 7.4, 1% TX-100, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10% glycerol) with protease and phosphatase inhibitor cocktail. Protein samples were analyzed by Western blot using antibodies as indicated at a 1:1000 dilution and signals were detected by Odyssey scanner (Li-cor).

Cell-attached Patch Clamp Electrophysiology

Primary rat and mouse hippocampal neurons were used on 7-10 DIV. Cell-attached
patch clamp recordings were performed on an Olympus IX70 inverted microscope in a 15-mm culture coverslip at room temperature (22-25 °C). Signals were recorded at 10 kHz and low-pass filtered at 2 kHz with an Axopatch 200B amplifier and digitized with a Digidata 1440 (Molecular Devices). Recording pipettes were pulled from borosilicate capillary glass (0.86 OD) with a Flaming micropipette puller (Model P-97, Sutter Instruments) and polished (polisher from World Precision Instruments). Pipette resistances were strictly maintained between 6-7 MΩ to ameliorate variations in number of channels in the patch pipette. The patch transmembrane potential was zeroed by perfusing cells with a high K⁺ extracellular solution containing (in mM) 145 KCl, 10 NaCl, and 10 HEPES, pH 7.4 (NaOH). The pipette solution contained (in mM) 20 tetraethylammonium chloride (TEA-Cl), 110 BaCl₂ (as charge carrier), and 10 HEPES, pH 7.3 (TEA-OH). This pipette solution was supplemented with 1 µM ω-conotoxin GVIA and 1 µM ω-conotoxin MCVIIC to block N and P/Q-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 as previously performed by our group and others (12, 30, 57-64). In a subset of experiments, BayK was left out of the pipette solution. Note that ISO and CAR had similar effects on channel activity whether BayK was included or not in the pipette solution. To examine the effects of β-adrenergic stimulation on the L-type Caᵥ1.2 single-channel activity, 1 µM isoproterenol was added to the pipette solution in independent experiments. Note that we have previously used the L-type Caᵥ1.2 channel blocker nifedipine (1 µM) to confirm the recording of L-type Caᵥ1.2 currents under control conditions and in the presence of isoproterenol (51). Single-channel activity was recorded during a single pulse protocol (2 seconds) from a holding potential of -80 mV to 0 mV every 5 seconds. An average of >50 sweeps were collected with each recording file under all experimental conditions. The half-amplitude event-detection algorithm of pClamp10 was used to measure overall single-channel L-type Caᵥ1.2 activity as nPo, where n is the number of channels in the patch and Po is the open probability. Because the variability of nPo can be a critical element to interpret single channel data due to overstating open probability based on a high n number, we corrected this parameter
by the number of channels (n) describing channel open probability and availability as
well as calculating the the mean ensemble average current. Data 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.e.m.
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 or the
Kruskal-Wallis test with Dunn’s post hoc test. P < 0.05 was considered statistically
significant (denoted by * or # in figures).

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

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were recipients of AHA postdoctoral fellowship. Y.K.X. is an established AHA
investigator.

Author contributions
A.S. and Y.K.X. conceived and designed experiments. A.S. generated DNA constructs
and stable cells, did mouse neuron culture, imaging, FRET and Western blot. M.F.N.,
M.N.C. and P.B. designed, performed and analyzed single channel recording. D.C.
and B.X. helped Western blot. M.K. and Q.S. helped DNA constructs and FRET.
J.M.M. helped Western blot and did MTT assay. K.M.M. did rat neuron cultures. A.S.
and Y.K.X. interpreted all the data and wrote the manuscript with inputs from X.-Y.Y.,
J.W.H. and M.F.N. Y.K.X. provided overall project supervision.
Figure Legends

Figure 1. Carvedilol and alprenolol selectively promote phosphorylation of \( \beta_2 \)AR at PKA sites. HEK293 cells stably expressing FLAG-tagged \( \beta_2 \)AR were either directly stimulated for 5 minutes with the \( \beta \)AR agonist ISO or different \( \beta \)-blockers at indicated concentrations (A, \( n=4 \)), or pretreated for 15 minutes with 1 \( \mu M \) \( \beta_1 \)AR antagonist CGP20712A (B, \( n=5 \)) or 10 \( \mu M \) \( \beta_2 \)AR antagonist ICI118551 (C, \( n=4 \)) before the treatment. The phosphorylation of \( \beta_2 \)AR on its PKA and GRK sites were determined with phospho-specific antibodies, and signals were normalized to total \( \beta_2 \)AR detected with anti-FLAG antibody. NT, no treatment; ISO, isoproterenol; TIM, timolol; ALP, alprenolol; CAR, carvedilol; ICI, ICI118551; PRO, propranolol; MET, metoprolol; 177, CGP12177; CGP, CGP20712A. Error bars denote s.e.m., \( P \) values are computed by one-way ANOVA followed by Tukey's test between NT and other groups.

Figure 1–figure supplement 1. Uncropped blots for Figure 1. (A) Uncropped blots corresponding to Figure 1A. (B) Uncropped blots corresponding to Figure 1B. (C) Uncropped blots corresponding to Figure 1C. Red box indicates the crop region displayed in main figure.

Figure 2. Carvedilol and alprenolol induce concentration-dependent PKA phosphorylation of \( \beta_2 \)AR in HEK293 and hippocampal neurons. HEK293 cells stably expressing FLAG-tagged \( \beta_2 \)AR were treated with increasing concentrations of CAR (A, \( n=4 \)) and ALP (B, \( n=3 \)), or pretreated for 15 minutes with 10 \( \mu M \) \( \beta_2 \)AR antagonist ICI118551 (C, \( n=4 \)) and PKA inhibitor H89 (D, \( n=3 \)) before stimulated with 1 \( \mu M \) indicated drugs for 5 minutes. The phosphorylation of \( \beta_2 \)AR on its PKA and GRK sites were determined with phospho-specific antibodies, and signals were normalized
to total β₂AR detected with anti-FLAG antibody. Experiments were performed in the
presence of 1 μM β₁AR-selective antagonist CGP20712A to block endogenous β₁AR
signaling. NT, no treatment; ISO, isoproterenol; ALP, alprenolol; CAR, carvedilol; ICI,
ICI118551. Error bars denote s.e.m., P values are computed by one-way ANOVA
followed by Tukey’s test between NT and other groups. (E) Rat hippocampal neurons
expressing β₂AR were treated for 5 minutes with 10 nM or 1 μM indicated drugs on 12
days in vitro (DIV), and immuno-stained for PKA-phosphorylated β₂AR. Confocal
images show PKA-phosphorylated β₂AR in agonist- or β-blocker-stimulated neurons
have similar distribution. Scale bar, 10 μm. Representative of 6 images for each
condition, three experiments.

**Figure 2–figure supplement 1. Uncropped blots for Figure 2.** (A) Uncropped blots
corresponding to Figure 2A. (B) Uncropped blots corresponding to Figure 2B. (C)
Uncropped blots corresponding to Figure 2C. (D) Uncropped blots corresponding
to Figure 2D. Red box indicates the crop region displayed in main figure.

**Figure 2–figure supplement 2. Phosphorylation of ERK and β₂AR at different
drug concentrations.** HEK293 cells stably expressing FLAG-tagged β₂AR were
stimulated by isoproterenol (ISO) or carvedilol (CAR) for 30 minutes with indicated
concentrations. Cell lysates were analyzed for PKA-phosphorylated β₂AR,
GRK-phosphorylated β₂AR, total β₂AR, pERK, and total ERK by Western blot. P
values are computed by one-way ANOVA followed by Tukey’s test between NT and
other groups. Data are the mean ± s.e.m. of three experiments.

**Figure 2–figure supplement 3. GRK-phosphorylation of β₂AR at different
carvedilol treated times.** HEK293 cells stably expressing FLAG-tagged β₂AR were
stimulated by 1 μM isoproterenol (ISO) or carvedilol (CAR) for indicated times. Cell
lysates were analyzed for PKA-phosphorylated β₂AR, GRK-phosphorylated β₂AR,
total β₂AR by Western blot. P values are computed by one-way ANOVA followed by
Tukey’s test between NT and other groups. Data are the mean ± s.e.m. of four
Figure 3. Carvedilol and alprenolol promote Gsα recruitment to β2AR and increase spatially restricted cAMP signal. (A) HEK293 cells co-expressing FLAG-tagged β2AR, HA-tagged Gsα and EGFP were stimulated with 100 nM ISO or indicated β-blockers for 5 minutes. In proximity ligation assay (PLA), cells were immuno-stained with HA and β2AR antibody, nuclei were counterstained with DAPI. The green EGFP signal represents transfected cells, and red PLA signal represents Gsα and β2AR interactions. Carvedilol and alprenolol promoted Gsα recruitment to β2AR, but timolol could not. Scale bar, 10 μm. Representative of n=15, 16, 16, 17, 18 and 18 images respectively, three experiments. (B–C) HEK293 cells expressing ICUE3 biosensor were treated with 1 μM ISO or indicated β-blockers (B), or together with 100 μM phosphodiesterase inhibitor IBMX (C). (D–E) HEK293 cells expressing β2AR-ICUE3 biosensor were treated with indicated concentration of ISO or β-blockers. In some cases, cells were pretreated for 30 minutes with the β2AR antagonist ICI (10 μM) or the adenylate cyclase inhibitor ddA (50 μM) before adding β-blockers. Changes in ICUE3 FRET ratio (an indication of cAMP activity) were measured. Experiments were performed in the presence of 1 μM β1AR-selective antagonist CGP20712A to block endogenous β1AR signaling. Mock, no primary antibody; NT, no treatment; ISO, isoproterenol; TIM, timolol; ALP, alprenolol; CAR, carvedilol; ICI, ICI118551; PRO, propranolol; MET, metoprolol; 177, CGP12177, IBMX, 3-isobutyl-1-methylxanthine; ddA, 2',5'-dideoxyadenosine. Each dot in the scatter dot plot in B–E represents a value from an individual tested cell. Error bars denote s.e.m., P values are computed by one-way ANOVA followed by Tukey’s test between NT (A) or TIM (B–E) and other groups.

Figure 3–figure supplement 1. Carvedilol-induced β2AR phosphorylation is AC-dependent. HEK293 cells stably expressing FLAG-tagged β2AR were pretreated with the Gi inhibitor pertussis toxin (PTX, 200 ng/ml, 16 hours) or the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (ddA, 50 μM, 30 minutes) and then
stimulated with 100 nM isoproterenol (ISO) or carvedilol (CAR) for 5 minutes. The phosphorylation levels of β₂AR on its PKA and GRK sites were determined with phospho-specific antibodies, and signals were normalized to total β₂AR detected with anti-FLAG antibody. Experiments were performed in the presence of 1 μM β₁AR-selective antagonist CGP20712A to block endogenous β₁AR signaling. P values are computed by one-way ANOVA followed by Tukey’s test between no drug (ND) and other drugs within the same group. Data are the mean ± s.e.m. of three experiments.

**Figure 3–figure supplement 2. Carvedilol- and alprenolol-induced cAMP can be abolished by β₂AR or AC inhibition.** The cAMP biosensor ICUE3 and FLAG-β₂AR were co-expressed in HEK293 cells. Cells were treated with 100 nM βAR agonist ISO or different β-blockers, and changes in cAMP FRET ratio were measured. In some cases, cells were pretreated for 30 minutes with the β₂AR antagonist ICI (10 μM) or the adenylate cyclase inhibitor ddA (50 μM) before adding β-blockers. Experiments were performed in the presence of 1 μM β₁AR-selective antagonist CGP20712A to block endogenous β₁AR signalling. ISO, isoproterenol; TIM, timolol; ALP, alprenolol; CAR, carvedilol; ICI, ICI118551; PRO, propranolol; MET, metoprolol; 177, CGP12177; ddA, 2’,5’-dideoxyadenosine. Each dot in the scatter dot plot represents a value from an individual tested cell. Error bars denote s.e.m. P values are computed by one-way ANOVA followed by Tukey’s test between TIM and other groups.

**Figure 3–figure supplement 3. Carvedilol- and alprenolol-induced cAMP are highly restricted.** HEK293 cells expressing either CAAX-ICUE3 targeted to non-rafts regions of the plasma membrane (A) or LYN-ICUE3 targeted to rafts regions of the plasma membrane (B) were treated with 1 μM ISO or indicated β-blockers. Changes in ICUE3 FRET ratio (an indication of cAMP activity) were measured. Carvedilol- and alprenolol-induced cAMP could be detected neither by CAAX-ICUE3 nor LYN-ICUE3. ISO, isoproterenol; TIM, timolol; ALP, alprenolol; CAR, carvedilol; ICI, ICI118551; PRO, propranolol; MET, metoprolol; 177, CGP12177. Each dot in the scatter dot plot represents a value from an individual tested cell. Error bars denote s.e.m., P values
Figure 4. Carvedilol promotes endogenous β₂AR-dependent phosphorylation of LTCC α₁.2 by PKA in neurons. (A) Rat neurons on 10-14 days in vitro (DIV) were treated for 5 minutes with 1 μM indicated drugs. The phosphorylation of endogenous LTCC α₁.2 subunit was determined with phospho-specific antibodies, and normalized to total α₁.2, n=3. (B) Rat neurons on 10-14 DIV were treated for 5 minutes with 1 μM indicated drugs. The phosphorylation of endogenous AMPAR GluA1 subunit was determined with phospho-specific antibodies, and normalized to total GluA1, n=4. (C) Neurons were pretreated for 30 minutes with 10 μM β₂AR inhibitor ICI, 50 μM AC inhibitor ddA, 10 μM PKA inhibitor H89 or 10 μM CaMKII inhibitor KN93 and then stimulated with 1 μM CAR for 5 minutes. Carvedilol-induced LTCC phosphorylation depends on endogenous β₂AR, AC and PKA, but not CaMKII, n=5. NT, no treatment; ISO, isoproterenol; TIM, timolol; ALP, alprenolol; CAR, carvedilol. Error bars denote s.e.m., P values are computed by one-way ANOVA followed by Tukey’s test between NT and other groups.

Figure 4–figure supplement 1. Uncropped blots for Figure 4. (A) Uncropped blots corresponding to Figure 4A. (B) Uncropped blots corresponding to Figure 4B. (C) Uncropped blots corresponding to Figure 4C. Red box indicates the crop region displayed in main figure.

Figure 5. Carvedilol augments LTCC Ca₁.2 channel activity in neurons. (A) Representative single channel recordings of LTCC Ca₁.2 currents using 110 mM Ba²⁺ as charge carrier in rat hippocampal neurons on 7-10 days in vitro (DIV) after depolarization from −80 (hp) to 0 mV (tp) in control patches (NT), patches containing 1 μM isoproterenol (ISO), 1 μM carvedilol (CAR) or 1 μM timolol (TIM) in the patch pipette or after addition of 1 μM CAR to the bath while the patch pipette contained a control pipette solution (CAR_out). Shown are 20 consecutive sweeps from
representative experiments. Arrows throughout the figure indicate the 0-current level (closed channel). Scale bar denotes 2 pA and 200 ms. (B) Ensemble average currents as determined from all sweeps recorded for all the experimental conditions. Scale bar denotes 50 fA and 400 ms. (C-E) Mean ± s.e.m. for (C) P_o (%), (D) availability (i.e. likelihood that a sweep had at least one event) (%) and (E) the mean ensemble average current (fA) for each experimental condition. *P < 0.05 with Kruskal Wallis – Dunn’s multiple comparison test. Sweep and n numbers as well as summary statistics are in Supplementary file 1. (F) Ensemble P_o versus time measurements obtained with a pipette backfilled with 1 μM CAR. The solid dark line represents the mean P_o over time and the gray area is the s.e.m. at each time point. The mean line was smoothed to 15 neighbors on each size with a second order polynomial smoothing in PRISM for representation purposes only. (G) Mean Po of the first 30 traces versus the last 30 traces obtained with a pipette backfilled with 1 μM CAR. The gray boxes highlight the mean on each group. n = 11 patches. *P < 0.05 with Mann-Whitney test.

**Figure 5–figure supplement 1.** Over-time effect of carvedilol on LTCC of neurons recorded in the cell attached configuration without using BayK. Cells were depolarized with 110 mM Ba^{2+} for 2 seconds from a holding potential of -80 mV to 0 mV and the NPopen was determined over time. (A) Diagram reflecting our patch statistics without using BayK in the recording pipette. Only about 30% of our patches showed channel activity. Cells with >5% channel availability within the first 50 sweeps were considered for our analysis. Cells with <5% availability were consequently excluded for pharmacological testing. (B) Control experiment demonstrating the effect of 1 μM isoproterenol applied through bath perfusion (n=11 cells). (C) Over-time effect of 1 μM carvedilol applied through backfilling of the recording pipette (n=8 cells). Tip filling was performed with pipette solution containing no carvedilol. (D) Experiment showing the effect of 1 μM carvedilol perfused into the bath solution (n=8 cells).

**Figure 5–figure supplement 2.** Inhibition of β_2_AR or LTCC counteracts
carvedilol-induced cell death of cultured cortical neurons. Rat cortical neurons on 7 days in vitro (DIV) were incubated overnight in fresh medium with or without 2 mM Ca\(^{2+}\) (no Ca\(^{2+}\)), cells were then either mock treated (NT), or treated with 1 μM indicated drugs. CAR, carvedilol; CGP, CGP20712A; TIM, timolol; ISR, isradipine. MTT assay was carried out at 48 hours post drug treatment and absorbance at 540 nM was measured. The first NT well in each plate was set as basal and all the other groups were normalized to that well. The lysed group had medium removed to represent nearly 100% cell death condition. CAR show decreased cell viability, β\(_1\)AR antagonist CGP does not prevent the carvedilol-induced reduction of cell viability but β\(_1\)AR/β\(_2\)AR antagonist TIM or LTCC blocker ISR does. Data are the mean ± s.e.m. of three individual plates. \(P\) values are computed by one-way ANOVA followed by Tukey’s test between NT and other groups.

Figure 6. A mutant β\(_2\)AR is selectively activated by carvedilol but not isoproterenol. (A) Schematic of an engineered β\(_2\)AR with S204/207A double serine mutations that loses high affinity binding to ISO but not CAR at nanomolar range. (B) cAMP biosensor ICUE3 and β\(_2\)AR wild-type (WT) or mutant were co-expressed in MEF cells lacking both β\(_1\)AR and β\(_2\)AR. Changes of cAMP FRET ratio by increasing concentrations of ISO or CAR were measured. n=5-29 cells. (C-D) HEK293 cells stably expressing FLAG-tagged β\(_2\)AR WT or mutant were stimulated for 5 minutes with increasing concentrations of ISO (C, n=7) or CAR (D, n=5). The phosphorylation of β\(_2\)AR on its PKA and GRK sites were determined with phospho-specific antibodies, and signals were normalized to total β\(_2\)AR detected with anti-FLAG antibody. Experiments were performed in the presence of 1 μM β\(_1\)AR-selective antagonist CGP20712A to block endogenous β\(_1\)AR signaling. NT, no treatment; ISO, isoproterenol; CAR, carvedilol. Error bars denote s.e.m., \(P\) values are computed by one-way ANOVA followed by Tukey’s test between NT and other concentrations.

Figure 6–figure supplement 1. Uncropped blots for Figure 6C and D. (A) Uncropped blots corresponding to Figure 6C. (B) Uncropped blots corresponding
to Figure 6D. Red box indicates the crop region displayed in main figure.

Figure 7. The β2AR mutant selectively supports carvedilol-induced augmentation of LTCC activity in neurons. (A-B) β1AR/β2AR double knockout (DKO) mouse hippocampal neurons on 7-10 days in vitro (DIV) were cotransfected with FLAG-tagged β2AR WT (A) or mutant (B) and HA-tagged LTCC α1.2 subunit, 24 hours later cells were either mock treated (NT), or treated for 5 minutes with 10 nM isoproterenol (ISO) or carvedilol (CAR), fixed and labeled with anti-FLAG and a phospho-specific antibody for S1928 phosphorylated α1.2. Confocal images show mutant β2AR lost the ability of promoting LTCC phosphorylation upon ISO stimulation but remained the ability upon CAR stimulation in neurons. Scale bar, 10 μm. Representative of 6 images for each condition, three experiments. (C) Representative single channel recordings of LTCC CaV1.2 currents using 110 mM Ba2+ as charge carrier in DKO neurons on 7-10 days DIV expressing mutant β2AR after depolarization from −80 to 0mV in in control patches (mutant) and patches containing 1 μM isoproterenol (ISO) or 1 μM carvedilol (CAR) in the patch pipette. Shown are 20 consecutive sweeps from representative experiments. Arrows throughout the figure indicate the 0-current level (closed channel). Scale bar denotes 2 pA and 200 ms. (D) Ensemble average currents as determined from all sweeps recorded for all the experimental conditions. Scale bar denotes 50 fA and 400 ms. (E-G) Mean ± s.e.m. for (E) P0 (%), (F) availability (i.e. likelihood that a sweep had at least one event) (%) and (G) the mean ensemble average current (fA) for each experimental condition. *P < 0.05 with Kruskal Wallis – Dunn’s multiple comparison test. Sweep and n numbers as well as summary statistics are in Supplementary file 2.

Figure 7–figure supplement 1. The mutant β2AR is selectively activated by carvedilol and promotes LTCC phosphorylation in neurons. β1AR/β2AR double knockout (DKO) hippocampal mouse neurons at 7-10 days in vitro (DIV) were cotransfected with FLAG-tagged β2AR WT (A) or mutant (B) and HA-tagged LTCC α1.2 subunit, 24 hours later cells were treated for 5 minutes with 1 μM ISO or CAR,
fixed and labeled with anti-FLAG and a phospho-specific antibody for S1928 phosphorylated α1.2. Confocal images show mutant β2AR lost the ability of promoting LTCC phosphorylation upon ISO stimulation but remained the ability upon CAR stimulation in mouse neurons. Scale bar, 10 μm. Representative of n= 4, 6, 5 and 6 cells, respectively, three experiments.

Legend for Supplementary file 1

Biophysical properties of L-type Ca\textsuperscript{2+} currents in the neurons recorded in Figure 5A-5E. Values are mean ± SEM. *P < 0.05 with Kruskal Wallis – Dunn’s multiple comparison test.

Legend for Supplementary file 2

Biophysical properties of L-type Ca\textsuperscript{2+} currents in the neurons recorded in Figure 7C-7G. Values are mean ± SEM. *P < 0.05 with Kruskal Wallis – Dunn’s multiple comparison test.

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Figure 1. Carvedilol and alprenolol selectively promote phosphorylation of $\beta_2$AR at PKA sites.
Figure 1–figure supplement 1. Uncropped blots for Figure 1.
Figure 2. Carvedilol and alprenolol induce concentration-dependent PKA phosphorylation of β2AR in HEK293 and hippocampal neurons.
Figure 2–figure supplement 1. Uncropped blots for Figure 2.
Figure 3. Carvedilol and alprenolol promote Gsα recruitment to β2AR and increase spatially restricted cAMP signal.
Figure 3–figure supplement 1. Carvedilol-induced β₂AR phosphorylation is AC-dependent.
Figure 3—figure supplement 2. Carvedilol- and alprenolol-induced cAMP can be abolished by β₂AR or AC inhibition.
Figure 3–figure supplement 3. Carvedilol- and alprenolol-induced cAMP are highly restricted.
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Figure 7. The β₂AR mutant selectively supports carvedilol-induced augmentation of LTCC activity in neurons.
Figure 7–figure supplement 1. The mutant β₂AR is selectively activated by carvedilol and promotes LTCC phosphorylation in neurons.