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Research Article

**5-HT_{2A} receptor-dependent phosphorylation of mGlu₂ receptor at Serine 843 promotes
mGlu₂ receptor-operated G_{i/o} signaling**

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Running title: mGlu₂ phosphorylation in 5-HT_{2A}/mGlu₂ crosstalk

Abstract

The serotonin 5-HT_{2A} and glutamate mGlu₂ receptors continue to attract particular attention given their implication in psychosis associated with schizophrenia and the mechanism of action of atypical antipsychotics and a new class of antipsychotics, respectively. A large body of evidence indicates a functional crosstalk between both receptors in the brain, but the underlying mechanisms are not entirely elucidated. Here, we have explored the influence of 5-HT_{2A} receptor upon the phosphorylation pattern of mGlu₂ receptor in light of the importance of specific phosphorylation events in regulating G protein-coupled receptor signaling and physiological outcomes. Among the five mGlu₂ receptor phosphorylated residues identified in HEK-293 cells, the phosphorylation of Ser⁸⁴³ was enhanced upon mGlu₂ receptor stimulation by the orthosteric agonist LY379268 only in cells co-expressing the 5-HT_{2A} receptor. Likewise, administration of LY379268 increased mGlu₂ receptor phosphorylation at Ser⁸⁴³ in prefrontal cortex of wild type mice but not 5-HT_{2A}^{-/-} mice. Exposure of HEK-293 cells co-expressing mGlu₂ and 5-HT_{2A} receptors to 5-HT also increased Ser⁸⁴³ phosphorylation state to a magnitude similar to that measured in LY379268-treated cells. In both HEK-293 cells and prefrontal cortex, Ser⁸⁴³ phosphorylation elicited by 5-HT_{2A} receptor stimulation was prevented by the mGlu₂ receptor antagonist LY341495, while the LY379268-induced effect was abolished by the 5-HT_{2A} receptor antagonist M100907. Mutation of Ser⁸⁴³ into alanine strongly reduced G_{i/o} signaling elicited by mGlu₂ or 5-HT_{2A} receptor stimulation in cells co-expressing both receptors. Collectively, these findings identify mGlu₂ receptor phosphorylation at Ser⁸⁴³ as a key molecular event that underlies the functional crosstalk between both receptors.

Introduction

Amongst the 14 5-hydroxytryptamine (serotonin, 5-HT) receptor subtypes, the 5-HT_{2A} receptor still raises special interest given its role in the psychotropic effects of psychedelic hallucinogens, which are often used to probe the psychosis associated with schizophrenia, and in the therapeutic effects of atypical antipsychotics like clozapine, olanzapine and risperidone, which act as antagonists or inverse agonists of the receptor (1,2). Atypical antipsychotics are efficient against positive symptoms of schizophrenia (hallucinations, delusions and disorder of thought), but they poorly control negative symptoms (social isolation, anhedonia and inactivity) and are often not efficient against cognitive symptoms (poor "executive functioning", lack of attention, impaired working memory) (3). During the 90ies, a new class of antipsychotics acting as orthosteric agonists (LY354740, LY379268, LY404039) or positive allosteric modulators (PAMs, LY487379, BINA) of group II metabotropic glutamate receptors (mGlu_{2/3}) has been developed. Subsequent preclinical studies indicated that mGlu₂ receptor activation accounts for their antipsychotic activity (4–6) and showed overall superiority over 5-HT_{2A} receptor antagonists in alleviating negative symptoms. Furthermore, an integrated study combining five placebo-controlled trials of pomaglometad, the oral prodrug of LY404039, demonstrated clinical efficacy of glutamate-based pharmacotherapy in schizophrenia in specific patient subgroups, such as patients at early disease stage and, interestingly, patients who had never been treated with 5-HT_{2A} receptor antagonists (7).

5-HT_{2A} and mGlu₂ receptors are canonically coupled to G_q and G_{i/o} proteins, respectively, and thus induce different signaling cascades, but they do not function as independent targets of antipsychotics. Electrophysiological and behavioral studies have demonstrated a functional antagonism between 5-HT_{2A} and mGlu₂ receptors: pharmacological activation of mGlu₂ receptor inhibits glutamate release and excitatory postsynaptic currents (EPSCs) elicited by 5-HT_{2A} receptor stimulation in the prefrontal cortex, and attenuates behavioral effects of hallucinogens, while mGlu₂ receptor antagonists further enhance the frequency and amplitude of 5-HT_{2A} receptor-induced EPSCs (7, 8). It has recently been proposed that

the functional crosstalk between both receptors does not solely occur at the level of neuronal networks or intracellular signaling cascades, but that it involves the formation of 5-HT_{2A}-mGlu₂ heteromers, which integrate inputs from ligands of each protomer to trigger unique signaling and behavioral outcomes (9). *In vitro* and *in vivo* data identified the 5-HT_{2A}-mGlu₂ heterocomplex as a possible target of hallucinogenic 5-HT_{2A} receptor agonists that mediates engagement of G_{i/o} signaling and the associated psychotropic effects (9). Conversely, 5-HT_{2A}-mGlu₂ heteromers are necessary for the stimulation of G_q signaling by mGlu₂ receptor agonists, which is altered in patients with schizophrenia (10). This particular signaling also depends on the ability of mGlu₂ receptor to couple to and activate G_{i/o} proteins (10). Finally, studies showed that 5-HT_{2A}-mGlu₂ heteromers serve as an integration point that determines the balance between G_{i/o} and G_q-dependent signaling, and pro-psychotic (increase in G_q vs. G_{i/o} signaling) vs. antipsychotic (increase in G_{i/o} vs. G_q signaling) outcome (11). These findings pinpoint the role of 5-HT_{2A}-mGlu₂ heteromers in the homeostatic equilibrium between G_{i/o} and in G_q signaling that ensures a normal non-psychotic state. However, the existence of 5-HT_{2A}-mGlu₂ heteromers and their physiological relevance still remain controversial in absence of unequivocal demonstration of the co-localization of both receptors in neurons (12). There is even predominant evidence for a presynaptic localization of mGlu₂ receptors while 5-HT_{2A} receptors are mainly localized at the post-synapse (12).

Given the importance of specific phosphorylation events in determining the signaling and physiological outcomes of G protein-coupled receptors (GPCRs) (13), phosphorylation of 5-HT_{2A} and mGlu₂ receptors is another mechanism that might contribute to the functional crosstalk between both receptors, irrespective of heteromer formation. In a previous study, we extensively characterized 5-HT_{2A} receptor phosphorylated sites and identified a serine residue in the third receptor intracellular loop (Ser²⁸⁰) phosphorylated in response to receptor activation by hallucinogenic 5-HT_{2A} receptor agonists, such as lysergic acid diethylamide (LSD) or 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), but not by non-hallucinogenic agonists, such as lisuride or ergotamine (14). This study also showed that the phosphorylation of Ser²⁸⁰ underlies the lower propensity of hallucinogens to induce receptor

desensitization and internalization, compared with non-hallucinogenic agonists (14). These data suggest that biased phosphorylation of 5-HT_{2A} receptor by hallucinogenic vs. non-hallucinogenic agonists might account for their distinct behavioural outcomes upon long-term treatment. Using an *in vitro* phosphorylation assay, another study also identified several residues phosphorylated in the mGlu₂ receptor C-terminal domain and showed that the phosphorylation of one of them (Ser⁸⁴³) by protein kinase A (PKA) affects receptor coupling to G proteins (15). However, the reciprocal influence of 5-HT_{2A} and mGlu₂ receptors on their phosphorylation state and the role of their phosphorylation in the functional crosstalk between both receptors remain unexplored.

Here, we characterized phosphorylated residues of mGlu₂ receptor expressed in HEK-293 cells and investigated the influence of 5-HT_{2A} receptor expression/stimulation upon their phosphorylation state using high-resolution mass spectrometry. Among the identified residues, we show that Ser⁸⁴³ phosphorylation elicited by agonist stimulation of mGlu₂ receptor depends on 5-HT_{2A} receptor co-expression and demonstrate a reciprocal interplay between the activation of both receptors to promote Ser⁸⁴³ phosphorylation. Characterization of the signaling outcome of Ser⁸⁴³ phosphorylation revealed its key role in G_{i/o} signaling elicited by 5-HT_{2A} and mGlu₂ receptors agonists in cells co-expressing both receptors.

Materials and Methods

Materials

Human Embryonic Kidney-293 (HEK-293) N type cells (RRID:CVCL_0045) were from the American Type Culture Collection (Anassas, VI, ATCC, CRL-1573). All experiments of the study were performed using a previously characterized batch of cells (Isolate #3) (16). Their negative mycoplasma status was analyzed every month using the MycoAlert Mycoplasma Detection Kit (Lonza, Switzerland). Culture media were from Invitrogen. M100907 maleate was a generous gift from Dr Jean-Michel Rivet (Institut de Recherches Servier, Croissy-sur-Seine, France). LY379268 (mGlu₂ receptor orthosteric agonist), LY341495 (mGlu₂ receptor antagonist), KT5720 (PKA inhibitor) and D4476 (casein kinase 1 inhibitor) were from Tocris, lisuride maleate from Santa Cruz Biotechnologies, 5-HT and DOI from Sigma Aldrich. Pertussis Toxin (PTX) was from Calbiochem[®] and FR900359 was produced as previously described (17).

The rat anti-Hemagglutinin (HA) antibody (11867423001) was from Roche, the rabbit anti-Flag antibody (F7425) and the mouse anti-Flag M2 antibody conjugated to magnetic beads (M8823) were from Sigma Aldrich. The rabbit anti-CK1 ϵ (#12448) was from Cell Signaling and the mouse anti-Actin (#MA5-11869) was from ThermoScientific. The anti-phospho-Ser⁸⁴³ antibody was generated by immunizing rabbits with the synthetic CAAPRASANLGQG peptide coupled to KLH (Eurogentec[®]). Antibodies were purified by two consecutive affinity chromatographies. A first chromatography against the immobilized phosphorylated CAAPRASANLGQG peptide as bait bound antibodies recognizing both phosphorylated and unphosphorylated forms of the peptide. The retained fraction was then eluted and used for a second chromatography against the immobilized non-phosphorylated CAAPRASANLGQG peptide. We considered that the non-retained fraction of the second chromatography step contains antibodies recognizing mGlu₂ receptor phosphorylated at Ser⁸⁴³.

The PRK5 plasmid encoding the HA-tagged 5-HT_{2A} receptor was described elsewhere (18). The cDNA encoding HA-tagged 5-HT_{2A}(D¹²⁰N) was generated by site-directed mutagenesis from this plasmid. The pcDNA3 plasmids encoding the Flag-tagged human and rat mGlu₂

receptors were generously provided by Dr. Philippe Rondard (Institut de Génomique Fonctionnelle, Montpellier, France). The cDNAs encoding human and rat S⁸⁴³A mGlu₂ receptors and human S⁸⁴³D and S⁸⁴⁴A mGlu₂ receptor were obtained by gene synthesis (Eurogentec[®]) and subcloned in pcDNA3. The pcDNA3 plasmid encoding human Cav2.2 calcium channel isoform and the single dual promotor plasmid (pDuo) encoding rat Cav α ₂- δ _{1-b} and Cav β _{1b} ancillary subunits cloned in a single dual promotor plasmid (pDuo) were described elsewhere (19). The peGFPC1 plasmid was from Clontech.

Cell cultures and transfection

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX (Life Technologies) supplemented with 10% dialyzed, heat-inactivated fetal calf serum (BioWhittaker) and antibiotics in a humidified 5% CO₂ incubator at 37°C. They were transfected at 40-50% confluence using polyethyleneimine (PEI, Sigma-Aldrich), as previously described (20), and used 48 h after transfection. CKI ϵ siRNA (FlexiTube siRNA CKI ϵ ; Cat.N° SI00287735 / SI00287728 / SI04434843 / SI04434836) and control siRNA (AllStars Negative Control siRNA; Cat.N° 107280) were purchased from QIAGEN. siRNA transfection in HEK-293 cells (at 40-50% confluence) was performed with Lipofectamine[®] 2000 (Invitrogen) according to the manufacturer's protocol using a final siRNA concentration of 10 nM. Cells were used 48 h after transfection.

Quantitative mass spectrometry analysis of mGlu₂ receptor phosphorylation

Transfected cells were starved of serum for 4 h, challenged with either vehicle or LY379268 (1 μ M) for 15 min and lysed in Triton X-100 (2%), sodium chloride (150 mM), Tris-HCl (50 mM, pH 7.5), EDTA (2 mM), EGTA (2 mM), SDS (0.2%), sodium orthovanadate (1 mM), sodium fluoride (50 mM), sodium β -glycerophosphate (25 mM), sodium pyrophosphate (5 mM) and a protease inhibitor cocktail (Roche). The samples were incubated under rotation for 1 h at 4°C and centrifuged for 15 min at 16,000 x g to eliminate insoluble material. Flag-tagged mGlu₂ receptor was immunoprecipitated from 10 mg protein extracts using the anti-Flag M2 antibody immobilized on magnetic beads. After reduction with dithiothreitol (10 mM,

10 min at 37°C) and alkylation with iodoacetamide (50 mM, 45 min at 25°C), immunoprecipitated mGlu₂ receptors were resolved by SDS-PAGE and digested *in-gel* with trypsin (10 ng/mL, Gold, Promega) or with chymotrypsin (10 ng/mL, Gold, Promega) overnight at 25°C. Peptides were analyzed by nano-flow liquid chromatography coupled to Fourier transform tandem mass spectrometry (nanoLC-FT-MS/MS) using a LTQ Velos Pro Orbitrap Elite mass spectrometer coupled to an Ultimate 3000 (Thermo Fisher Scientific). Desalting and pre-concentration of samples were performed on-line on a Pepmap precolumn (0.3 mm 10 mm, Dionex) in buffer A (2% acetonitrile, 0.1% formic acid). A gradient consisting of 2–40% buffer B (B = 99.9% acetonitrile with 0.1% formic acid) (3–33 min), 40–80% B (33–34 min), 80–0% B (49–50 min), and equilibrated for 20 min in 0% B (50–70 min) was used to elute peptides at 300 nL/min from a Pepmap capillary reversed-phase column (0.075 mm x 150 mm, Thermo Fisher Scientific). Mass spectra were acquired using a top-10 collision-induced dissociation (CID) data-dependent acquisition (DDA) method. The LTQ-Orbitrap was programmed to perform a FT full scan (60,000 resolution) on 400–1,400 Th mass range with the top ten ions from each scan selected for LTQ-MS/MS with multistage activation on the neutral loss of 24.49, 32.66 and 48.99 Th. FT spectra were internally calibrated using a single lock mass (445.1200 Th). Target ion numbers were 500,000 for FT full scan on the Orbitrap and 10,000 MSn on the LTQ. Precursor mass and top 6 per 30 Da windows peak lists were extracted from MS2 using MSconvert 3.0 and searched with Mascot 2.6 against the human or rat Complete Proteome Set database (<http://www.uniprot.org>), cysteine carbamidomethylation as a fixed modification and phosphorylation of Ser, Thr and Tyr as variable modifications, 7 ppm precursor mass tolerance, 0.5 Da fragment mass tolerance and trypsin/P or chymotrypsin digestion. MS2 spectra matching phosphorylated peptides with ion score over 15 were manually inspected for unique transitions that pinpoint the position of phosphorylation sites. Ion signals corresponding to phosphorylated peptides were quantified from the maximal intensities measured in their ion chromatograms manually extracted using Qual browser v2.1 (Thermo Fisher Scientific) with a tolerance of 5 ppm for mass deviation, and normalized to signals of their non-phosphorylated counterparts. For

each identified phosphorylated residue, a phosphorylation index [phosphorylated peptide ion signal intensity / (phosphorylated peptide ion signal intensity + non-phosphorylated peptide ion signal intensity)], was calculated.

Western Blotting

Proteins, resolved onto 10% polyacrylamide gels, were transferred electrophoretically to nitrocellulose membranes (Biorad). Membranes were immunoblotted with primary antibodies (anti phospho-Ser⁸⁴³ mGlu₂ receptor, 1:2,000; anti-Flag, 1:1,000; Anti-HA, 1:1,000) and then with either anti-rat (Jackson ImmunoResearch 112-035-003) or anti-rabbit (Sigma Aldrich GENA934V) or anti-mouse (Sigma Aldrich GENA931V) horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000). Immunoreactivity was detected with an enhanced chemiluminescence method (Western lightning® Plus-ECL, Perkin Elmer) on a ChemiDoc™ Touch Imaging System (Biorad). Immunoreactive bands were quantified by densitometry using the ImageJ software (v.1.50). In protein phosphorylation analyses, the amount of each phosphoprotein was normalized to the amount of the corresponding total protein detected in the sample.

Analysis of Ser⁸⁴³ phosphorylation in the mouse prefrontal cortex

Experiments on mice (C57/Bl6J, eight weeks old, male) conformed to European ethics standards (86/609-EEC) and to decrees of the French National Ethics Committee (N°87/848) for the care and use of laboratory animals. Protocols were approved by regional ethic committee for animal use (CEEA LR 34, #7251). A minimum of four animals was used for each group. Animals were randomly allocated into the different groups by an observer who was blind to the experimental conditions. Prior to immunochemistry, brains were randomly numbered in order to perform immunochemistry and image acquisition in a blind way and reassign the samples to their group afterwards. Mice (about 20 g) were injected i.p. with either vehicle (5% DMSO/5% Tween-80) or LY379268 (10 mg/kg) or DOI (10 mg/kg) or lisuride (10 mg/kg). M100907 (5 mg/kg) or LY341495 (3 mg/kg) were injected 15 min prior to vehicle or agonists. LY379268 was also administered to 5-HT_{2A}^{-/-} (21) and mGlu₂^{-/-} (22) mice

(originally provided by Prof. Shigetada Nakanishi at Kyoto University, Japan), and DOI to mGlu₂^{-/-} mice. Thirty min after agonist injection, mice were anesthetized with pentobarbital (100 mg/kg i.p., Ceva SA) and perfused transcardially with fixative solution containing 4% w/v paraformaldehyde, 0.1 M sodium phosphate buffer (pH 7.5), sodium fluoride (10 mM) and sodium orthovanadate (2 mM). Brains were post-fixed for 48 h in the same solution and stored at 4°C. Thirty µm-thick sections were cut with a vibratome (Leica). Sections were incubated with a solution containing 0.2% Triton X-100, 3% bovine serum albumin (BSA) and 2% goat serum in Tris buffer saline (TBS) for 30 min. They were then incubated for 24 h at 4°C with the anti-phospho-Ser⁸⁴³ antibody (1:500) in TBS containing 3% BSA. After four washes, they were incubated for 1 h with an Alexa Fluor® 488-conjugated anti-rabbit antibody (1:2,000, Invitrogen) in TBS containing 2.5% BSA and 0.08% Triton X-100. Immunofluorescent staining was observed with a Zeiss Axioimager Z1 microscope equipped with apotome (Carl Zeiss). Images were acquired using the Zen2 (blue edition) software driving an AxioCam MRm CCD camera (Carl Zeiss). Image analysis was performed with the Image J software (v1.50) and results were expressed in percentage of anti-phospho-Ser⁸⁴³-positive cells relative to DAPI-stained cells.

ELISA

Quantification of cell surface expression of HA-tagged 5-HT_{2A} receptors and Flag-tagged wild-type, S⁸⁴³A and S⁸⁴³D mGlu₂ receptors was performed by ELISA under non-permeabilized conditions, as previously described (23). Dilutions for primary antibodies were 1:400 (Anti-HA) and 1:1,000 (anti-Flag). Secondary antibodies (donkey anti-mouse or anti-rabbit conjugated to HRP, GE Healthcare) were used at 1:5,000 dilution. Immunoreactive signal was detected with a M200 Infinite plate reader (Tecan), using the SuperSignal ELISA Femto chromogenic substrate (Thermo Scientific). Control experiments were performed using cells transfected with empty vectors.

Electrophysiology

HEK-293 cells were co-transfected with plasmids encoding Flag-tagged wild-type or S⁸⁴³A or S⁸⁴³D mGlu₂ receptor, HA-tagged 5-HT_{2A} receptor, Cav2.2, Cavβ_{1b} and Cavα_{2-δ1-b} ancillary subunits and GFP (to detection of transfected cells). Twenty-four hours after transfection, cells were dissociated with Versene (Invitrogen), and plated at low density in poly-ornithine-coated 35-mm Petri dishes for 24 h. Cells were incubated with extracellular solution containing 125 mM NaCl, 20 mM TEACl, 10 mM HEPES, 5mM BaCl₂, 1 mM MgCl₂ and pH was adjusted to 7.3 with TEAOH. Borosilicate glass pipettes (2–4 MΩ, Sutter instrument) were filled with internal solution containing 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM CaCl₂, 1 mM MgCl₂, 4 mM MgATP, 0.3 mM Tris-GTP (pH adjusted to 7.3 with NaOH). Currents were elicited from a holding potential of -80 mV to 0 mV during 10 msec repeated each 1 sec. Data were plotted as means ± SEM. Statistical difference (p < 0.05) was determined using one-way analysis of variance.

Statistics

Statistical analyses were performed using Prism (v. 7.0, GraphPad Software Inc.). Histograms show means ± SEMs. One-way ANOVA followed by Tukey's and Dunnett's tests were performed for multiple comparisons to multiple groups or to a single group, respectively. For each statistical test, F value was calculated in order to determine how the variance between the mean values obtained in different populations is significantly different. F value, number of independent biological replicates and sample size (or number of animals per condition) are indicated in the corresponding figure legends. MS peptide identification probabilities were calculated using the Mascot algorithm. Mascot score represents -10*LOG₁₀(p-value), where p-value is the absolute probability of peptide wrong assignment.

Results

Agonist-stimulated mGlu₂ receptor phosphorylation at Ser⁸⁴³ requires co-expression of 5-HT_{2A} receptor in HEK-293 cells

To identify novel molecular substrates of the functional crosstalk between 5-HT_{2A} and mGlu₂ receptors, we first examined the phosphorylation pattern of Flag-tagged human mGlu₂ receptor co-expressed with HA-tagged 5-HT_{2A} receptor in HEK-293 cells following exposure to vehicle or a maximally-effective concentration of the mGlu₂ receptor orthosteric agonist LY379268 (1 μM) (24). mGlu₂ receptors were purified by immunoprecipitation followed by SDS-PAGE. They were then digested *in-gel* with trypsin. Analysis of peptides from vehicle or LY379268-treated cells by nano-flow liquid chromatography coupled to high-resolution tandem mass spectrometry (nano-LC-MS/MS) yielded 57 % coverage of the entire receptor sequence and 75% coverage of intracellular domains, with a p-value threshold of 0.05 for peptide identification (Supplementary Figure1). LC-MS/MS analysis identified one phosphorylated residue located in the third extracellular domain of the receptor (Thr⁷¹⁸) and four residues in the C-terminal domain (Ser⁸³³, Ser⁸³⁷, Ser^{843/844} and Ser⁸⁷¹, Table 1, Figure 1A, Supplementary Figures 2-5). Note that MS/MS fragmentation did not discriminate between a phosphorylation at Ser⁸⁴³ or Ser⁸⁴⁴, due to the absence of site-specific fragment ions in MS/MS spectra (Supplementary Figure 4). In an effort to distinguish Ser⁸⁴³ from Ser⁸⁴⁴ phosphorylation, purified mGlu₂ receptor was digested with chymotrypsin to generate a proteolytic peptide with the two serine residues located in the central part of the sequence, which should be enriched in discriminant fragment ions. Unfortunately, chymotrypsin digestion yielded only 31 % receptor sequence coverage (Supplementary Figure 6A) and the two theoretically discriminant ions were not detected in the corresponding fragmentation spectra (Supplementary Figure 6B).

Alignment of human, mouse and rat mGlu₂ receptor sequences revealed that the region encompassing Ser⁸⁴³ and Ser⁸⁴⁴ is highly conserved in the three species (Figure 1B). Specifically, Ser⁸⁴³ of the human receptor is conserved in rat and mouse orthologs, whereas Ser⁸⁴⁴ is replaced by an alanine (Figure 1B). We thus renewed the experiments in cells

expressing the rat mGlu₂ receptor and clearly identified a peptide phosphorylated at Ser⁸⁴³ (Supplementary Figure 7). Consistent with these findings, a peptide comprising phosphorylated Ser⁸⁴³ was likewise identified in cells expressing the S⁸⁴⁴A mutant of the human mGlu₂ receptor (Supplementary Figure 8), while no peptide phosphorylated at Ser⁸⁴⁴ was detected in cells expressing the S⁸⁴³A mutant. Collectively, these results unambiguously identify Ser⁸⁴³ as the phosphorylated residue in the human and murine receptors.

To explore the impact of agonist stimulation of mGlu₂ receptor and 5-HT_{2A} receptor expression upon mGlu₂ receptor phosphorylation, we next compared the phosphorylation index of each identified phosphorylated residue in cells exposed or not to LY379268, in the presence or absence of 5-HT_{2A} receptor. The phosphorylation index of Thr⁷¹⁸, Ser⁸³³, Ser⁸³⁷ and Ser⁸⁷¹ did not display any significant variation upon exposure of cells to LY379268 or co-expression of 5-HT_{2A} receptor (Table 1 and Supplementary Figures 2B, 3B, 5B). In contrast, LY379268 induced a marked increase in Ser⁸⁴³ phosphorylation in cells co-expressing human mGlu₂ and 5-HT_{2A} receptors while it did not significantly affect its phosphorylation state in cells expressing mGlu₂ receptor alone (Figure 1C). Likewise, LY379268 exposure of cells expressing the rat mGlu₂ receptor increased Ser⁸⁴³ phosphorylation only when the 5-HT_{2A} receptor was co-expressed (Figure 1C and Supplementary Figure 7). Collectively, these findings demonstrate that agonist stimulation of mGlu₂ receptor induces the phosphorylation of a conserved serine residue in human and murine receptors in HEK-293 cells, an effect dependent of the presence of 5-HT_{2A} receptor.

Agonist-stimulated mGlu₂ receptor phosphorylation at Ser⁸⁴³ in the mouse prefrontal cortex requires co-expression of 5-HT_{2A} receptor

A polyclonal antibody against the mGlu₂ receptor phosphorylated at Ser⁸⁴³ was obtained by immunizing a rabbit with the synthetic CAAPRASANLGQG peptide encompassing a phosphorylated serine residue corresponding to Ser⁸⁴³ in the entire murine receptor. We checked the specificity of the antibody for the phosphorylated site by immunoblotting on HEK-293 cell protein extracts. No immunoreactive signal was observed in blots from cells

transfected with empty vector, but a clear signal was detected at the expected molecular weight in blots obtained from cells expressing the rat mGlu₂ receptor (Supplementary Figure 9). Furthermore, LY379268 treatment strongly enhanced this immunoreactive signal only in cells co-expressing the 5-HT_{2A} receptor, consistent with the results of MS analysis (Figure 1C and Supplementary Figure 9). Mutation of Ser⁸⁴³ into alanine abolished this immunoreactive signal, confirming the specificity of the antibody for phosphorylated Ser⁸⁴³ (Supplementary Figure 9). The maximal signal intensity was reached at LY379268 concentration as low as 1 nM (Supplementary Figure 10A). Notably, expression of 5-HT_{2A} receptor at various densities (in absence of LY379268) did not enhance phosphorylation of Ser⁸⁴³ of mGlu₂ receptor (Supplementary Figure 11). As expected, the increase in Ser⁸⁴³ phosphorylation induced by LY379268 was prevented by the co-application of the mGlu₂ receptor antagonist LY341495 (10 μM, Supplementary Figure 12). An increase in the anti-phospho-Ser⁸⁴³ antibody immunoreactivity was also observed in Western blots from cells expressing the human mGlu₂ receptor, though the intensity of the immunoreactive signal was much lower than that measured in cells expressing the rat receptor (Supplementary Figure 13), suggesting a higher affinity of our anti-phospho-Ser⁸⁴³ antibody for the murine phosphorylated receptor.

We next took advantage of this antibody to investigate the phosphorylation of native mGlu₂ receptor in the mouse prefrontal cortex, a brain region where mGlu₂/5-HT_{2A} heteromers have been identified (9). We injected mice with either vehicle of LY379268 (10 mg/kg, 30 min) and assessed mGlu₂ phosphorylation by immunohistochemistry. Reminiscent of the data obtained in HEK-293 cells, LY379268 administration strongly increased immunostaining with the anti-phospho-Ser⁸⁴³ antibody (Figure 1D). The most intense signal was observed in middle layers of prefrontal cortex, which express the highest density of 5-HT_{2A} receptor (25). As observed in HEK-293 cells, Ser⁸⁴³ phosphorylation elicited in mouse prefrontal cortex by injecting LY379268 was also prevented by the administration of LY341495, which alone did not affect Ser⁸⁴³ phosphorylation (Figure 1D). Confirming the specificity of our antibody for phosphorylated mGlu₂, no signal was detected in prefrontal cortex of mGlu₂ receptor

knockout (mGlu₂^{-/-}) mice treated with either vehicle or LY379268 (Figure 1D). Moreover, administration of LY379268 to 5-HT_{2A} receptor knockout (5-HT_{2A}^{-/-}) mice did not increase the phospho-Ser⁸⁴³ immunoreactive signal in prefrontal cortex (Figure 1D), indicating that 5-HT_{2A} receptor expression is also necessary for LY379268-induced phosphorylation of native mGlu₂ receptor at Ser⁸⁴³.

Ser⁸⁴³ phosphorylation is mediated by casein kinase 1 (CK1)

In order to identify protein kinase(s) involved in Ser⁸⁴³ phosphorylation, mGlu₂ receptor sequence surrounding Ser⁸⁴³ was uploaded into Group-based Prediction System (GPS[®], v3.0), a software for kinase consensus search that ranks protein kinases into a hierarchical tree with four levels and builds its algorithm against the PhosphoELM database, in order to calculate false discovery rates for each of them (26). GPS search revealed that Ser⁸⁴³ is a good consensus for phosphorylation by casein kinase 1 (CK1, GPS[®] score 9.3), and cyclic AMP-dependent protein kinase (PKA, GPS[®] score 4.1), consistent with previous findings (15). Pretreatment of HEK-293 cells co-expressing mGlu₂ and 5-HT_{2A} receptor with the CK1 inhibitor D4476 (10 μM, 60 min) (27) abolished LY379268-induced phosphorylation of Ser⁸⁴³, whereas the PKA pharmacological inhibitor KT5720 (1 μM, 60 min) (28) did not affect the level of Ser⁸⁴³ phosphorylation (Figure 2A), suggesting that CK1 rather than PKA promotes Ser⁸⁴³ phosphorylation in living cells. Treatment of cells with D4476 did not affect the low Ser⁸⁴³ phosphorylation level measured in cells expressing mGlu₂ receptor alone and challenged with LY379268 (Supplementary Figure 14).

Among the CK1 isoforms, GPS search pointed CK1δ and CK1ε as the best candidates able to phosphorylate Ser⁸⁴³. Given the high CK1ε expression level and the very low expression of CK1δ in prefrontal cortex (<http://mouse.brain-map.org/>), we targeted specifically the ε isoform using RNA interference. Silencing CK1ε expression abolished LY379268-induced Ser⁸⁴³ phosphorylation in cells co-expressing 5-HT_{2A} receptor (Figure 2B and Supplementary Figure 15), reminiscent of the effect of cell exposure to D4476 (Figure 2A).

G_{i/o} and G_q proteins are involved in Ser⁸⁴³ phosphorylation elicited by mGlu₂ receptor stimulation in cells co-expressing 5-HT_{2A} receptor

In line with the canonical coupling of mGlu₂ and 5-HT_{2A} receptors to G_{i/o} and G_q proteins, respectively, we next explored their influence on LY379268-induced Ser⁸⁴³ phosphorylation in cells co-expressing both receptors. LY379268 exposure did not increase Ser⁸⁴³ phosphorylation in cells pretreated with the G_{i/o} inhibitor Pertussis toxin (PTX, 100 ng/mL, 18 h) or the pharmacological G_{q/11} inhibitor FR900359 (1 μM, 2 h) (17), indicating that the activation of both G_{i/o} and G_{q/11} is necessary to LY379268-induced Ser⁸⁴³ phosphorylation (Supplementary Figure 16A). Further supporting the role of G_{q/11} activation by unliganded 5-HT_{2A} receptor, LY379268 exposure did not promote Ser⁸⁴³ phosphorylation in cells expressing the signaling-dead 5-HT_{2A}(D¹²⁰N) receptor (Supplementary Figure 17A) (29).

Agonist-elicited phosphorylation of mGlu₂ receptor at Ser⁸⁴³ promotes receptor-operated G_{i/o} signaling

To determine the role of Ser⁸⁴³ phosphorylation in mGlu₂ receptor-mediated signal transduction, we measured mGlu₂ receptor-operated inhibition of N-type voltage-gated calcium channels, a read-out commonly used to study engagement of G_{i/o} signaling by GPCRs (30,31) (Figure 3A).

As expected, LY379268 inhibited N-type calcium channel-mediated current in a concentration-dependent manner (pEC₅₀ = 8.81 ± 0.16, n = 10 cells) in HEK-293 cells transiently expressing mGlu₂ receptor (Figure 3B). No effect of LY379268 was seen in cells transfected with an empty vector or transiently expressing the 5-HT_{2A} receptor alone, but co-expression of 5-HT_{2A} receptor with mGlu₂ receptor increased the amplitude of LY379268-induced inhibition of N-type calcium channels without affecting its apparent affinity (pEC₅₀ = 8.66 ± 0.07, n = 10 cells, Figure 3B). In contrast, co-expression of 5-HT_{2A} receptor with S⁸⁴³A mGlu₂ receptor mutant did not enhance the LY379268-induced inhibition of N-type calcium channels, while mutation of Ser⁸⁴³ into aspartate, to mimic permanent phosphorylation of this residue, had no consequence on the level of inhibition of the N-type calcium channels by LY379268 (Figure 3C). Furthermore, pre-treatment of cells with the CK1 inhibitor D4476 (10

μM), which prevented Ser⁸⁴³ phosphorylation, also abolished the potentiation of the LY379268 response elicited by 5-HT_{2A} receptor expression in cells co-expressing wild type mGlu₂ receptor but not in cells co-expressing S⁸⁴³D mGlu₂ receptors (Figure 3C). D4476 exposure did not affect the amplitude of LY379268-induced inhibition of N-type calcium channels in cells expressing mGlu₂ receptor alone (Figure 3C). Collectively, these results suggest that Ser⁸⁴³ phosphorylation is a necessary step of the potentiation of mGlu₂ receptor-operated G_{i/o} signaling induced by 5-HT_{2A} receptor expression. This potentiation was not due to an enhanced expression of mGlu₂ receptor at the plasma membrane, as ELISA experiments showed that co-expression of 5-HT_{2A} receptors did not change the cell surface expression of mGlu₂ receptor (Supplementary Figure 18). Further supporting the absence of influence of Ser⁸⁴³ phosphorylation on mGlu₂ receptor plasma membrane localization, wild type, S⁸⁴³A and S⁸⁴³D mGlu₂ receptors exhibited similar cell surface expression (Supplementary Figure 18).

5-HT_{2A} receptor stimulation promotes phosphorylation of mGlu₂ receptor at Ser⁸⁴³

Exposure of HEK-293 cells co-expressing mGlu₂ and 5-HT_{2A} receptors to 5-HT increased Ser⁸⁴³ phosphorylation state in a concentration-dependent manner and to a magnitude similar to that measured in LY379268-treated cells (Figures 1C and 4A, Supplementary Figure 10B), an effect prevented by the 5-HT_{2A} receptor antagonist M100907 (100 nM, Figure 4A). Reminiscent of the LY379268-induced response, the 5-HT-induced Ser⁸⁴³ phosphorylation was strongly reduced in cells expressing the G_q-dead 5-HT_{2A}(D¹²⁰N) receptor (Supplementary Figure 17B). Likewise, the 5-HT-elicited Ser⁸⁴³ phosphorylation was abolished in cells pretreated with FR900359 but it persisted in cells pretreated with PTX (Supplementary Figure 16B). The hallucinogenic 5-HT_{2A} receptor agonist DOI (10 μM) and the non-hallucinogenic agonist lisuride (10 μM) induced increases in Ser⁸⁴³ phosphorylation comparable to that measured in cells challenged with 5-HT (Figure 4B). Furthermore, no additional increase in Ser⁸⁴³ phosphorylation state was measured in cells exposed to both LY379268 and 5-HT_{2A} receptor agonists (5-HT, DOI or lisuride, Figure 4B).

Corroborating results obtained in transfected HEK-293 cells, administration of DOI (10 mg/kg, 30 min) or lisuride (10 mg/kg, 30 min) to mice induced elevations of Ser⁸⁴³ phosphorylation in prefrontal cortex similar to those measured in mice injected with LY379268 (Figure 4C). As expected, Ser⁸⁴³ phosphorylation elicited by DOI injection was prevented by the administration of M100907, which alone did not affect Ser⁸⁴³ phosphorylation (Figure 4C). Collectively, these results indicate that Ser⁸⁴³ phosphorylation can be triggered with similar efficiencies by agonist stimulation of either 5-HT_{2A} or mGlu₂ receptors in cells co-expressing both receptor subtypes.

Interestingly, treatment of HEK-293 cells co-expressing 5-HT_{2A} and mGlu₂ receptors with LY341495, which alone did not affect Ser⁸⁴³ phosphorylation, inhibited the 5-HT-induced effect (Figure 5A). Conversely, M100907 prevented the LY379268-induced increase in Ser⁸⁴³ phosphorylation, while it slightly but not significantly decreased basal Ser⁸⁴³ phosphorylation (Figure 5B). mGlu₂ and 5-HT_{2A} receptor ligands induced similar regulations of Ser⁸⁴³ phosphorylation in mouse prefrontal cortex: Ser⁸⁴³ phosphorylation elicited by injecting LY379268 was prevented by the administration of M100907 and, reciprocally, the DOI-induced effect was prevented by the administration of LY341495 (Figure 5C). Collectively, these observations indicate that phosphorylation of Ser⁸⁴³ elicited by the stimulation of 5-HT_{2A} receptor depends on the co-expression of mGlu₂ receptor at active conformation and *vice versa*.

Impact of 5-HT_{2A} receptor activation on G_{i/o}-dependent signaling under the control of mGlu₂ receptor

Given the role of Ser⁸⁴³ phosphorylation in mGlu₂ receptor-operated activation of G_{i/o} signaling and the critical influence of the 5-HT_{2A} receptor upon this phosphorylation, we also investigated the effect of 5-HT_{2A} receptor stimulation on G_{i/o} signaling by measuring the activity of N-type calcium channels. In cells expressing 5-HT_{2A} receptor alone, 5-HT (1 μM) already induced a weak inhibition of N-type calcium channels (Figure 5D). This inhibition was more pronounced in cells co-expressing wild type or S⁸⁴³D mGlu₂ receptors, but not in cells co-expressing S⁸⁴³A mGlu₂ receptor (Figure 5D). Reminiscent of their influence upon

Ser⁸⁴³ phosphorylation, both LY341495 and M100907 prevented LY379268- and 5-HT-induced inhibition of N-type calcium channels (Figure 5E). Collectively, these data suggest a reciprocal interplay between 5-HT_{2A} and mGlu₂ receptors in engagement of G_{i/o} signaling, which depends on Ser⁸⁴³ phosphorylation.

Discussion

Phosphorylation of GPCRs is classically considered as a mechanism initiating receptor desensitization and β -arrestin-dependent receptor internalization following sustained agonist exposure (32). More recently, subtle modifications of GPCR phosphorylation at specific sites have emerged as key molecular substrates that determine the nature of GPCR-operated signaling, depending on the ligand (functional selectivity) or the cell type, that can be tailored to meet a specific physiological function (33–38). It has also been suggested that GPCR heteromerization can influence the phosphorylation status of the different heteromer constituents, which in turn modulates signaling and physiological outcome of the heteromer. For instance, a recent study has shown that the RF-amide peptide NPF₂, by acting at NPF₂ receptors, induces a transphosphorylation of Mu-opioid receptor within Mu/NPF₂ heteromers, a process responsible for a loss of function of Mu receptors and contributing to the tolerance to morphine analgesia induced by NPF₂ (39). These observations highlight the functional and physiological impact of GPCR phosphorylation within heterocomplexes and underscore the potential of deciphering the phosphorylation events on each subunit of GPCR heteromers to elucidate the mechanism of action of psychotropic drugs. They provided the impetus for the present phosphoproteomics screen aimed at characterizing the influence of 5-HT_{2A} receptor expression and stimulation upon mGlu₂ receptor phosphorylation pattern.

We identified three (Ser⁸³³, Ser⁸⁴³ and Ser⁸⁷¹) out of the 12 putative phosphorylated residues (Ser¹⁰⁹, Ser¹¹¹, Ser²⁴⁶, Thr⁴⁵⁷, Ser⁵⁰¹, Tyr⁷³⁴, Tyr⁷⁴⁵, Tyr⁷⁴⁹, Thr⁸³², Ser⁸³³, Ser⁸⁴³ and Ser⁸⁷¹) referenced in the PhosphoSitePlus database (<http://www.phosphosite.org>) and two previously not described phosphorylated sites (Ser⁷¹⁸ and Ser⁸³⁷) in human mGlu₂ receptor co-expressed with 5-HT_{2A} receptor in HEK-293 cells challenged with a mGlu₂ receptor agonist. The absence of manual inspection of MS/MS spectra pinpointing the phosphorylated sites referenced in the PhosphoSitePlus database suggests that the majority of the previously described sites are actually not fully validated, though one cannot rule out

that the difference between our results and the previously reported ones might reflect the distinct biological samples and experimental conditions used.

Among the five sites identified in the present study, only the phosphorylation of a serine residue (Ser⁸⁴³) located in the receptor C-terminal domain and conserved in the human and rat sequences was up-regulated upon agonist receptor stimulation. Moreover, Ser⁸⁴³ phosphorylation depended on the co-expression of 5-HT_{2A} receptor. Likewise, phosphorylation of mGlu₂ receptor at Ser⁸⁴³ elicited in mice prefrontal cortex by peripheral administration of the mGlu₂ receptor agonist LY379268 (assessed by immunohistochemistry using a newly-generated phospho-Ser⁸⁴³ antibody) was not seen in 5-HT_{2A}^{-/-} mice. This result indicates that the joint influence of 5-HT_{2A} and mGlu₂ receptors upon Ser⁸⁴³ phosphorylation can be observed on both recombinant receptors and native receptors expressed in prefrontal cortex, a brain structure co-expressing both receptors and important for the behavioral responses to 5-HT_{2A} and mGlu₂ receptor ligands (9,40). Intriguingly, 5-HT_{2A} receptor stimulation by 5-HT promoted Ser⁸⁴³ phosphorylation as efficiently as that induced by mGlu₂ receptor stimulation, reminiscent of the transphosphorylation of Mu-opioid receptor elicited by NPF receptor activation (39). Moreover, Ser⁸⁴³ phosphorylation induced by 5-HT (or the synthetic 5-HT_{2A} receptor agonist DOI) was impaired by the mGlu₂ receptor antagonist LY341495, while the LY379268-induced effect was prevented by the 5-HT_{2A} receptor antagonist M100907, thus demonstrating a positive reciprocal influence of both receptor activities upon mGlu₂ receptor phosphorylation at Ser⁸⁴³ in HEK-293 cells and mice prefrontal cortex. Notably, the LY379268-induced Ser⁸⁴³ phosphorylation depends on both G_{i/o} and G_q activation, whereas the 5-HT-induced response is only G_q-dependent. Collectively, these findings suggest that Ser⁸⁴³ phosphorylation can result from two types of allosteric modulation exerted by different active conformations of the 5-HT_{2A} receptor within heteromers: i) an allosteric modulation by unliganded (constitutively active) 5-HT_{2A} receptor of agonist-stimulated mGlu₂ receptor (bound to activated G_{i/o} protein) and ii) an allosteric modulation by 5-HT-stimulated 5-HT_{2A} receptor that is independent of mGlu₂ receptor

stimulation by its own agonist and of $G_{i/o}$ protein activation. However, one cannot rule out that 5-HT_{2A} receptor-dependent Ser⁸⁴³ phosphorylation can also be induced indirectly, *via* a 5-HT_{2A} receptor-operated $G_{q/11}$ signaling pathway. Irrespective of the mechanism underlying Ser⁸⁴³ phosphorylation, our results identify a novel functional crosstalk mechanism between 5-HT_{2A} and mGlu₂ receptors at the receptor level. The demonstration of this crosstalk in mouse prefrontal cortex strongly supports the co-localization of at least a fraction of both receptors expressed in this brain area and, possibly, the formation of 5-HT_{2A}-mGlu₂ heteromers *in vivo*.

The difference in signal transduction elicited by hallucinogenic and non-hallucinogenic agonists of the 5-HT_{2A} receptor represents one of the best characterized examples of functional selectivity translated into distinct behaviors (21,41). Furthermore, we previously showed that the two categories of 5-HT_{2A} receptor agonists differ in their capacities to promote 5-HT_{2A} receptor phosphorylation at Ser²⁸⁰ (14). Such a biased phosphorylation was not retrieved for Ser⁸⁴³: treatment of cells with the hallucinogenic agonist DOI and the non-hallucinogenic agonist lisuride induced comparable increases in Ser⁸⁴³ phosphorylation to that elicited by 5-HT. It is thus unlikely that Ser⁸⁴³ phosphorylation underlies the distinct behavioral responses induced by both ligand categories.

Among the two kinases possibly involved in Ser⁸⁴³ phosphorylation suggested by the GPS algorithm, namely CK1 and PKA, our data based on pharmacological inhibitors and CK1 expression silencing suggest that this phosphorylation is mediated by CK1. This contrasts with previous findings, which demonstrated that Ser⁸⁴³ can be phosphorylated *in vitro* by PKA or following a treatment of cells expressing recombinant mGlu₂ receptor with the adenylyl cyclase activator forskolin or cell-permeable cAMP analogs. Hence, CK1 rather than PKA seems to be involved in the more physiological context of agonist mGlu₂ receptor stimulation in cells co-expressing 5-HT_{2A} receptors, consistent with the canonical coupling of mGlu₂ receptors to $G_{i/o}$ proteins and their ability to inhibit rather than stimulate cAMP production (42). CK1 activity generally depends on its expression level or the expression of

regulators, which are likely not affected by acute extracellular stimuli (43,44). In light of the well-established formation of 5-HT_{2A}-mGlu₂ heteromers, at least in recombinant cells (9, 34), we hypothesize that Ser⁸⁴³ might be accessible for CK1 only in a mGlu₂ receptor conformation stabilized by its association with 5-HT_{2A} receptor and agonist stimulation of either mGlu₂ or 5-HT_{2A} receptor within the heterocomplex.

Consistent with the localization of Ser⁸⁴³ in mGlu₂ receptor C-terminal domain, a region important for coupling to G proteins, several lines of evidence suggest that its phosphorylation is an essential step in engagement of G_{i/o} signaling not only upon mGlu₂ receptor activation but also following 5-HT_{2A} receptor activation in cells co-expressing both receptors: *i*) corroborating previous findings indicating that heteromeric assembly of mGlu₂ and 5-HT_{2A} receptors enhances glutamate-elicited G_{i/o} signaling (11), co-expression of 5-HT_{2A} receptor with mGlu₂ receptor enhanced LY379268-induced inhibition of N-type channels (used as readout of activation of G_{i/o}-dependent signaling) (30,31), an effect suppressed by mutating Ser⁸⁴³ into alanine; *ii*) reminiscent of their influence upon Ser⁸⁴³ phosphorylation, the 5-HT_{2A} receptor antagonist M100907 prevented enhancement of LY379268-induced inhibition of N-type channels induced by the co-expression of 5-HT_{2A} receptor, while the mGlu₂ receptor antagonist LY341495 blocked the 5-HT-evoked response. These findings thus identify Ser⁸⁴³ phosphorylation as a novel molecular substrate underlying the functional crosstalk between 5-HT_{2A} and mGlu₂ receptors, which determines the level of G_{i/o} signaling under the control of both receptors. It has been proposed that the balance between G_{i/o} and G_q-dependent signaling determines pro-psychotic vs. antipsychotic activity of 5-HT_{2A} and mGlu₂ receptors ligands (11). Given the critical influence of Ser⁸⁴³ phosphorylation level on mGlu₂ and 5-HT_{2A} receptor-dependent G_{i/o} signaling, its fine-tuning might be an essential mechanism contributing to the homeostatic balance that ensures a normal non-psychotic state.

In conclusion, the present study identifies Ser⁸⁴³ phosphorylation as an early molecular substrate of the functional crosstalk between 5-HT_{2A} and mGlu₂ receptors that determines

the level of $G_{i/o}$ signaling under the control of both receptors, which itself might be predictive of antipsychotic or pro-psychotic effects of drugs targeting each receptor. Accordingly, the anti-phospho-Ser⁸⁴³ antibody developed in this study might be a relevant tool for the screening of potential antipsychotic drugs in preclinical models of schizophrenia. Several studies also reported changes in 5-HT_{2A} and mGlu₂ receptors densities in patients with schizophrenia, which might contribute to the pathogenicity of the disease (9,12,46). Though the data show some discrepancies and must be considered cautiously due to the potential impact of antipsychotic drug treatment on 5-HT_{2A} receptor expression, the majority of post-mortem studies demonstrate a decrease in cortical 5-HT_{2A} receptor density in patients. In line with these findings and the strong influence of 5-HT_{2A} receptor expression/stimulation on Ser⁸⁴³ phosphorylation, analysis of its phosphorylation status in post-mortem brain of patients with schizophrenia might be of great interest.

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Author Contributions: SM performed biochemical and electrophysiology experiments, some LC-MS/MS analyses and participated in manuscript writing. MB and JC performed some biochemical experiments and LC-MS/MS analyses. PM conceived the study, supervised experiments and wrote the manuscript. FV conceived the study, performed LC-MS/MS analyses and some biochemical experiments, supervised experiments and wrote the manuscript. JB conceived the study and participated in manuscript writing. EB supervised electrophysiology experiments. GK and EK extracted and purified FR900359 from *Ardisia crenata* plant. GB and FN participated in experiments on mGlu₂^{-/-} mice.

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Supplementary information is available at MP's website.

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Figure legends

Figure 1. Agonist-stimulated mGlu₂ receptor phosphorylation at Ser⁸⁴³ requires co-expression of 5-HT_{2A} receptor in HEK-293 cells and mouse prefrontal cortex. **A**, Schematic overview of the position of phosphorylated residues identified by MS/MS in human mGlu₂ receptor. **B**, Sequence alignment of the receptor region containing Ser⁸⁴³ in human, mouse and rat mGlu₂ orthologs. Note that Ser⁸⁴³ is conserved in the three orthologs, while Ser⁸⁴⁴ in the human receptor is replaced by an alanine in murine orthologs. The sequences in bold correspond to peptides identified by MS/MS. **C**, HEK-293 cells transiently expressing human or rat mGlu₂ receptor in the absence or presence of 5-HT_{2A} receptor were exposed to vehicle or LY379268 (LY37, 1 μM) for 15 min. The histograms represent the means ± SEM of ion signal intensities (expressed in % of signal intensity measured in cells co-expressing mGlu₂ and 5-HT_{2A} receptors and treated with LY379268) measured in three replicates. One-way ANOVA (Post-hoc: Tukey's range test): F(3,12)=14.91, p=0.0002 and F(3,8)=5.558, p=0.0234, in cells expressing human and rat mGlu₂, respectively. *p<0.05; **p<0.01; ***p<0.001. **D**, Wild type, 5-HT_{2A}^{-/-} and mGlu₂^{-/-} mice were injected with either vehicle or LY379268 (10 mg/kg, i.p) for 30 min. LY341495 (3 mg/kg i.p.) was injected 15 min before vehicle/LY379268 administration. Representative images showing phospho-Ser⁸⁴³-mGlu₂ immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean ± SEM of values (expressed in % of anti-phospho-Ser⁸⁴³-positive cells relative to DAPI-stained cells) obtained in four animals per group. One-way ANOVA (Post-hoc: Tukey's range test): F(5,18)=8.967, p=0.0002. *p<0.05; **p<0.01, ***p<0.001. Scale bar: 100 μm. ns: not significant.

Figure 2. 5-HT_{2A} receptor-dependent phosphorylation of Ser⁸⁴³ elicited by LY379268 is mediated by casein kinase 1. **A**, HEK-293 cells co-expressing rat Flag-tagged mGlu₂ and HA-tagged 5-HT_{2A} receptors were pre-incubated with either vehicle or the CK1 inhibitor (D4476, 10 μM) or the PKA inhibitor (KT5720, 1 μM) for 60 min and then challenged with vehicle or LY379268 (LY37, 1 μM) for 15 min. **B**, HEK-293 cells co-expressing Flag-tagged

rat mGlu₂ and HA-tagged 5-HT_{2A} receptors were transfected with either control or CK1ε siRNA and challenged for 15 min with vehicle or LY379268 (LY37, 1 μM). Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means ± SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.) obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): A, F(5,12)=10.23, p=0.0005. B, F(3,8)=8.114, p=0.0082. *p<0.05, **p<0.01 vs. corresponding vehicles. ns: not significant.

Figure 3. Potentiation of mGlu₂ receptor-operated G_{i/o} signaling elicited by 5-HT_{2A} receptor expression depends on Ser⁸⁴³ phosphorylation. **A**, Schematic overview of N-type calcium channel inhibition elicited upon activation of G_{i/o}-coupled receptors. **B**, Left panel: representative current traces obtained in response to vehicle (open circles) or 1 μM LY379268 (filled circles) in HEK-293 cells expressing Flag-tagged wild type mGlu₂ receptor alone or in combination with HA-tagged 5-HT_{2A} receptor. Right panel: HEK-293 cells, transiently expressing Flag-tagged wild type mGlu₂ receptor alone or in combination with HA-tagged 5-HT_{2A} receptor, were exposed to increasing concentrations of LY379268. Each point represents the means ± SEM of percentage of N-type calcium channel current inhibition measured in 10 cells from three different sets of cultured cells. **C**, Left panel: cells, expressing Flag-tagged mGlu₂ receptor alone or co-expressing HA-tagged 5-HT_{2A} receptor and either wild type or S⁸⁴³A or S⁸⁴³D Flag-tagged mGlu₂ receptor, were exposed to 1 μM LY379268. Right panel: HEK-293 cells, expressing Flag-tagged mGlu₂ receptor alone or co-expressing HA-tagged 5-HT_{2A} receptor and either wild type or S⁸⁴³D mGlu₂ receptor, were treated with vehicle or D4476 (10 μM, delivered *via* the patch pipette 5 min before the LY379268 challenge). Representative traces of N-type calcium channel currents in conditions not depicted in B are illustrated. Histograms represent the means ± SEM of N-

type calcium channel current inhibition induced by LY379268 in 10 cells from three independent cultures. One-way ANOVA (Post-hoc: Tukey's range test): $F(3,36)=16.94$, $p<0.0001$; $F(5,54)=17.15$, $p<0.0001$ in left and right panels, respectively. $***p<0.001$.

Figure 4. 5-HT_{2A} receptor stimulation promotes phosphorylation of mGlu₂ at Ser⁸⁴³ in HEK-293 cells and mouse prefrontal cortex. **A**, HEK-293 cells co-expressing Flag-tagged mGlu₂ receptor and HA-tagged 5-HT_{2A} receptor were exposed to vehicle or 5-HT (1 μ M, 15 min) in the absence or presence of M100907 (100 nM). **B**, Cells were exposed to either vehicle or 5-HT (1 μ M), or DOI (10 μ M) or lisuride (10 μ M) in the absence or presence of LY379268 (1 μ M) for 15 min. Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and the anti-Flag antibody. In A and B, representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means \pm SEM of anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units) obtained in the three experiments. A, One-way ANOVA (Post-hoc: Tukey's range test): $F(2,6)=16.63$, $p<0.0036$. B, One-way ANOVA (Post-hoc: Dunnett's range test): $F(6,14)=7.779$, $p<0.0008$. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs. Vehicle. § $p<0.05$ vs. 5-HT. **C**, Wild type mice were injected with either vehicle or DOI (10 mg/kg, i.p) or lisuride (10 mg/kg, i.p). mGlu₂^{-/-} mice were injected with DOI (10 mg/kg, i.p). M100907 (5 mg/kg, i.p.) was injected 15 min before vehicle/DOI administration. Representative images showing phospho-Ser⁸⁴³-mGlu₂ immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean \pm SEM of values (expressed in % of anti-phospho-Ser⁸⁴³-positive cells relative to DAPI-stained cells) obtained in four animals per group. One-way ANOVA (Post-hoc: Tukey's range test): $F(4,15)=33.62$, $p<0.0001$. *** $p<0.001$. Scale bar: 100 μ m.

Figure 5. Reciprocal influence of mGlu₂ and 5-HT_{2A} receptor stimulation upon G_{i/o} signaling depends on Ser⁸⁴³ phosphorylation. **A**, HEK-293 cells co-expressing Flag-tagged mGlu₂ receptor and HA-tagged 5-HT_{2A} receptor were exposed to vehicle or 5-HT (1 μM), alone or in combination with LY341495 (10 μM), or to LY341495 alone (10 μM) for 15 min. **B**, HEK-293 cells co-expressing Flag-tagged mGlu₂ receptor and HA-tagged 5-HT_{2A} receptor were exposed for 15 min to vehicle or LY379268 (10 nM), in the absence or presence of M100907 (100 nM). In A and B, mGlu₂ receptor phosphorylation was analyzed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³ antibody and the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means ± SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.) obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): F(3,8)=22.49, p=0.0003 and F(3,8)=28.97, p=0.0001 in A and B, respectively. **p<0.01, ***p<0.001. **C**, Mice were injected with either DOI (10 mg/kg, i.p), LY379268 (10 mg/kg, i.p), M100907 (5 mg/kg, i.p.) and LY341495 (3 mg/kg, i.p.) were injected 15 min before vehicle/LY379268/DOI administration. Representative images showing phospho-Ser⁸⁴³-mGlu₂ immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean ± SEM of values (expressed in % of anti-phospho-Ser⁸⁴³-positive cells relative to DAPI-stained cells) obtained in four animals per group. One-way ANOVA (Post-hoc: Tukey's range test): F(5,18)=24.57, p<0.0001. ***p<0.001. Scale bar: 100 μm. **D**, HEK-293 cells, transiently expressing 5-HT_{2A} receptor alone or in combination with either wild type or S⁸⁴³A or S⁸⁴³D mGlu₂ receptor, were exposed to 1 μM 5-HT. Representative traces of N-type calcium channel currents obtained after exposure to vehicle or 5-HT are shown. The histograms represent the means ± SEM of N-type calcium channel current inhibition measured for each treatment in 10 cells originating from three independent cultures. One-way ANOVA (Post-hoc: Tukey's range test): F(3,36)=13.45, p<0.0001. ***p<0.001. **E**, Left panels: HEK-293 cells co-expressing 5-HT_{2A} and mGlu₂ receptors were sequentially challenged with LY3792968 (10 nM), LY341495 (10 μM) and LY3792968 + LY341495 or with 5-HT (1 μM), M100907 (100 nM) and 5-HT +

M100907. Right panels: cells were sequentially challenged with LY3792968, M100907 and LY3792968 + M100907 or with 5-HT, LY341495 and 5-HT + LY341495. Representative traces of N-type calcium channel currents obtained after 5-HT or LY3792968 challenges (in absence or presence of M100907 or LY341495) are illustrated. The histograms represent the means \pm SEM of N-type calcium channel current inhibition measured for each condition in 10 cells originating from three independent cultures. Note that M100907 or LY341495 alone did not alter the N-type calcium channel current. One-way ANOVA (Post-hoc: Tukey's range test): $F(3,36)=89.9$, $p<0.0001$; $F(3,36)=78.18$, $p<0.0001$ in left and right panels, respectively. *** $p<0.001$.

Modified sequence	Species	Start-end	Experimental m/z (Th)	Theoretical mass (Da)	Δ mass (ppm)	Mascot score	Phosphorylation site occupancy index			
							mGlu ₂		mGlu ₂ + 5-HT _{2A}	
							Vehicle	LY37	Vehicle	LY37
REVVt ⁷¹⁸ LR + Phospho (ST)	Human	714-720	477,2515	951,4903	-5,40	23	0,059 ± 0.03	0,057 ± 0.03	0,028 ± 0.02	0,042 ± 0.02
APTs ⁸³³ RFGs ⁸³⁷ AAAR + 2 Phospho (ST)	Human	830-841	676,2813	1350,5482	-0,18	31	0,222 ± 0.10	0,237 ± 0.12	0,228 ± 0.10	0,276 ± 0.10
AS ⁸⁴³ S ⁸⁴⁴ SLGQGSGSQFVPTVCNGR + Phospho (ST)	Human	842-862	1088,476	2174,9416	-1,93	136	0,004 ± 0.001	0,010 ± 0.001	0,006 ± 0.0007	0,025 ± 0.004***
EVVDSTTSs ⁸⁷¹ L + Phospho (ST)	Human	863-872	559,2383	1116,4588	2,9	53	0,052 ± 0.02	0,041 ± 0.01	0,052 ± 0.01	0,047 ± 0.003
As ⁸⁴³ ANLGQGSGSQFVPTVCNGR + Phospho (ST)	Rat	842-862	1093,9878	2185,9576	1,57	60	0,040 ± 0.01	0,041 ± 0.01	0,032 ± 0.01	0,112 ± 0.02*

Table 1. List of phosphorylated peptides identified from purified mGlu₂ receptors by nano-LC-MS/MS. HEK-293 cells transiently expressing human or rat Flag-tagged mGlu₂ receptor, with or without HA-tagged 5-HT_{2A} receptor, were exposed to either Vehicle or LY379268 (1 μ M, 15 min). Solubilized receptors were immunoprecipitated with the anti-Flag M2 antibody, resolved by SDS-PAGE and digested in-gel with trypsin. Peptides were analyzed by nano-LC-MS/MS using multistage activation on the neutral loss of phosphoric acid. MS/MS spectra were manually interpreted. For each peptide, the position of modified residue(s), the position in the protein sequence, experimental mass/charge, theoretical mass, mass deviation, Mascot score, and relative abundance compared with the non-phosphorylated peptide (site occupancy index: maximal intensity observed in the phosphorylated peptide extracted ion chromatogram/sum of the maximal intensities observed in the phosphorylated and the non-phosphorylated peptide extracted ion chromatograms) are indicated. The data are the means of values calculated from three independent experiments performed on different sets of cultured cells. *p<0.05, *** p<0.001 vs. corresponding vehicles.

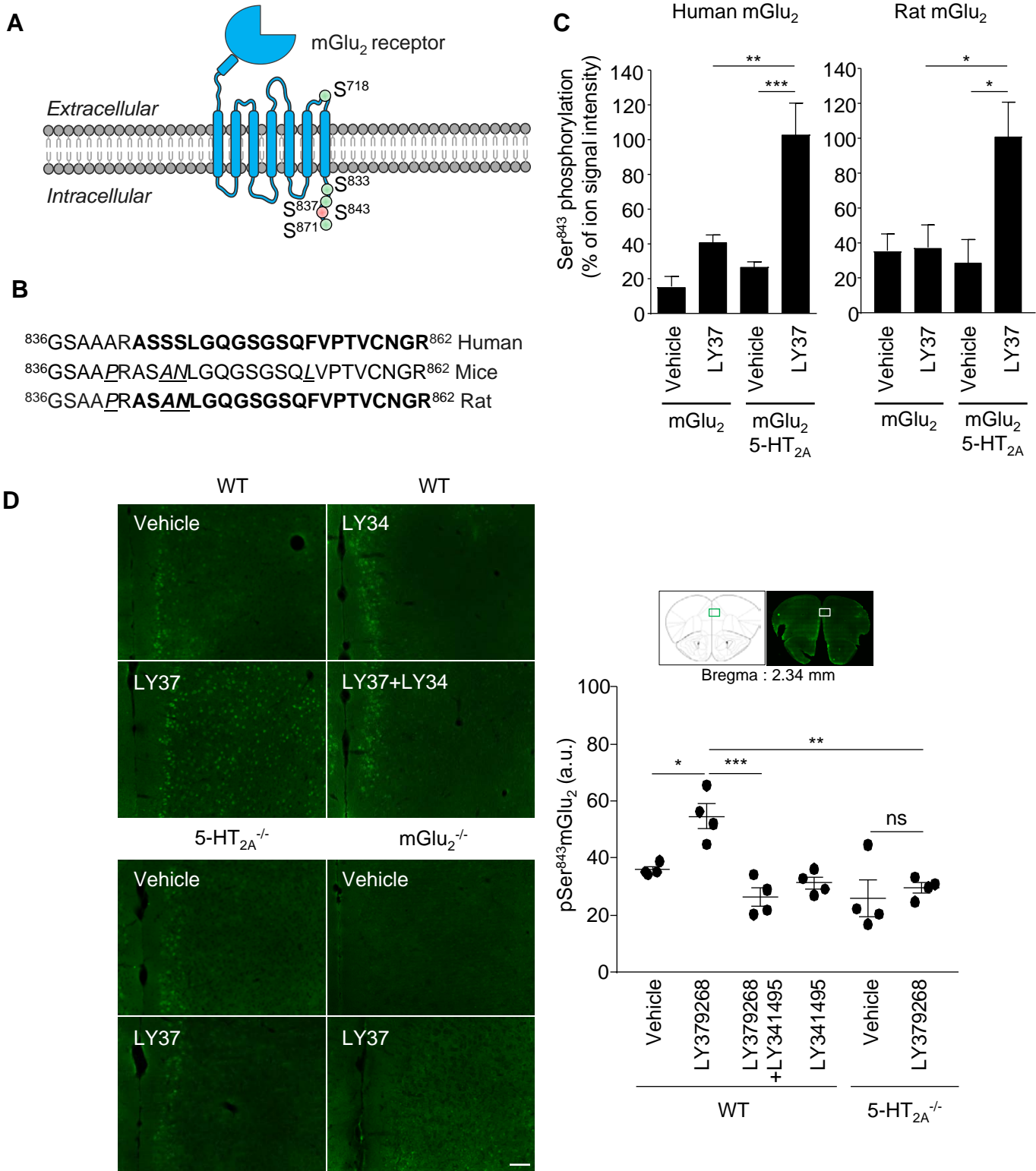


Figure 1

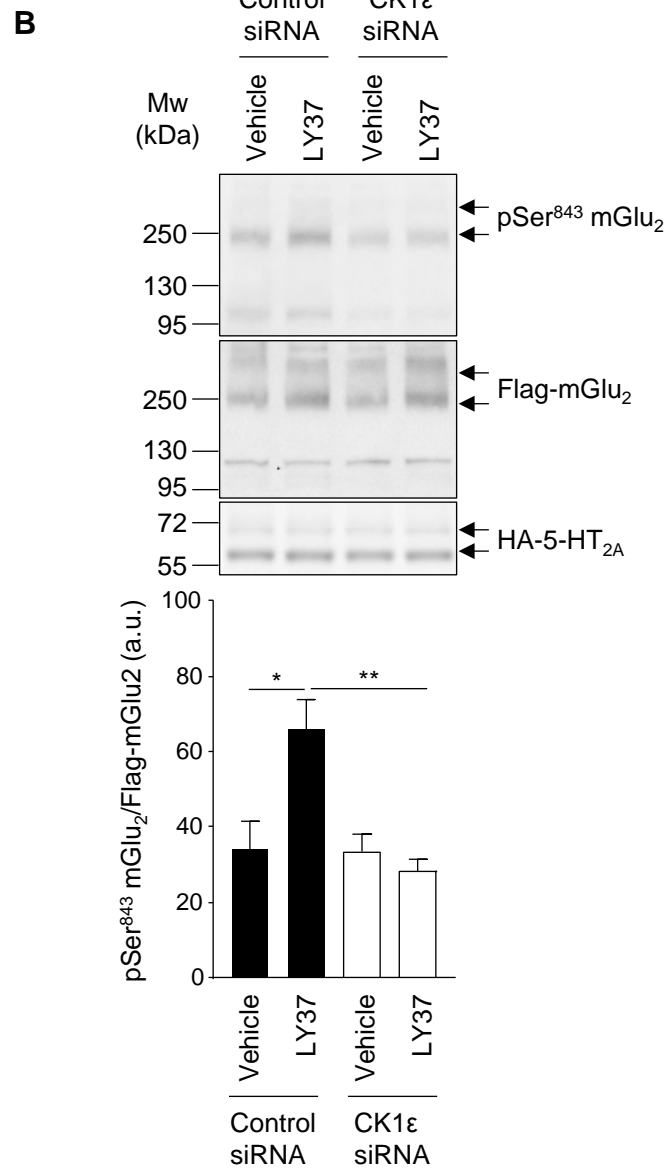
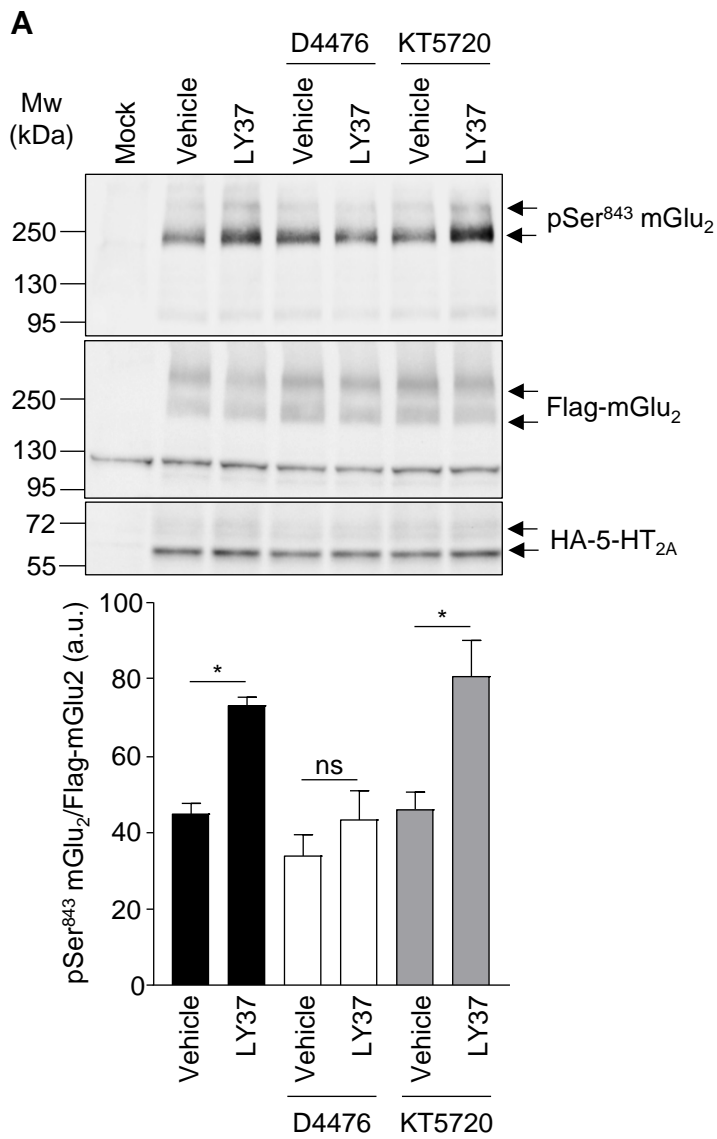


Figure 2

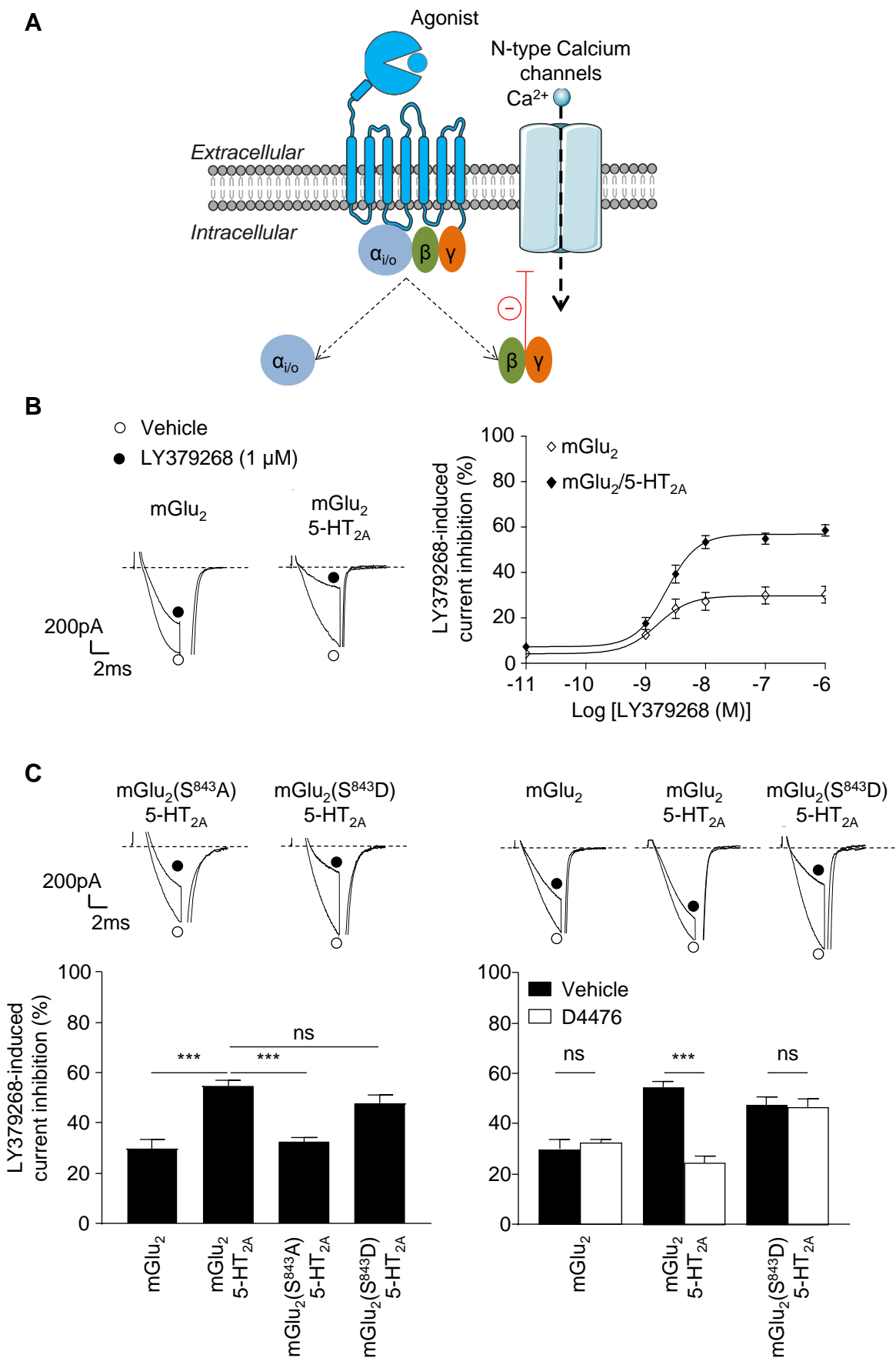


Figure 3

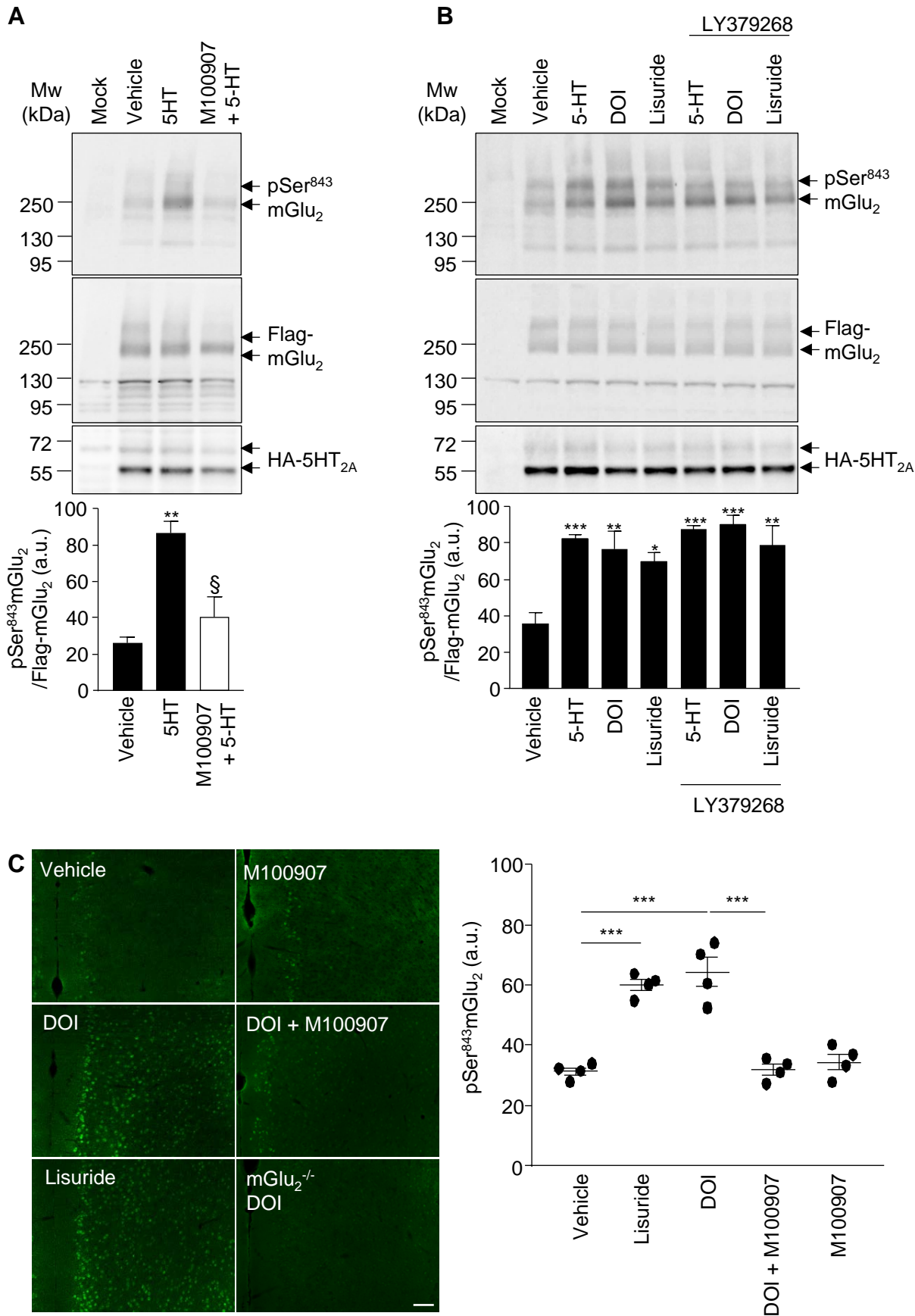


Figure 4

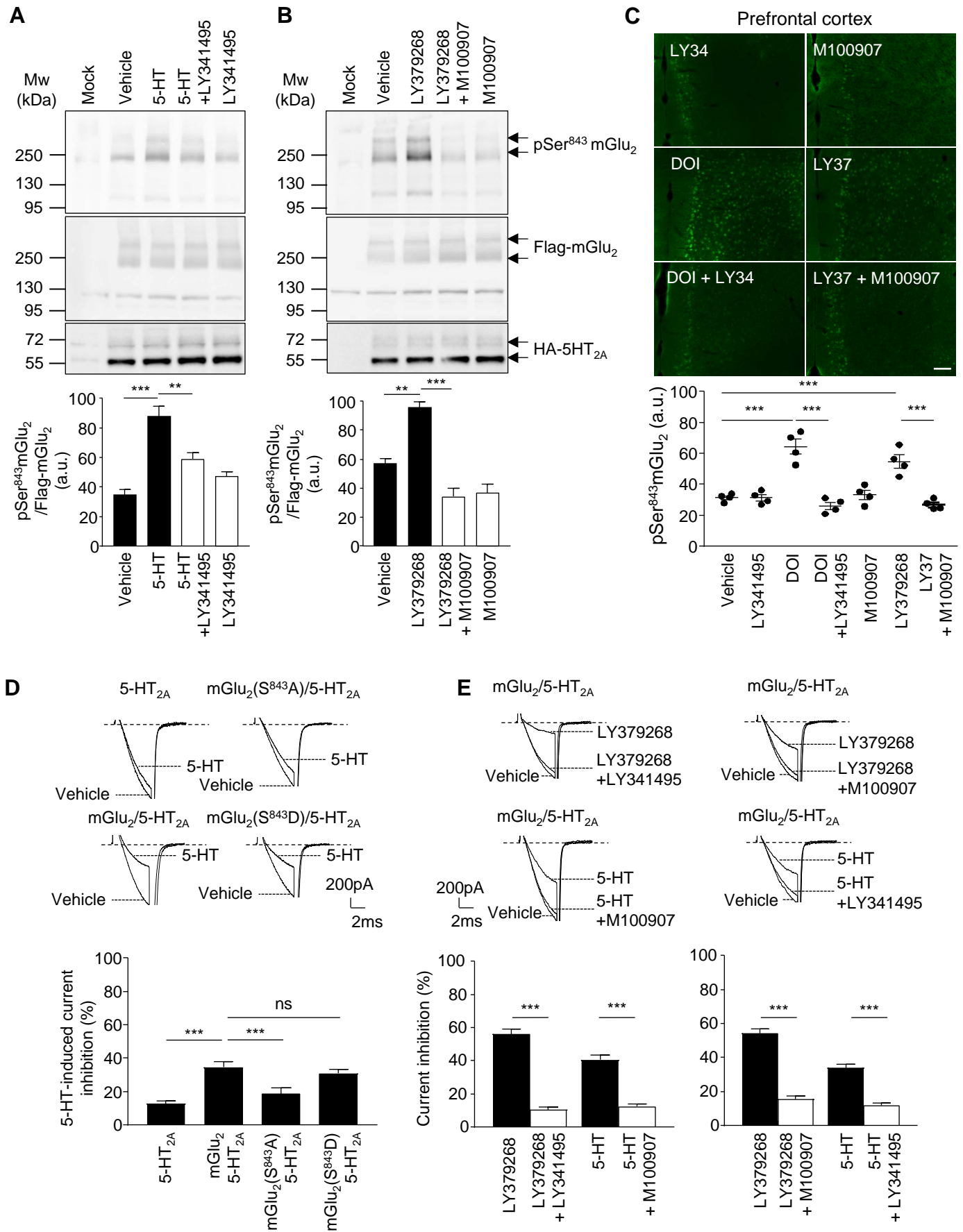


Figure 5

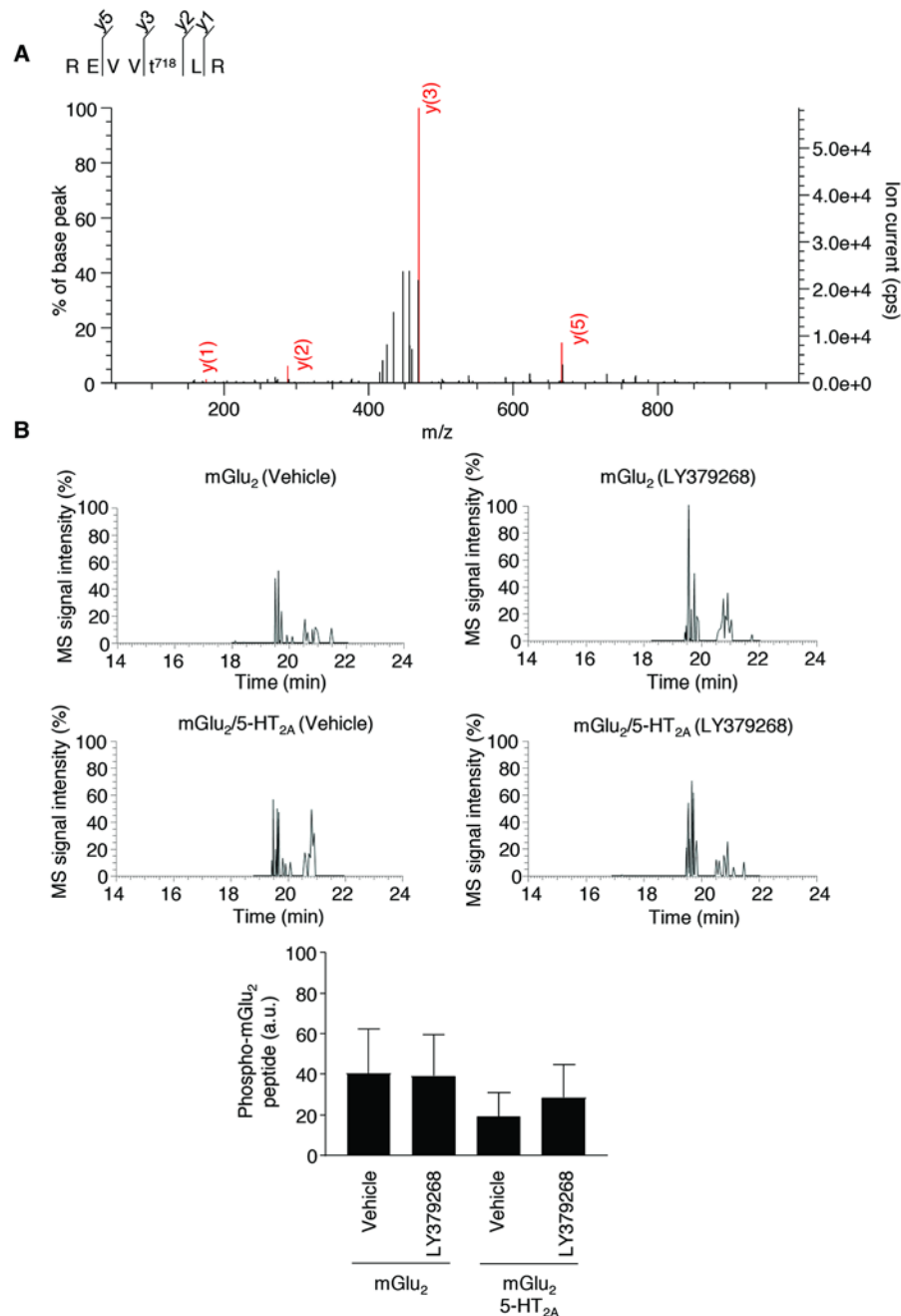
mGlu₂ receptor coverage (Human) : 57% (exp pvalue cut off <0.05)

1	MGSLLALLAL	LLLWGAVAEG	PAK KVLTLEG	DLVLGGLFPV	HQKGGPAEDC
51	GPVNEHRGIQ	RLEAMLFALD	RINRDPHLLP	GVRLGAHILD	SCSKDTHALE
101	QALDFVRASL	SRGADGSRHI	CPDGSYATHG	DAPTAITGVI	GGSYSDVSIQ
151	VANLLRLFQI	PQISYASTSA	KLSDKSRDYD	FARTVPPDF	QAKAMAEILR
201	FFNWTYVSTV	ASEGDYGETG	IEAFELEARA	RNICVATSEK	VGRAMSRAAF
251	EGVVRALLQK	PSARVAVLFT	RSEDARELLA	ASQRLNASFT	WVASDGGWAL
301	ESVAGSEGA	AEGAITIELA	SYPISEFASY	FQSLDPWNNS	RNPWFREFWE
351	QRFRCFSRQR	DCAAHSLRAV	PFEQESKIMF	VVNAVYAMAH	ALHNMHRALC
401	PNTRRLCDAM	RPVNGRRLYK	DFVLNVKFDA	PFRPADTHNE	VRFRDFGDGI
451	GRYNIFTYLR	AGSGRYRYQK	VGWAEGLTL	DTSLIPWASP	SAGPLPASRC
501	SEPCLQNEVK	SVQPGEVCCW	LCIPCQPYEY	RLDEFTCADC	GLGYWPNASL
551	TGCFELPQEY	IRWGDAWAVG	PVTIACLGAL	ATLFVLGVFV	RHNATPVVKA
601	SGRELCYILL	GGVFLCYCMT	FIFIAPKSTA	VCTLRRLGLG	TAFSVCYSAL
651	LTKTNRIARI	FGGAREGAQR	PRFISPASQV	AICLALISGQ	LLIVAWLVV
701	EAPGTGKETA	PERREVV⁷¹⁸LR	CNHRDASMLG	SLAYNVLLIA	LCTLYAFKTR
751	KCPENFNEAK	FIGFTMYTTC	IIWLAFLPIF	YVTSSDYRVQ	TTTMCVSVSL
801	SGSVVLGCLF	APKLHILFQ	PQKNVSHRA	PT^{S833}RFG^{S837}AAA	RA^{S843}S^{S844}SLGQGS
851	GSQFVPTVCN	GREVDSTTS	S⁸⁷¹L		

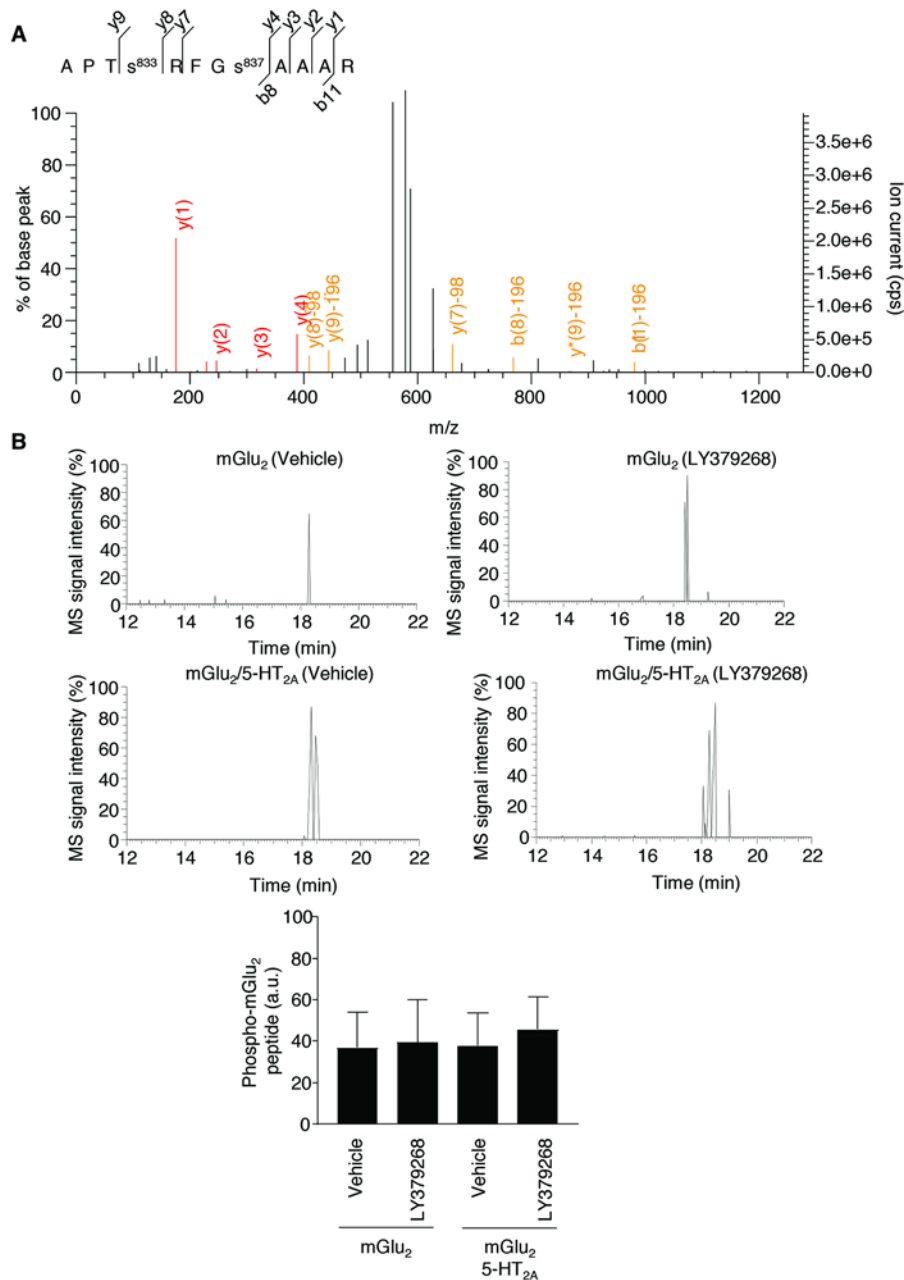
mGlu₂ receptor coverage (Rat) : 40% (exp pvalue cut off <0.05)

1	MESLLGFLAL	LLLWGAVAEG	PAK KVLTLEG	DLVLGGLFPV	HQKGGPAEEC
51	GPVNEHRGIQ	RLEAMLFALD	RINRDPHLLP	GVRLGAHILD	SCSKDTHALE
101	QALDFVRASL	SRGADGSRHI	CPDGSYATHS	DAPTAITGVI	GGSYSDVSIQ
151	VANLLRLFQI	PQISYASTSA	KLSDKSRDYD	FARTVPPDF	QAKAMAEILR
201	FFNWTYVSTV	ASEGDYGETG	IEAFELEARA	RNICVATSEK	VGRAMSRAAF
251	EGVVRALLQK	PSARVAVLFT	RSEDARELLA	ATQRLNASFT	WVASDGGWAL
301	ESVAGSERA	AEGAITIELA	SYPISEFASY	FQSLDPWNNS	RNPWFREFWE
351	ERFHCSFRQR	DCAAHSLRAV	PFEQESKIMF	VVNAVYAMAH	ALHNMHRALC
401	PNTTHLCDAM	RPVNGRRLYK	DFVLNVKFDA	PFRPADTDE	VRFRDFGDGI
451	GRYNIFTYLR	AGSGRYRYQK	VGWAEGLTL	DTSFIPWASP	SAGPLPASRC
501	SEPCLQNEVK	SVQPGEVCCW	LCIPCQPYEY	RLDEFTCADC	GLGYWPNASL
551	TGCFELPQEY	IRWGDAWAVG	PVTIACLGAL	ATLFVLGVFV	RHNATPVVKA
601	SGRELCYILL	GGVFLCYCMT	FVFIAPKSTA	VCTLRRLGLG	TAFSVCYSAL
651	LTKTNRIARI	FGGAREGAQR	PRFISPASQV	AICLALISGQ	LLIVAWLVV
701	EAPGTGKETA	PERREVVTLR	CNHRDASMLG	SLAYNVLLIA	LCTLYAFKTR
751	KCPENFNEAK	FIGFTMYTTC	IIWLAFLPIF	YVTSSDYRVQ	TTTMCVSVSL
801	SGSVVLGCLF	APKLHILFQ	PQKNVSHRA	PTSRFGSAAP	RA^{S843}ANLQGS
851	GSQFVPTVCN	GREVDSTTS	S⁸⁷¹L		

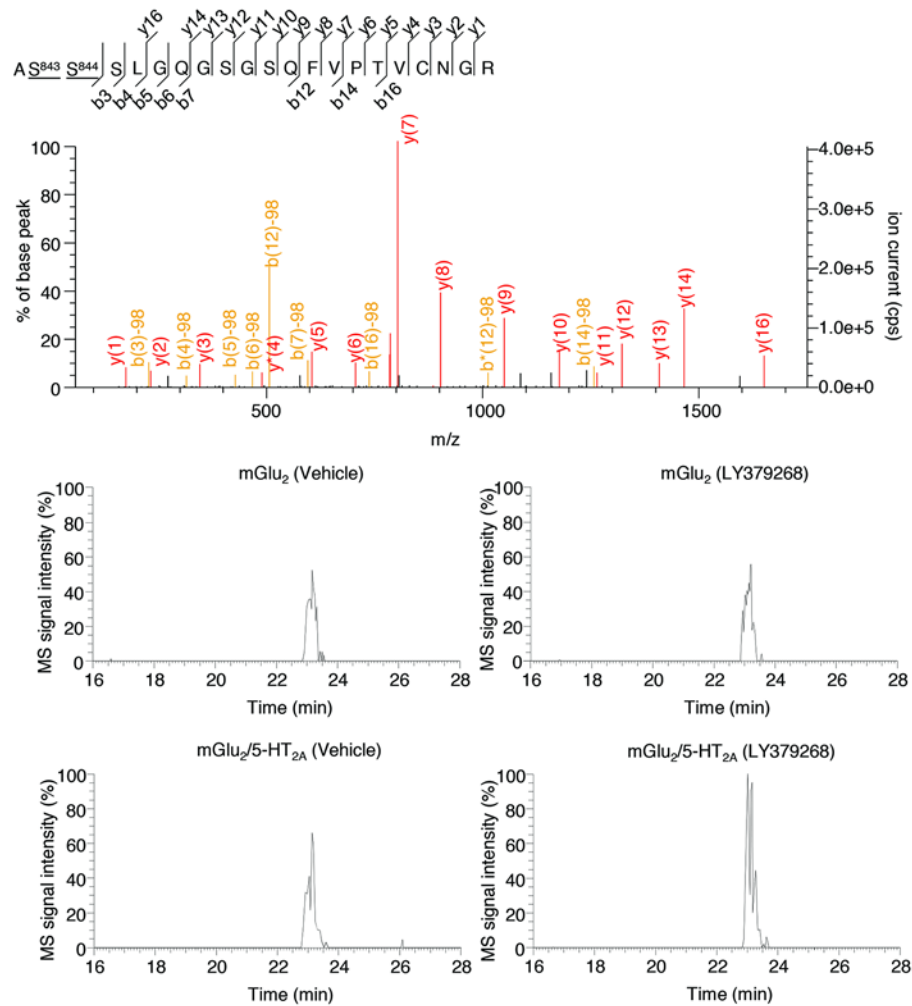
Supplementary Figure 1. Sequence coverage of human and rat mGlu₂ receptors obtained by LC-MS/MS. The sequence covered by LC-MS/MS analysis is highlighted in red. Identified phosphorylated residues in human mGlu₂ receptor (Thr⁷¹⁸, Ser⁸³³, Ser⁸³⁷, Ser^{843/844}, Ser⁸⁷¹) and rat mGlu₂ receptor (Ser⁸⁴³, Ser⁸⁷¹) and their positions are highlighted in blue.



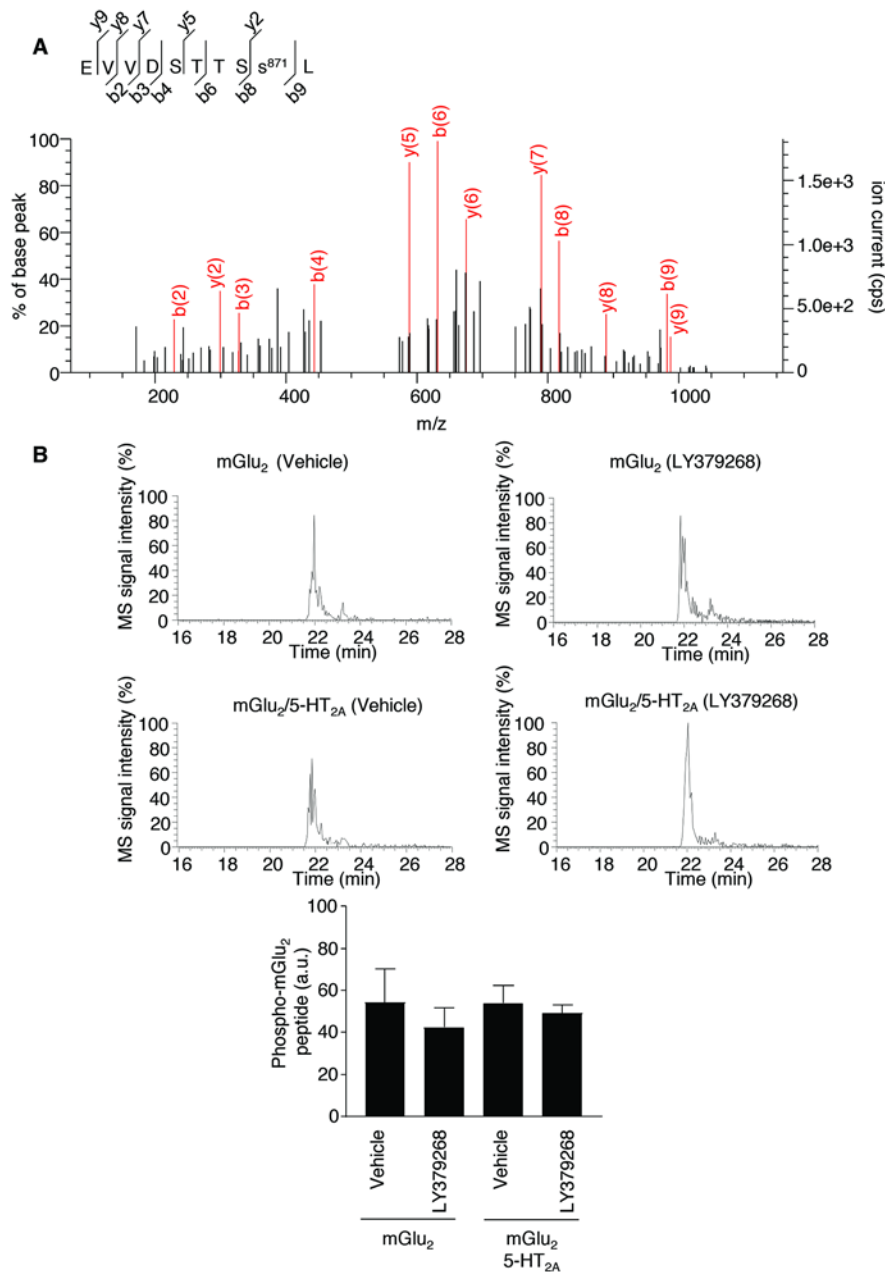
Supplementary Figure 2. Identification and quantification of the REVV^{t718}LR phosphorylated peptide by LC-MS. **A**, The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu₂ receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu₂ receptor alone or in combination with 5-HT_{2A} receptor, and exposed to either vehicle or LY379268 (1 μM) for 15 min. The histogram represents the means ± SEM of ion signal intensities of REVV^{t718}LR phosphorylated peptide obtained in three independent experiments performed on independent cultures. One-way ANOVA (Post-hoc: Tukey's range test): F(3,12)=0.3072, p=0.8197.



Supplementary Figure 3. Identification and quantification of the APTs⁸³³RFGs⁸³⁷AAAR phosphorylated peptide by LC-MS. **A**, The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu₂ receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu₂ receptor alone or in combination with 5-HT_{2A} receptor, and exposed to either vehicle or LY379268 (1 μM) for 15 min. The histogram represents the means ± SEM of ion signal intensities of APTs⁸³³RFGs⁸³⁷AAAR phosphorylated peptide obtained in three independent experiments performed on independent cultures. One-way ANOVA (Post-hoc: Tukey's range test): F(3,12)=0.05346, p=0.9829.



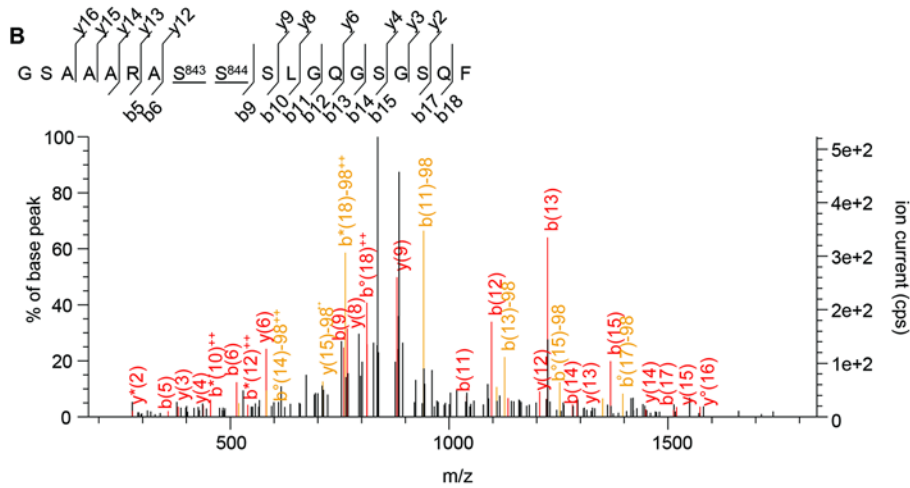
Supplementary Figure 4. Identification and quantification of the AS⁸⁴³S⁸⁴⁴SLGQGSGSQFVPTVCNGR phosphorylated peptide by LC-MS. The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu₂ receptor are illustrated (top panel). The bottom panels show representative extracted ion chromatograms of the peptide in cells expressing mGlu₂ receptor alone or in combination with 5-HT_{2A} receptor, and exposed to either vehicle or LY379268 (1 μM) for 15 min.



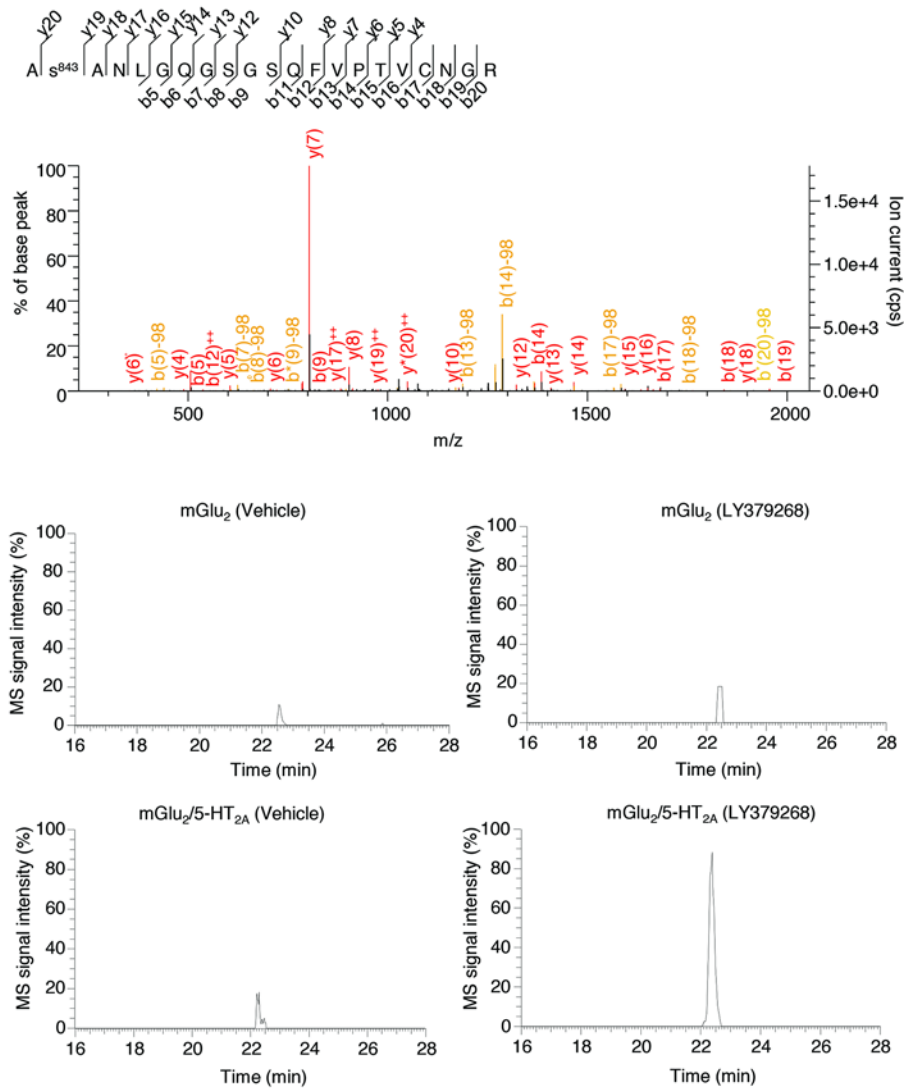
Supplementary Figure 5. Identification and quantification of the EVVDSTTS⁸⁷¹L phosphorylated peptide by LC-MS. **A**, The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu₂ receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu₂ receptor alone or in combination with 5-HT_{2A} receptor, and exposed to either vehicle or LY379268 (1 μM) for 15 min. The histogram represents the means ± SEM of ion signal intensities of EVVDSTTS⁸⁷¹L phosphorylated peptide obtained in three independent experiments performed on independent cultures. One-way ANOVA (Post-hoc: Tukey's range test): F(3,12)=0.2874, p=0.8337.

A mGlu₂ receptor coverage (Human) : 31% (exp pvalue cut off <0.05)

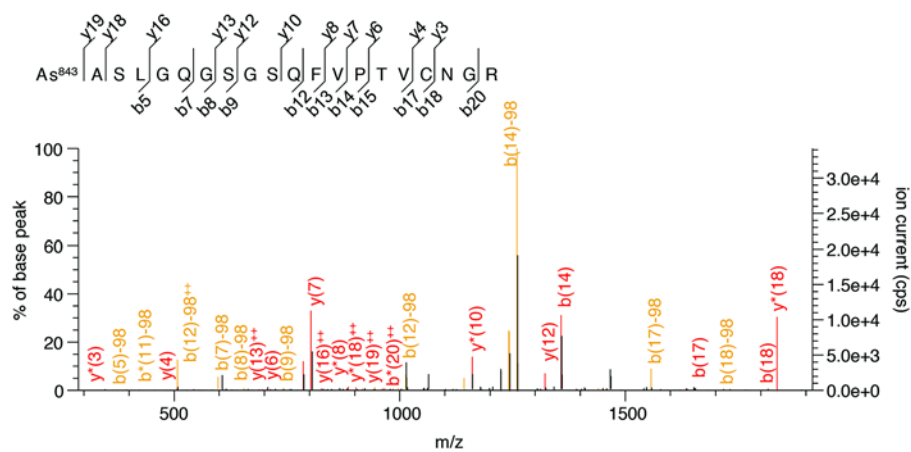
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51	GPVNEHRGIQ	RLEAMLFALD	RINRDPHLLP	GVRLGAHILD	SCSKDTHALE
101	QALDFVRASL	SRGADGSRHI	CPDGSYATHG	DAPTAITGVI	GGSYSDVSIQ
151	VANLLRLFQI	PQISYASTSA	KLSDKSRYDY	FARTVPPDFD	QAKAMAEILR
201	FFNWTYVSTV	ASEGDYGETG	IEAFELEARA	RNICVATSEK	VGRAMSRAAF
251	EGVVRALLQK	PSARVAVLFT	RSEDARELLA	ASQRLNASFT	WVASDGGWAL
301	ESVVAGSEGA	AEGAITIELA	SYPIDFASY	FQSLDPWNNS	RNPWFREFWE
351	QRFRCSFRQR	DCAAHSLRAV	PFEQESKIMF	VVNAVYAMAH	ALHNMHRALC
401	PNTTRLCDAM	RPVNGRRLYK	DFVLNVKFDA	PFRPADTHNE	VRFDRFGDGI
451	GRYNIFTYLR	AGSGRYRYQK	VGYWAEGLTL	DTSLIPWASP	SAGPLPASRC
501	SEPCLQNEVK	SVQPGEVCCW	LCIPCQPYEY	RLDEFTCADC	GLGYWPNASL
551	TGCFELPQEQ	IRWGDAAVAVG	PVTIACLGAL	ATLFLVGVFV	RHNATPVVKA
601	SGRELCYILL	GGVFLCYCMT	FIFIAPKSTA	VCTLRRLGLG	TAFSVCYSAL
651	LTKTNRIARI	FGGAREGAQR	PRFISPASQV	AICLALISGQ	LLIVVAWLTV
701	EAPGTGKETA	PERREVVTLR	CNHRDASMLG	SLAYNVLLIA	LCTLYAFKTR
751	KCPENFNEAK	FIGFTMYTTC	IIWLAFLPIF	YVTSSDYRVQ	TTTMCVSVSL
801	SGSVVLGCLF	APKLHILFQ	PQKNVSHRA	PTSRF GSAAA	RA <u>S⁸⁴³</u> <u>S⁸⁴⁴</u> <u>S⁸⁴⁵</u> LGQGS
851	GSQFVPTVCN	GREVVDSTTS	S⁸⁷L		



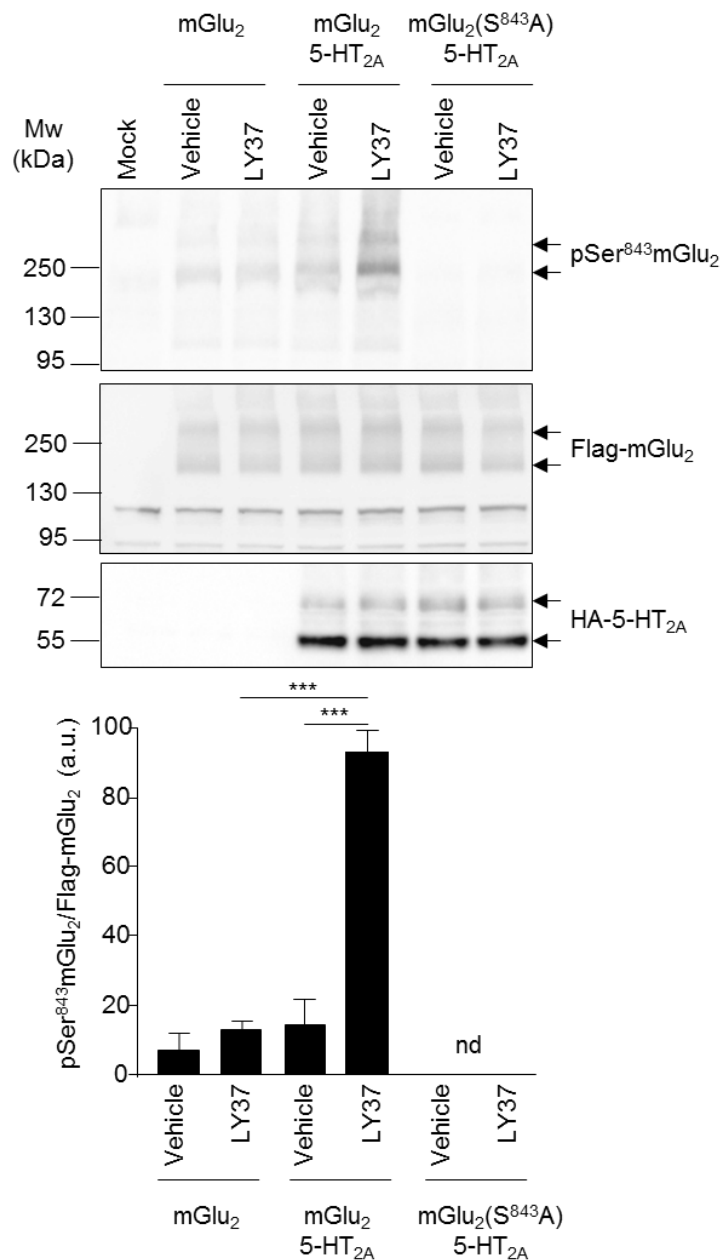
Supplementary Figure 6. LC-MS analysis of human mGlu₂ receptor digested with chymotrypsin. **A**, The sequence covered by LC-MS/MS analysis after *in-gel* digestion of human mGlu₂ receptor with chymotrypsin is highlighted in red. Identified phosphorylated residues and their positions in the protein sequence are highlighted in blue. **B**, The MS/MS spectrum of the GSAAARAS⁸⁴³S⁸⁴⁴SLGQGSQSQF phosphorylated peptide that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the possible phosphorylated residues in the full-length receptor are underlined.



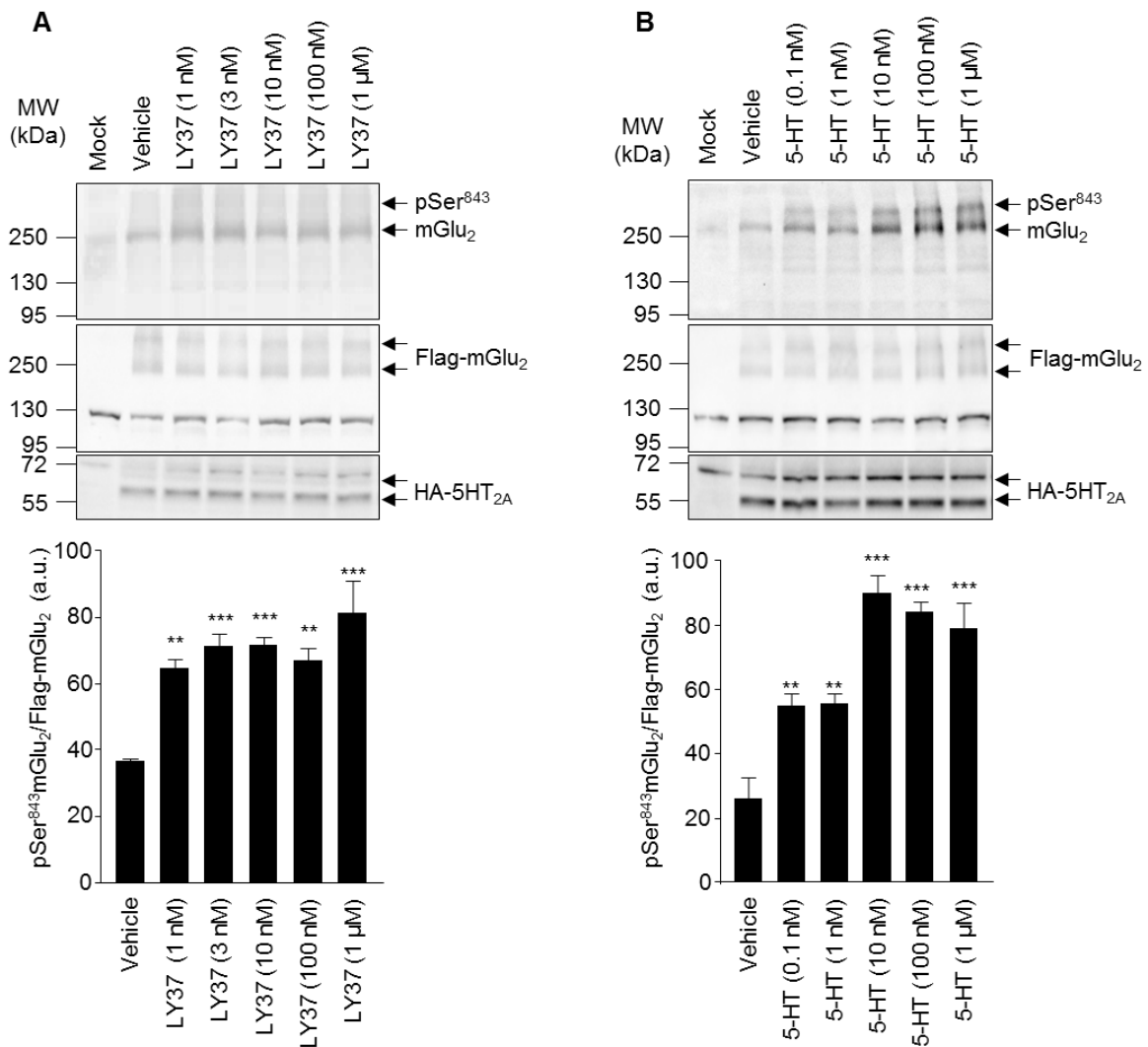
Supplementary Figure 7. Identification and quantification of the As⁸⁴³ANLGQSGSGSQFVPTVCNGR phosphorylated peptide by LC-MS. The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length rat mGlu₂ receptor are illustrated (top panel). The bottom panels show representative extracted ion chromatograms of the As⁸⁴³ANLGQSGSGSQFVPTVCNGR peptide in cells expressing mGlu₂ receptor alone or in combination with 5-HT_{2A} receptor and treated with either vehicle or LY379268 (1 μM) for 15 min.



Supplementary Figure 8. Identification of the $As^{843}ASLGQSGSQFVPTVCNGR$ phosphorylated peptide in $S^{844}A$ mGlu₂ receptor. The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human $S^{844}A$ mGlu₂ receptor are illustrated.



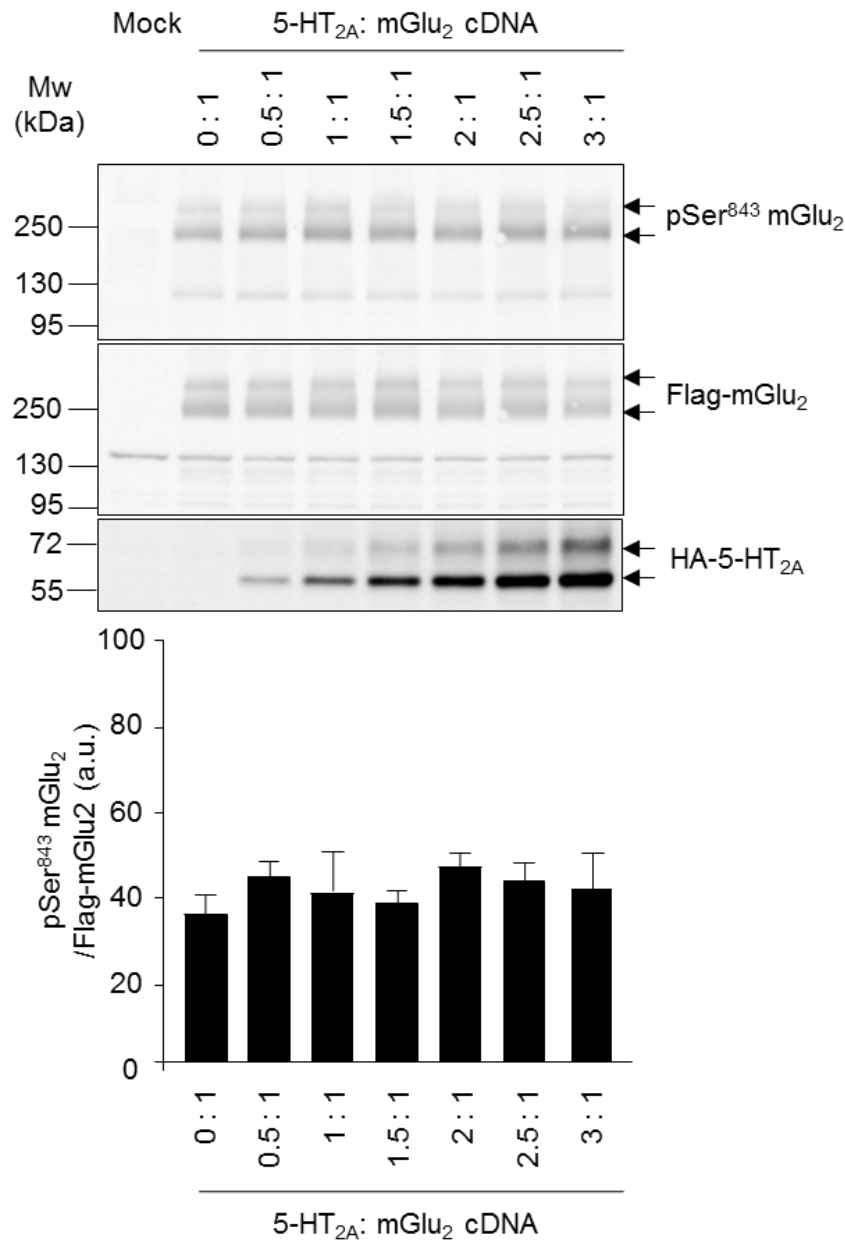
Supplementary Figure 9. Validation of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor antibody by Western blotting. HEK-293 cells transiently expressing rat Flag-tagged mGlu₂ receptor alone or in combination with HA-tagged 5-HT_{2A} receptor or co-expressing rat Flag-tagged S⁸⁴³A mGlu₂ receptor and HA-tagged 5-HT_{2A} receptor were exposed to vehicle or LY379268 (LY37, 1 μM) for 15 min. mGlu₂ receptor phosphorylation was analyzed by sequential immunoblotting with the antibody raised against the CAAPRASANLGQG peptide and the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The generated antibody provided an immunoreactive signal in Western blots from cells expressing Flag-tagged mGlu₂ receptors at a molecular weight corresponding to the signal obtained with the anti-Flag antibody. The histograms show the means ± SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.) obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): F(5,12)=58.02, p<0.0001. ***p<0.001. nd: non-detectable.



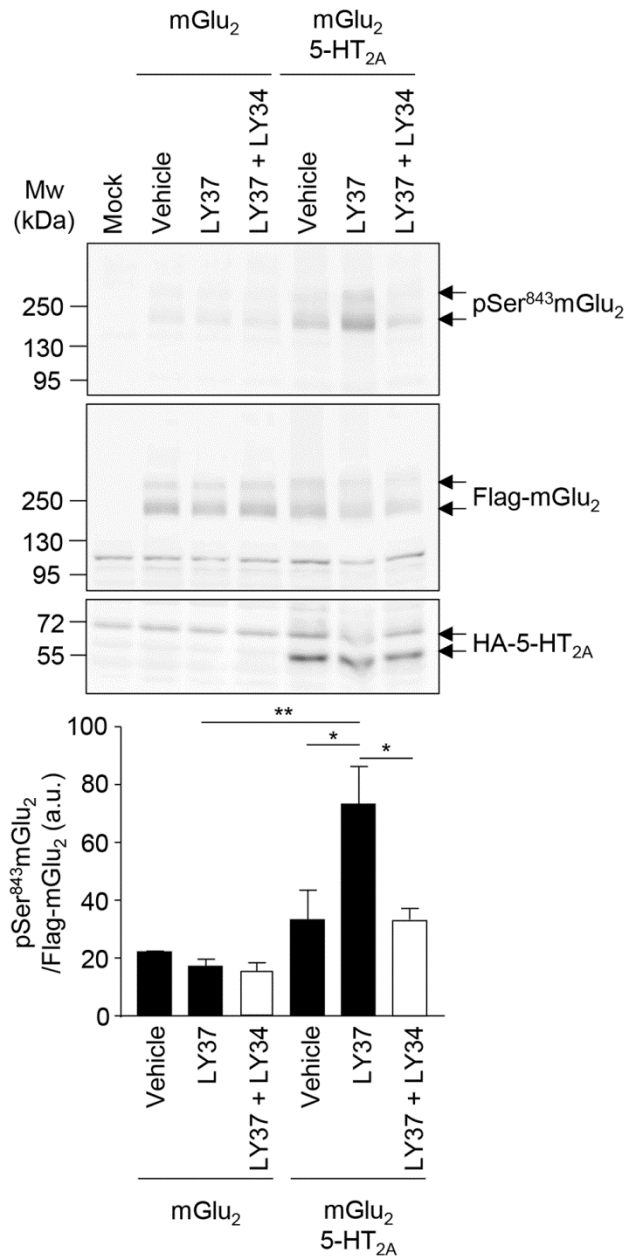
Supplementary Figure 10. Effects of increasing concentrations of LY379268 and 5-HT

on Ser⁸⁴³ phosphorylation. **A**, HEK-293 cells co-expressing Flag-tagged mGlu₂ receptor and HA-tagged 5-HT_{2A} receptor were exposed to vehicle or increasing concentrations of LY379268 (LY37, 1 nM, 3 nM, 10 nM, 100 nM, 1 μ M) for 15 min. **B**, Cells were exposed to vehicle or increasing concentrations of 5-HT (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μ M) for 15 min. Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and mGlu₂ receptor expression by immunoblotting with the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means \pm SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.). One-way ANOVA (Post-hoc: Dunnett's range test): A, F(5,12)=9.884, p=0.0005. B, F(5,12)=20.46, p<0.0001.

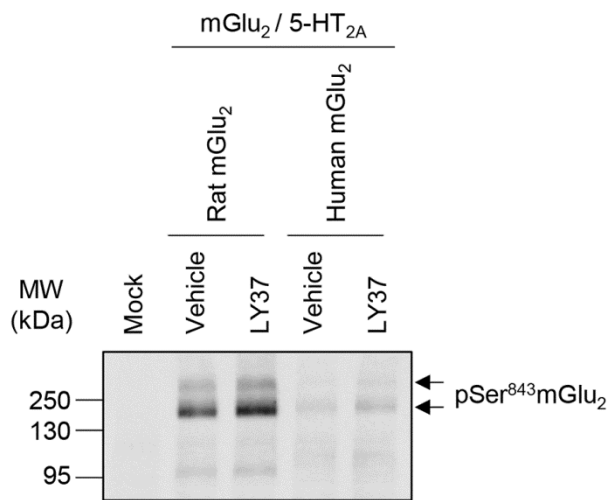
*p<0.05 vs. corresponding vehicle conditions.



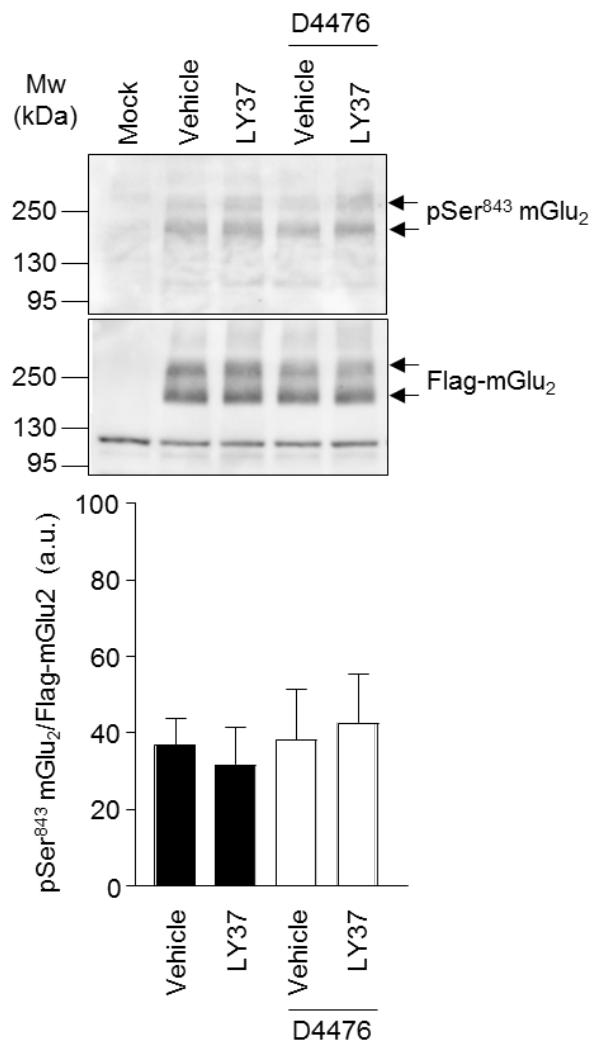
Supplementary Figure 11. Basal mGlu₂ receptor Ser⁸⁴³ phosphorylation level does not depend on 5-HT_{2A} receptor expression level. HEK-293 cells co-expressing rat Flag-tagged mGlu₂ receptor and increasing amounts of HA-tagged 5-HT_{2A} receptor were challenged with vehicle or LY379268 for 15 min. Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and with the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means \pm SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.). One-way ANOVA (Post-hoc: Tukey's range test): $F(6,14)=0.4493$, $p=0.8338$.



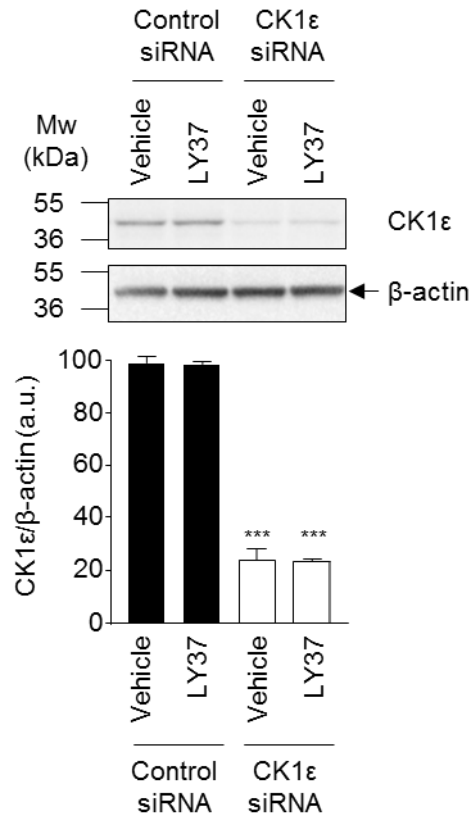
Supplementary Figure 12. LY379268-elicited mGlu₂ receptor phosphorylation at Ser⁸⁴³ in cells co-expressing 5-HT_{2A} receptor is prevented by the mGlu₂ receptor antagonist LY341495. HEK-293 cells transiently expressing rat Flag-tagged mGlu₂ receptor alone or in combination with HA-tagged 5-HT_{2A} receptor were exposed to vehicle or LY379268 (LY37, 10 nM), in absence or presence of LY341495 (LY34, 10 μM) for 15 min. mGlu₂ receptor phosphorylation was analyzed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³ antibody and the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means ± SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.) obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): F(5,12)=8.407, p=0.0013. *p<0.05; **p<0.01.



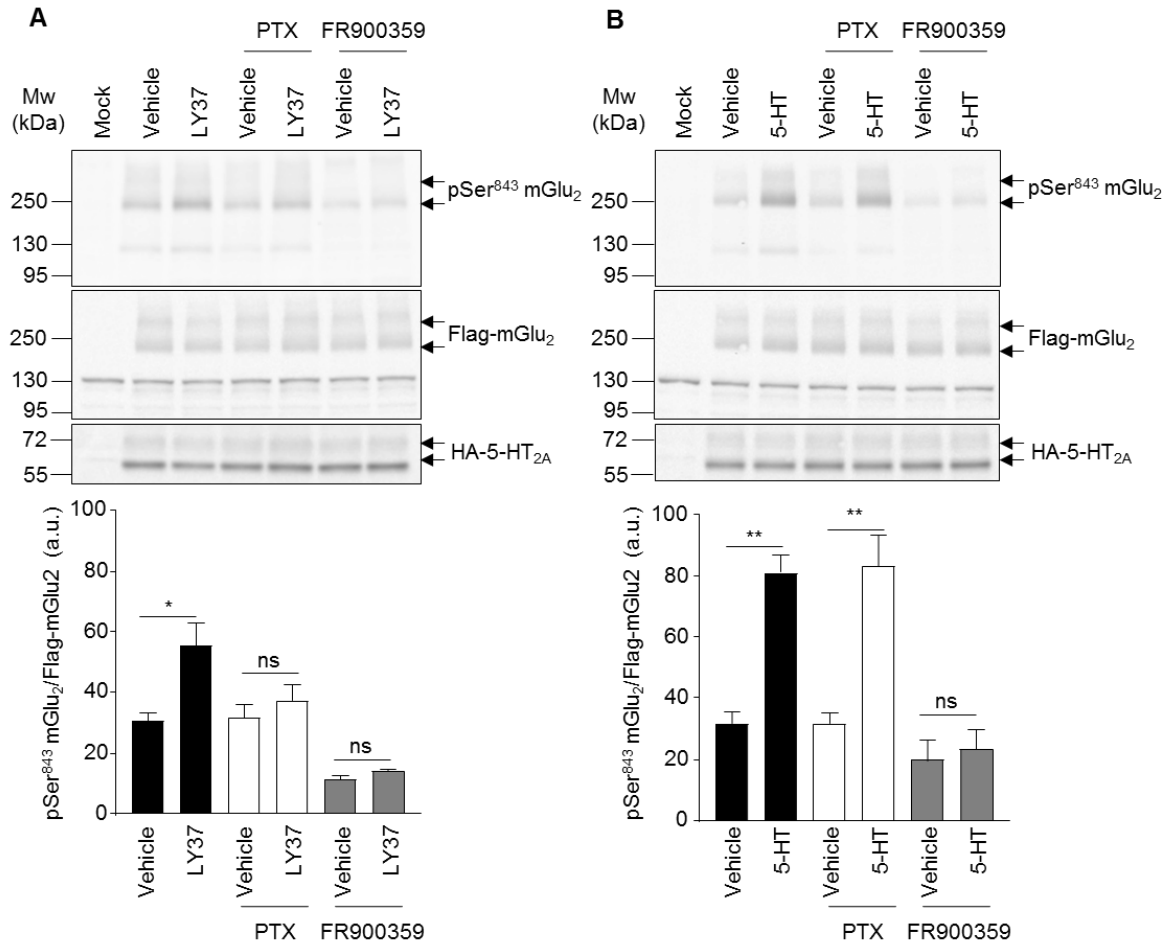
Supplementary Figure 13. Western blot analysis of rat and human mGlu₂ receptor phosphorylation at Ser⁸⁴³. HEK-293 cells co-expressing HA-tagged 5-HT_{2A} receptor and either rat or human Flag-tagged mGlu₂ receptor were exposed to vehicle or LY379268 (LY37, 1 μ M) for 15 min. The phosphorylation of rat and human mGlu₂ receptor at Ser⁸⁴³ was analyzed by Western blotting using the newly generated anti-phospho-Ser⁸⁴³ mGlu₂ receptor antibody. Note that LY379268 treatment increased the immunoreactive signal of both rat and human orthologs, but the signal intensity was much higher for the rat receptor.



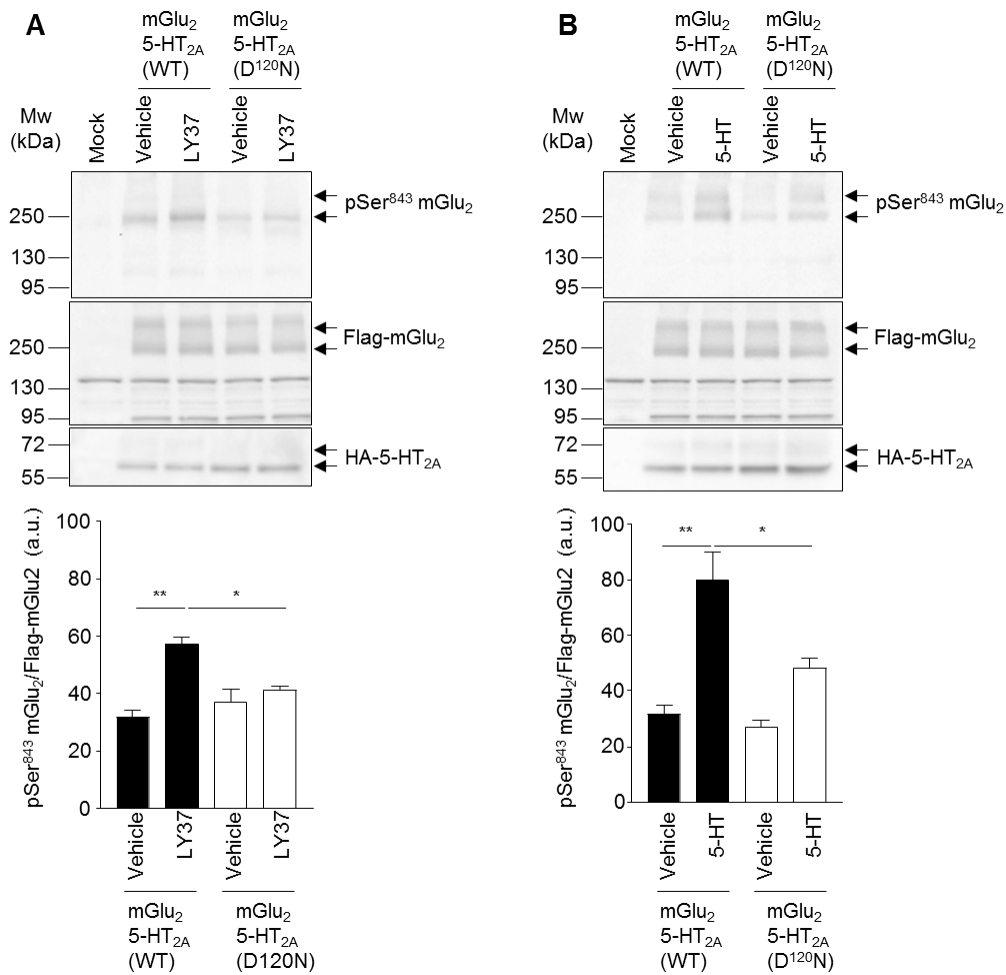
Supplementary Figure 14. LY379268-induced Ser⁸⁴³ phosphorylation in cells expressing mGlu₂ receptor alone is not mediated by casein kinase 1. HEK-293 cells expressing rat Flag-tagged mGlu₂ alone were pre-incubated with either vehicle or the CK1 inhibitor (D4476, 10 μ M) for 60 min and then challenged with vehicle or LY379268 (LY37, 1 μ M) for 15 min. Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by sequential immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means \pm SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.) obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): $F(3,8)=0.1738$, $p=0.9112$



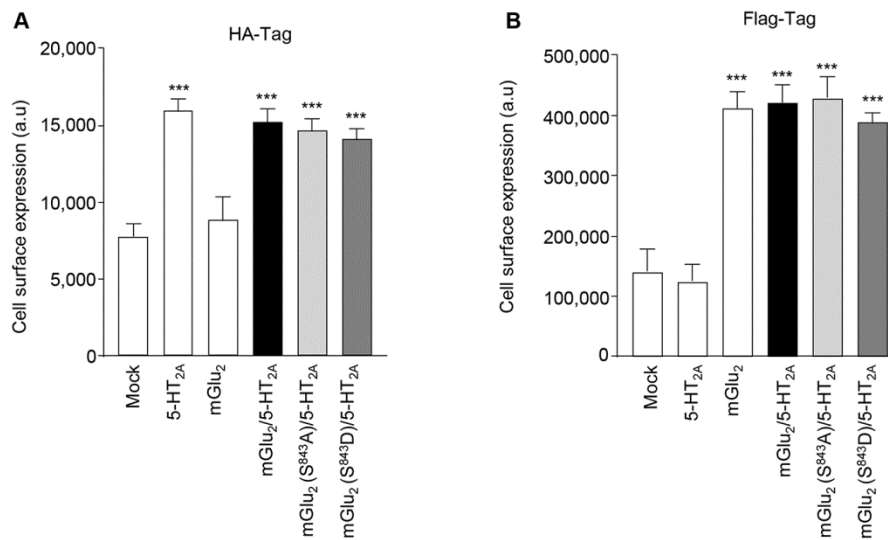
Supplementary Figure 15. Silencing casein kinase 1ε (CK1ε) expression in HEK-293 cells by RNA interference. HEK-293 cells co-expressing rat Flag-tagged mGlu₂ and HA-tagged 5-HT_{2A} receptors were transfected with either control or CK1ε siRNA and challenged with vehicle or LY379268 (LY37, 1 μM) for 15 min. CK1ε protein expression was assessed by sequential immunoblotting with the anti-CK1ε antibody and the anti-β-actin antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means ± SEM (expressed in arbitrary units, a.u.) of the anti-CK1ε immunoreactive signal relative to β-actin immunoreactive signal obtained in the three experiments. One-way ANOVA (Post-hoc: Tukey's range test): F(3, 8)=230, p<0.0001. *** p<0.001 vs. corresponding condition in presence of control siRNA.



Supplementary Figure 16. Role of $G_{i/o}$ and G_q proteins in the phosphorylation of $mGlu_2$ receptor at Ser^{843} . **A**, HEK-293 cells co-expressing rat Flag-tagged $mGlu_2$ and HA-tagged $5-HT_{2A}$ receptors were pre-incubated with either vehicle or Pertussis toxin (PTX, 0.1 μ g/mL, 18 h) or FR900359 (1 μ M, 2 h) and challenged with either vehicle or LY379268 (LY37, 1 μ M, 15 min). **B**, HEK-293 cells were challenged with either vehicle or 5-HT (1 μ M, 15 min). Phosphorylation of $mGlu_2$ receptor at Ser^{843} was assessed by immunoblotting with the anti-phospho- Ser^{843} - $mGlu_2$ receptor antibody and $mGlu_2$ receptor expression by immunoblotting with the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means \pm SEM of the anti-phospho- Ser^{843} $mGlu_2$ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.). One-way ANOVA (Post-hoc: Tukey's range test): A, $F(5,12)=14.82$, $p<0.0001$. B, $F(5,12)=17.37$, $p<0.0001$. * $p<0.05$; ** $p<0.01$ vs. corresponding vehicles.



Supplementary Figure 17. Neither LY379268 nor 5-HT promote Ser⁸⁴³ phosphorylation in cells expressing G_q signaling-dead D^{120N} 5-HT_{2A} receptor. **A**, HEK-293 cells co-expressing rat Flag-tagged mGlu₂ in combination with HA-tagged wild type or D^{120N} 5-HT_{2A} receptor were treated with either vehicle or LY379268 (LY37, 1 μM) for 15 min. **B**, HEK-293 cells were treated with either vehicle or 5-HT (1 μM) for 15 min. Phosphorylation of mGlu₂ receptor at Ser⁸⁴³ was assessed by immunoblotting with the anti-phospho-Ser⁸⁴³-mGlu₂ receptor antibody and mGlu₂ receptor expression by immunoblotting with the anti-Flag antibody. Representative blots of three independent experiments performed on different sets of cultured cells are illustrated. The histograms show the means ± SEM of the anti-phospho-Ser⁸⁴³ mGlu₂ receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.). One-way ANOVA (Post-hoc: Tukey's range test): A, F(3,8)=14.92, p=0.0012. B, F(3,8)=17.79, p=0.0007. *p<0.05; **p<0.01 vs. corresponding vehicles.



Supplementary Figure 18. Ser⁸⁴³ phosphorylation does not affect cell surface expression of mGlu₂ and 5-HT_{2A} receptors. Quantification of cell surface expression of HA-tagged 5-HT_{2A} receptors (A) and Flag-tagged mGlu₂ receptors (B) was performed by ELISA in non-permeabilizing conditions using anti-HA and anti-Flag antibodies, respectively, in cells expressing 5-HT_{2A} or wild type mGlu₂ receptor alone or 5-HT_{2A} receptor in combination with either wild type or S⁸⁴³A or S⁸⁴³D mGlu₂ receptor. Data are the means \pm SEM of values obtained in three independent experiments. One-way ANOVA (Post-hoc: Tukey's range test): A, F(5.48)=12.97, p<0.0001; B, F(5.48)=23.64, p<0.0001. ***p<0.001 vs. cells transfected with empty vectors (Mock).