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Samy Murat, Mathilde Bigot, Jonathan Chapron, Gabriele M König, Evi Kostenis, et al.. 5-HT<sub>2A</sub> receptor-dependent phosphorylation of mGlu<sub>2</sub> receptor at Serine 843 promotes mGlu<sub>2</sub> receptor-operated Gi/o signaling. *Molecular Psychiatry*, 2019, 24 (11), pp.1610-1626. 10.1038/s41380-018-0069-6 . hal-02356293

**HAL Id: hal-02356293**

**<https://hal.science/hal-02356293>**

Submitted on 8 Nov 2019

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**5-HT<sub>2A</sub> receptor-dependent phosphorylation of mGlu<sub>2</sub> receptor at Serine 843 promotes  
mGlu<sub>2</sub> receptor-operated G<sub>i/o</sub> signaling**

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Running title: mGlu<sub>2</sub> phosphorylation in 5-HT<sub>2A</sub>/mGlu<sub>2</sub> crosstalk

## Abstract

The serotonin 5-HT<sub>2A</sub> and glutamate mGlu<sub>2</sub> 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<sub>2A</sub> receptor upon the phosphorylation pattern of mGlu<sub>2</sub> receptor in light of the importance of specific phosphorylation events in regulating G protein-coupled receptor signaling and physiological outcomes. Among the five mGlu<sub>2</sub> receptor phosphorylated residues identified in HEK-293 cells, the phosphorylation of Ser<sup>843</sup> was enhanced upon mGlu<sub>2</sub> receptor stimulation by the orthosteric agonist LY379268 only in cells co-expressing the 5-HT<sub>2A</sub> receptor. Likewise, administration of LY379268 increased mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> in prefrontal cortex of wild type mice but not 5-HT<sub>2A</sub><sup>-/-</sup> mice. Exposure of HEK-293 cells co-expressing mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors to 5-HT also increased Ser<sup>843</sup> phosphorylation state to a magnitude similar to that measured in LY379268-treated cells. In both HEK-293 cells and prefrontal cortex, Ser<sup>843</sup> phosphorylation elicited by 5-HT<sub>2A</sub> receptor stimulation was prevented by the mGlu<sub>2</sub> receptor antagonist LY341495, while the LY379268-induced effect was abolished by the 5-HT<sub>2A</sub> receptor antagonist M100907. Mutation of Ser<sup>843</sup> into alanine strongly reduced G<sub>i/o</sub> signaling elicited by mGlu<sub>2</sub> or 5-HT<sub>2A</sub> receptor stimulation in cells co-expressing both receptors. Collectively, these findings identify mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> 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<sub>2A</sub> 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<sub>2/3</sub>) has been developed. Subsequent preclinical studies indicated that mGlu<sub>2</sub> receptor activation accounts for their antipsychotic activity (4–6) and showed overall superiority over 5-HT<sub>2A</sub> receptor antagonists in alleviating negative symptoms. Furthermore, an integrated study combining five placebo-controlled trials of pomaglumetad, 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<sub>2A</sub> receptor antagonists (7).

5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors are canonically coupled to G<sub>q</sub> and G<sub>i/o</sub> 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<sub>2A</sub> and mGlu<sub>2</sub> receptors: pharmacological activation of mGlu<sub>2</sub> receptor inhibits glutamate release and excitatory postsynaptic currents (EPSCs) elicited by 5-HT<sub>2A</sub> receptor stimulation in the prefrontal cortex, and attenuates behavioral effects of hallucinogens, while mGlu<sub>2</sub> receptor antagonists further enhance the frequency and amplitude of 5-HT<sub>2A</sub> 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<sub>2A</sub>-mGlu<sub>2</sub> 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<sub>2A</sub>-mGlu<sub>2</sub> heterocomplex as a possible target of hallucinogenic 5-HT<sub>2A</sub> receptor agonists that mediates engagement of G<sub>i/o</sub> signaling and the associated psychotropic effects (9). Conversely, 5-HT<sub>2A</sub>-mGlu<sub>2</sub> heteromers are necessary for the stimulation of G<sub>q</sub> signaling by mGlu<sub>2</sub> receptor agonists, which is altered in patients with schizophrenia (10). This particular signaling also depends on the ability of mGlu<sub>2</sub> receptor to couple to and activate G<sub>i/o</sub> proteins (10). Finally, studies showed that 5-HT<sub>2A</sub>-mGlu<sub>2</sub> heteromers serve as an integration point that determines the balance between G<sub>i/o</sub> and G<sub>q</sub>-dependent signaling, and pro-psychotic (increase in G<sub>q</sub> vs. G<sub>i/o</sub> signaling) vs. antipsychotic (increase in G<sub>i/o</sub> vs. G<sub>q</sub> signaling) outcome (11). These findings pinpoint the role of 5-HT<sub>2A</sub>-mGlu<sub>2</sub> heteromers in the homeostatic equilibrium between G<sub>i/o</sub> and in G<sub>q</sub> signaling that ensures a normal non-psychotic state. However, the existence of 5-HT<sub>2A</sub>-mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptors while 5-HT<sub>2A</sub> 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<sub>2A</sub> and mGlu<sub>2</sub> 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<sub>2A</sub> receptor phosphorylated sites and identified a serine residue in the third receptor intracellular loop (Ser<sup>280</sup>) phosphorylated in response to receptor activation by hallucinogenic 5-HT<sub>2A</sub> 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 Ser<sup>280</sup> 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<sub>2A</sub> 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 mGlu<sub>2</sub> receptor C-terminal domain and showed that the phosphorylation of one of them (Ser<sup>843</sup>) by protein kinase A (PKA) affects receptor coupling to G proteins (15). However, the reciprocal influence of 5-HT<sub>2A</sub> and mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptor expressed in HEK-293 cells and investigated the influence of 5-HT<sub>2A</sub> receptor expression/stimulation upon their phosphorylation state using high-resolution mass spectrometry. Among the identified residues, we show that Ser<sup>843</sup> phosphorylation elicited by agonist stimulation of mGlu<sub>2</sub> receptor depends on 5-HT<sub>2A</sub> receptor co-expression and demonstrate a reciprocal interplay between the activation of both receptors to promote Ser<sup>843</sup> phosphorylation. Characterization of the signaling outcome of Ser<sup>843</sup> phosphorylation revealed its key role in G<sub>i/o</sub> signaling elicited by 5-HT<sub>2A</sub> and mGlu<sub>2</sub> 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 (mGlu<sub>2</sub> receptor orthosteric agonist), LY341495 (mGlu<sub>2</sub> 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 $\epsilon$  (#12448) was from Cell Signaling and the mouse anti-Actin (#MA5-11869) was from ThermoScientific. The anti-phospho-Ser<sup>843</sup> 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 mGlu<sub>2</sub> receptor phosphorylated at Ser<sup>843</sup>.

The PRK5 plasmid encoding the HA-tagged 5-HT<sub>2A</sub> receptor was described elsewhere (18). The cDNA encoding HA-tagged 5-HT<sub>2A</sub>(D<sup>120</sup>N) was generated by site-directed mutagenesis from this plasmid. The pcDNA3 plasmids encoding the Flag-tagged human and rat mGlu<sub>2</sub>

receptors were generously provided by Dr. Philippe Rondard (Institut de Génomique Fonctionnelle, Montpellier, France). The cDNAs encoding human and rat S<sup>843</sup>A mGlu<sub>2</sub> receptors and human S<sup>843</sup>D and S<sup>844</sup>A mGlu<sub>2</sub> 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 Cavα<sub>2-δ</sub><sub>1-b</sub> and Cavβ<sub>1b</sub> 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% CO<sub>2</sub> 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. CKIε siRNA (FlexiTube siRNA CKIε; 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 mGlu<sub>2</sub> receptor phosphorylation**

Transfected cells were starved of serum for 4 h, challenged with either vehicle or LY379268 (1 μM) 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 mGlu<sub>2</sub> 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 mGlu<sub>2</sub> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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 Ser<sup>843</sup> 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 (CEEALR 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<sub>2A</sub><sup>-/-</sup> (21) and mGlu<sub>2</sub><sup>-/-</sup> (22) mice

(originally provided by Prof. Shigetada Nakanishi at Kyoto University, Japan), and DOI to mGlu<sub>2</sub><sup>-/-</sup> mice. Thirty min after agonist injection, mice were anesthetized with pentobarbital (100 mg/kg i.p., Ceva SA) and perfused transcardiacally 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-Ser<sup>843</sup> 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-Ser<sup>843</sup>-positive cells relative to DAPI-stained cells.

## **ELISA**

Quantification of cell surface expression of HA-tagged 5-HT<sub>2A</sub> receptors and Flag-tagged wild-type, S<sup>843</sup>A and S<sup>843</sup>D mGlu<sub>2</sub> 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 S<sup>843</sup>A or S<sup>843</sup>D mGlu<sub>2</sub> receptor, HA-tagged 5-HT<sub>2A</sub> receptor, Cav2.2, Cavβ<sub>1b</sub> and Cavα<sub>2</sub>-δ<sub>1-b</sub> 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 BaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> 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 CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 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 mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> requires co-expression of 5-HT<sub>2A</sub> receptor in HEK-293 cells**

To identify novel molecular substrates of the functional crosstalk between 5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors, we first examined the phosphorylation pattern of Flag-tagged human mGlu<sub>2</sub> receptor co-expressed with HA-tagged 5-HT<sub>2A</sub> receptor in HEK-293 cells following exposure to vehicle or a maximally-effective concentration of the mGlu<sub>2</sub> receptor orthosteric agonist LY379268 (1  $\mu$ M) (24). mGlu<sub>2</sub> 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 (Thr<sup>718</sup>) and four residues in the C-terminal domain (Ser<sup>833</sup>, Ser<sup>837</sup>, Ser<sup>843/844</sup> and Ser<sup>871</sup>, Table 1, Figure 1A, Supplementary Figures 2-5). Note that MS/MS fragmentation did not discriminate between a phosphorylation at Ser<sup>843</sup> or Ser<sup>844</sup>, due to the absence of site-specific fragment ions in MS/MS spectra (Supplementary Figure 4). In an effort to distinguish Ser<sup>843</sup> from Ser<sup>844</sup> phosphorylation, purified mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptor sequences revealed that the region encompassing Ser<sup>843</sup> and Ser<sup>844</sup> is highly conserved in the three species (Figure 1B). Specifically, Ser<sup>843</sup> of the human receptor is conserved in rat and mouse orthologs, whereas Ser<sup>844</sup> is replaced by an alanine (Figure 1B). We thus renewed the experiments in cells

expressing the rat mGlu<sub>2</sub> receptor and clearly identified a peptide phosphorylated at Ser<sup>843</sup> (Supplementary Figure 7). Consistent with these findings, a peptide comprising phosphorylated Ser<sup>843</sup> was likewise identified in cells expressing the S<sup>844</sup>A mutant of the human mGlu<sub>2</sub> receptor (Supplementary Figure 8), while no peptide phosphorylated at Ser<sup>844</sup> was detected in cells expressing the S<sup>843</sup>A mutant. Collectively, these results unambiguously identify Ser<sup>843</sup> as the phosphorylated residue in the human and murine receptors.

To explore the impact of agonist stimulation of mGlu<sub>2</sub> receptor and 5-HT<sub>2A</sub> receptor expression upon mGlu<sub>2</sub> 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<sub>2A</sub> receptor. The phosphorylation index of Thr<sup>718</sup>, Ser<sup>833</sup>, Ser<sup>837</sup> and Ser<sup>871</sup> did not display any significant variation upon exposure of cells to LY379268 or co-expression of 5-HT<sub>2A</sub> receptor (Table 1 and Supplementary Figures 2B, 3B, 5B). In contrast, LY379268 induced a marked increase in Ser<sup>843</sup> phosphorylation in cells co-expressing human mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors while it did not significantly affect its phosphorylation state in cells expressing mGlu<sub>2</sub> receptor alone (Figure 1C). Likewise, LY379268 exposure of cells expressing the rat mGlu<sub>2</sub> receptor increased Ser<sup>843</sup> phosphorylation only when the 5-HT<sub>2A</sub> receptor was co-expressed (Figure 1C and Supplementary Figure 7). Collectively, these findings demonstrate that agonist stimulation of mGlu<sub>2</sub> 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<sub>2A</sub> receptor.

### **Agonist-stimulated mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> in the mouse prefrontal cortex requires co-expression of 5-HT<sub>2A</sub> receptor**

A polyclonal antibody against the mGlu<sub>2</sub> receptor phosphorylated at Ser<sup>843</sup> was obtained by immunizing a rabbit with the synthetic CAAPRASANLGQG peptide encompassing a phosphorylated serine residue corresponding to Ser<sup>843</sup> 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 mGlu<sub>2</sub> receptor (Supplementary Figure 9). Furthermore, LY379268 treatment strongly enhanced this immunoreactive signal only in cells co-expressing the 5-HT<sub>2A</sub> receptor, consistent with the results of MS analysis (Figure 1C and Supplementary Figure 9). Mutation of Ser<sup>843</sup> into alanine abolished this immunoreactive signal, confirming the specificity of the antibody for phosphorylated Ser<sup>843</sup> (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<sub>2A</sub> receptor at various densities (in absence of LY379268) did not enhance phosphorylation of Ser<sup>843</sup> of mGlu<sub>2</sub> receptor (Supplementary Figure 11). As expected, the increase in Ser<sup>843</sup> phosphorylation induced by LY379268 was prevented by the co-application of the mGlu<sub>2</sub> receptor antagonist LY341495 (10 μM, Supplementary Figure 12). An increase in the anti-phospho-Ser<sup>843</sup> antibody immunoreactivity was also observed in Western blots from cells expressing the human mGlu<sub>2</sub> 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-Ser<sup>843</sup> antibody for the murine phosphorylated receptor.

We next took advantage of this antibody to investigate the phosphorylation of native mGlu<sub>2</sub> receptor in the mouse prefrontal cortex, a brain region where mGlu<sub>2</sub>/5-HT<sub>2A</sub> heteromers have been identified (9). We injected mice with either vehicle or LY379268 (10 mg/kg, 30 min) and assessed mGlu<sub>2</sub> phosphorylation by immunohistochemistry. Reminiscent of the data obtained in HEK-293 cells, LY379268 administration strongly increased immunostaining with the anti-phospho-Ser<sup>843</sup> antibody (Figure 1D). The most intense signal was observed in middle layers of prefrontal cortex, which express the highest density of 5-HT<sub>2A</sub> receptor (25). As observed in HEK-293 cells, Ser<sup>843</sup> phosphorylation elicited in mouse prefrontal cortex by injecting LY379268 was also prevented by the administration of LY341495, which alone did not affect Ser<sup>843</sup> phosphorylation (Figure 1D). Confirming the specificity of our antibody for phosphorylated mGlu<sub>2</sub>, no signal was detected in prefrontal cortex of mGlu<sub>2</sub> receptor

knockout (mGlu<sub>2</sub><sup>-/-</sup>) mice treated with either vehicle or LY379268 (Figure 1D). Moreover, administration of LY379268 to 5-HT<sub>2A</sub> receptor knockout (5-HT<sub>2A</sub><sup>-/-</sup>) mice did not increase the phospho-Ser<sup>843</sup> immunoreactive signal in prefrontal cortex (Figure 1D), indicating that 5-HT<sub>2A</sub> receptor expression is also necessary for LY379268-induced phosphorylation of native mGlu<sub>2</sub> receptor at Ser<sup>843</sup>.

### **Ser<sup>843</sup> phosphorylation is mediated by casein kinase 1 (CK1)**

In order to identify protein kinase(s) involved in Ser<sup>843</sup> phosphorylation, mGlu<sub>2</sub> receptor sequence surrounding Ser<sup>843</sup> was uploaded into Group-based Prediction System (GPS<sup>®</sup>, 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 Ser<sup>843</sup> is a good consensus for phosphorylation by casein kinase 1 (CK1, GPS<sup>®</sup> score 9.3), and cyclic AMP-dependent protein kinase (PKA, GPS<sup>®</sup> score 4.1), consistent with previous findings (15). Pretreatment of HEK-293 cells co-expressing mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptor with the CK1 inhibitor D4476 (10 μM, 60 min) (27) abolished LY379268-induced phosphorylation of Ser<sup>843</sup>, whereas the PKA pharmacological inhibitor KT5720 (1 μM, 60 min) (28) did not affect the level of Ser<sup>843</sup> phosphorylation (Figure 2A), suggesting that CK1 rather than PKA promotes Ser<sup>843</sup> phosphorylation in living cells. Treatment of cells with D4476 did not affect the low Ser<sup>843</sup> phosphorylation level measured in cells expressing mGlu<sub>2</sub> 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 Ser<sup>843</sup>. 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 Ser<sup>843</sup> phosphorylation in cells co-expressing 5-HT<sub>2A</sub> receptor (Figure 2B and Supplementary Figure 15), reminiscent of the effect of cell exposure to D4476 (Figure 2A).



### **G<sub>i/o</sub> and G<sub>q</sub> proteins are involved in Ser<sup>843</sup> phosphorylation elicited by mGlu<sub>2</sub> receptor stimulation in cells co-expressing 5-HT<sub>2A</sub> receptor**

In line with the canonical coupling of mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors to G<sub>i/o</sub> and G<sub>q</sub> proteins, respectively, we next explored their influence on LY379268-induced Ser<sup>843</sup> phosphorylation in cells co-expressing both receptors. LY379268 exposure did not increase Ser<sup>843</sup> phosphorylation in cells pretreated with the G<sub>i/o</sub> inhibitor Pertussis toxin (PTX, 100 ng/mL, 18 h) or the pharmacological G<sub>q/11</sub> inhibitor FR900359 (1  $\mu$ M, 2 h) (17), indicating that the activation of both G<sub>i/o</sub> and G<sub>q/11</sub> is necessary to LY379268-induced Ser<sup>843</sup> phosphorylation (Supplementary Figure 16A). Further supporting the role of G<sub>q/11</sub> activation by unliganded 5-HT<sub>2A</sub> receptor, LY379268 exposure did not promote Ser<sup>843</sup> phosphorylation in cells expressing the signaling-dead 5-HT<sub>2A</sub>(D<sup>120</sup>N) receptor (Supplementary Figure 17A) (29).

### **Agonist-elicited phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> promotes receptor-operated G<sub>i/o</sub> signaling**

To determine the role of Ser<sup>843</sup> phosphorylation in mGlu<sub>2</sub> receptor-mediated signal transduction, we measured mGlu<sub>2</sub> receptor-operated inhibition of N-type voltage-gated calcium channels, a read-out commonly used to study engagement of G<sub>i/o</sub> signaling by GPCRs (30,31) (Figure 3A).

As expected, LY379268 inhibited N-type calcium channel-mediated current in a concentration-dependent manner ( $pEC_{50} = 8.81 \pm 0.16$ ,  $n = 10$  cells) in HEK-293 cells transiently expressing mGlu<sub>2</sub> receptor (Figure 3B). No effect of LY379268 was seen in cells transfected with an empty vector or transiently expressing the 5-HT<sub>2A</sub> receptor alone, but co-expression of 5-HT<sub>2A</sub> receptor with mGlu<sub>2</sub> receptor increased the amplitude of LY379268-induced inhibition of N-type calcium channels without affecting its apparent affinity ( $pEC_{50} = 8.66 \pm 0.07$ ,  $n = 10$  cells, Figure 3B). In contrast, co-expression of 5-HT<sub>2A</sub> receptor with S<sup>843</sup>A mGlu<sub>2</sub> receptor mutant did not enhance the LY379268-induced inhibition of N-type calcium channels, while mutation of Ser<sup>843</sup> 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

$\mu\text{M}$ ), which prevented Ser<sup>843</sup> phosphorylation, also abolished the potentiation of the LY379268 response elicited by 5-HT<sub>2A</sub> receptor expression in cells co-expressing wild type mGlu<sub>2</sub> receptor but not in cells co-expressing S<sup>843</sup>D mGlu<sub>2</sub> receptors (Figure 3C). D4476 exposure did not affect the amplitude of LY379268-induced inhibition of N-type calcium channels in cells expressing mGlu<sub>2</sub> receptor alone (Figure 3C). Collectively, these results suggest that Ser<sup>843</sup> phosphorylation is a necessary step of the potentiation of mGlu<sub>2</sub> receptor-operated G<sub>i/o</sub> signaling induced by 5-HT<sub>2A</sub> receptor expression. This potentiation was not due to an enhanced expression of mGlu<sub>2</sub> receptor at the plasma membrane, as ELISA experiments showed that co-expression of 5-HT<sub>2A</sub> receptors did not change the cell surface expression of mGlu<sub>2</sub> receptor (Supplementary Figure 18). Further supporting the absence of influence of Ser<sup>843</sup> phosphorylation on mGlu<sub>2</sub> receptor plasma membrane localization, wild type, S<sup>843</sup>A and S<sup>843</sup>D mGlu<sub>2</sub> receptors exhibited similar cell surface expression (Supplementary Figure 18).

#### **5-HT<sub>2A</sub> receptor stimulation promotes phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup>**

Exposure of HEK-293 cells co-expressing mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors to 5-HT increased Ser<sup>843</sup> 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<sub>2A</sub> receptor antagonist M100907 (100 nM, Figure 4A). Reminiscent of the LY379268-induced response, the 5-HT-induced Ser<sup>843</sup> phosphorylation was strongly reduced in cells expressing the G<sub>q</sub>-dead 5-HT<sub>2A</sub>(D<sup>120</sup>N) receptor (Supplementary Figure 17B). Likewise, the 5-HT-elicited Ser<sup>843</sup> phosphorylation was abolished in cells pretreated with FR900359 but it persisted in cells pretreated with PTX (Supplementary Figure 16B). The hallucinogenic 5-HT<sub>2A</sub> receptor agonist DOI (10  $\mu\text{M}$ ) and the non-hallucinogenic agonist lisuride (10  $\mu\text{M}$ ) induced increases in Ser<sup>843</sup> phosphorylation comparable to that measured in cells challenged with 5-HT (Figure 4B). Furthermore, no additional increase in Ser<sup>843</sup> phosphorylation state was measured in cells exposed to both LY379268 and 5-HT<sub>2A</sub> 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 Ser<sup>843</sup> phosphorylation in prefrontal cortex similar to those measured in mice injected with LY379268 (Figure 4C). As expected, Ser<sup>843</sup> phosphorylation elicited by DOI injection was prevented by the administration of M100907, which alone did not affect Ser<sup>843</sup> phosphorylation (Figure 4C). Collectively, these results indicate that Ser<sup>843</sup> phosphorylation can be triggered with similar efficiencies by agonist stimulation of either 5-HT<sub>2A</sub> or mGlu<sub>2</sub> receptors in cells co-expressing both receptor subtypes.

Interestingly, treatment of HEK-293 cells co-expressing 5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors with LY341495, which alone did not affect Ser<sup>843</sup> phosphorylation, inhibited the 5-HT-induced effect (Figure 5A). Conversely, M100907 prevented the LY379268-induced increase in Ser<sup>843</sup> phosphorylation, while it slightly but not significantly decreased basal Ser<sup>843</sup> phosphorylation (Figure 5B). mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptor ligands induced similar regulations of Ser<sup>843</sup> phosphorylation in mouse prefrontal cortex: Ser<sup>843</sup> 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 Ser<sup>843</sup> elicited by the stimulation of 5-HT<sub>2A</sub> receptor depends on the co-expression of mGlu<sub>2</sub> receptor at active conformation and *vice versa*.

### **Impact of 5-HT<sub>2A</sub> receptor activation on G<sub>i/o</sub>-dependent signaling under the control of mGlu<sub>2</sub> receptor**

Given the role of Ser<sup>843</sup> phosphorylation in mGlu<sub>2</sub> receptor-operated activation of G<sub>i/o</sub> signaling and the critical influence of the 5-HT<sub>2A</sub> receptor upon this phosphorylation, we also investigated the effect of 5-HT<sub>2A</sub> receptor stimulation on G<sub>i/o</sub> signaling by measuring the activity of N-type calcium channels. In cells expressing 5-HT<sub>2A</sub> 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 S<sup>843</sup>D mGlu<sub>2</sub> receptors, but not in cells co-expressing S<sup>843</sup>A mGlu<sub>2</sub> receptor (Figure 5D). Reminiscent of their influence upon

Ser<sup>843</sup> 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<sub>2A</sub> and mGlu<sub>2</sub> receptors in engagement of G<sub>i/o</sub> signaling, which depends on Ser<sup>843</sup> phosphorylation.

## Discussion

Phosphorylation of GPCRs is classically considered as a mechanism initiating receptor desensitization and  $\beta$ -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 NPFF, by acting at NPFF<sub>2</sub> receptors, induces a transphosphorylation of Mu-opioid receptor within Mu/NPFF<sub>2</sub> heteromers, a process responsible for a loss of function of Mu receptors and contributing to the tolerance to morphine analgesia induced by NPFF (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<sub>2A</sub> receptor expression and stimulation upon mGlu<sub>2</sub> receptor phosphorylation pattern.

We identified three (Ser<sup>833</sup>, Ser<sup>843</sup> and Ser<sup>871</sup>) out of the 12 putative phosphorylated residues (Ser<sup>109</sup>, Ser<sup>111</sup>, Ser<sup>246</sup>, Thr<sup>457</sup>, Ser<sup>501</sup>, Tyr<sup>734</sup>, Tyr<sup>745</sup>, Tyr<sup>749</sup>, Thr<sup>832</sup>, Ser<sup>833</sup>, Ser<sup>843</sup> and Ser<sup>871</sup>) referenced in the PhosphoSitePlus database (<http://www.phosphosite.org>) and two previously not described phosphorylated sites (Ser<sup>718</sup> and Ser<sup>837</sup>) in human mGlu<sub>2</sub> receptor co-expressed with 5-HT<sub>2A</sub> receptor in HEK-293 cells challenged with a mGlu<sub>2</sub> 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 (Ser<sup>843</sup>) located in the receptor C-terminal domain and conserved in the human and rat sequences was up-regulated upon agonist receptor stimulation. Moreover, Ser<sup>843</sup> phosphorylation depended on the co-expression of 5-HT<sub>2A</sub> receptor. Likewise, phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> elicited in mice prefrontal cortex by peripheral administration of the mGlu<sub>2</sub> receptor agonist LY379268 (assessed by immunohistochemistry using a newly-generated phospho-Ser<sup>843</sup> antibody) was not seen in 5-HT<sub>2A</sub><sup>-/-</sup> mice. This result indicates that the joint influence of 5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors upon Ser<sup>843</sup> 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<sub>2A</sub> and mGlu<sub>2</sub> receptor ligands (9,40). Intriguingly, 5-HT<sub>2A</sub> receptor stimulation by 5-HT promoted Ser<sup>843</sup> phosphorylation as efficiently as that induced by mGlu<sub>2</sub> receptor stimulation, reminiscent of the transphosphorylation of Mu-opioid receptor elicited by NPFF receptor activation (39). Moreover, Ser<sup>843</sup> phosphorylation induced by 5-HT (or the synthetic 5-HT<sub>2A</sub> receptor agonist DOI) was impaired by the mGlu<sub>2</sub> receptor antagonist LY341495, while the LY379268-induced effect was prevented by the 5-HT<sub>2A</sub> receptor antagonist M100907, thus demonstrating a positive reciprocal influence of both receptor activities upon mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> in HEK-293 cells and mice prefrontal cortex. Notably, the LY379268-induced Ser<sup>843</sup> phosphorylation depends on both G<sub>i/o</sub> and G<sub>q</sub> activation, whereas the 5-HT-induced response is only G<sub>q</sub>-dependent. Collectively, these findings suggest that Ser<sup>843</sup> phosphorylation can result from two types of allosteric modulation exerted by different active conformations of the 5-HT<sub>2A</sub> receptor within heteromers: i) an allosteric modulation by unliganded (constitutively active) 5-HT<sub>2A</sub> receptor of agonist-stimulated mGlu<sub>2</sub> receptor (bound to activated G<sub>i/o</sub> protein) and ii) an allosteric modulation by 5-HT-stimulated 5-HT<sub>2A</sub> receptor that is independent of mGlu<sub>2</sub> receptor

stimulation by its own agonist and of  $G_{i/o}$  protein activation. However, one cannot rule out that 5-HT<sub>2A</sub> receptor-dependent Ser<sup>843</sup> phosphorylation can also be induced indirectly, *via* a 5-HT<sub>2A</sub> receptor-operated  $G_{q/11}$  signaling pathway. Irrespective of the mechanism underlying Ser<sup>843</sup> phosphorylation, our results identify a novel functional crosstalk mechanism between 5-HT<sub>2A</sub> and mGlu<sub>2</sub> 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<sub>2A</sub>-mGlu<sub>2</sub> heteromers *in vivo*.

The difference in signal transduction elicited by hallucinogenic and non-hallucinogenic agonists of the 5-HT<sub>2A</sub> 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<sub>2A</sub> receptor agonists differ in their capacities to promote 5-HT<sub>2A</sub> receptor phosphorylation at Ser<sup>280</sup> (14). Such a biased phosphorylation was not retrieved for Ser<sup>843</sup>: treatment of cells with the hallucinogenic agonist DOI and the non-hallucinogenic agonist lisuride induced comparable increases in Ser<sup>843</sup> phosphorylation to that elicited by 5-HT. It is thus unlikely that Ser<sup>843</sup> phosphorylation underlies the distinct behavioral responses induced by both ligand categories.

Among the two kinases possibly involved in Ser<sup>843</sup> 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 Ser<sup>843</sup> can be phosphorylated *in vitro* by PKA or following a treatment of cells expressing recombinant mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptor stimulation in cells co-expressing 5-HT<sub>2A</sub> receptors, consistent with the canonical coupling of mGlu<sub>2</sub> 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<sub>2A</sub>-mGlu<sub>2</sub> heteromers, at least in recombinant cells (9, 34), we hypothesize that Ser<sup>843</sup> might be accessible for CK1 only in a mGlu<sub>2</sub> receptor conformation stabilized by its association with 5-HT<sub>2A</sub> receptor and agonist stimulation of either mGlu<sub>2</sub> or 5-HT<sub>2A</sub> receptor within the heterocomplex.

Consistent with the localization of Ser<sup>843</sup> in mGlu<sub>2</sub> 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<sub>i/o</sub> signaling not only upon mGlu<sub>2</sub> receptor activation but also following 5-HT<sub>2A</sub> receptor activation in cells co-expressing both receptors: *i*) corroborating previous findings indicating that heteromeric assembly of mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors enhances glutamate-elicited G<sub>i/o</sub> signaling (11), co-expression of 5-HT<sub>2A</sub> receptor with mGlu<sub>2</sub> receptor enhanced LY379268-induced inhibition of N-type channels (used as readout of activation of G<sub>i/o</sub>-dependent signaling) (30,31), an effect suppressed by mutating Ser<sup>843</sup> into alanine; *ii*) reminiscent of their influence upon Ser<sup>843</sup> phosphorylation, the 5-HT<sub>2A</sub> receptor antagonist M100907 prevented enhancement of LY379268-induced inhibition of N-type channels induced by the co-expression of 5-HT<sub>2A</sub> receptor, while the mGlu<sub>2</sub> receptor antagonist LY341495 blocked the 5-HT-evoked response. These findings thus identify Ser<sup>843</sup> phosphorylation as a novel molecular substrate underlying the functional crosstalk between 5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors, which determines the level of G<sub>i/o</sub> signaling under the control of both receptors. It has been proposed that the balance between G<sub>i/o</sub> and G<sub>q</sub>-dependent signaling determines pro-psychotic vs. antipsychotic activity of 5-HT<sub>2A</sub> and mGlu<sub>2</sub> receptors ligands (11). Given the critical influence of Ser<sup>843</sup> phosphorylation level on mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptor-dependent G<sub>i/o</sub> 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 Ser<sup>843</sup> phosphorylation as an early molecular substrate of the functional crosstalk between 5-HT<sub>2A</sub> and mGlu<sub>2</sub> 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-Ser<sup>843</sup> 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<sub>2A</sub> and mGlu<sub>2</sub> 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<sub>2A</sub> receptor expression, the majority of post-mortem studies demonstrate a decrease in cortical 5-HT<sub>2A</sub> receptor density in patients. In line with these findings and the strong influence of 5-HT<sub>2A</sub> receptor expression/stimulation on Ser<sup>843</sup> phosphorylation, analysis of its phosphorylation status in post-mortem brain of patients with schizophrenia might be of great interest.

## **Acknowledgements**

**Funding:** This work was supported by grants from la Fondation pour la Recherche Médicale, ANR (Contract n° ANR-08-MNPS-0011), CNRS, INSERM and University of Montpellier to PM and FV. EB is supported by the LABEX Ion Channel Science and Therapeutics (ICST). Mass spectrometry experiments were carried out using facilities of the Functional Proteomic Platform of Montpellier Languedoc-Roussillon. SM was a recipient of a fellowship from the French Ministry for Research.

**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 mGlu<sub>2</sub><sup>-/-</sup> mice.

**Competing interests:** The authors declare that they have no conflict of interest.

**Supplementary information** is available at MP's website.

## References

1. Gray JA, Roth BL. Molecular targets for treating cognitive dysfunction in schizophrenia. *Schizophr Bull.* 2007 Sep;33(5):1100–19.
2. Meltzer HY, Massey BW, Horiguchi M. Serotonin receptors as targets for drugs useful to treat psychosis and cognitive impairment in schizophrenia. *Curr Pharm Biotechnol.* 2012 Jun;13(8):1572–86.
3. Keefe RSE, Bilder RM, Davis SM, Harvey PD, Palmer BW, Gold JM, et al. Neurocognitive effects of antipsychotic medications in patients with chronic schizophrenia in the CATIE Trial. *Arch Gen Psychiatry.* 2007 Jun;64(6):633–47.
4. Ellaithy A, Younkin J, González-Maeso J, Logothetis DE. Positive allosteric modulators of metabotropic glutamate 2 receptors in schizophrenia treatment. *Trends Neurosci.* 2015 Aug;38(8):506–16.
5. Gibert-Rahola J, Villena-Rodriguez A. Glutamatergic drugs for schizophrenia treatment. *Actas Esp Psiquiatr.* 2014 Oct;42(5):234–41.
6. Fell MJ, McKinzie DL, Monn JA, Svensson KA. Group II metabotropic glutamate receptor agonists and positive allosteric modulators as novel treatments for schizophrenia. *Neuropharmacology.* 2012 Mar;62(3):1473–83.
7. Kinon BJ, Millen BA, Zhang L, McKinzie DL. Exploratory analysis for a targeted patient population responsive to the metabotropic glutamate 2/3 receptor agonist pomaglumetad methionil in schizophrenia. *Biol Psychiatry.* 2015 Dec 1;78(11):754–62.
8. Marek GJ, Wright RA, Schoepp DD, Monn JA, Aghajanian GK. Physiological antagonism between 5-hydroxytryptamine(2A) and group II metabotropic glutamate receptors in prefrontal cortex. *J Pharmacol Exp Ther.* 2000 Jan;292(1):76–87.

9. González-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, López-Giménez JF, et al. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature*. 2008 Mar 6;452(7183):93–7.
10. Moreno JL, Miranda-Azpiaz P, García-Bea A, Younkin J, Cui M, Kozlenkov A, et al. Allosteric signaling through an mGlu2 and 5-HT<sub>2A</sub> heteromeric receptor complex and its potential contribution to schizophrenia. *Sci Signal*. 2016 Jan 12;9(410):ra5.
11. Fribourg M, Moreno JL, Holloway T, Provasi D, Baki L, Mahajan R, et al. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell*. 2011 Nov 23;147(5):1011–23.
12. Delille HK, Mezler M, Marek GJ. The two faces of the pharmacological interaction of mGlu2 and 5-HT<sub>2A</sub> - relevance of receptor heterocomplexes and interaction through functional brain pathways. *Neuropharmacology*. 2013 Jul;70:296–305.
13. Butcher AJ, Kong KC, Prihandoko R, Tobin AB. Physiological role of G-protein coupled receptor phosphorylation. *Handb Exp Pharmacol*. 2012;(208):79–94.
14. Karaki S, Becamel C, Murat S, Mannoury la Cour C, Millan MJ, Prézeau L, et al. Quantitative phosphoproteomics unravels biased phosphorylation of serotonin 2A receptor at Ser280 by hallucinogenic versus nonhallucinogenic agonists. *Mol Cell Proteomics MCP*. 2014 May;13(5):1273–85.
15. Schaffhauser H, Cai Z, Hubalek F, Macek TA, Pohl J, Murphy TJ, et al. cAMP-dependent protein kinase inhibits mGluR2 coupling to G-proteins by direct receptor phosphorylation. *J Neurosci Off J Soc Neurosci*. 2000 Aug 1;20(15):5663–70.
16. Lefkowitz RJ, Pierce KL, Luttrell LM. Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol Pharmacol*. 2002 Nov;62(5):971–4.

17. Schrage R, Schmitz A-L, Gaffal E, Annala S, Kehraus S, Wenzel D, et al. The experimental power of FR900359 to study Gq-regulated biological processes. *Nat Commun*. 2015 Dec 14;6:10156.
18. Bécamel C, Gavarini S, Chanrion B, Alonso G, Galéotti N, Dumuis A, et al. The serotonin 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors interact with specific sets of PDZ proteins. *J Biol Chem*. 2004 May 7;279(19):20257–66.
19. Gaillard S, Lo Re L, Mantilleri A, Hepp R, Urien L, Malapert P, et al. GINIP, a Gαi-interacting protein, functions as a key modulator of peripheral GABAB receptor-mediated analgesia. *Neuron*. 2014 Oct 1;84(1):123–36.
20. Dubois F, Vandermoere F, Gernez A, Murphy J, Toth R, Chen S, et al. Differential 14-3-3 affinity capture reveals new downstream targets of phosphatidylinositol 3-kinase signaling. *Mol Cell Proteomics MCP*. 2009 Nov;8(11):2487–99.
21. González-Maeso J, Yuen T, Ebersole BJ, Wurmbach E, Lira A, Zhou M, et al. Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *J Neurosci Off J Soc Neurosci*. 2003 Oct 1;23(26):8836–43.
22. Yokoi M, Kobayashi K, Manabe T, Takahashi T, Sakaguchi I, Katsuura G, et al. Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science*. 1996 Aug 2;273(5275):645–7.
23. Chanrion B, Mannoury la Cour C, Gavarini S, Seimandi M, Vincent L, Pujol J-F, et al. Inverse agonist and neutral antagonist actions of antidepressants at recombinant and native 5-hydroxytryptamine<sub>2C</sub> receptors: differential modulation of cell surface expression and signal transduction. *Mol Pharmacol*. 2008 Mar;73(3):748–57.

24. Delille HK, Becker JM, Burkhardt S, Bleher B, Terstappen GC, Schmidt M, et al. Heterocomplex formation of 5-HT<sub>2A</sub>-mGlu<sub>2</sub> and its relevance for cellular signaling cascades. *Neuropharmacology*. 2012 Jun;62(7):2184–91.
25. Miner L a. H, Backstrom JR, Sanders-Bush E, Sesack SR. Ultrastructural localization of serotonin<sub>2A</sub> receptors in the middle layers of the rat prelimbic prefrontal cortex. *Neuroscience*. 2003;116(1):107–17.
26. Xue Y, Ren J, Gao X, Jin C, Wen L, Yao X. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol Cell Proteomics MCP*. 2008 Sep;7(9):1598–608.
27. Rena G, Bain J, Elliott M, Cohen P. D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO Rep*. 2004 Jan;5(1):60–5.
28. Russo LC, Castro LM, Gozzo FC, Ferro ES. Inhibition of thimet oligopeptidase by siRNA alters specific intracellular peptides and potentiates isoproterenol signal transduction. *FEBS Lett*. 2012 Sep 21;586(19):3287–92.
29. Sealfon SC, Chi L, Ebersole BJ, Rodic V, Zhang D, Ballesteros JA, et al. Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT<sub>2A</sub> receptor. *J Biol Chem*. 1995 Jul 14;270(28):16683–8.
30. Chen W-P, Kirchgessner AL. Activation of group II mGlu receptors inhibits voltage-gated Ca<sup>2+</sup> currents in myenteric neurons. *Am J Physiol Gastrointest Liver Physiol*. 2002 Dec;283(6):G1282-1289.
31. Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP. Crosstalk between G proteins and protein kinase C mediated by the calcium channel  $\alpha$ 1 subunit. *Nature*. 1997 Jan 30;385(6615):442–6.

32. Premont RT, Gainetdinov RR. Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol.* 2007;69:511–34.
33. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular mechanism of  $\beta$ -arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol.* 2012;52:179–97.
34. Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, et al. Distinct phosphorylation sites on the  $\beta(2)$ -adrenergic receptor establish a barcode that encodes differential functions of  $\beta$ -arrestin. *Sci Signal.* 2011 Aug 9;4(185):ra51.
35. Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, et al. Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J Biol Chem.* 2011 Apr 1;286(13):11506–18.
36. Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J Biol Chem.* 2010 Mar 5;285(10):7805–17.
37. Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, et al. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci U S A.* 2007 Oct 16;104(42):16657–62.
38. Tobin AB, Butcher AJ, Kong KC. Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci.* 2008 Aug;29(8):413–20.
39. Moulédous L, Froment C, Dauvillier S, Burlet-Schiltz O, Zajac J-M, Mollereau C. GRK2 protein-mediated transphosphorylation contributes to loss of function of  $\mu$ -opioid receptors induced by neuropeptide FF (NPFF2) receptors. *J Biol Chem.* 2012 Apr 13;287(16):12736–49.

40. Moreno JL, Holloway T, Albizu L, Sealfon SC, González-Maeso J. Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT<sub>2A</sub> receptor agonists. *Neurosci Lett*. 2011 Apr 15;493(3):76–9.
41. González-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R, et al. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. *Neuron*. 2007 Feb 1;53(3):439–52.
42. Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, et al. Metabotropic glutamate receptors: from the workbench to the bedside. *Neuropharmacology*. 2011 Jun;60(7–8):1017–41.
43. Knippschild U, Gocht A, Wolff S, Huber N, Löhler J, Stöter M. The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. *Cell Signal*. 2005 Jun 1;17(6):675–89.
44. Yang Y, Xu T, Zhang Y, Qin X. Molecular basis for the regulation of the circadian clock kinases CK1 $\delta$  and CK1 $\epsilon$ . *Cell Signal*. 2017 Feb 1;31:58–65.
45. El Moustaine D, Granier S, Doumazane E, Scholler P, Rahmeh R, Bron P, et al. Distinct roles of metabotropic glutamate receptor dimerization in agonist activation and G-protein coupling. *Proc Natl Acad Sci U S A*. 2012 Oct 2;109(40):16342–7.
46. Dean B. The cortical serotonin<sub>2A</sub> receptor and the pathology of schizophrenia: a likely accomplice. *J Neurochem*. 2003 Apr;85(1):1–13.



## Figure legends

**Figure 1. Agonist-stimulated mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> requires co-expression of 5-HT<sub>2A</sub> receptor in HEK-293 cells and mouse prefrontal cortex.** **A**, Schematic overview of the position of phosphorylated residues identified by MS/MS in human mGlu<sub>2</sub> receptor. **B**, Sequence alignment of the receptor region containing Ser<sup>843</sup> in human, mouse and rat mGlu<sub>2</sub> orthologs. Note that Ser<sup>843</sup> is conserved in the three orthologs, while Ser<sup>844</sup> 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 mGlu<sub>2</sub> receptor in the absence or presence of 5-HT<sub>2A</sub> receptor were exposed to vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. The histograms represent the means  $\pm$  SEM of ion signal intensities (expressed in % of signal intensity measured in cells co-expressing mGlu<sub>2</sub> and 5-HT<sub>2A</sub> 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 mGlu<sub>2</sub>, respectively. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. **D**, Wild type, 5-HT<sub>2A</sub><sup>-/-</sup> and mGlu<sub>2</sub><sup>-/-</sup> 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-Ser<sup>843</sup>-mGlu<sub>2</sub> immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean  $\pm$  SEM of values (expressed in % of anti-phospho-Ser<sup>843</sup>-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  $\mu$ m. ns: not significant.

**Figure 2. 5-HT<sub>2A</sub> receptor-dependent phosphorylation of Ser<sup>843</sup> elicited by LY379268 is mediated by casein kinase 1.** **A**, HEK-293 cells co-expressing rat Flag-tagged mGlu<sub>2</sub> and HA-tagged 5-HT<sub>2A</sub> receptors were pre-incubated with either vehicle or the CK1 inhibitor (D4476, 10  $\mu$ M) or the PKA inhibitor (KT5720, 1  $\mu$ M) for 60 min and then challenged with vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. **B**, HEK-293 cells co-expressing Flag-tagged

rat mGlu<sub>2</sub> and HA-tagged 5-HT<sub>2A</sub> receptors were transfected with either control or CK1ε siRNA and challenged for 15 min with vehicle or LY379268 (LY37, 1 μM). Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptor-operated G<sub>i/o</sub> signaling elicited by 5-HT<sub>2A</sub> receptor expression depends on Ser<sup>843</sup> phosphorylation.** **A**, Schematic overview of N-type calcium channel inhibition elicited upon activation of G<sub>i/o</sub>-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 mGlu<sub>2</sub> receptor alone or in combination with HA-tagged 5-HT<sub>2A</sub> receptor. Right panel: HEK-293 cells, transiently expressing Flag-tagged wild type mGlu<sub>2</sub> receptor alone or in combination with HA-tagged 5-HT<sub>2A</sub> 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 mGlu<sub>2</sub> receptor alone or co-expressing HA-tagged 5-HT<sub>2A</sub> receptor and either wild type or S<sup>843</sup>A or S<sup>843</sup>D Flag-tagged mGlu<sub>2</sub> receptor, were exposed to 1 μM LY379268. Right panel: HEK-293 cells, expressing Flag-tagged mGlu<sub>2</sub> receptor alone or co-expressing HA-tagged 5-HT<sub>2A</sub> receptor and either wild type or S<sup>843</sup>D mGlu<sub>2</sub> 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<sub>2A</sub> receptor stimulation promotes phosphorylation of mGlu<sub>2</sub> at Ser<sup>843</sup> in HEK-293 cells and mouse prefrontal cortex.** **A**, HEK-293 cells co-expressing Flag-tagged mGlu<sub>2</sub> receptor and HA-tagged 5-HT<sub>2A</sub> receptor were exposed to vehicle or 5-HT (1  $\mu$ M, 15 min) in the absence or presence of M100907 (100 nM). **B**, Cells were exposed to either vehicle or 5-HT (1  $\mu$ M), or DOI (10  $\mu$ M) or lisuride (10  $\mu$ M) in the absence or presence of LY379268 (1  $\mu$ M) for 15 min. Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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). mGlu<sub>2</sub><sup>-/-</sup> 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-Ser<sup>843</sup>-mGlu<sub>2</sub> immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean  $\pm$  SEM of values (expressed in % of anti-phospho-Ser<sup>843</sup>-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  $\mu$ m.

**Figure 5. Reciprocal influence of mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptor stimulation upon G<sub>i/o</sub> signaling depends on Ser<sup>843</sup> phosphorylation.** **A**, HEK-293 cells co-expressing Flag-tagged mGlu<sub>2</sub> receptor and HA-tagged 5-HT<sub>2A</sub> receptor were exposed to vehicle or 5-HT (1  $\mu$ M), alone or in combination with LY341495 (10  $\mu$ M), or to LY341495 alone (10  $\mu$ M) for 15 min. **B**, HEK-293 cells co-expressing Flag-tagged mGlu<sub>2</sub> receptor and HA-tagged 5-HT<sub>2A</sub> 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, mGlu<sub>2</sub> receptor phosphorylation was analyzed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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-Ser<sup>843</sup>-mGlu<sub>2</sub> immunofluorescent staining in prefrontal cortex are illustrated. The histogram shows the mean  $\pm$  SEM of values (expressed in % of anti-phospho-Ser<sup>843</sup>-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  $\mu$ m. **D**, HEK-293 cells, transiently expressing 5-HT<sub>2A</sub> receptor alone or in combination with either wild type or S<sup>843</sup>A or S<sup>843</sup>D mGlu<sub>2</sub> receptor, were exposed to 1  $\mu$ M 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  $\pm$  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<sub>2A</sub> and mGlu<sub>2</sub> receptors were sequentially challenged with LY3792968 (10 nM), LY341495 (10  $\mu$ M) and LY3792968 + LY341495 or with 5-HT (1  $\mu$ M), 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)	$\Delta$ mass (ppm)	Mascot score	Phosphorylation site occupancy index			
							mGlu <sub>2</sub>		mGlu <sub>2</sub> + 5-HT <sub>2A</sub>	
							Vehicle	LY37	Vehicle	LY37
REVVt <sup>718</sup> 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 <sup>833</sup> RFGs <sup>837</sup> 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 <sup>843</sup> S <sup>844</sup> 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***
EVVDSTTs <sup>871</sup> 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 <sup>843</sup> 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 mGlu<sub>2</sub> receptors by nano-LC-MS/MS.** HEK-293 cells transiently expressing human or rat Flag-tagged mGlu<sub>2</sub> receptor, with or without HA-tagged 5-HT<sub>2A</sub> receptor, were exposed to either Vehicle or LY379268 (1  $\mu$ 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.

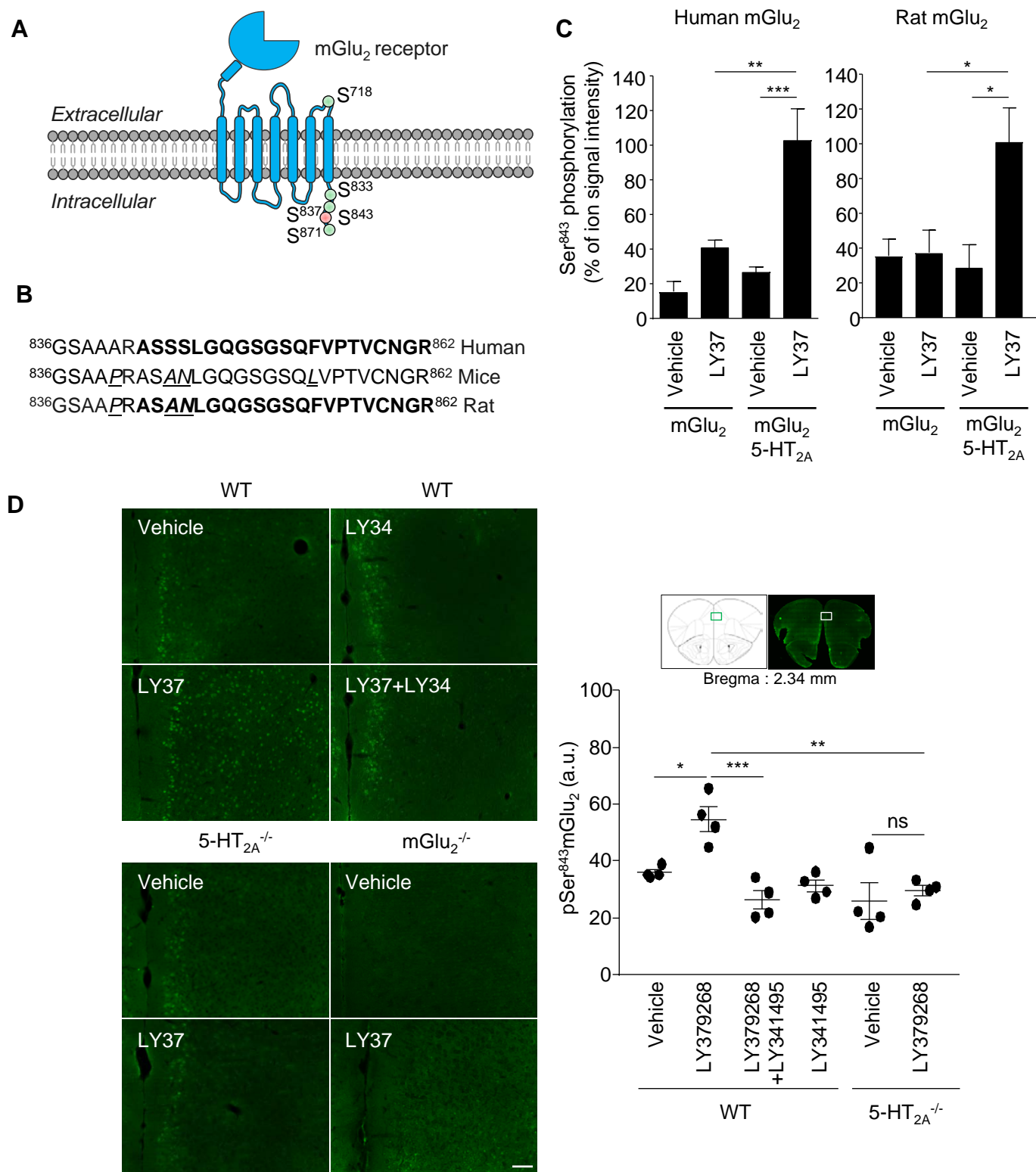


Figure 1

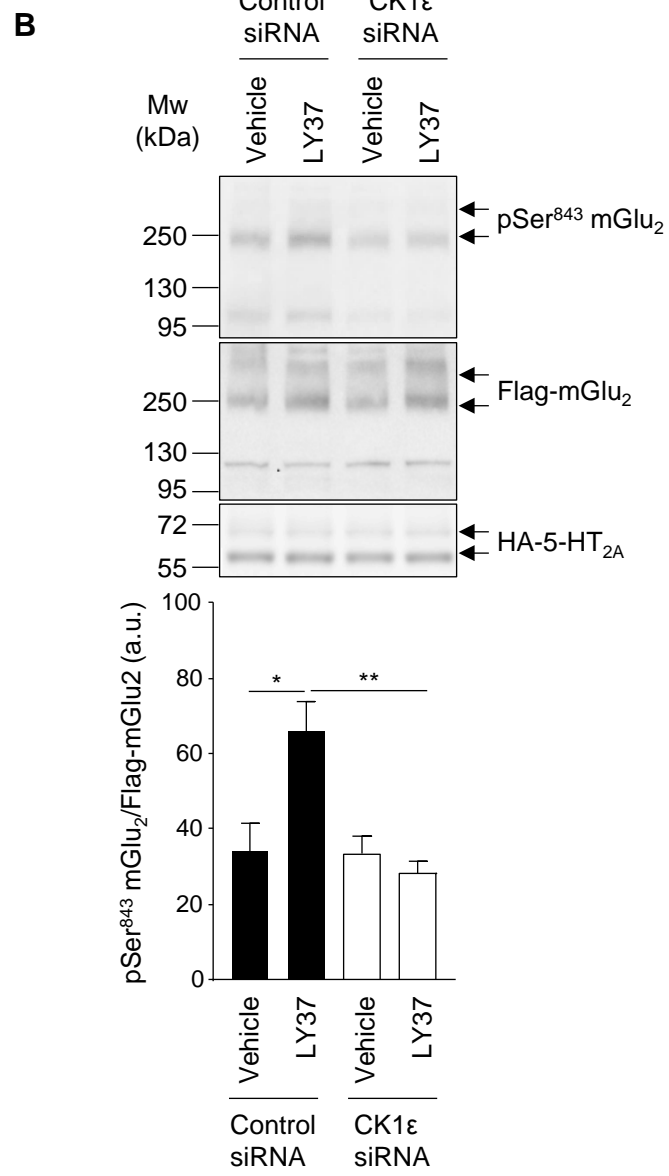
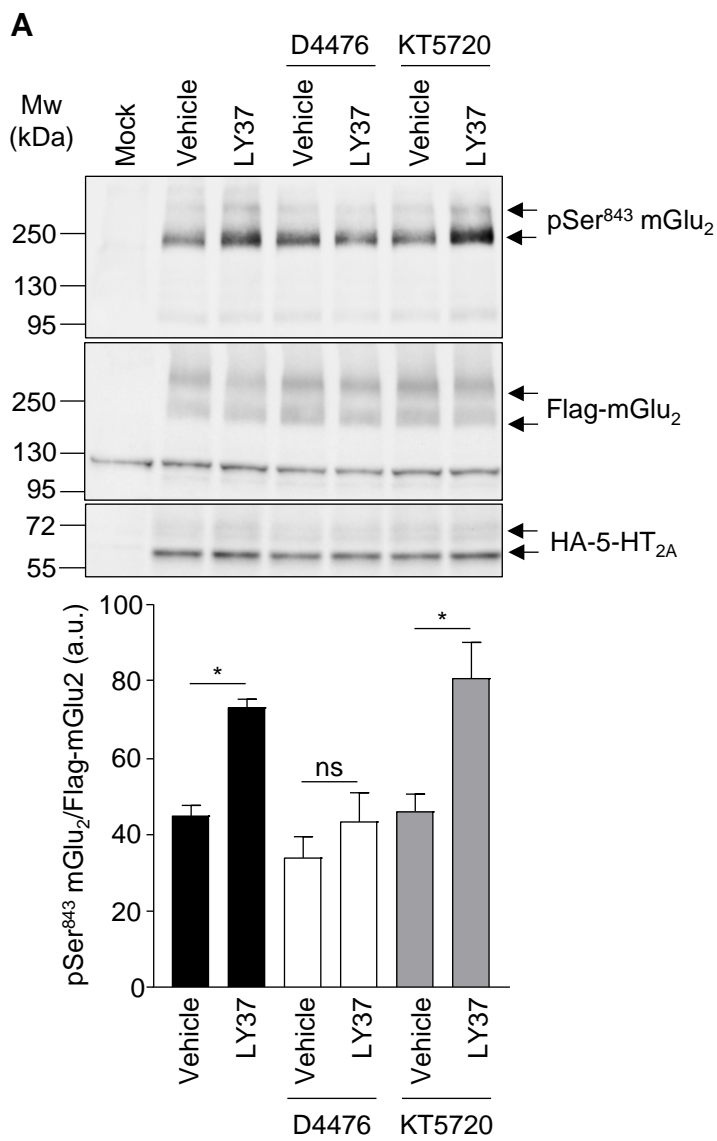


Figure 2



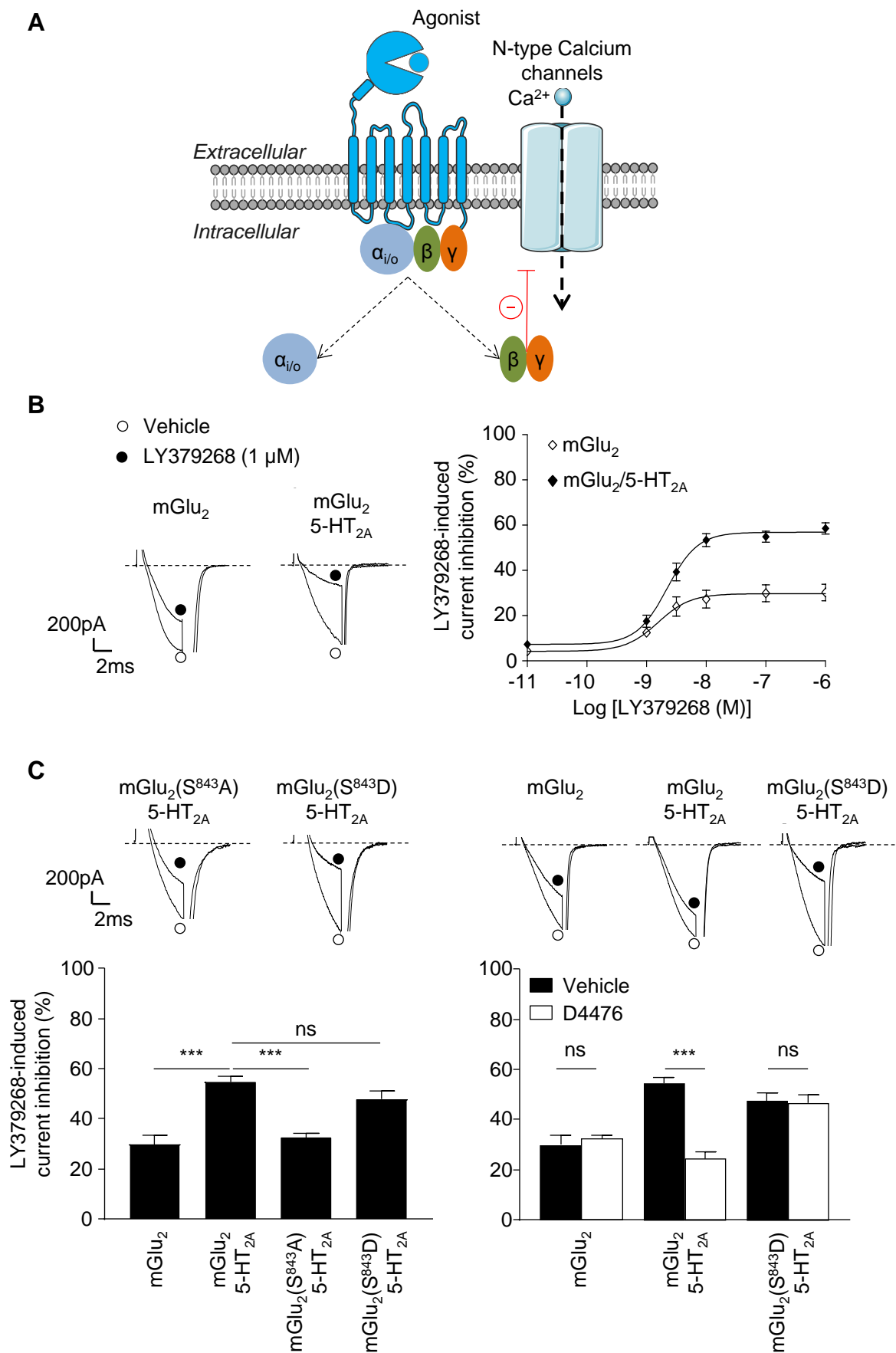


Figure 3

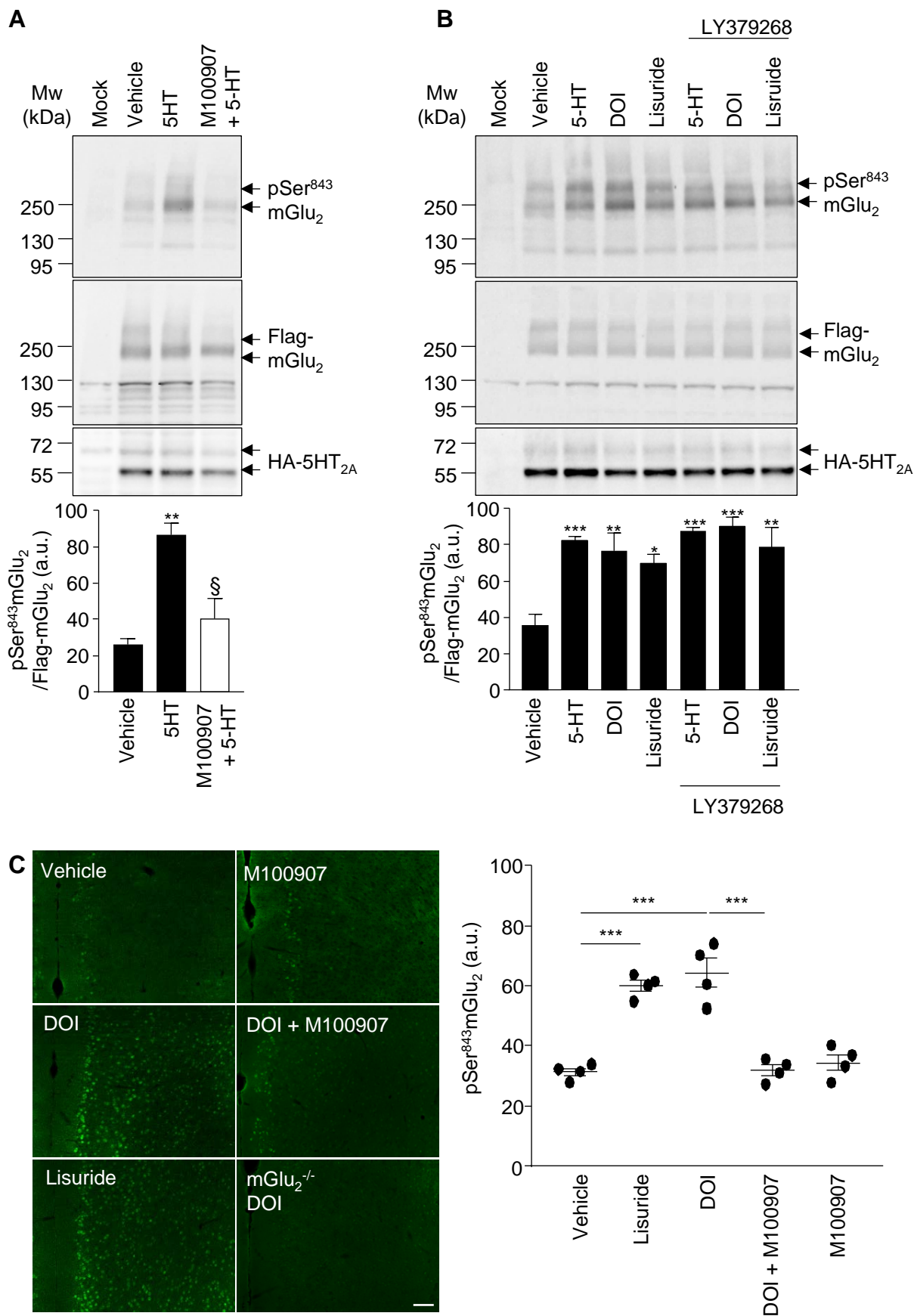


Figure 4

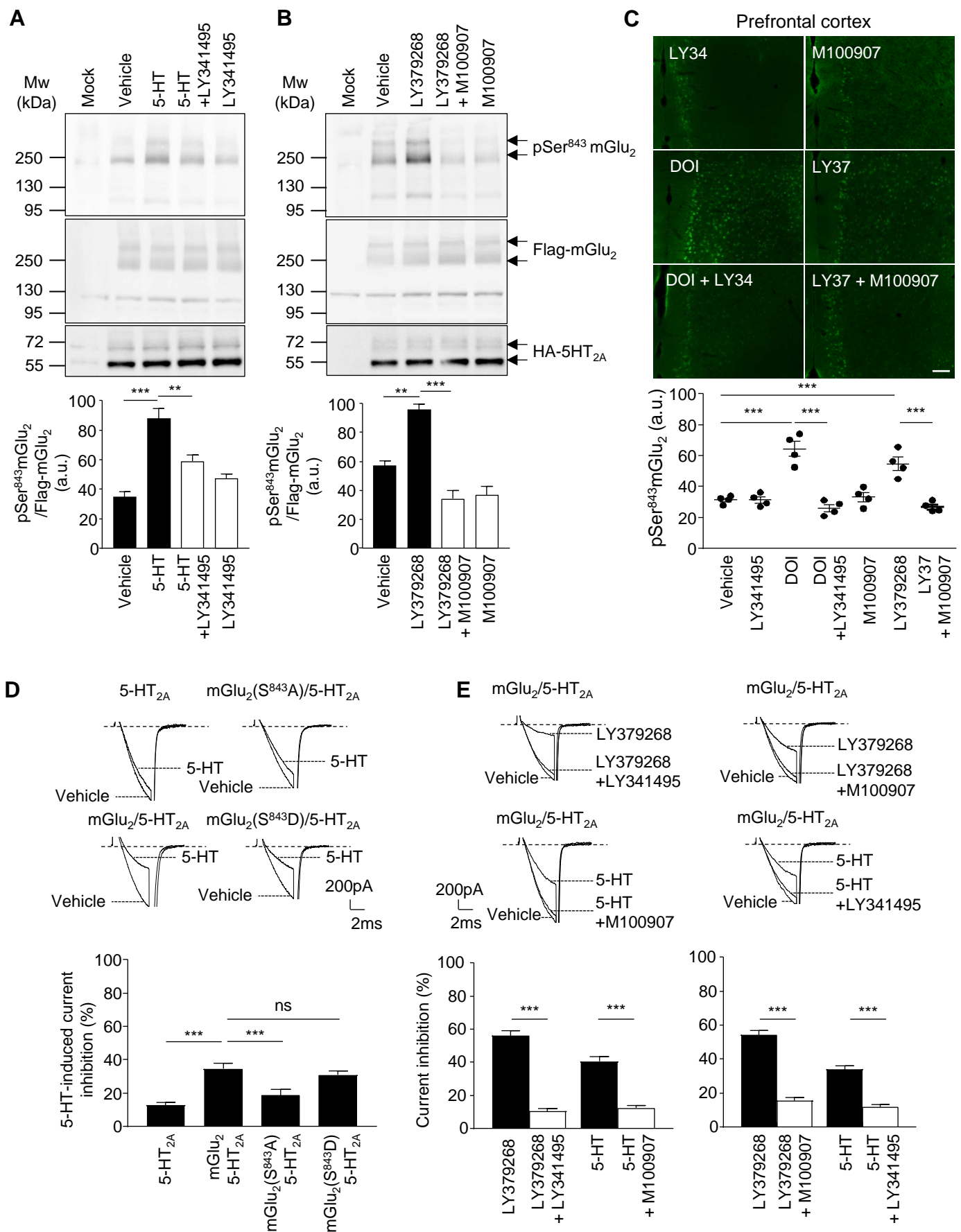


Figure 5

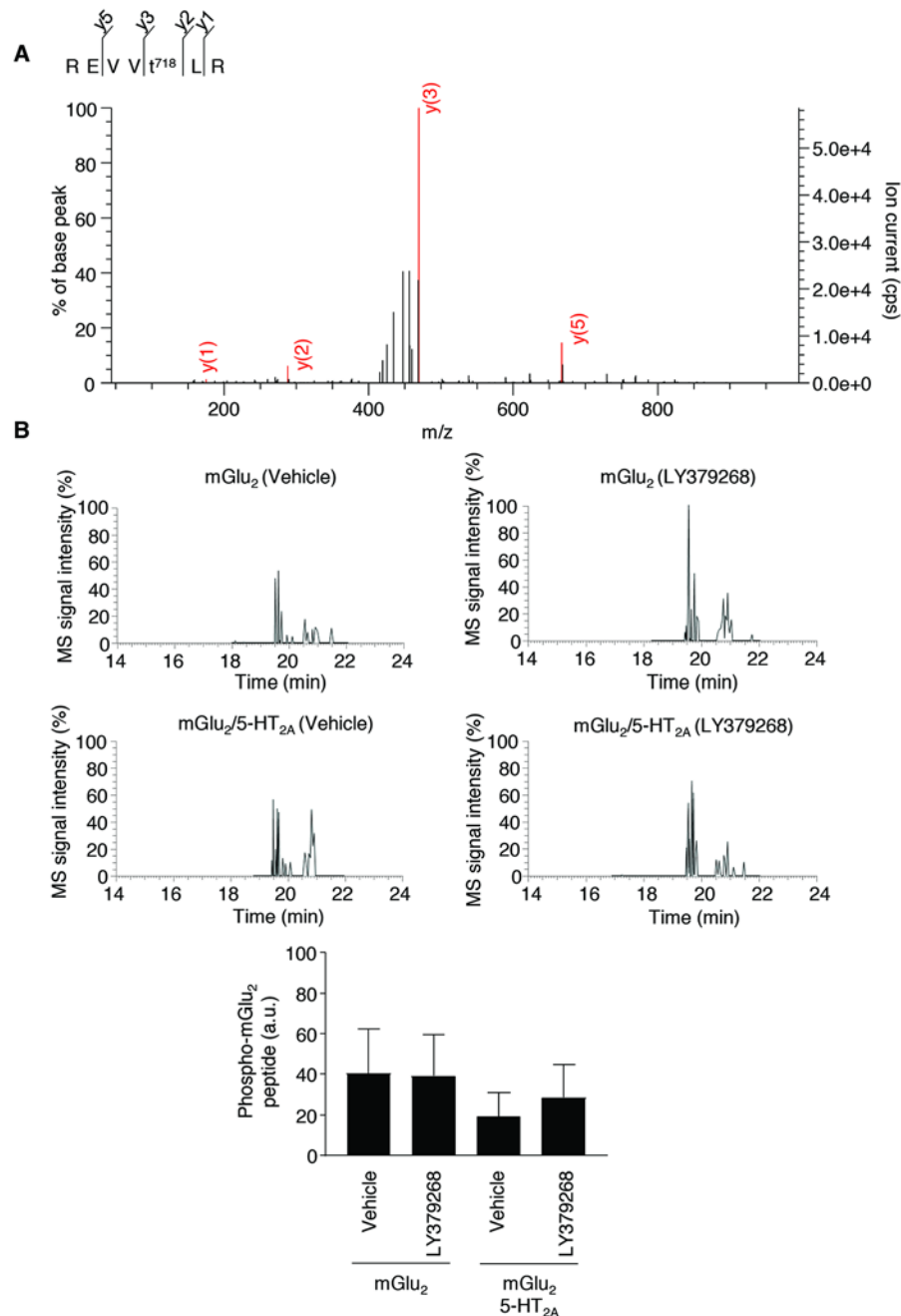
mGlu<sub>2</sub> receptor coverage (Human) : 57% (exp pvalue cut off <0.05)

1	MGSLALLAL	LLWGAFAEG	PAK <b>KVLTLEG</b>	<b>DLVLGGLFPV</b>	<b>HQKGGPAEDC</b>
51	<b>GPVNEHRGIQ</b>	<b>RLEAMLFALD</b>	<b>RINRDPHLLP</b>	<b>GVRLGAHILD</b>	<b>SCSKDTHALE</b>
101	<b>QALDFVRASL</b>	<b>SRGADGSRHI</b>	<b>CPDGSYATHG</b>	<b>DAPTAITGVI</b>	<b>GGSYSDVSIQ</b>
151	<b>VANLLRLFQI</b>	<b>PQISYASTSA</b>	<b>KLSDKSRYDY</b>	<b>FARTVPPDFF</b>	<b>QAKAMAEILR</b>
201	FFNWTYVSTV	ASEGDYGETG	IEAFELEARA	RNICVATSEK	VGRAMSRAAF
251	<b>EGVVRALLQK</b>	<b>PSARVAVLFT</b>	<b>RSEDARELLA</b>	<b>ASQRLNASFT</b>	WVASDGGWAL
301	ESVVASSEGA	AEGAITIELA	SYPIDFASY	FQSLDPWNNS	RNPWFREFWE
351	<b>QRFRCFSFRQR</b>	<b>DCAAHSLRAV</b>	<b>PFEQESKIMF</b>	<b>VVNAVYAMAH</b>	<b>ALHNMHRALC</b>
401	PNTT <b>RLCDAM</b>	<b>RPVNGRRLYK</b>	<b>DFVLNVKFDA</b>	<b>PFRPADTHNE</b>	<b>VRFDRLFQGI</b>
451	<b>GRYNIFTYLR</b>	AGSGRYRYQK	<b>VGWAEGLTL</b>	<b>DTSLIPWASP</b>	<b>SAGPLPASRC</b>
501	<b>SEPCLQNEVK</b>	<b>SVQPGEVCCW</b>	<b>LCIPCQPYEY</b>	<b>RLDEFTCADC</b>	GLGYWPNASL
551	TGCFELPQY	<b>IRWGDAWAVG</b>	<b>PVTIACLGAL</b>	<b>ATLFVLGVFV</b>	<b>RHNATPVVKA</b>
601	SGRELCYILL	GGVFLCYCMT	FIFIAKPSTA	VCTLRRLGLG	<b>TAFSVCYSAL</b>
651	<b>LTKTNRIARI</b>	FGGAREGAQR	PRFISPASQV	AICLALISGQ	LLIVVAWLIV
701	EAPGTGKETA	PER <b>REV</b> <sup>718</sup> <b>LR</b>	CNHRDASMLG	SLAYNVLLIA	LCTLYAFKTR
751	<b>KCPENFNEAK</b>	FIGFTMYTTC	IIWLAFLPIF	YVTSSDYRVQ	TTTMCVSVSL
801	SGSVVLGCLF	APKL <b>HILFQ</b>	<b>PQKNVVSRA</b>	<b>PT</b> <sup>833</sup> <b>RFG</b> <sup>837</sup> <b>AAA</b>	<b>RA</b> <sup>843</sup> <b>S</b> <sup>844</sup> <b>SLGQGS</b>
851	<b>GSQFVPTVCN</b>	<b>GREVDSTTS</b>	<b>S</b> <sup>871</sup> <b>L</b>		

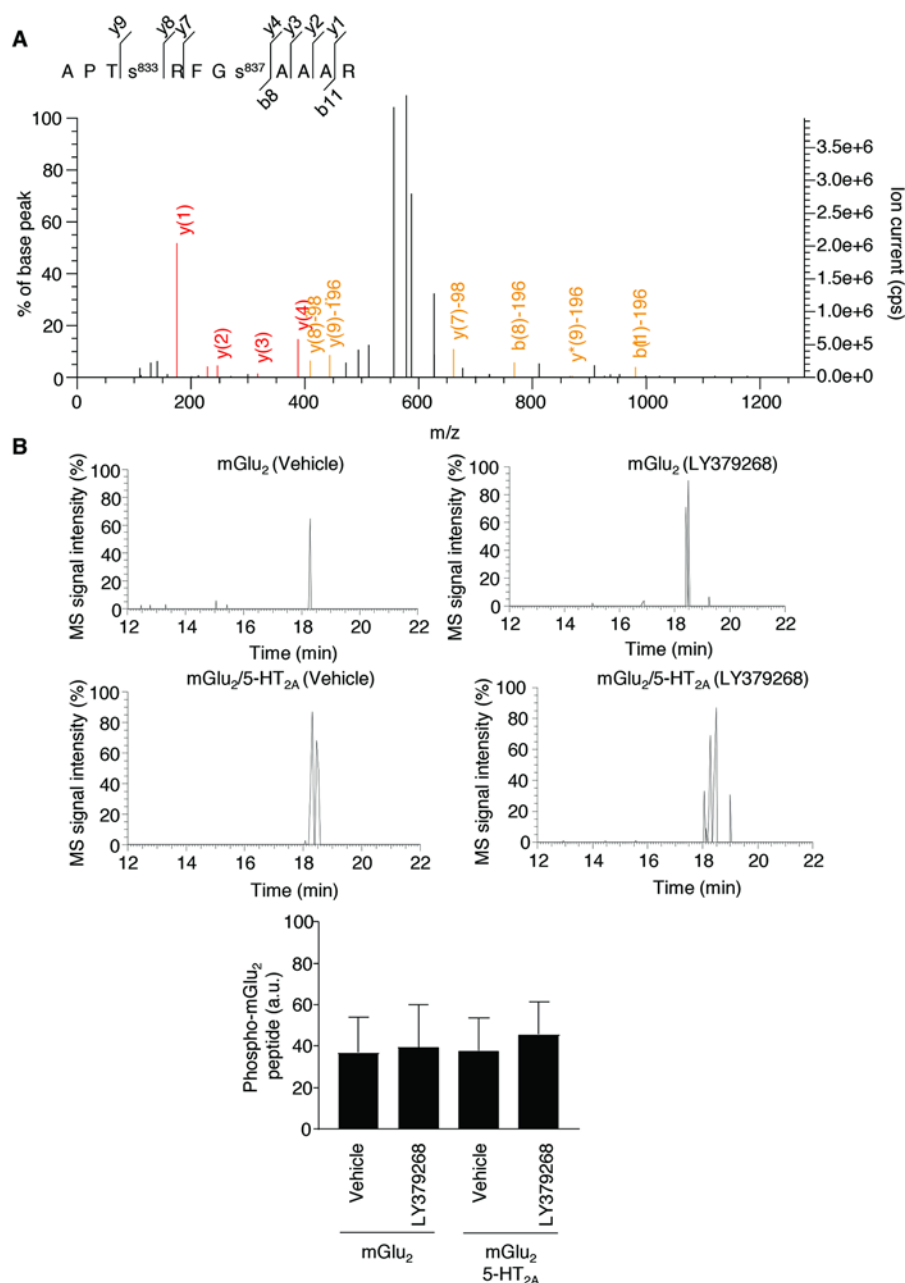
mGlu<sub>2</sub> receptor coverage (Rat) : 40% (exp pvalue cut off <0.05)

1	MESLLGFLAL	LLWGAFAEG	PAK <b>KVLTLEG</b>	<b>DLVLGGLFPV</b>	<b>HQKGGPAEEC</b>
51	<b>GPVNEHRGIQ</b>	<b>RLEAMLFALD</b>	<b>RINRDPHLLP</b>	<b>GVRLGAHILD</b>	<b>SCSKDTHALE</b>
101	<b>QALDFVRASL</b>	<b>SRGADGSRHI</b>	<b>CPDGSYATHS</b>	<b>DAPTAITGVI</b>	<b>GGSYSDVSIQ</b>
151	<b>VANLLRLFQI</b>	<b>PQISYASTSA</b>	<b>KLSDKSRYDY</b>	<b>FARTVPPDFF</b>	<b>QAKAMAEILR</b>
201	FFNWTYVSTV	ASEGDYGETG	IEAFELEARA	RNICVATSEK	VGRAMSRAAF
251	<b>EGVVRALLQK</b>	<b>PSARVAVLFT</b>	<b>RSEDARELLA</b>	<b>ATQRLNASFT</b>	WVASDGGWAL
301	ESVVASSEGA	AEGAITIELA	SYPIDFASY	FQSLDPWNNS	RNPWFREFWE
351	<b>ERFHCSFRQR</b>	<b>DCAAHSLRAV</b>	<b>PFEQESKIMF</b>	<b>VVNAVYAMAH</b>	<b>ALHNMHRALC</b>
401	PNTT <b>HLCDAM</b>	<b>RPVNGRRLYK</b>	<b>DFVLNVKFDA</b>	<b>PFRPADTDDE</b>	<b>VRFDRLFQGI</b>
451	<b>GRYNIFTYLR</b>	AGSGRYRYQK	<b>VGWAEGLTL</b>	<b>DTSFIPWASP</b>	<b>SAGPLPASRC</b>
501	<b>SEPCLQNEVK</b>	<b>SVQPGEVCCW</b>	<b>LCIPCQPYEY</b>	<b>RLDEFTCADC</b>	GLGYWPNASL
551	TGCFELPQY	<b>IRWGDAWAVG</b>	<b>PVTIACLGAL</b>	<b>ATLFVLGVFV</b>	<b>RHNATPVVKA</b>
601	SGRELCYILL	GGVFLCYCMT	FVFIAPSTA	VCTLRRLGLG	TAFSVCYSAL
651	LTKTNRIARI	FGGAREGAQR	PR <b>FISPASQV</b>	<b>AICLALISGQ</b>	<b>LLIVAAWLIV</b>
701	<b>EAPGTGKETA</b>	PERREVVTLR	CNHRDASMLG	SLAYNVLLIA	LCTLYAFKTR
751	<b>KCPENFNEAK</b>	FIGFTMYTTC	IIWLAFLPIF	YVTSSDYRVQ	TTTMCVSVSL
801	SGSVVLGCLF	APKL <b>HILFQ</b>	<b>PQKNVVSRA</b>	<b>PTSRFGSAAP</b>	<b>RA</b> <sup>843</sup> <b>ANLGQGS</b>
851	<b>GSQFVPTVCN</b>	<b>GREVDSTTS</b>	<b>S</b> <sup>871</sup> <b>L</b>		

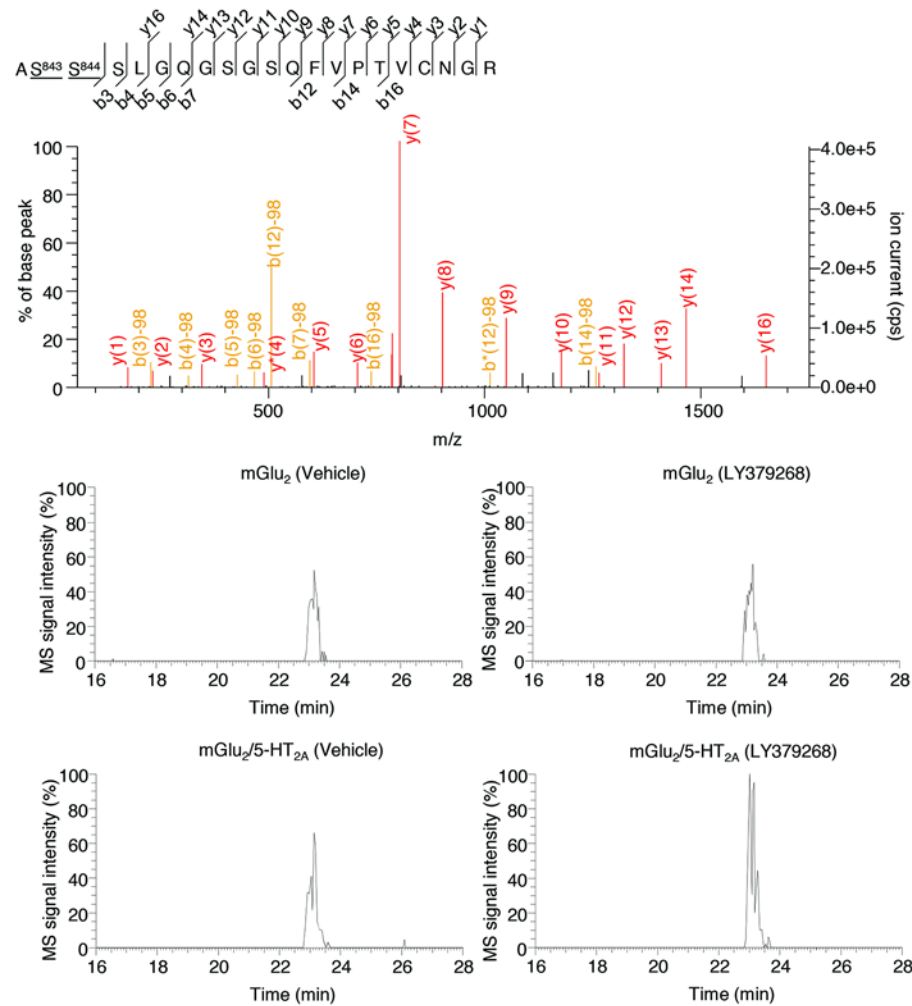
**Supplementary Figure 1. Sequence coverage of human and rat mGlu<sub>2</sub> receptors obtained by LC-MS/MS.** The sequence covered by LC-MS/MS analysis is highlighted in red. Identified phosphorylated residues in human mGlu<sub>2</sub> receptor (Thr<sup>718</sup>, Ser<sup>833</sup>, Ser<sup>837</sup>, Ser<sup>843/844</sup>, Ser<sup>871</sup>) and rat mGlu<sub>2</sub> receptor (Ser<sup>843</sup>, Ser<sup>871</sup>) and their positions are highlighted in blue.



**Supplementary Figure 2. Identification and quantification of the REVVt<sup>718</sup>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 mGlu<sub>2</sub> receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu<sub>2</sub> receptor alone or in combination with 5-HT<sub>2A</sub> receptor, and exposed to either vehicle or LY379268 (1  $\mu$ M) for 15 min. The histogram represents the means  $\pm$  SEM of ion signal intensities of REVVt<sup>718</sup>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.

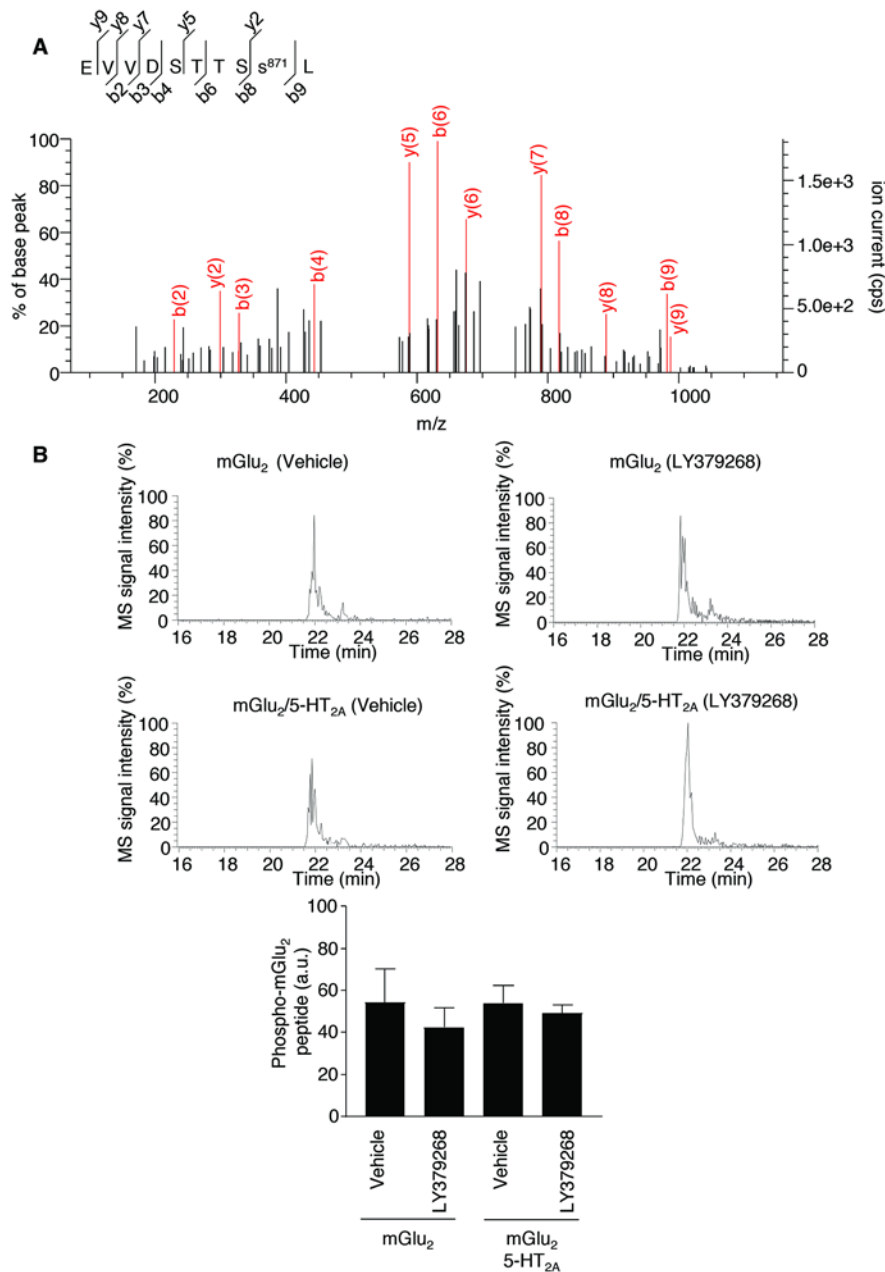


**Supplementary Figure 3. Identification and quantification of the APTs<sup>833</sup>RFGs<sup>837</sup>AAAR phosphorylated peptide by LC-MS.** **A**, The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu<sub>2</sub> receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu<sub>2</sub> receptor alone or in combination with 5-HT<sub>2A</sub> receptor, and exposed to either vehicle or LY379268 (1 μM) for 15 min. The histogram represents the means ± SEM of ion signal intensities of APTs<sup>833</sup>RFGs<sup>837</sup>AAAR 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.



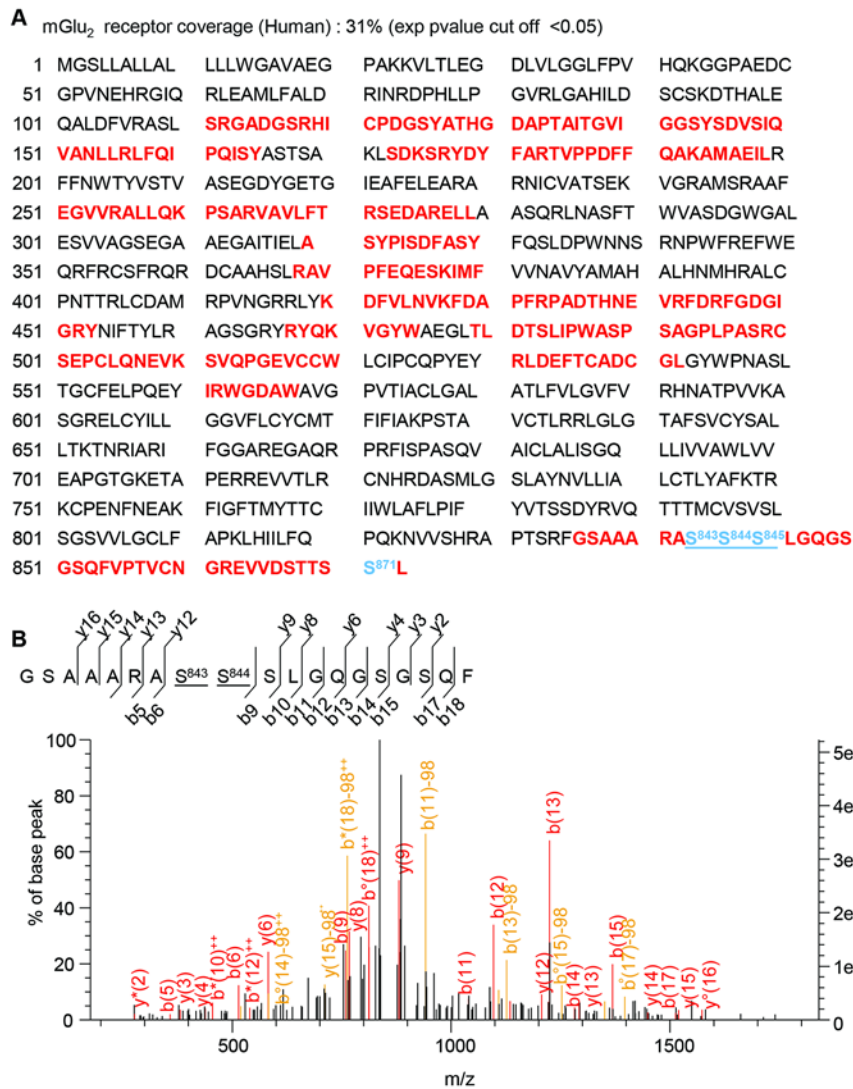
**Supplementary Figure 4. Identification and quantification of the AS<sup>843</sup>S<sup>844</sup>SLGQGSGSQFVPTVCNGR phosphorylated peptide by LC-MS.** The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu<sub>2</sub> receptor are illustrated (top panel). The bottom panels show representative extracted ion chromatograms of the peptide in cells expressing mGlu<sub>2</sub> receptor alone or in combination with 5-HT<sub>2A</sub> receptor, and exposed to either vehicle or LY379268 (1  $\mu$ M) for 15 min.



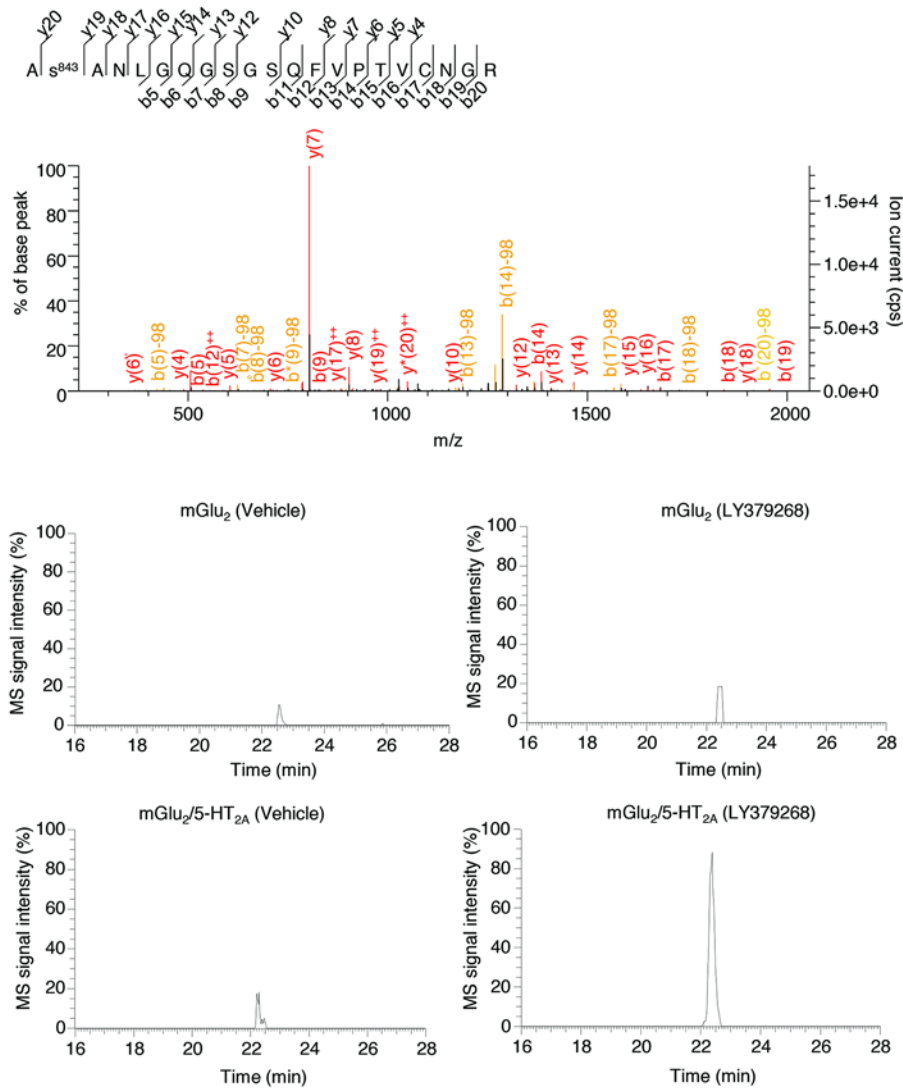


**Supplementary Figure 5. Identification and quantification of the EVVDSTTs<sup>871</sup>L phosphorylated peptide by LC-MS.** **A**, The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length human mGlu<sub>2</sub> receptor are illustrated. **B**, Representative extracted ion chromatograms of this peptide in cells expressing mGlu<sub>2</sub> receptor alone or in combination with 5-HT<sub>2A</sub> receptor, and exposed to either vehicle or LY379268 (1  $\mu$ M) for 15 min. The histogram represents the means  $\pm$  SEM of ion signal intensities of EVVDSTTs<sup>871</sup>L 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.

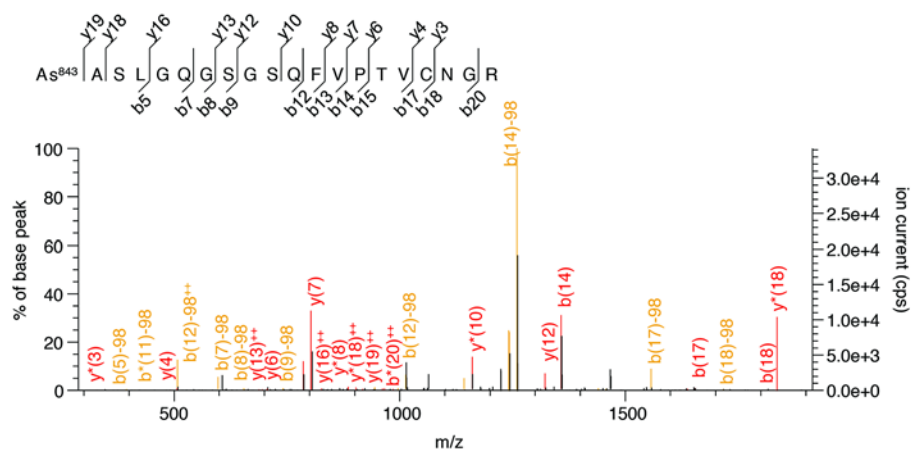




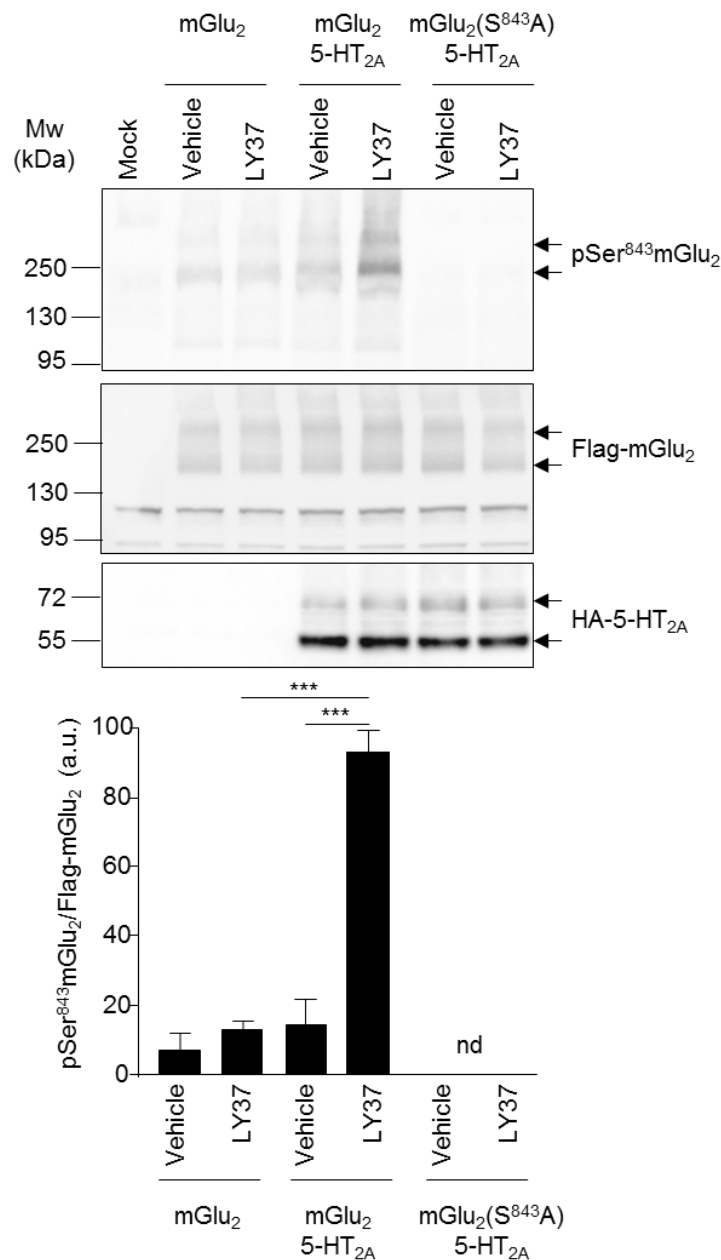
**Supplementary Figure 6. LC-MS analysis of human mGlu<sub>2</sub> receptor digested with chymotrypsin.** **A**, The sequence covered by LC-MS/MS analysis after *in-gel* digestion of human mGlu<sub>2</sub> receptor with chymotrypsin is highlighted in red. Identified phosphorylated residues and their positions in the protein sequence are highlighted in blue. **B**, The MS/MS spectrum of the GSAAARAS<sup>843</sup>S<sup>844</sup>SLGQGSGSQF 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.



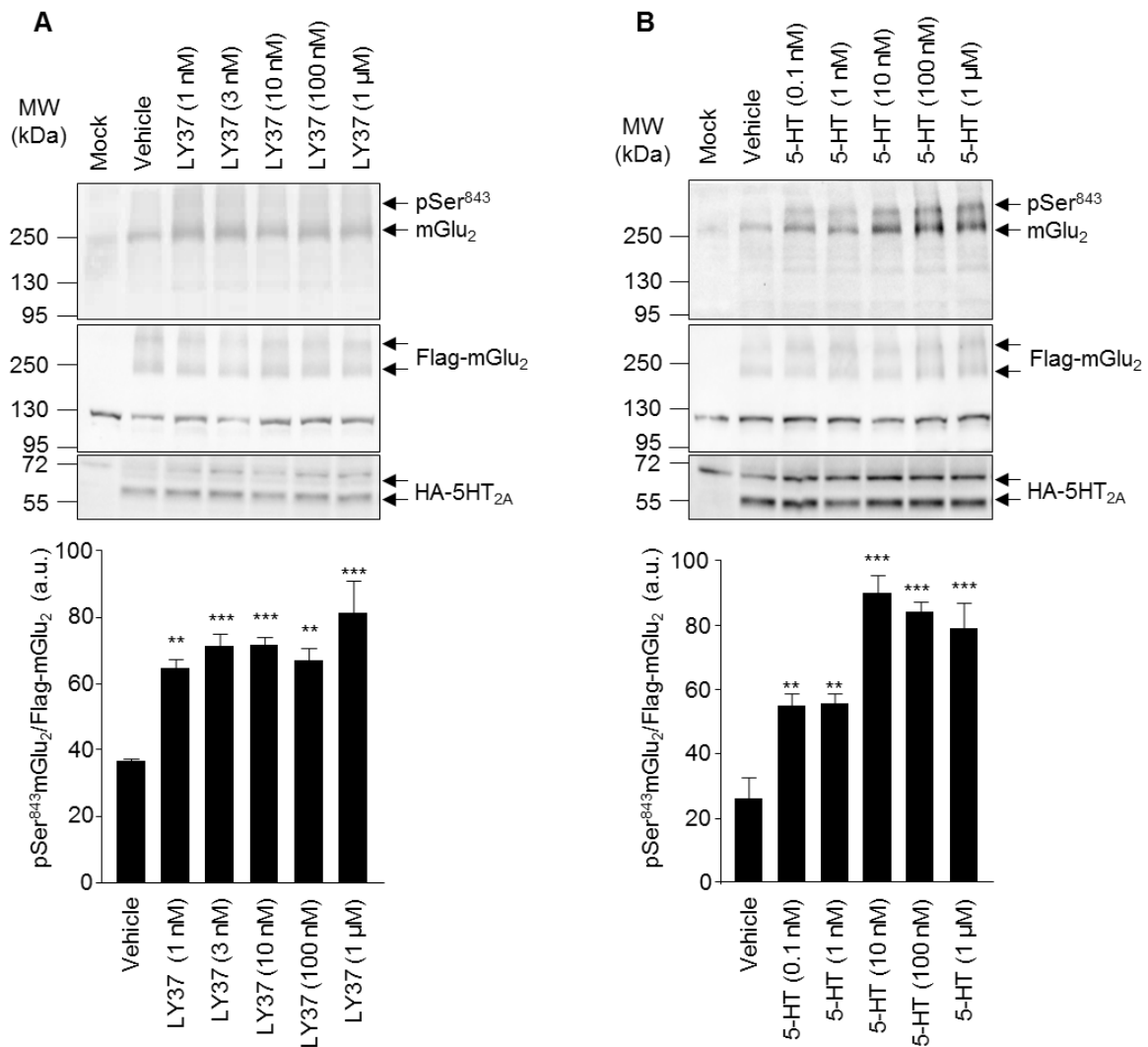
**Supplementary Figure 7. Identification and quantification of the  $\text{As}^{843}\text{ANLGQSGSGSQFVPTVCNGR}$  phosphorylated peptide by LC-MS.** The MS/MS spectrum that yielded the highest Mascot score, matched b and y ions, peptide sequence and position of the phosphorylated residue in the full-length rat mGlu<sub>2</sub> receptor are illustrated (top panel). The bottom panels show representative extracted ion chromatograms of the  $\text{As}^{843}\text{ANLGQSGSGSQFVPTVCNGR}$  peptide in cells expressing mGlu<sub>2</sub> receptor alone or in combination with 5-HT<sub>2A</sub> receptor and treated with either vehicle or LY379268 (1  $\mu\text{M}$ ) for 15 min.



**Supplementary Figure 8. Identification of the As<sup>843</sup>ASLGQSGSQFVPTVCNGR phosphorylated peptide in S<sup>844</sup>A mGlu<sub>2</sub> 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<sup>844</sup>A mGlu<sub>2</sub> receptor are illustrated.



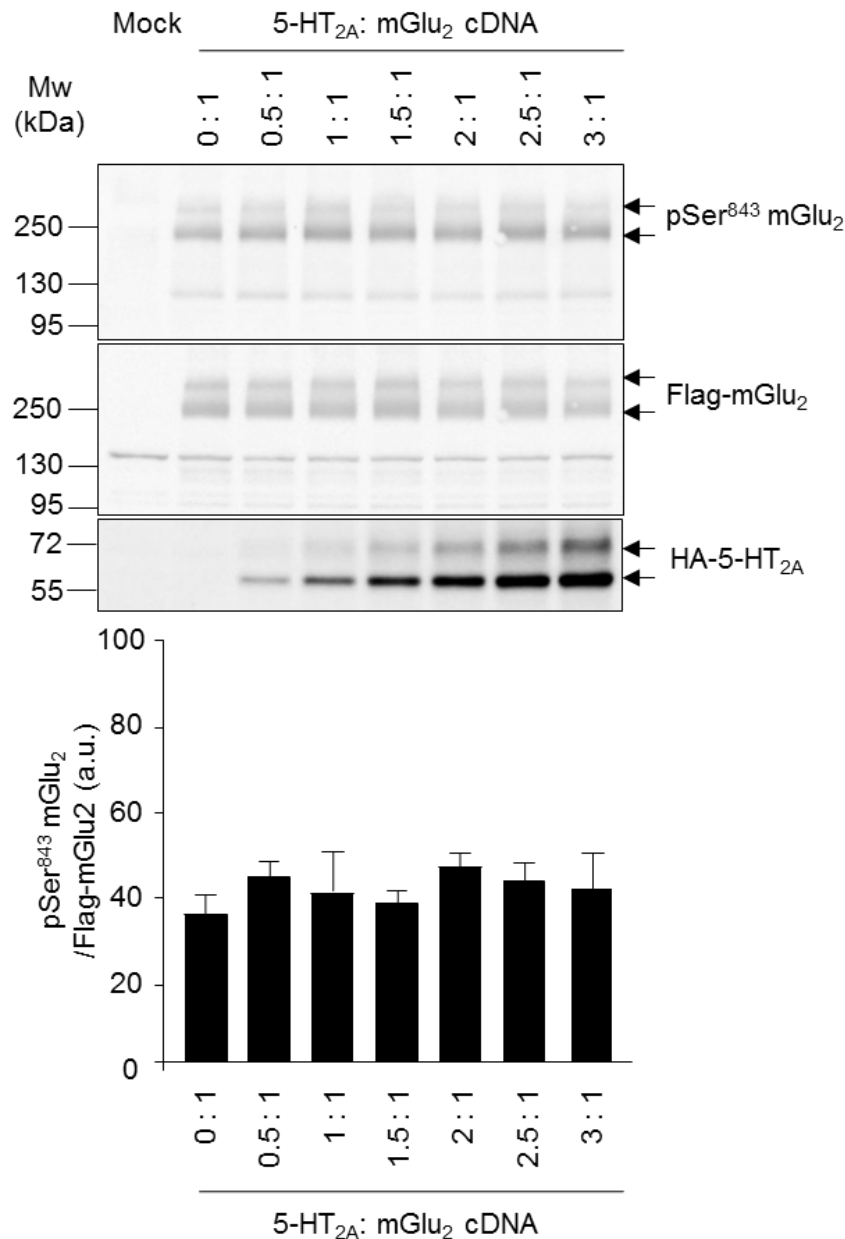
**Supplementary Figure 9. Validation of the anti-phospho-Ser<sup>843</sup> mGlu<sub>2</sub> receptor antibody by Western blotting.** HEK-293 cells transiently expressing rat Flag-tagged mGlu<sub>2</sub> receptor alone or in combination with HA-tagged 5-HT<sub>2A</sub> receptor or co-expressing rat Flag-tagged S<sup>843</sup>A mGlu<sub>2</sub> receptor and HA-tagged 5-HT<sub>2A</sub> receptor were exposed to vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. mGlu<sub>2</sub> 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 mGlu<sub>2</sub> receptors at a molecular weight corresponding to the signal obtained with the anti-Flag antibody. The histograms show the means  $\pm$  SEM of the anti-phospho-Ser<sup>843</sup> mGlu<sub>2</sub> 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.



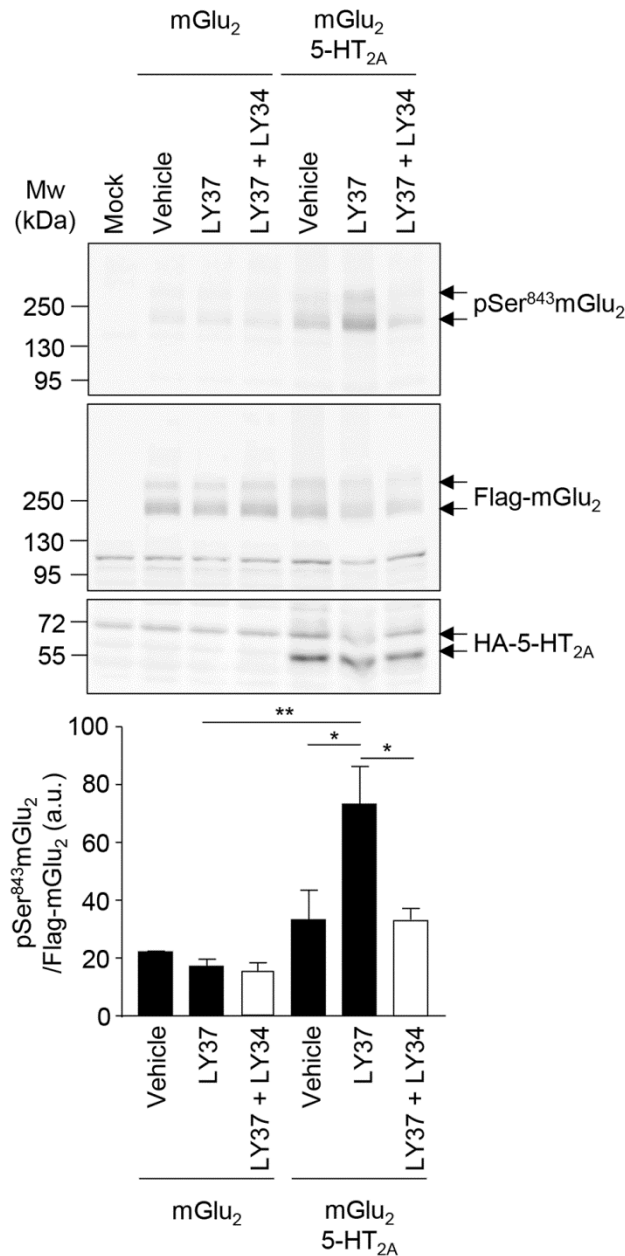
### Supplementary Figure 10. Effects of increasing concentrations of LY379268 and 5-HT

**on Ser<sup>843</sup> phosphorylation.** **A**, HEK-293 cells co-expressing Flag-tagged mGlu<sub>2</sub> receptor and HA-tagged 5-HT<sub>2A</sub> receptor were exposed to vehicle or increasing concentrations of LY379268 (LY37, 1 nM, 3 nM, 10 nM, 100 nM, 1  $\mu$ 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  $\mu$ M) for 15 min. Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> receptor antibody and mGlu<sub>2</sub> 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<sup>843</sup> mGlu<sub>2</sub> 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.

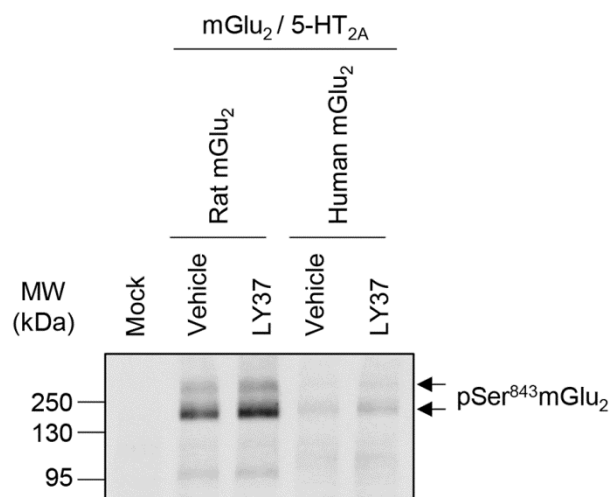
\*p<0.05 vs. corresponding vehicle conditions.



**Supplementary Figure 11. Basal mGlu<sub>2</sub> receptor Ser<sup>843</sup> phosphorylation level does not depend on 5-HT<sub>2A</sub> receptor expression level.** HEK-293 cells co-expressing rat Flag-tagged mGlu<sub>2</sub> receptor and increasing amounts of HA-tagged 5-HT<sub>2A</sub> receptor were challenged with vehicle or LY379268 for 15 min. Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> 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-Ser<sup>843</sup> mGlu<sub>2</sub> receptor immunoreactive signal relative to Flag immunoreactive signal (expressed in arbitrary units, a.u.). One-way ANOVA (Post-hoc: Tukey's range test):  $F(6,14)=0.4493$ ,  $p=0.8338$ .

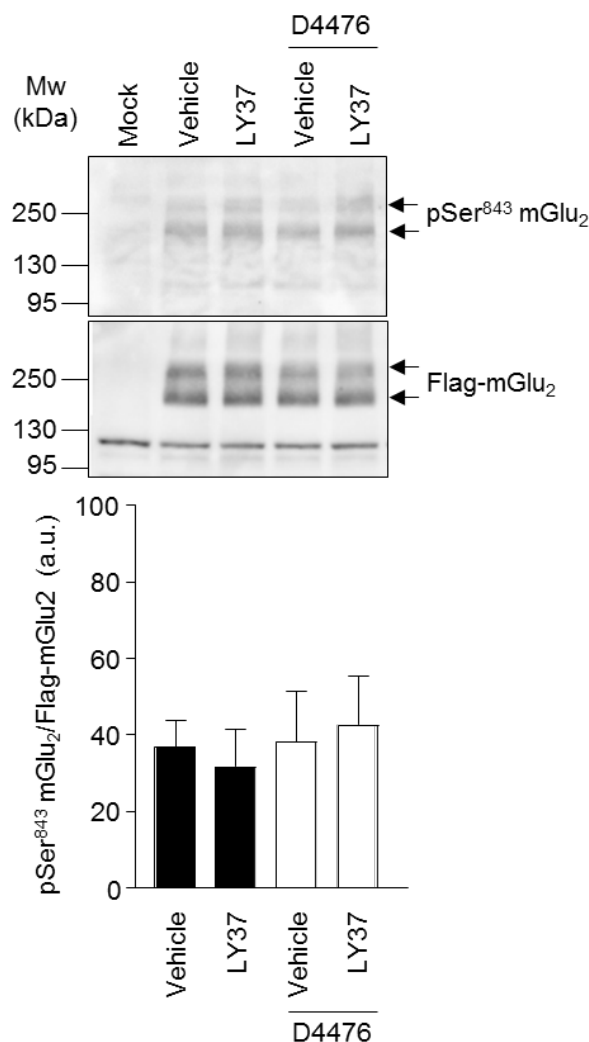


**Supplementary Figure 12. LY379268-elicited mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup> in cells co-expressing 5-HT<sub>2A</sub> receptor is prevented by the mGlu<sub>2</sub> receptor antagonist LY341495.** HEK-293 cells transiently expressing rat Flag-tagged mGlu<sub>2</sub> receptor alone or in combination with HA-tagged 5-HT<sub>2A</sub> receptor were exposed to vehicle or LY379268 (LY37, 10 nM), in absence or presence of LY341495 (LY34, 10  $\mu$ M) for 15 min. mGlu<sub>2</sub> receptor phosphorylation was analyzed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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):  $F(5,12)=8.407$ ,  $p=0.0013$ . \* $p<0.05$ ; \*\* $p<0.01$ .

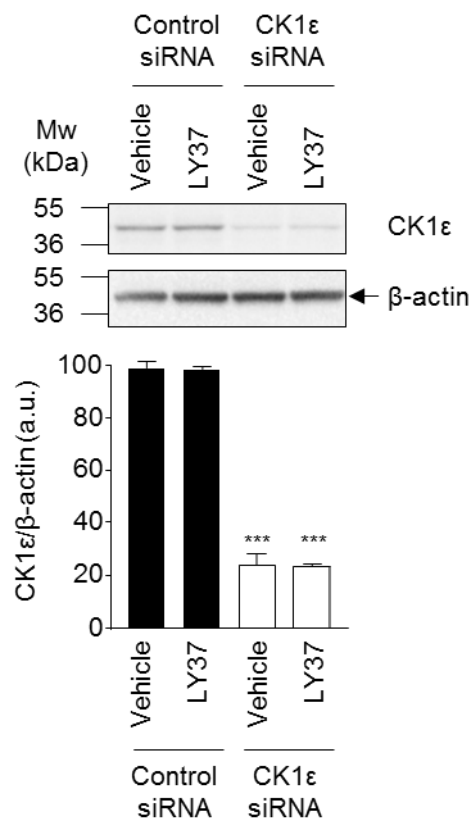


**Supplementary Figure 13. Western blot analysis of rat and human mGlu<sub>2</sub> receptor phosphorylation at Ser<sup>843</sup>.** HEK-293 cells co-expressing HA-tagged 5-HT<sub>2A</sub> receptor and either rat or human Flag-tagged mGlu<sub>2</sub> receptor were exposed to vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. The phosphorylation of rat and human mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was analyzed by Western blotting using the newly generated anti-phospho-Ser<sup>843</sup> mGlu<sub>2</sub> 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.

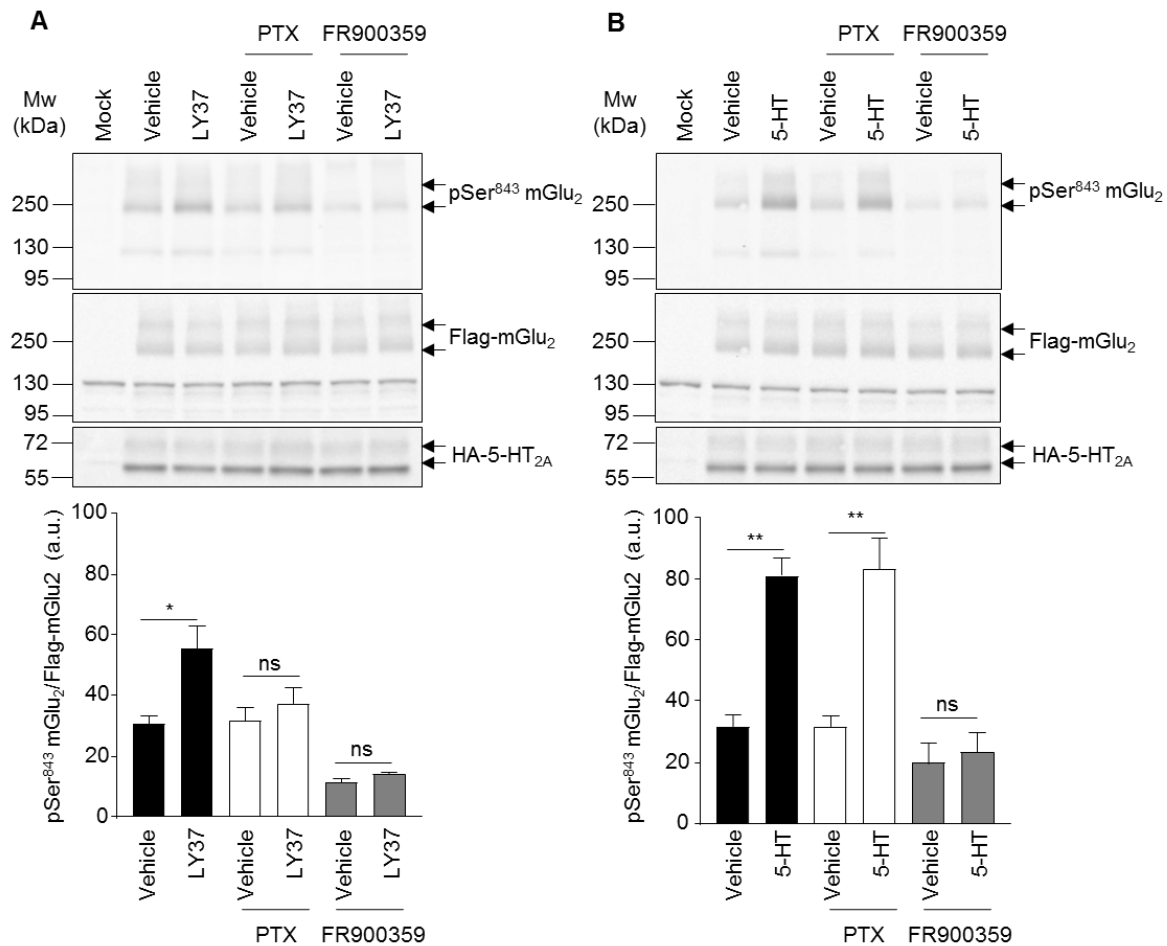




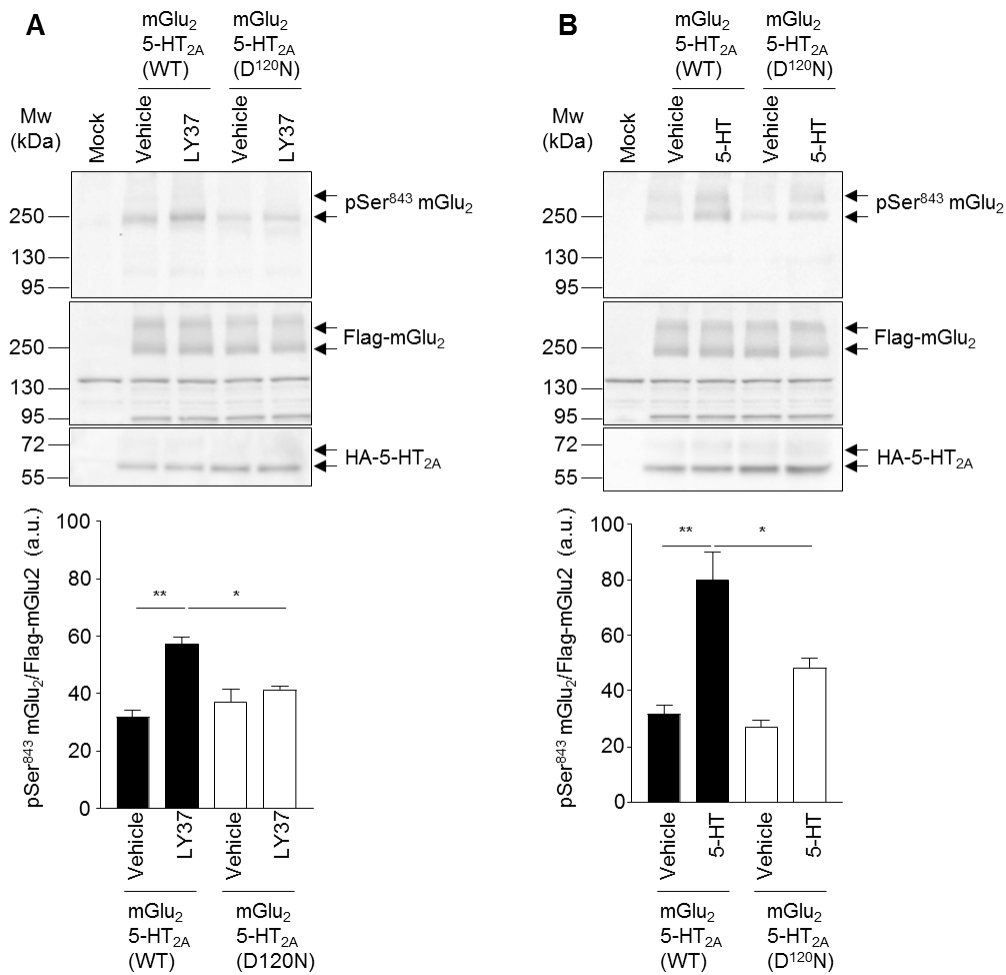
**Supplementary Figure 14. LY379268-induced Ser<sup>843</sup> phosphorylation in cells expressing mGlu<sub>2</sub> receptor alone is not mediated by casein kinase 1.** HEK-293 cells expressing rat Flag-tagged mGlu<sub>2</sub> alone were pre-incubated with either vehicle or the CK1 inhibitor (D4476, 10  $\mu$ M) for 60 min and then challenged with vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by sequential immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> 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-Ser<sup>843</sup> mGlu<sub>2</sub> 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):  $F(3,8)=0.1738$ ,  $p=0.9112$



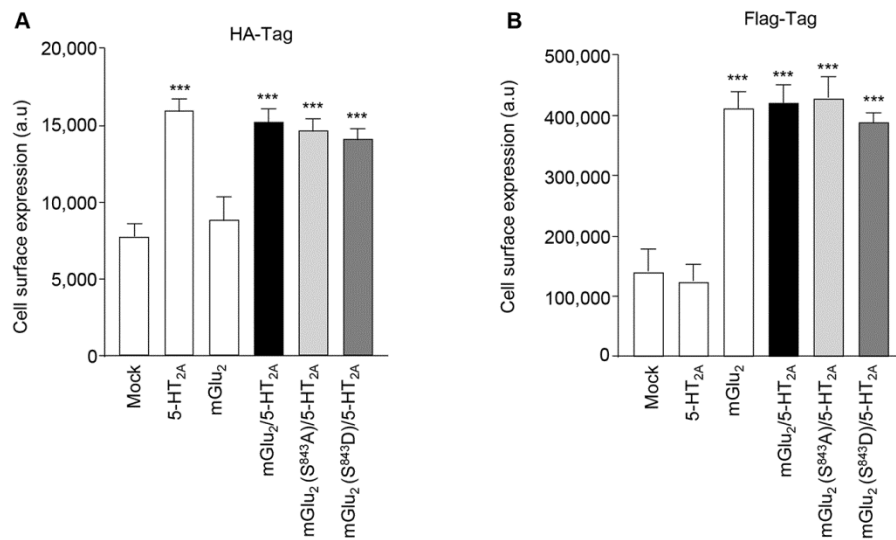
**Supplementary Figure 15. Silencing casein kinase 1ε (CK1ε) expression in HEK-293 cells by RNA interference.** HEK-293 cells co-expressing rat Flag-tagged mGlu<sub>2</sub> and HA-tagged 5-HT<sub>2A</sub> receptors were transfected with either control or CK1ε siRNA and challenged with vehicle or LY379268 (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<sub>2</sub> receptor at Ser<sup>843</sup>.** **A**, HEK-293 cells co-expressing rat Flag-tagged mGlu<sub>2</sub> and HA-tagged 5-HT<sub>2A</sub> receptors were pre-incubated with either vehicle or Pertussis toxin (PTX, 0.1  $\mu$ g/mL, 18 h) or FR900359 (1  $\mu$ M, 2 h) and challenged with either vehicle or LY379268 (LY37, 1  $\mu$ M, 15 min). **B**, HEK-293 cells were challenged with either vehicle or 5-HT (1  $\mu$ M, 15 min). Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> receptor antibody and mGlu<sub>2</sub> 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<sup>843</sup> mGlu<sub>2</sub> 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 Ser<sup>843</sup> phosphorylation in cells expressing G<sub>q</sub> signaling-dead D<sup>120N</sup> 5-HT<sub>2A</sub> receptor.** **A**, HEK-293 cells co-expressing rat Flag-tagged mGlu<sub>2</sub> in combination with HA-tagged wild type or D<sup>120N</sup> 5-HT<sub>2A</sub> receptor were treated with either vehicle or LY379268 (LY37, 1  $\mu$ M) for 15 min. **B**, HEK-293 cells were treated with either vehicle or 5-HT (1  $\mu$ M) for 15 min. Phosphorylation of mGlu<sub>2</sub> receptor at Ser<sup>843</sup> was assessed by immunoblotting with the anti-phospho-Ser<sup>843</sup>-mGlu<sub>2</sub> receptor antibody and mGlu<sub>2</sub> 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<sup>843</sup> mGlu<sub>2</sub> 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. Ser<sup>843</sup> phosphorylation does not affect cell surface expression of mGlu<sub>2</sub> and 5-HT<sub>2A</sub> receptors.** Quantification of cell surface expression of HA-tagged 5-HT<sub>2A</sub> receptors (A) and Flag-tagged mGlu<sub>2</sub> receptors (B) was performed by ELISA in non-permeabilizing conditions using anti-HA and anti-Flag antibodies, respectively, in cells expressing 5-HT<sub>2A</sub> or wild type mGlu<sub>2</sub> receptor alone or 5-HT<sub>2A</sub> receptor in combination with either wild type or S<sup>843</sup>A or S<sup>843</sup>D mGlu<sub>2</sub> 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).