Biochemical Pharmacology 185 (2021) 114440

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

His452Tyr polymorphism in the human 5-HT$_{2A}$ receptor affects clozapine-induced signaling networks revealed by quantitative phosphoproteomics

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ARTICLE INFO

Keywords:
5-HT$_{2A}$ receptor
G protein-coupled receptor (GPCR)
Polymorphism
Antipsychotics
Clozapine
Schizophrenia
Phosphoproteomics

ABSTRACT

Antipsychotic drugs remain the current standard for schizophrenia treatment. Although they directly recognize the orthosteric binding site of numerous monoaminergic G protein-coupled receptors (GPCRs), these drugs, and particularly second-generation antipsychotics such as clozapine, all have in common a very high affinity for the serotonin 5-HT$_{2A}$ receptor (5-HT$_{2A}$R). Using classical pharmacology and targeted signaling pathway assays, previous findings suggest that clozapine and other atypical antipsychotics behave principally as 5-HT$_{2A}$R neutral antagonists and/or inverse agonists. However, more recent findings showed that antipsychotics may also behave as pathway-specific agonists. Reversible phosphorylation is a common element in multiple signaling networks. Combining a quantitative phosphoproteomic method with signaling network analysis, we tested the effect of clozapine treatment on the overall level of protein phosphorylation and signal transduction cascades *in vitro* in mammalian cell lines induced to express either the human 5-HT$_{2A}$R or the H452Y variant of the gene encoding the *5-HT$_{2A}$R* receptor. This naturally occurring variation within the *5-HT$_{2A}$R* gene was selected because it has been repeatedly associated with schizophrenia patients who do not respond to clozapine treatment. Our data show that short time exposure (5 or 10 min) to clozapine (10^{-5} M) led to phosphorylation of numerous signaling components of pathways involved in processes such as endocytosis, ErbB signaling, insulin signaling or estrogen signaling. Cells induced to express the H452Y variant showed a different basal phosphoproteome, with increases in the phosphorylation of mTOR signaling components as a translationally relevant example. However, the effect of clozapine on the functional landscape of the phosphoproteome was significantly reduced in cells expressing the 5-HT$_{2A}$R-H452Y construct. Together, these findings suggest that clozapine behaves as an agonist inducing phosphorylation of numerous pathways downstream of the 5-HT$_{2A}$R, and that the single nucleotide polymorphism encoding 5-HT$_{2A}$R-H452Y affects these clozapine-induced phosphorylation-dependent signaling networks.

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https://doi.org/10.1016/j.bcp.2021.114440
Received 24 November 2020; Received in revised form 19 January 2021; Accepted 21 January 2021
Available online 1 February 2021

1. Introduction

G protein-coupled receptors (GPCRs) are plasma membrane proteins composed of seven transmembrane-spanning domains that transmit signaling inputs including light, hormones, peptides and neurotransmitters from the extracellular milieu to a profusion of intracellular signaling cascades [1]. Most of the previous studies related to GPCR-dependent signaling focused their interest on their principal immediate downstream target: the heterotrimeric guanine nucleotide proteins (G proteins). Heterotrimeric G proteins are divided into four families according to their primary sequence and functional properties (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$). The G_s proteins behave as stimulators of adenylyl cyclases (AC), resulting in increased levels of cyclic AMP (cAMP) and activation of protein kinase A (PKA), whereas the $G_{i/o}$ proteins are usually defined as inhibitory with AC and potassium channels as their main targets. The $G_{q/11}$ proteins stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and Ca^{2+} release from intracellular stores via the phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP_3) signaling pathway, whereas $G_{12/13}$ proteins act as activators of the RhoA small GTP binding protein and phospholipase D (PLD), which ultimately regulate cell shape and motility. Although these G protein-dependent mechanisms play fundamental roles in multiple cell processes, other pathways such as Akt, JAK-STAT, MAPK, mTOR, NF-κB, and TGF-β, are also crucial components of signaling cascades in multicellular organisms. It is then clear that the use of classical biochemical tools to study GPCR-dependent signaling, such as [^{35}S]GTPγS binding, AC activity, or Ca^{2+} release from the endoplasmic reticulum, may not provide sufficient information about how GPCR activation upon agonist binding affects the activity of multiple signaling cascades within individual cells. This is particularly relevant in light of relatively recent findings suggesting that GPCRs may also signal through G protein-independent pathways such as those regulated by β-arrestin [2]. The primary role of β-arrestin is to bind phosphorylated GPCRs to induce receptor uncoupling from heterotrimeric G proteins. More recent findings also suggest that β-arrestin can also function as an adaptor protein that associates the tyrosine kinase Src via SH_3 domain interactions, which leads to activation of the MAPK (mitogen-activated protein kinase) signaling cascade. However, most of the studies focused on GPCRs and β-arrestin use as readouts experimental assays that test direct binding between GPCR and β-arrestin using biophysical assays such bioluminescence resonance energy transfer (BRET), yet do not directly test whether this recruitment of β-arrestin affects signaling downstream [3]. Together, these findings point to the need of further investigations about signaling networks affected by GPCRs in living mammalian cells [1].

The serotonin (or 5-hydroxytryptamine, 5-HT) 5-HT_{2A} receptor (5-HT_{2A}R) is a class A GPCR whose canonical pathway upon agonist administration involves activation of $G_{q/11}$ proteins. This receptor is the target responsible for most of the signaling and behavioral effects induced by psychedelic drugs, such as lysergic acid diethylamide (LSD) and psilocybin and its active compound psilocin [4]. Additionally, drugs clinically used to reduce psychosis in patients with neuropsychiatric conditions, such as schizophrenia, Parkinson's disease and bipolar disorder, show a high affinity for the 5-HT_{2A}R [5,6]. When testing canonical pathways downstream of 5-HT_{2A}R, most of these second generation of atypical antipsychotic medications, such as clozapine, olanzapine and risperidone, behave as either neutral antagonists or inverse agonists [7–9]. However, most recent findings have suggested that, either indirectly through a physical interaction with other GPCRs, or directly via the 5-HT_{2A}R, clozapine may show agonistic properties in both heterologous expression systems and in vivo in rodent models. Thus, clozapine was able to potentiate $G_{i/o}$ protein-dependent signaling in cells expressing 5-HT_{2A}R and the $G_{i/o}$ protein-coupled metabotropic glutamate receptor 2 (mGluR2) as a GPCR heterocomplex [10]. Similarly, clozapine acts as an agonist at 5-HT_{2A}R to activate Akt via a β-arrestin-independent mechanism [11].

Clozapine was described in 1958 as a "tricyclic antidepressant but with neuroleptic properties". Today, clozapine is still an atypical antipsychotic that has been demonstrated to be superior for the treatment of refractory schizophrenia patients [7]. Interestingly, a naturally occurring single nucleotide polymorphism (SNP) within the *5-HT_{2A}R* (*Htr2a*) gene has repeatedly been associated with poor clozapine treatment response. Thus, schizophrenia patients carrying a H452Y polymorphism (rs6314) at the *5-HT_{2A}R* gene responded less to clozapine treatment, demonstrating that the Tyr452 allele occurred more frequently in the non-responders that in the responders [12,13]. Using a proteomic approach based on peptide affinity chromatography followed by mass spectrometry and immunoblotting, previous findings by other groups showed a battery of proteins that interact with the C-terminal tail of the 5-HT_{2C}R in mouse choroid plexus samples [14]. Although interesting, these studies were carried out under steady-state conditions, and therefore the effect of drug administration to the signaling machinery downstream of 5-HT_{2A}R remains largely unexplored. Using transfected NIH3T3 mammalian cells as an experimental system, previous findings convincingly demonstrated that the H452Y polymorphism affects canonical pathways downstream 5-HT_{2A}R, such as heterotrimeric G protein activation and phosphoinositide hydrolysis [15]. Herein we extend previous findings regarding the molecular pharmacology characterization of 5-HT_{2A}R-H452Y expressed in mammalian cells. In addition, using a mass-spectometry (MS)-based phosphoproteomics method that enables the identification of thousands of phosphorylated peptides in a single experiment, we tested the effect of clozapine on kinase network statuses in HEK293 cells stably expressing 5-HT_{2A}R or the variant 5-HT_{2A}R-H452Y.

2. Materials and methods

2.1. Materials

All the reagents for tissue culture procedures were obtained from Life Technologies (Waltham, MA, USA). [^{3}H]ketanserin, [^{3}H]*myo*-inositol (20.3 Ci/mmol) and RNA Binding YSi SPA Beads were purchased from PerkinElmer Life Science (Waltham, MA, USA). Doxycycline and lysergic acid diethylamide (LSD) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 5-hydroxytryptamine (5-HT), (±)2,5-dimethoxy-4-iodoamphetamine (DOI), mianserin and clozapine were purchased from Tocris (Minneapolis, MN, USA).

2.2. Receptor fusions with fluorescent proteins and site-directed mutagenesis

Modifications and subcloning of the human version of 5-HT_{2A}R was essentially as previously described [16]. Briefly, a c-Myc epitope was tagged at the amino terminus of the receptor by PCR techniques using a forward primer containing the sequence of the c-Myc (EQLISEEDL). Fusion of the enhanced cyan fluorescent protein (eCFP) to the C-terminus of 5-HT_{2A}R was made by removing the stop codon of the receptor sequence by PCR to subsequently proceed to the ligation of this fragment into the eCFP sequence in order to obtain a unique open reading frame encoding the receptor-fluorescent protein fusion. The polymorphic version of the human 5-HT_{2A}R consisting in the substitution of a histidine at position 452 by a tyrosine was generated from the original c-Myc-5-HT_{2A}R-eCFP construct. Firstly, the sequence corresponding to eCFP was removed and replaced by an equivalent PCR product containing the sequence of the enhanced yellow fluorescent protein (eYFP). Secondly, to produce the H452Y amino acid substitution in the primary structure of the c-Myc-5-HT_{2A}R-eYFP fusion, site-directed mutagenesis of the encoding nucleotide sequence was performed using the QuickChange II site-directed mutagenesis kit (Agilent Technologies. Santa Clara, CA, USA) according to the manufacturer's instructions. The following primers were designed for the mutagenesis of 5-HT_{2A}R: GTTGCTCTAGGAAAGCAGTATTCTGAAGAGGCTTCTAAA (forward) and TTTAAAAGCCTCTTCAGAATACTGCTTTCCTAGAGCAAC (reverse)

that substitute His (CAT) by Tyr (TAT) at position 452 in the C-terminus of the human 5-HT_{2A}R. Both constructs, i.e. c-Myc-5-HT_{2A}R-eCFP (from now on referred as 5-HT_{2A}R) and c-Myc-5-HT_{2A}R-H452Y-eYFP (from now on referred as 5-HT_{2A}R-H452Y), were subcloned into the vector pcDNA5/FRT/TO (Life Technologies. Waltham, MA, USA) for the subsequent generation of Flp-In T-Rex HEK293 cell lines. All the constructs were verified by DNA sequencing.

2.3. Generation of stable Flp-In T-Rex HEK293 cell lines

Generation of the stable cell line permanently expressing 5-HT_{2A}R in an inducible manner was previously described [16]. Similarly, in order to obtain an equivalent cell line expressing 5-HT_{2A}R-H452Y host cells were co-transfected with a mixture of pcDNA5/FRT/TO vector containing the c-Myc-5-HT_{2A}R-H452Y-eYFP construct and pOG44 vector at a 1:9 ratio using Effectene transfection reagent (QIAGEN. Hilden, Germany). pOG44 plasmid encodes for the expression of a recombinase that facilitates the integration of 5-HT_{2A}R-H452Y gene into the Flp recombination target (FRT) of the host cell line. Cell maintenance and selection was carried out as previously described [16]. Selection of positive clones harboring the inducible gene was made by resistance to blasticidin and expression of 5-HT_{2A}R-H452Y were screened by both fluorescence microscopy and Western blotting. To induce expression of 5-HT_{2A}R-H452Y cells were treated with varying concentrations of doxycycline for different periods. The optimal expression of 5-HT_{2A}R-H452Y was observed after 24 h of treatment using 0.01 μg of doxycycline per ml in growth medium. Dialyzed fetal calf serum was used for cell growth to avoid activation of 5-HT_{2A}R and 5-HT_{2A}R-H452Y by 5-HT that is routinely present in serum.

2.4. Living cell epifluoresence microscopy

Cells expressing receptor constructs fused to either eCFP or eYFP were grown on poly-D-lysine-treated coverslips. The coverslips were placed into a microscope chamber containing physiological solution (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES and 10 mM D-glucose, pH 7.4). Fluorescent images of the cells were acquired using a TE2000-E inverted microscope (Nikon. Minato, Tokio, Japan) equipped with a 40× (numerical aperture, 1.3) oil immersion Plan Fluor lens and a cooled digital CoolSNAP$_{HQ}$ charge-coupled device camera (Photometrics. Thousand Oaks, CA, USA).

2.5. $[Ca^{2+}]_i$ ratio imaging

Cells were plated onto poly-D-lysine-coated coverslips and fed with the Ca^{2+} sensitive dye Fura-2 acetoxymethyl ester (1.5 μM) (Invitrogen, Molecular Probes. Waltham, MA, USA) by incubation for 30 min at 37 °C under reduced light conditions in Dulbecco's modified Eagle's medium. In the epifluorescence microscope system, Optoscan monochromator was used to rapidly alternate the excitation wavelength between 340 nm and 380 nm and to control the excitation bandpass (340 nm, band pass = 10 nm; 380 nm, band pass = 8 nm). Fura-2 fluorescence emission at 510 nm was monitored using a cooled digital CoolSNAP$_{HQ}$ charge-coupled device camera (Photometrics. Thousand Oaks, CA, USA). Metafluor imaging software was used for control of all electronic hardware and image-data processing. Sequential images (3 × 3 binning) were collected every 2 s, exposure to excitation light was 100 ms/image and all experiments were undertaken in HEPES-buffered saline solution (see composition above). Image analysis was performed as described previously [17].

2.6. Measurement of inositol phosphates accumulation

Cells were seeded in 96-well plates at a density of 6.5 × 10^4 cells/well. After 24 h, the medium was replaced by another one containing 10 μCi/ml of [^{3}H]*myo*-inositol (20.3 Ci/mmol) and 10 ng/ml doxycycline. 24 h later, cells were washed with experimental medium (Hank's Balanced Salt Solution supplemented with 20 mM HEPES, 20 mM LiCl and 2% BSA, pH 7.55) for 10 min, and then were incubated 20 min with the different drugs. Subsequently cells were lysed with 200 μl of 100 mM formic acid for 30 min at 4 °C, and an aliquot of 20 μl of lysate was mixed with 80 μl of a solution of RNA Binding YSi SPA beads. The radioactivity was quantified with a liquid scintillation counter (Microbeta^2 microplate counter, PerkinElmer. Waltham, MA, USA).

2.7. Cell membrane preparation

Harvested pellets from both cell lines kept at −80 °C were thawed and resuspended in 10 mM Tris and 0.1 mM EDTA, pH 7.4 (Tris/EDTA buffer). Cells were homogenized by 25 passes of a glass-on Teflon homogenizer. The resulting suspension was centrifuged at 1200×*g* for 10 min in order to remove unbroken cells and nuclei. The supernatant was subsequently centrifuged at 218,000×*g* for 30 min in an Optima TLX ultracentrifuge (Beckman Coulter. Brea, CA, USA). Resulting pellets were resuspended in Tris/EDTA buffer and passed 10 times through a 25-gauge needle. Protein concentration was determined and the resulting membranes stored at −80 °C until used.

2.8. [^{3}H]ketanserin binding assays

Binding assays were initiated by the addition of 15 to 20 μg of cell membranes to assay buffer (50 mM Tris-HCl, 100 mM Na Cl and 3 mM $MgCl_2$, pH 7.4) containing [^{3}H]ketanserin (0.2–20 nM). Non-specific binding was determined in the presence of 10 μM mianserin. Reactions were incubated for 60 min at 25 °C and bound ligand was separated from free ligand by vacuum filtration through GF/B filters (Whatman. Maidstone, UK) by using a cell harvester (Brandel Inc. Gaithersburg, MD, USA). The filters were washed twice with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4 and 8 mM Na_2HPO_4) and bound ligand was estimated by liquid scintillation spectrometry.

2.9. Measurement of ERK stimulation

Cells were plated on poly-D-lysine-coated 96-well plates at 4 × 10^4 cells/well and simultaneous induced with doxycycline as required. Before stimulation, cells were serum starved for 4 h in cell culture medium devoid of dialyzed fetal bovine serum. Stimulation of cells with the different drugs was arrested on ice and cells were washed twice with ice-cold phosphate-buffered saline. For Alpha SureFire pERK determination (Perkin Elmer. Waltham, MA, USA) cells were lysed in appropriate lysis buffer provided with the kits and samples were processed according to the manufacturer instructions. Time course experiments were conducted by stimulating cells with 5-HT (10^{-6} M) or DOI (10^{-6} M) during 5, 10, 15 30, 45, 60, 90, 120 and 180 min. In the case of clozapine (10^{-5} M), cells were treated for 5, 10 and 30 min. Each point was done by duplicate and at least five independent assays per agonist were carried out.

2.10. Cell treatments and sample preparations for phosphoproteomics analysis

Phosphoproteomics experiments were carried out as described before ([18]). Before treatments with clozapine, doxycyclin (10 ng/ml) was added and incubated for 24 h to induce the expression of both receptors. Expression of both receptor forms was evaluated by fluorescence microscope visualization. Next, cells were incubated for 6 h in free-serum medium and clozapine at 10^{-5} M was added for 5 or 10 min. Cells treated with the same concentration of vehicle (DMSO) were used as controls. In brief, cells from four independent experiments were lysed in urea buffer (8 M urea in 200 mM HEPES pH 8.0) containing phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF, 1 mM β-glycerol-phosphate and 2.5 mM Na_4P_2O7) and homogenized by sonication (30 cycles

of 30 s on 30 s off; Diagenode Bioruptor® Plus, Liege, Belgium). Insoluble material was removed by centrifugation at 20,000×*g* for 10 min at 5 °C and protein in the cell extracts was quantified by bicinchoninic acid (BCA) analysis. 200 μg of extracted proteins were reduced with dithiotreitol (DTT, 10 mM) for 1 h at 25 °C and alquilated with Iodoacetamide (IAM, 16.6 mM) for 30 min at 25 °C. Then, samples were diluted with 20 mM HEPES (pH 8.0) to a final concentration of 2 M urea and digested with equilibrated trypsin beads (100 μl of 50% slurry of TLCK-trypsin per sample, Thermo-Fisher Scientific. Waltham, MA, USA) overnight at 37 °C (Trypsin beads were equilibrated by three washes with 20 mM HEPES; pH 8.0). After that, trypsin beads were removed by centrifugation (2000×*g* for 5 min at 5 °C) and samples were desalted using OASIS cartridges (10 mg OASIS-HLB cartridges, Waters, Manchester, UK). Columns were activated with acetonitrile (ACN) and equilibrated with a desalting washing solution (1% ACN, 0.1% trifluoroacetic acid, TFA), then, samples were loaded into the cartridges and washed twice with the desalting washing solution. Finally, peptides were eluted with glycolic solution 1 (1 M Glycolic acid, 50% ACN, 5% TFA) and subjected to phosphoenrichment with TiO_2. Sample volumes were normalized to 1 ml with glycolic solution 2 (1 M Glycolic acid, 80% ACN, 5% TFA) and incubated for 5 min with TiO_2 (50 μl per sample of 500 μg/ml of TiO_2 in 1% TFA). Afterwards, samples were loaded in empty spin tips by centrifugation for 30 s at 1500×*g*. Samples were sequentially washed by centrifugation with glycolic solution 2 and phosphoenrichment washing solutions 1 (100 mM ammonium acetate in 25% ACN) and 2 (10% ACN). After that, phosphopeptides were eluted 4 times with elution solution (5% NH4OH). Finally, eluents were snap frozen in dry ice for 15 min, dried in a speed vac overnight and phosphopeptide pellets stored at −80 °C. Peptide pellets were resuspended in 13 μl of reconstitution buffer (20 fmol/μl enolase in 3% ACN, 0.1% TFA), and 5 μl were loaded in an LC-MS platform consisting in a DionexUltiMate 3000 RSLC directly coupled to an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific. Waltham, MA, USA). Samples were run in two technical replicates using described settings [19].

2.11. Phosphoproteomic analysis

Mascot Demon was used to automate the identification of phosphopeptides [20]. Thus, Mascot Distiller (version 2.5.0) was used to generate the peak lists that were used by Mascot search engine. Searches were performed against the Swiss-Prot database (uniprot_sprot_2014_08.fasta) considering 2 trypsin missed cleavages, mass tolerance of ±10 ppm for the MS scans and ±25 mmu for the MS/MS scans, carbamidomethyl Cys as a fixed modification, PyroGlu on N-terminal Gln, oxidation of Met and phosphorylation on Ser, Thr, and Tyr as variable modifications. Pescal software (version 2.5.0) was used for quantification of label free phosphopeptides across experimental conditions. For the determination of intensity values, Pescal generated extracted ion chromatograms for all identified phosphopeptides across all conditions and quantified the area under the curve using the parameters described previously [19]. Data was normalized to the sum of all peptide intensities derived from a sample (column). To determine phosphorylation changes induced by treatments, the signals from treated cells were divided by those of the respective untreated control samples and given as Fold Changes in Log2 Ratio. Thus, Fold Change = log2 Ratio (Ratio = mean of normalized phosphopeptide signals across biological replicates in treated conditions/mean of normalized phosphopeptide signals across replicates in untreated cells). To determine differences in protein phosphorylation between both receptors, the signals from cells expressing c-Myc-5-HT_{2A}H452Y-eYFP receptors were divided by those of the respective cells expressing c-Myc-5-HT_{2A}-eCFP receptors and given as Fold Changes in Log2 Ratio. Significant phosphopeptides with a fold change higher than 0.8 or smaller than −0.8 in each analyzed comparison were used for further analysis. KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway analysis from selected phosphopeptides in each condition were performed using DAVID Bioinformatic Tool [21] (version 6.8). KEGG pathway terms obtained were then sorted by the significance (EASE score or Modified Fisher's Exact Test P value) and number of genes associated to each term using Rstudio (Version 3.5.2, Boston, MA, USA). The degree of phosphorylation across conditions of significant phosphopeptides were represented using heatmaps with the Heatmapper tool [22].

2.12. Data analysis

Data were analyzed using Prism (GraphPad Software Inc., San Diego, CA, USA), and statistical significance was determined using either Student's *t*-test or two-way ANOVA followed by the post hoc analysis as appropriate. In order to determine differences in the phosphorylation degree between control and clozapine treatment or between receptors, two tail unpaired Students *t*-test were used. For detailed analysis of the significant changes between receptors, one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Tests were used. $P < 0.05$ determined statistical significance.

3. Results

3.1. Subcellular distribution in living cells and density of 5-HT_{2A}R and 5-HT_{2A}R-H452Y

Previous observations of the Flp-In T-Rex HEK293 cell line inducibly expressing 5-HT_{2A}R by epifluorescence microscopy revealed a peculiar distribution of the fluorescent signal in the form of punctate aggregates all through the cytoplasmic area of the cells [16,23]. This particular cellular distribution was confirmed in the present set of experiments as shown in Fig. 1A. By contrast, cells expressing 5-HT_{2A}R-H452Y

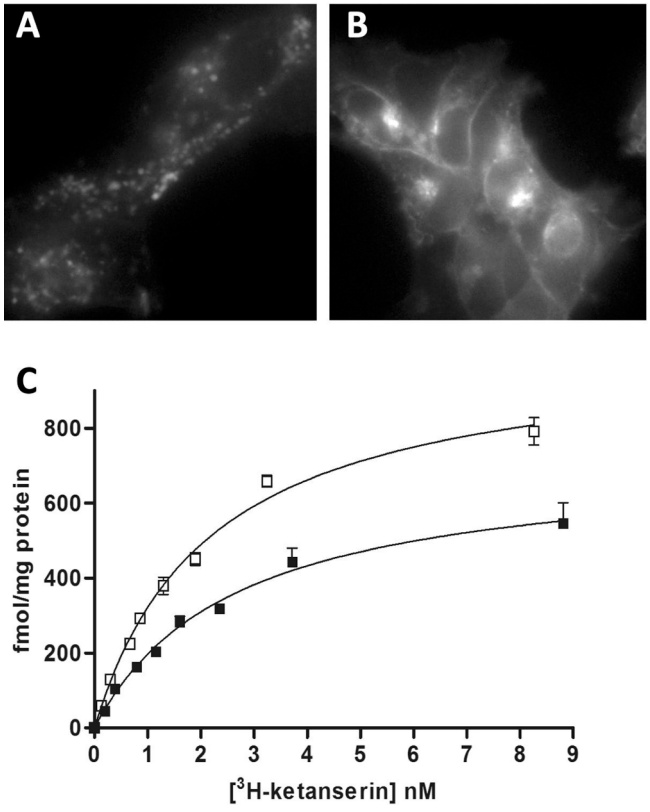

Fig. 1. Fluorescence microscopy images of Flp-In T-Rex HEK293 living cells expressing A: 5-HT_{2A}R or B: 5-HT_{2A}R-H452Y. C: Graph plot displaying [^{3}H] ketanserin specific binding from saturation binding assays performed with membranes from cells expressing 5-HT_{2A}R (filled squares) or 5-HT_{2A}R-H452Y (open squares). Each point represents mean ± SEM (n = 3).

presented a fluorescent signal more homogeneously distributed and concentrated in some intracellular compartments as well as at the plasma membrane (Fig. 1B).

Radioligand binding experiments performed with cell membrane preparations resulted in monophasic saturation curves for [^{3}H]ketanserin binding in both cell lines (Fig. 1C). Non-linear regression analysis demonstrated no significant differences between wild type and mutant receptors in relation to the equilibrium dissociation constant (K_D) obtained: 2.60 nM ± 0.20 (n = 3) versus 2.17 nM ± 0.17 (n = 3) respectively (P > 0.05, Student's *t*-test). On the other hand, the maximum density of 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y quantified as [^{3}H]ketanserin binding sites were 665 ± 89 versus 1021 ± 35 (fmol/mg prot., n = 3) respectively (P < 0.05, Student's *t*-test), being the mutant receptor expressed some 35% higher than the wild type genotype.

3.2. Functional evaluation of 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y

Measurements of inositol phosphates accumulation in cells treated with 5-HT, DOI or LSD were performed in order to evaluate the functionality of the 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y constructs. Concentration-response curves obtained in cells expressing 5-HT$_{2A}$R demonstrated equivalent potencies for the three assessed agonists within the range of 10^{-9} M and 10^{-7} M (Fig. 2 and Table 1) and no significant differences were observed between both receptor genotypes in this respect. In relation to the efficacy of the maximum response in terms of fold over basal, 5-HT elicited the highest response, whereas DOI and LSD behaved as partial agonists showing maximal responses of some 65% and 40%, respectively, of that observed for 5-HT (Fig. 2 and Table 1). When comparing agonist efficacies observed in both receptor genotypes, 5-HT$_{2A}$R-H452Y showed a significant reduction of some 33% of the maximal response obtained with the three different drugs in relation to 5-HT$_{2A}$R (P < 0.05, Student's *t*-test) (Table 1). No significant differences were detected with regards to the basal level of accumulated inositol phosphates in the absence of any agonist drug in both cell lines (data not shown).

Intracellular calcium mobilization assays in single cells by epifluorescence microscopy were conducted in order to further evaluate the functionality of both receptor genotypes in response to 5-HT. According to previous studies performed with human platelets derived from subjects carrying either 452H or 452Y alleles [24], we considered three parameters resulting from the calcium mobilization peak in order to compare the response of 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y, i.e., the peak amplitude measured as fold over basal, the latency time of the response to appear and the half time of the response to recover from the maximum peak to the basal level (Fig. 3A). Five consecutives challenges with 5-HT at 10^{-7} M during 30 s followed by a washing period of 2.5 min with assay buffer between pulses were made to evaluate the desensitization

Table 1
Agonist functional evaluation of 5-HT$_{2A}$R (5-HT$_{2A}$) and 5-HT$_{2A}$R-H452Y (5-HT$_{2A}$H452Y) expressed in Flp-In T-Rex HEK293 cells by measurement of inositol phosphate accumulation. Values represent mean ± SEM of three independent experiments. *P < 0.05, student's *t*-test compared to the 5-HT$_{2A}$ corresponding value.

	pEC$_{50}$ (M)		*Emax* (fold over basal)	
	5-HT$_{2A}$	5-HT$_{2A}$H452Y	5-HT$_{2A}$	5-HT$_{2A}$H452Y
5-HT	7.2 ± 0.1	7.4 ± 0.1	13.0 ± 0.2	8.7 ± 0.1*
DOI	7.6 ± 0.1	7.9 ± 0.1	8.4 ± 0.2	5.8 ± 0.2*
LSD	8.5 ± 0.2	8.8 ± 0.4	5.5 ± 0.2	3.4 ± 0.1*

process of this response upon repeated stimulation (Fig. 3A). *Two-way* ANOVA analysis revealed no significant differences when comparing both receptor genotypes with regards the latency time of the response and the half time of recovery (data not shown). Conversely and regarding the peak amplitude of the different 5-HT pulses, a significant reduction was observed in the H452Y genotype response when compared to the 5-HT$_{2A}$R construct either for the pulse (F[4,15] = 64.93; P < 0.0001) or for the genotype (F[1,15] = 29.34; P < 0.0001) variables. No significant differences were found in relation to the interaction of both variables (F[4,15] = 3.02; P > 0.05). Bonferroni's multiple comparison test revealed that the efficacy of 5-HT to reach the maximum response was statistically different in cells expressing the non-mutated and mutant 5-HT$_{2A}$R constructs only from the third pulse (Fig. 3B). The efficiency of the response corresponding to the first agonist stimulation was 3.57 ± 0.20 (n = 5) and 3.69 ± 0.21 (n = 5) fold over basal for 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y respectively resulting in no significant differences between them (P > 0.05, Student's *t*-test).

3.3. Measurement of ERK stimulation by 5-HT$_{2A}$R and 5-HT$_{2A}$R-H452Y

In vitro AlphaScreen™ technology was used to quantify the generation of phospho-ERK upon MAPK signaling pathway activation following exposure to agonists 5-HT or DOI, or the antagonist/inverse agonist clozapine. It has been widely described for most GPCRs a distinctive biphasic curve when analyzing the time course of phospho-ERK response [25], consisting in an early and transient peak followed by a more flat and sustained response that terminates at the end of the signaling process. Time course experiments including ten different time points up to 180 min were designed with both cell lines in order to further analyze the kinetics of phospho-ERK response mediated by 5-HT$_{2A}$R in the presence of 5-HT, DOI or clozapine. Fig. 4 shows the different curves obtained for each drug and receptor genotype from a representative experiment. Fetal bovine serum was used to determine the maximal generation of phospho-ERK species in this experimental model and the effect of each drug is represented as the percentage of this

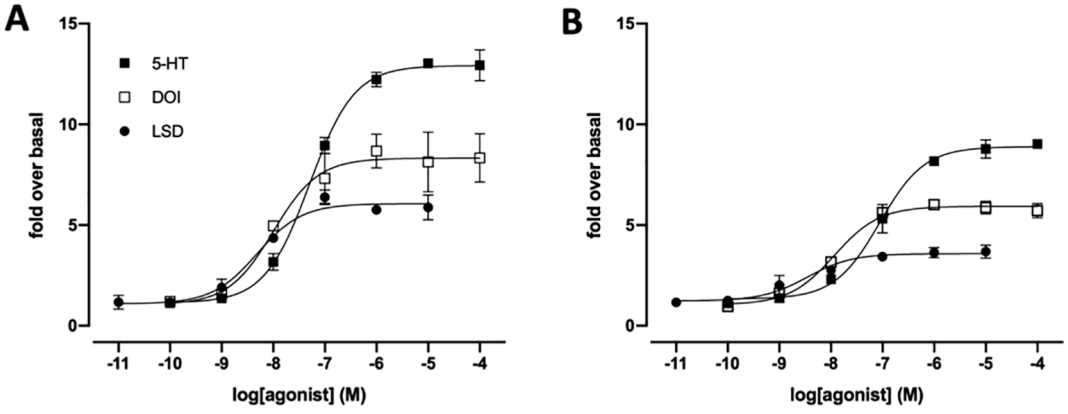

Fig. 2. Concentration-response curves generated by 5-HT (filled squares), DOI (open squares) and LSD (filled circles) when measuring inositol phosphates accumulation in Flp-In T-Rex HEK293 cells expressing A: 5-HT$_{2A}$R or B: 5-HT$_{2A}$R-H452Y. Each point represents mean ± SEM (n = 3).

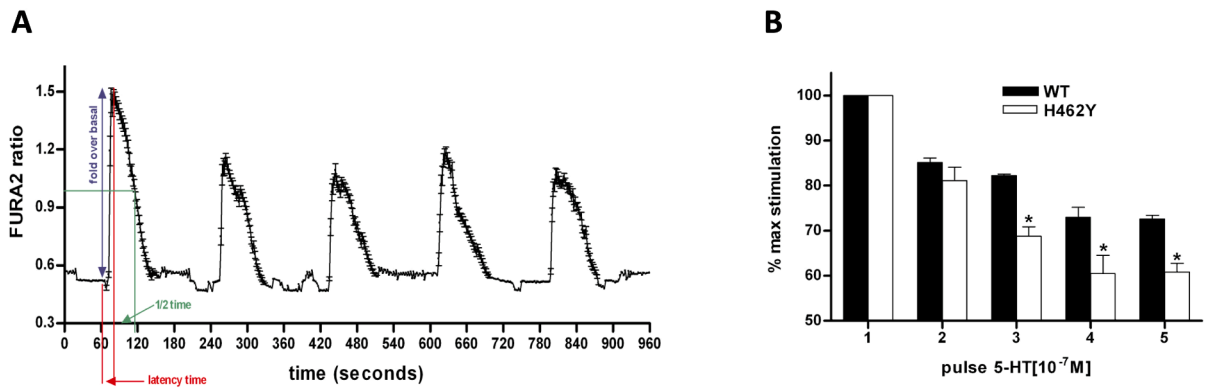

Fig. 3. A: Graph plot showing a representative result from an intracellular calcium mobilization assay in single cells expressing 5-HT_{2A}R by epifluorescence microscopy consisting in five consecutive pulses of 5-HT at 10^{-7} M. The three parameters considered for the response analysis are indicated as "fold over basal", "1/2 time" of the response recovery and "latency time" of the response to appear. B: Bars graph showing the peak amplitude of the calcium mobilization response obtained upon stimulation with 5-HT at 10^{-7} M in Flp-In T-Rex HEK293 cells expressing 5-HT_{2A}R (filled bars, referred as WT) or 5-HT_{2A}R-H452Y (open bars, referred as H452Y). Results are expressed as the percentage of the maximal response observed in the first pulse. Each bar represent the mean of three independent experiments ±SEM. *P < 0.05 in Bonferroni's multiple comparison test.

maximal response. Either 5-HT (Fig. 4A) or DOI (Fig. 4B) promoted a substantial formation of phospho-ERK in cells expressing 5-HT_{2A}R or 5-HT_{2A}R-H452Y displaying in all the cases a peak of maximal response at 5 min within the time course. No substantial differences were observed between 5-HT and DOI in the maximal response resulting after 5 min of treatment in both allelic forms of the receptors when original AlphaScreen™ fluorescent units were compared (data not shown). In the case of 5-HT_{2A}R, the time course curves corresponding to 5-HT and DOI stimulation are substantially different, especially during the period comprised within the first 60 min of treatment (Fig. 4C). Thus, two-way ANOVA revealed a significant difference in time variable ($F[9,18] = 128.46$; $P < 0.005$), drug variable ($F[1,2] = 27.04$; $P < 0.05$) and interaction between both variables ($F[9,18] = 7.18$; $P < 0.0005$). This disparity resides in the intensity of the response which is higher and more sustained between 5 and 45 min when receptors are stimulated with 5-HT than when stimulated with DOI during the same period of time (Fig. 4C). Conversely, equivalent experiments performed with 5-HT_{2A}R-H452Y resulted in no statistical differences in the time course curves obtained with 5-HT and DOI (Fig. 4D): time variable ($F[9,18] = 205.37$; $P < 0.005$), drug variable ($F[1,2] = 0.80$; $P > 0.05$) and interaction between both variables ($F[9,18] = 2.19$; $P > 0.05$). When comparing 5-HT_{2A}R genotypes, no differences were observed between 5-HT responses (Fig. 4A): time variable ($F[9,18] = 110.33$; $P < 0.005$), genotype variable ($F[1,2] = 5.59$; $P > 0.05$) and interaction between both variables ($F[9,18] = 2.10$; $P > 0.05$), whereas the equivalent comparison made with DOI responses resulted in a substantial difference between both genotypes consisting in a more sustained DOI response in the case of 5-HT_{2A}R-H452Y (Fig. 4B): time variable ($F[9,18] = 368.2$; $P < 0.005$), genotype variable ($F[1,2] = 82.83$; $P < 0.05$) and interaction between both variables ($F[9,18] = 19.38$; $P < 0.0005$). Finally, clozapine failed to generate any significant response after 5, 10 and 30 min of treatment in both cell lines (Fig. 4E) behaving therefore as an antagonist for this functional assay.

3.4. *Phosphoproteomics analysis of clozapine treatment in cells expressing 5-HT_{2A}R and 5-HT_{2A}R-H452Y*

Once both cell lines were pharmacologically characterized in terms of receptor functionality, we next analyzed the response to clozapine in cells expressing 5-HT_{2A}R or 5-HT_{2A}R-H452Y using phosphoproteomics analysis. Treatment of cells expressing 5-HT_{2A}R with clozapine (10^{-5} M) for 5 or 10 min resulted in an alteration in the phosphorylation status of several proteins compared to vehicle-treated cells referred to control (Fig. 5A). Interestingly, the treatment of cells with clozapine for 5 min or 10 min significantly increased the phosphorylation of 67 and 187 peptides, respectively (fold change ≥0.8 compared to control, $P \leq 0.05$, Fig. 5B). Clozapine also decreased the phosphorylation of 11 and 21 peptides upon treatment for 5 min and 10 min, respectively (fold change ≤−0.8 compared to control, $P \leq 0.05$, Fig. 5B). These results indicate that clozapine treatment in cells expressing 5-HT_{2A}R increases the phosphorylation status of several proteins. In contrast, cells expressing 5-HT_{2A}R-H452Y showed fewer differences in the phosphorylation status (Fig. 5C). Thus, treatment with clozapine for 5 min or 10 min only increased the phosphorylation of 32 and 43 peptides, respectively (fold change ≥0.8 compared to control, $P \leq 0.05$, Fig. 5D). Additionally, the treatment with clozapine for 5 min increased the number of phosphopeptides with reduced phosphorylation (64 phosphopeptides showing a fold change ≤−0.8 compared to control, Fig. 5D). This situation was not so evident in the treatment for 10 min with clozapine (Fig. 5D).

As treatment with clozapine for 10 min exhibited the most relevant changes in the phosphorylation of several peptides, and basically increased the phosphorylation of 187 peptides in cells expressing 5-HT_{2A}R, we selected this condition to evaluate the biological relevance of the phosphopeptides modified in both cell lines using ontology analysis. To further explore this issue, a KEGG pathways analysis was performed using DAVID Bioinformatic tools [21]. Concretely, we selected and analyzed the phosphopeptides modified significatively by clozapine after 10 min of treatment, i.e., peptides with increased and decreased phosphorylation indicated in Fig. 5B and D. In cells expressing 5-HT_{2A}R, KEGG analysis showed that 9 pathways were enriched ($P \leq 0.05$, number of genes [corresponding to the proteins identified] per term ≥5) upon clozapine treatment (Fig. 6A): "spliceosome", "insulin signaling pathway", "ErbB signaling pathway", "estrogen signaling pathway", "choline metabolism in cancer", "RNA transport", "Epstein-Barr virus infection", "Endocytosis" and "Herpes simplex infection". The respective phosphopeptides identified for each term showed that clozapine treatment mainly significantly increased the phosphorylation of them compared to vehicle-treated cells (Fig. 6B). On the other hand, KEGG analysis in cells expressing 5-HT_{2A}R-H452Y showed a reduction in the number of enriched terms and also in the number of genes associated to each term (Fig. 6C) ($P \leq 0.05$, number of genes [corresponding to the proteins identified] per term ≥5). Thus, we only identified four enriched terms: "ErbB signaling pathway", "thyroid hormone signaling pathway", "proteoglycans in cancer" and "pathways in cancer" and all these terms only contains five genes per term (Fig. 6C). When the respective peptides to those genes and its phosphorylation status were analyzed, we observed that the number of phosphopeptides was markedly reduced and the number of phosphopeptides with decreased phosphorylation

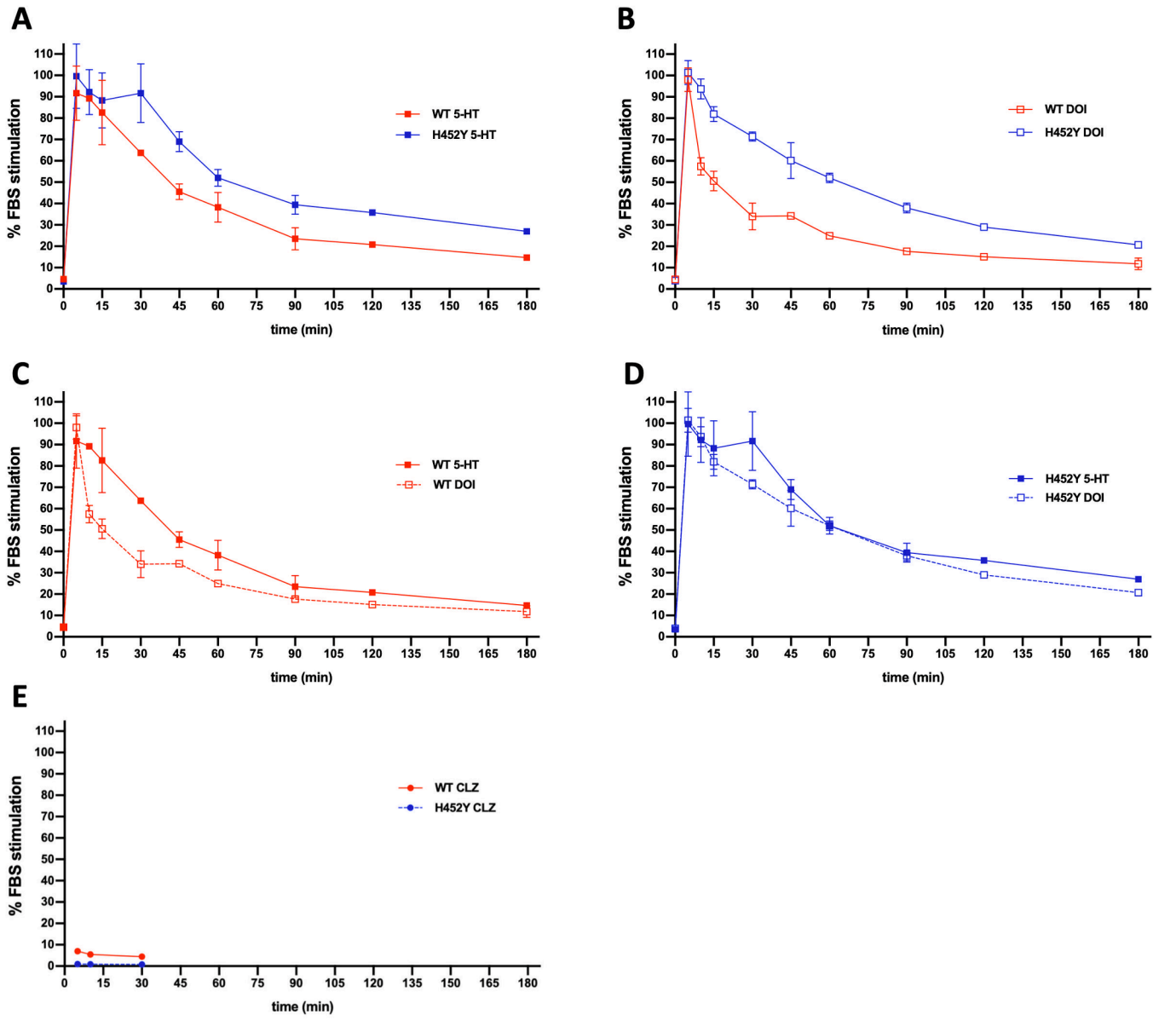

Fig. 4. Representative graph plots displaying the time course of the phosphoERK response stimulated by 5-HT (filled squares) DOI (open squares) and clozapine (circles) in Flp-In T-Rex HEK293 cells expressing 5-HT_{2A}R (referred as WT) or 5-HT_{2A}R-H452Y (referred as H452Y). Upper panels: comparison of the responses elicited by 5-HT (A) and DOI (B) in both receptor genotypes. Middle panels: comparison of the responses elicited by 5-HT and DOI in 5-HT_{2A}R (C) and 5-HT_{2A}R-H452Y (D). Lower panel: Responses generated by clozapine in both receptor genotypes (E).

increased (Fig. 6D).

Different authors have shown that clozapine treatment can activate Akt [26–28]. Since clozapine treatment for 10 min in cells expressing 5-HT_{2A}R increased the phosphorylation of multiple proteins, we evaluated whether Akt was also activated in our model. Phosphorylation of Akt1 at Ser473, Thr308, and Ser477/Thr479 are implicated in its activation [29]. In our phosphoproteomics data, we did not observe a significant activation of Akt1 after clozapine treatment as reflected by the phosphopeptides AKT1 pS473 pT479 and AKT1 pT479 (Fig. 7A, left and middle graphs). Similarly, in cells expressing 5-HT_{2A}R-H452Y clozapine treatment did not show a significant increase in the phosphorylation of those phosphopeptides (Fig. 7B, left and middle graphs), which suggest that clozapine did not induce the activation of Akt1. Interestingly, the phosphorylation of Akt2 at Ser474, site which is related with its activation [30], showed a significant increase of its phosphorylation after 10 min of clozapine treatment in cells expressing 5-HT_{2A}R and 5-HT_{2A}R-H452Y (Fig. 7A and B, right graphs), indicating that clozapine activates Akt2 but not Akt1 after 10 min treatment.

3.5. *5-HT_{2A}R presents a different basal phosphorylation pattern compared to 5-HT_{2A}R-H452Y*

As our current data showed a different pattern in the phosphoproteome upon clozapine treatment, we compared the phosphoproteome of cells expressing 5-HT_{2A}R to those expressing 5-HT_{2A}R-H452Y, in absence (vehicle treatment, referred as control) and in presence of clozapine at 10^{-5} M for 5 and 10 min. Interestingly, cells expressing either 5-HT_{2A}R or 5-HT_{2A}R-H452Y without exposure to clozapine showed differences in their phosphoproteome (Fig. 8A). In this sense, cells expressing the 452Y allele showed 328 peptides whose phosphorylation was significantly increased compared to cells expressing 5-HT_{2A}Rs (Fold change $\geq$0.8 compared to 5-HT_{2A} receptors, $P \leq 0.05$), and 47 peptides with decreased phosphorylation (Fold change ≤ -0.8 compared to 5-HT2A receptors, $P \leq 0.05$,) (Fig. 8B). These results indicate that both

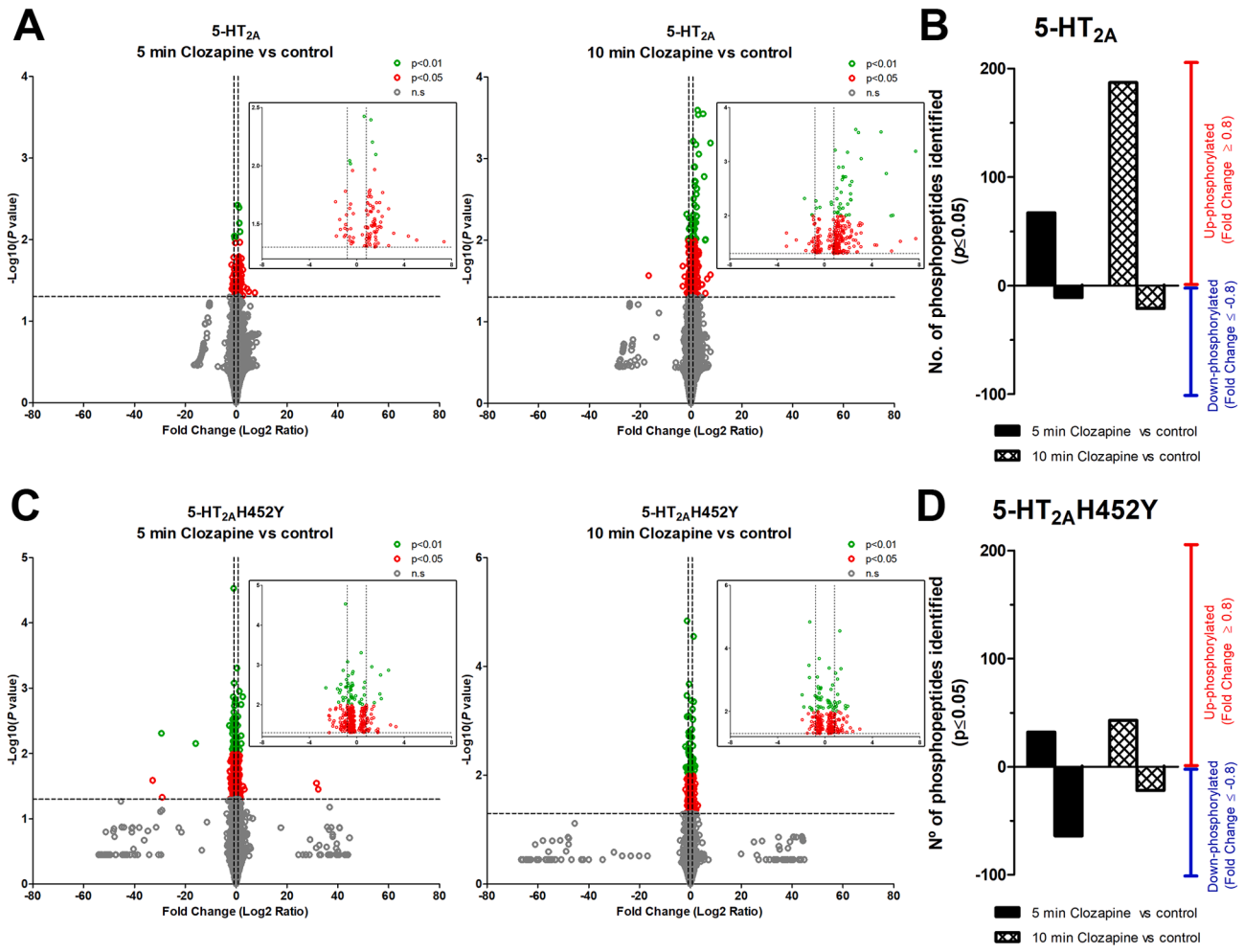

Fig. 5. Flp-In T-Rex HEK293 cells expressing 5-HT_{2A}R (referred as 5-HT_{2A}) or 5-HT2AR-H452Y (referred as 5-HT_{2A}H452Y) were treated with clozapine (10^{-5} M) for 5 and 10 min, and then processed for phosphoproteome analysis. Cells expressing 5-HT_{2A}R or 5-HT_{2A}R-H452Y and treated with DMSO (vehicle) were used as control cells. The average signal intensities of each phosphopeptide in treated cells were divided by those of the respective control cells and expressed in Log2 (Log2 Ratio, referred as Fold Change). The degree of phosphorylation (Fold Change) of each phosphopeptide identified by LC–MS/MS was obtained from four independent experiments (n = 4). Significant differences were determined using two tail unpaired *t*-test. Phosphopeptides identified in cells expressing 5-HT_{2A}R (A) and 5-HT_{2A}R-H452Y (C) treated with clozapine for 5 min (left panels in A and C) and 10 min (right panels in A and C) were represented in volcano plots according to their statistical P value (−Log P value, y-axis) and their Fold Change (x-axis). Horizontal and vertical dashed lines indicate the filtering criteria (P = 0.05 and Fold Change = ±0.8, respectively). Red and green dots correspond to phosphopeptides that changed significantly respect to control cells (P ≤ 0.05 and P ≤ 0.01, respectively), while grey dots represent phosphopeptides that did not match with the filtering criteria (P ≥ 0.05, Fold Change >−0.8 or Fold Change <0.8). The embedded graph in each volcano plot shows a magnification of the x-axis between −8 and 8. The number of phosphopeptides with significant changes (P ≤ 0.05) in cells expressing 5-HT_{2A}R and 5-HT_{2A}R-H452Y after the treatment with clozapine are shown in bar graphs in (B) and (D), respectively. The positive and negative part of y-axis indicates the number of phosphopeptides that showed a significant increase (P ≤ 0.05, Fold Change ≥0.8) or decrease (P ≤ 0.05, Fold Change ≤−0.8) in their phosphorylation compared to control cells after the treatment with clozapine for 5 min (black bars) and 10 min (bars with pattern). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

type of cells may have a different basal phosphoproteome under the experimental control condition consisting in treatment with vehicle, which may be traduced in a different intracellular signaling pattern. Interestingly, when clozapine was added the number of peptides up-phosphorylated decreased. The cells expressing 5-HT_{2A}R-H452Y showed 168 peptides with increased phosphorylation compared to cells expressing 5-HT_{2A}R, upon treatment with clozapine for 5 min (Fold change ≥0.8 compared to 5-HT_{2A}R, P ≤ 0.05, Fig. 8B). In clozapine treatment for 10 min, we observed only 93 peptides with increased phosphorylation when both cells were compared (Fold change ≥0.8 compared to 5-HT_{2A}R, P ≤ 0.05, Fig. 8B). As quantification of phosphopeptides indicated, the number of phosphorylated peptides decreased with clozapine treatment and this reduction was higher if the time of exposure to clozapine was longer (Fig. 8B). In regards to peptides with decreased phosphorylation, clozapine treatment for 5 and 10 min reduced the phosphorylation of 111 and 27 peptides, respectively, when both receptor genotypes were compared (Fig. 8B).

Since both cell lines showed a different phosphorylation pattern in absence of clozapine treatment, we inquired in which pathways these modified proteins are involved. With that aim, we performed KEGG analysis of the proteins that showed a significant differential phosphorylation status between both receptor genotypes (−0.8 ≥ fold change ≥ 0.8, P ≤ 0.05, proteins that belongs to the phosphopeptides indicated in the "control condition" in Fig. 8B). We found that ten terms were enriched (P ≤ 0.05, number of genes per term ≥5, Fig. 8C): "RNA transport", "spliceosome", "insuling signaling pathway", "mTOR signaling pathway", "ErbB signaling pathway", "AMPK signaling", " cell cycle", "ribosome biogenesis in eukaryotes", "viral carcinogenesis" and "chronic myeloid leukemia".

A different phosphorylation pattern was also observed between both type of receptors when the phosphopeptides associated to these pathways were analyzed. For instance, Figs. 9A, 10A, 11A and 12A show

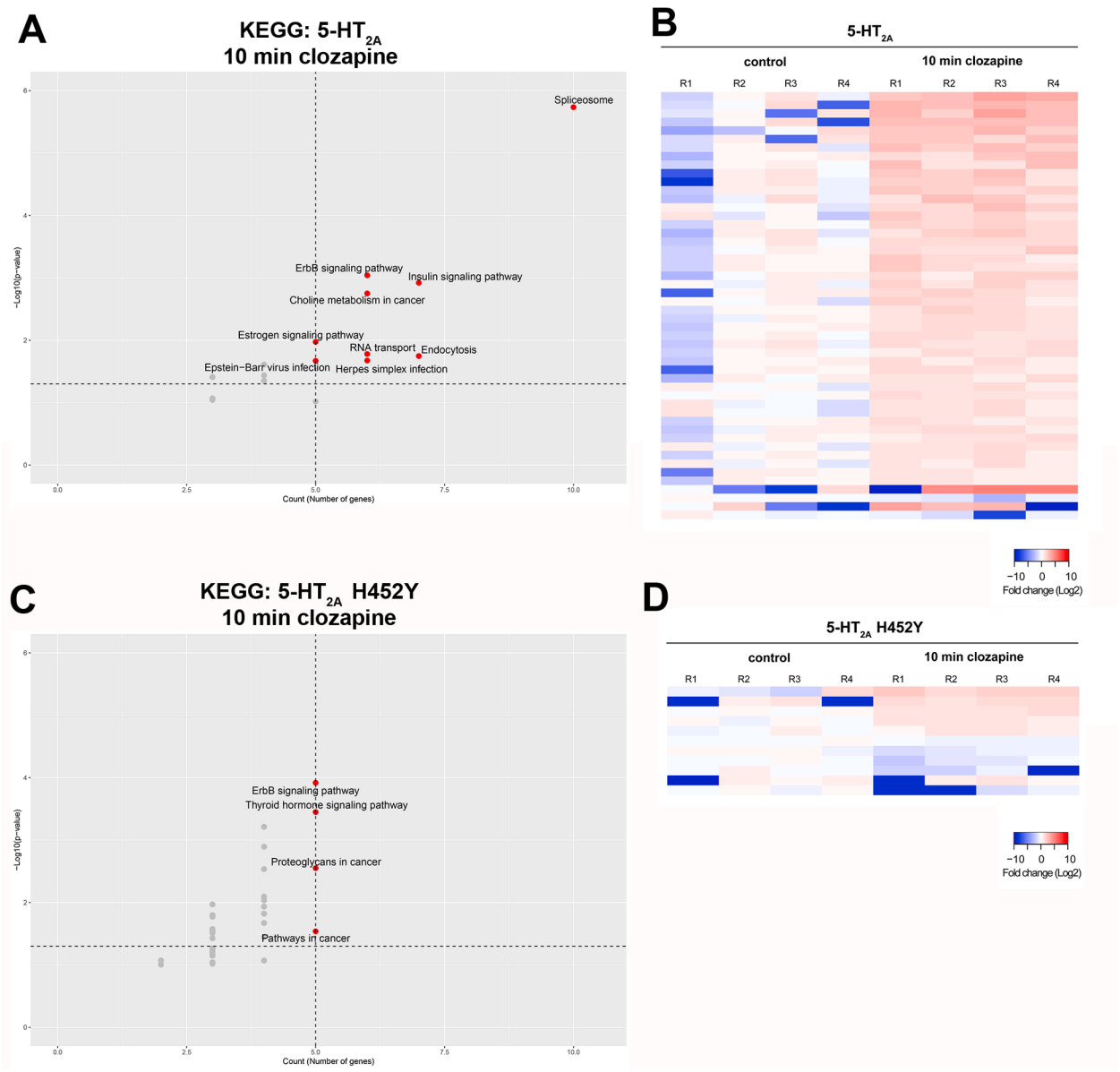

Fig. 6. Phosphopeptides that showed a significant increase ($P \leq 0.05$, Fold Change ≥ 0.8) or decrease ($P \leq 0.05$, Fold Change ≤ -0.8) in their phosphorylation after treatment with clozapine for 10 min in cells expressing 5-HT_{2A}R (referred as 5-HT_{2A} receptor, A) or 5-HT_{2A}R-H452Y (referred as 5-HT_{2A} H452Y receptor, C) were subjected to KEGG pathway enrichment analysis. The KEGG pathway terms obtained were then sorted by their significance (Modified Fisher's Exact Test P value) and the number of genes (corresponding to the proteins identified) associated to each term. Grey dots represent terms that showed less than five genes per term or showed no significant enrichment ($P \geq 0.05$). Red dots represent significant terms with more than five genes. (B, D) Heatmaps showing the effect of the treatment with clozapine for 10 min in cells expressing 5-HT_{2A}R (B) or 5-HT_{2A}R-H452Y (D). The phosphopeptides indicated in the heatmaps belong to those included in the significant terms obtained in A and C, respectively. The color scale is based on the phosphorylation degree of each phosphopeptide (Fold Change), where blue indicate a decrease in the phosphorylation and red indicates an increase of the phosphorylation. Four independent experiments are shown and indicated as R1-R4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these peptides and their phosphorylation (normalized signal intensity obtained from LC–MS/MS analysis), belonging to the "mTOR signaling", "Insulin signaling pathway", "AMPK signaling pathway" and "ErbB signaling pathway", terms identified by KEGG analysis. As volcano plot has previously shown, cells expressing 5-HT_{2A}R-H452Y showed an increase in the phosphorylation of these peptides in the absence of clozapine treatment compared to cells expressing 5-HT_{2A}R (Figs. 9A, 10A, 11A, 12A). We found a significant increase in the phosphorylation of Ribosomal protein S6 kinase beta-1 (RPS6KB1 or p70 S6K-alpha) at Thr 444, Tuberous sclerosis 1 protein (TSC1) at Ser 505, Rapamycin-insensitive companion of mTOR (RICTOR) at Ser 1388, the Eukaryotic translation initiation factor 4B (EIF4B) at Ser 422 and Ser 424, Eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1) at Thr 50 and Ser 96, and in Proline-rich AKT1 substrate 1 (AKT1S1 or also known as PRAS40) at Ser 88 and 92 (Fig. 9A). Other identified phosphopeptides belonging to the terms "AMPK signaling pathway", "ErbB signaling pathway" and "Insulin signaling pathway" also showed a differential pattern of phosphorylation under the experimental control condition between both receptors (Figs. 10A, 11A, 12A). In this case, Serine/threonine-protein kinase PAK 4 or RAF proto-oncogene serine/threonine-protein kinase (RAF1) showed an increase in the basal phosphorylation of untreated 5-HT_{2A}R-H452Y compared to untreated 5-HT_{2A}R. Otherwise, the protein 6-phosphofructokinase type C (PFKP) phosphorylated at Ser386 and the insulin receptor substrate 4 (IRS4) phosphorylated at Ser872 presented a different phosphorylation pattern between both receptor genotypes, i.e., cells expressing the 452Tyr allele

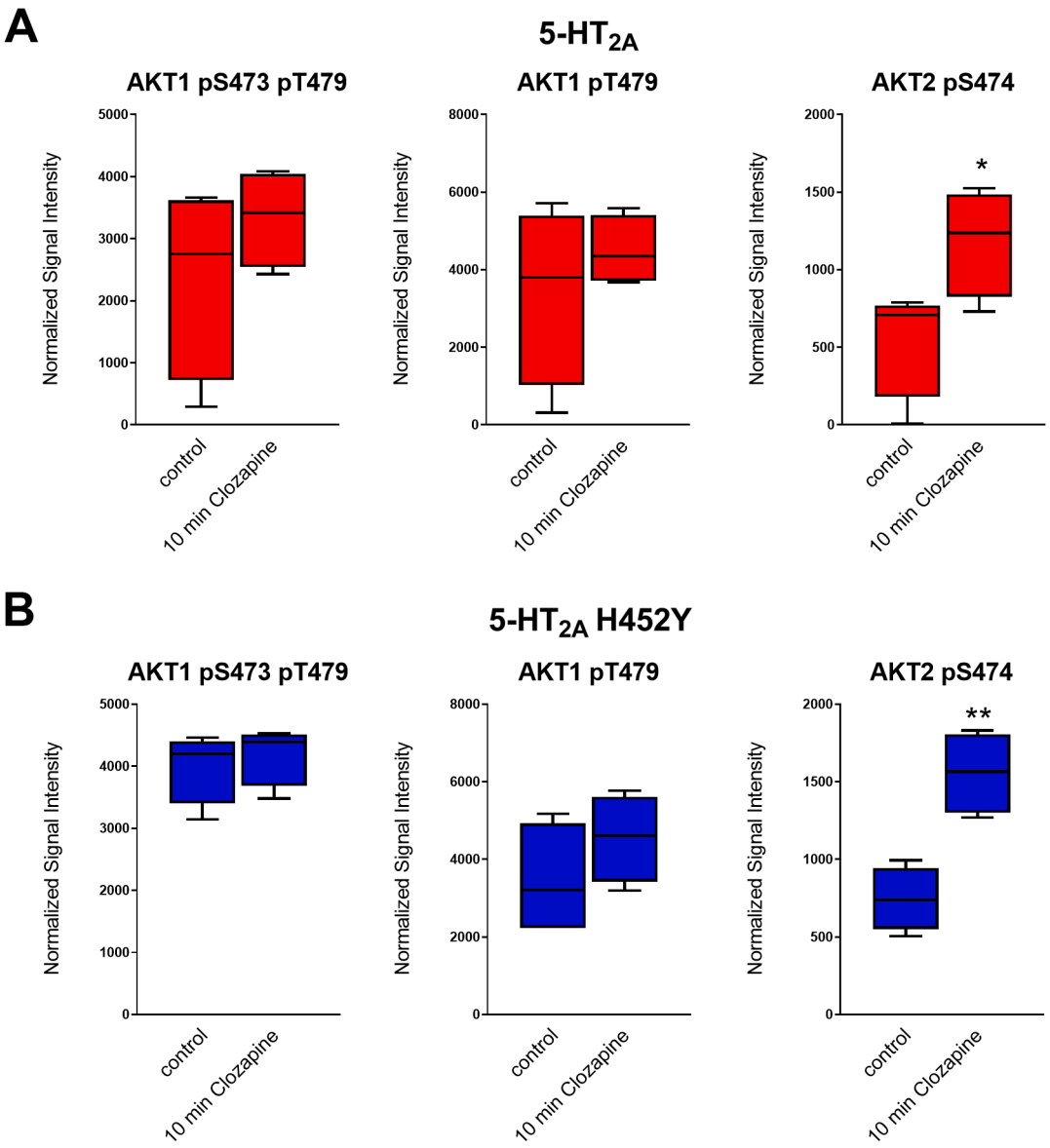

Fig. 7. Phosphorylation of AKT1 and AKT2 at sites linked to their activation in cells expressing 5-HT2AR (referred as 5-HT_{2A} receptor, A) and 5-HT_{2A}R-H452Y (referred as 5-HT_{2A}H452Y receptor, B) after treatment with clozapine for 10 min. The normalized signal intensity of each phosphopeptide is represented with box and whiskers (median values ± SEM). The normalized signal intensity was obtained from four independent experiments (n = 4). Statistical significance was calculated using two tail unpaired *t*-test (*: $P \leq 0.05$ and **: $P \leq 0.01$ respect to control).

showed less phosphorylation that those expressing non-mutated receptors.

Interestingly, clozapine treatment did not modify the phosphorylation status of these proteins in comparison to the control condition in cells expressing 5-HT_{2A}R-H452Y (Figs. 9C, 10C, 11C, 12C). In contrast, clozapine treatment in cells expressing 5-HT_{2A}R seemed to increase the basal phosphorylation of some of them, specifically in treatment with clozapine for 10 min (e.g. EIF4B at S422 and S424, Fig. 9B).

4. Discussion

Single nucleotide polymorphisms resulting in the substitution of an amino acid in the primary structure of a given protein may confer alterations in its functionality at the physiological level. Particularly in the case of GPCRs, these polymorphisms could involve pharmacological implications such as alterations in the risk of developing a disease and/or alterations of the response to therapy [31]. One of the genetic variants of the human 5-HT_{2A}R is a non-synonymous single nucleotide polymorphism consisting in the mutation C/T (rs6314) that results in the substitution His/Tyr at the position 452 of the amino acidic sequence of this protein. This mutation has an allele frequency of 9% and originally was described as not associated to the development of schizophrenia [32]. Nevertheless, subsequent clinical investigations compiled in two comprehensive meta-analyses described a significant association between schizophrenic carriers of Tyr452 allele and poor response to clozapine [12,13]. Herein we report on results that extend previous investigations characterizing the molecular pharmacology profile of 5-HT_{2A}R-H452Y variant in comparison to 5-HT_{2A}R using as experimental model inducible cell lines heterologously expressing them in a permanent manner. In addition, an extensive phosphoproteomics analysis was carried out with both cell lines in order to infer into the different signaling pathways activated upon treatment with clozapine.

Epifluorescence microscopy visualization in living cells of both receptor variants tagged with fluorescent proteins revealed a substantial difference in their subcellular distribution. In this sense, 5-HT_{2A}R distributes in punctuate forms showing a constitutive trafficking as

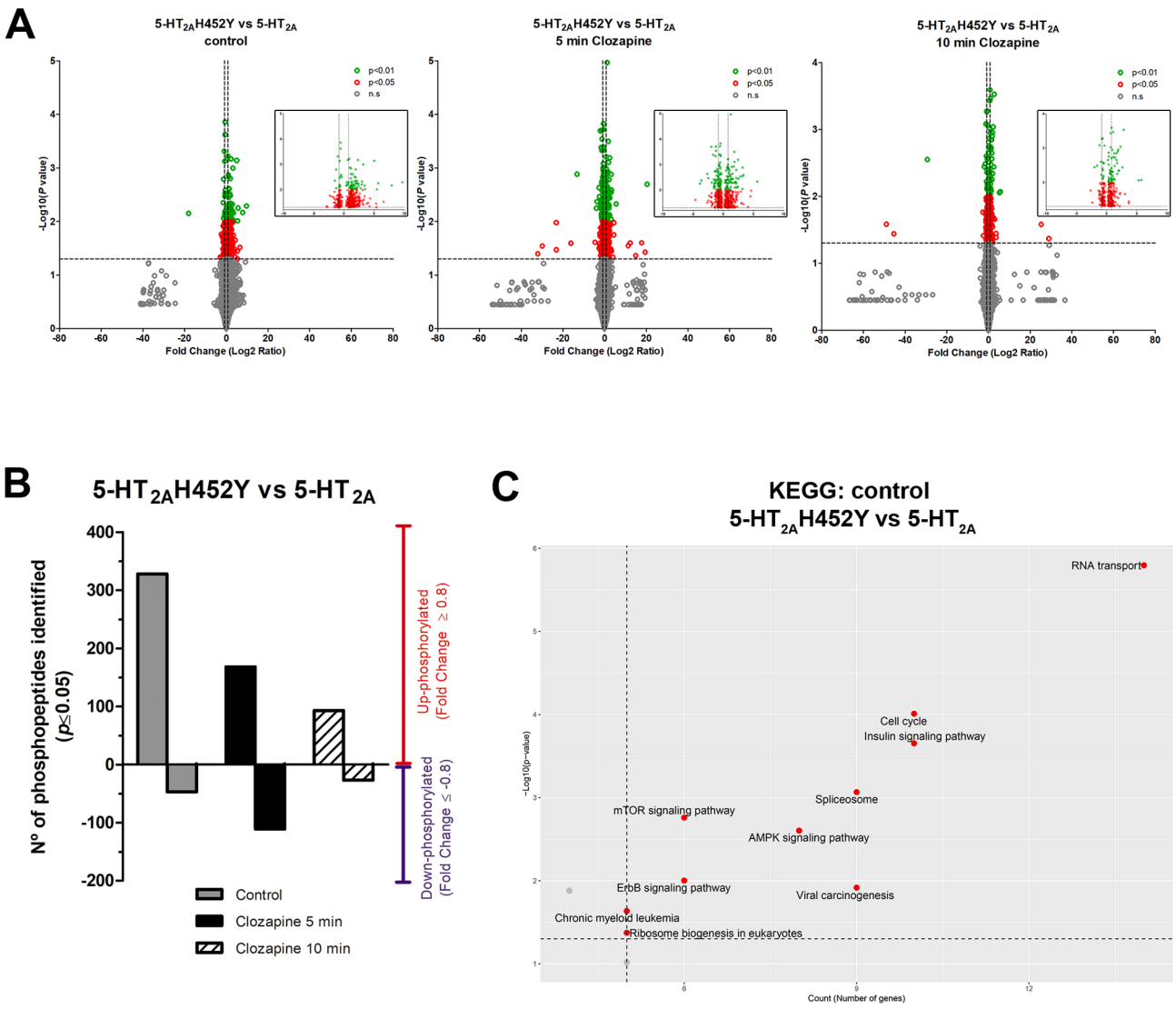

Fig. 8. (A) Volcano plots showing the phosphorylation pattern of cells expressing 5-HT_{2A}R-H452Y (referred as 5-HT_{2A}H452Y) compared to cells expressing 5-HT_{2A}R (referred as 5-HT_{2A}) treated only with DMSO (control, left) or treated with clozapine for 5 min (middle) and 10 min (right). The x-axis shows the Fold Change calculated as the ratio of the average of signal intensities of each phosphopeptide in 5-HT_{2A}R-H452Y cells and those respective in 5-HT_{2A}R cells, and expressed in Log2 (Log2 Ratio, Fold Change). y-axis shows their statistical P value (−Log P value). Horizontal and vertical dashed lines indicate the filtering criteria (P = 0.05 and Fold Change = ±0.8, respectively). Red and green dots correspond to phosphopeptides that changed significantly respect to control cells ($P \leq 0.05$ and $P \leq 0.01$, respectively), while grey dots represent phosphopeptides that did not match with the filtering criteria ($P \geq 0.05$, Fold Change >−0.8 or Fold Change <0.8). The embedded graph in each volcano plot shows a magnification of the x-axis between −10 and 10. (B) Number of phosphopeptides with significant changes ($P \leq 0.05$) in cells expressing 5-HT2AR-H452Y compared to cells expressing 5-HT_{2A}R. The positive and negative part of y-axis indicate the number of phosphopeptides that showed a significant increase ($P \leq 0.05$, Fold Change ≥0.8) or decrease ($P \leq 0.05$, Fold Change ≤−0.8) in their phosphorylation in absence of clozapine (treated only with DMSO, referred as control, grey bars) or treated with clozapine for 5 min (black bars) and 10 min (bars with pattern). (C) Phosphopeptides that showed a significant increase ($P \leq 0.05$, Fold Change ≥0.8) or decrease ($P \leq 0.05$, Fold Change ≤−0.8) in their phosphorylation in absence of clozapine (referred as control, grey bars in B) were subjected to KEGG pathway enrichment analysis. The KEGG pathway terms obtained were then sorted by their significance (Modified Fisher's Exact Test P value) and the number of genes (corresponding to the proteins identified) identified in each term. Grey dots represent terms that showed less than five genes per term or showed no significant enrichment ($P \geq 0.05$). Red dots represent significant terms with more than five genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

previously described when this cell line was originally characterized and, interestingly, this vesicle trafficking was abolished after pretreatment of cells with the antagonist/inverse agonist mianserin [16,23]. In contrast, 5-HT_{2A}R-H452Y presented a more homogeneous distribution either at the plasma membrane or within intracellular compartments and no evidence of vesicle trafficking was observed. This divergence is indicative of a different basal or quiescent state of both receptor variants when they are expressed in the same cellular environment. Equivalent studies reported previously did not observe such discrepancy between both receptor genotypes in relation to the targeting to the plasma membrane [15,33], probably because a different experimental approach was used, *i.e.*, biotinylation of the plasma membrane and [^{3}H]ketanserin binding in intact cells, and different host cell lines, NIH3T3 and COS7, were employed to heterologously express 5-HT_{2A}R and 5-HT_{2A}R-H452Y.

The *in vitro* pharmacological characterization of 5-HT_{2A}R-H452Y and 5-HT_{2A}R assessed by [^{3}H]ketanserin binding resulted in equivalent affinity values of this antagonist at the nanomolar range in agreement with previous studies reported by others [15,33–35]. Regarding the expression levels of both receptors evaluated by maximum [^{3}H]ketanserin specific binding, we observed an increase of some 35% in the case of the

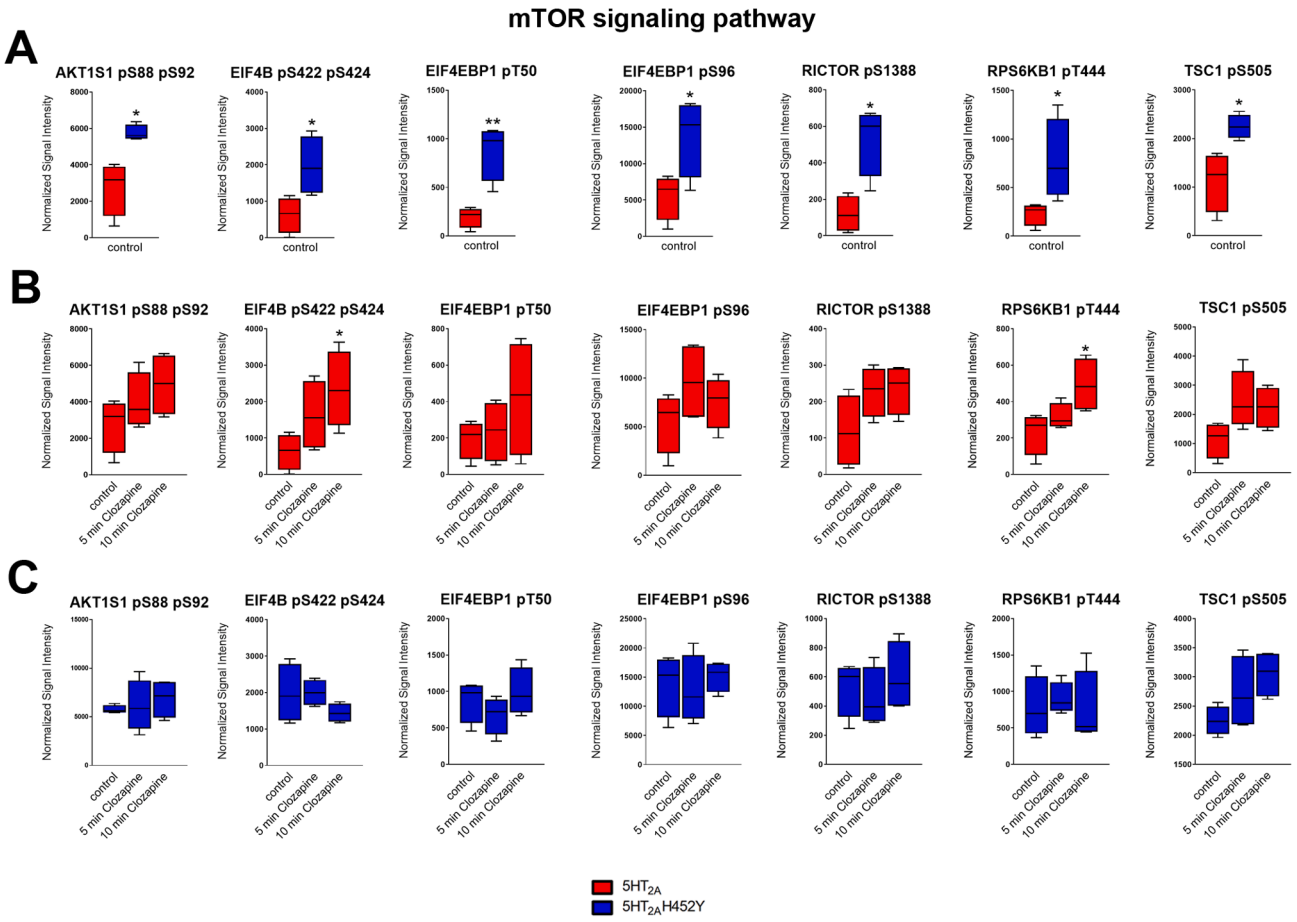

Fig. 9. Normalized signal intensity for the phosphopeptides included in the KEGG terms "mTOR signaling pathway" (Fig. 8C). (A) Normalized signal intensity for each phosphopeptide in both type of receptors under control conditions (vehicle-treated only). (B) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R after the treatment with clozapine for 5 and 10 min. (C) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R-H452Y after the treatment with clozapine for 5 and 10 min. In A, B and C the y axis represents the normalized signal intensity (median values ± SEM) for each indicated phosphopeptide in the condition and receptor indicated. The normalized signal intensity was obtained from four independent experiments (n = 4). Statistical significance was calculated using two tail unpaired *t*-test (*: $P \leq 0.05$ and **: $P \leq 0.01$ respect to 5-HT_{2A} receptor) in A, and one-way ANOVA test with Tukey post-test analysis (*: $P \leq 0.05$ respect to corresponding control) in B and C. AKTS1, proline-rich AKT1 substrate 1 or PRAS40; EIF4B, eukaryotic translation initiation factor 4B; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; RICTOR, rapamycin-insensitive companion of mTOR; RPS6KB1, ribosomal protein S6 kinase beta-1, p70-S6K 1 or S6K1; TSC1, hamartin or tuberous sclerosis 1 protein.

mutant receptor in contrast to other equivalent studies where no substantial differences were found in this respect even in those cases where HEK293 cells were used as experimental model [34,35], although in those cases the receptors were transiently transfected and not stably expressed as they are in the present study.

The traditional approach to evaluate the functionality of 5-HT_{2A}Rs *in vitro* consists in the measurements of second messengers intracellularly generated by the activation of this receptor and its subsequent coupling to $G_{q/11}$ proteins – this process considered as the canonical signaling pathway of the 5-HT_2R family. Essentially, $G_{q/11}$ subunit is responsible for the direct activation of phospholipase C (PLC) leading to the accumulation of inositol phosphates and, indirectly, for the calcium mobilization from intracellular stores. Measurement of inositol phosphates accumulation resulted in equivalent potencies of the three agonists assayed, i.e., 5-HT, DOI and LSD, showing no significant differences between both receptor genotypes. In relation to efficacy, that is the maximum response generated in this experimental system, DOI and LSD behave as partial agonists with respect to 5-HT in cells expressing either 5-HT_{2A}R or 5-HT_{2A}R-H452Y. When comparing both receptors, the mutant receptor presented a significant reduction of the maximal response observed with the three agonists of some 33% in relation to the native receptor. These results are in complete agreement with those ones previously reported from equivalent experiments and the diminishment of receptor efficacy have also been observed in the activity of other phospholipases such as phospholipase D [15,33]. Analogous studies to determine the functionality of 5-HT_{2A}R analyzing calcium mobilization as indicator of receptor activation resulted in comparable observations, i.e., no substantial differences in potency among the agonists assayed was observed in relation to both receptor genotypes and a notable reduction of their efficacy in 5-HT_{2A}R-H452Y receptors was confirmed [34,36].

Initial calcium mobilization experiments reported by Ozaki and co-workers to evaluate the potential influence of the His/Tyr substitution on cellular receptor function using platelets from His/His homozygous and His/Tyr heterozygous subjects demonstrated a weak response in samples from Tyr carriers reflected as a blunting of the shape of the calcium mobilization peak, particularly a smaller peak amplitude and a different time course of the response [24]. This attenuation of calcium release was later confirmed by others with equivalent experiments and including one available Tyr/Tyr homozygote [37]. One speculation to conjecture about this blunted response of 5-HT when stimulating 5-HT_{2A}R-H452Y was to consider this mutant receptor in a state of relative desensitization resulting from the phosphorylation of the Tyr residue [24]. However, this hypothesis was subsequently investigated by others substituting the Tyr mutation by Phe, an amino acid not susceptible to be phosphorylated [15]; this new mutation did not change the effect of the

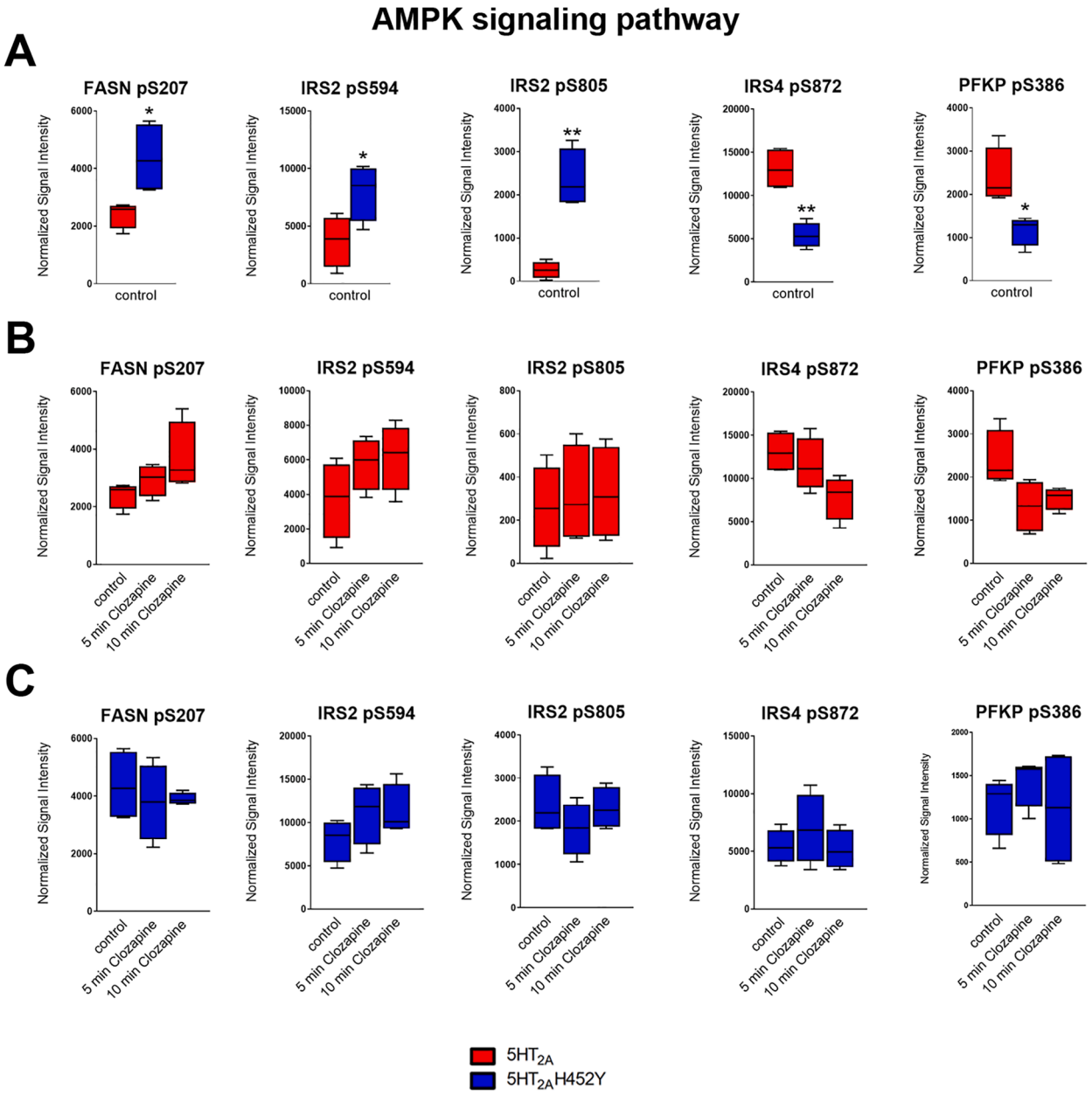

Fig. 10. Normalized signal intensity for the phosphopeptides included in the KEGG terms "AMPK signaling pathway" (Fig. 8C). (A) Normalized signal intensity for each phosphopeptide in both type of receptors under control conditions (vehicle-treated only). (B) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R after the treatment with clozapine for 5 and 10 min. (C) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R-H452Y after the treatment with clozapine for 5 and 10 min. In A, B and C the y axis represents the normalized signal intensity (median values ± SEM) for each indicated phosphopeptide in the condition and receptor indicated. The normalized signal intensity was obtained from four independent experiments (n = 4). Statistical significance was calculated using two tail unpaired *t*-test (*: $P \leq 0.05$ and **: $P \leq 0.01$ respect to 5-HT_{2A} receptor) in A, and one-way ANOVA test with Tukey post-test analysis in B and C. FASN, fatty acid synthase; IRS2, insulin receptor substrate 2; IRS4, insulin receptor substrate 4; PFKP, ATP-dependent 6-phosphofructokinase (platelet type) or 6-phosphofructokinase type C.

reduction on the efficacy of the maximal response observed in inositol phosphate hydrolysis, therefore excluding the possibility that the Tyr residue provides a novel phosphorylation site that justifies the desensitized status of 5-HT_{2A}R-H452Y. In order to further explore into the differences in the time course of this functional response between both receptor genotypes, intracellular calcium mobilization experiments in single cells by epifluorescence microscopy were carried out with the cell lines heterologously expressing each of these receptors. When tested separately, 5-HT_{2A}R and 5-HT_{2A}R-H452Y presented no differences in their calcium response profiles when comparing peak amplitude, latency time of the response and half time of recovery of the maximal response. This disparity in relation with previous results obtained from experiments conducted with human platelets could be due either to the different host cell expressing these receptors, i.e. platelets versus HEK293 cells, or to the agonist concentration used in the assay, 5-HT at 10^{-5} M in platelet experiments [24,37] against to 10^{-7} M in the present investigations. In furtherance of inquiring about the possibility that 5-HT_{2A}R-H452Y remains in a basal state that makes it more prone to be desensitized, desensitization experiments consisting in consecutive 5-HT acute challenges at a submaximal concentration (10^{-7} M) were carried out. Our results reflecting a significant reduction in the peak amplitude of the response of 5-HT_{2A}R-H452Y upon the third agonist pulse confirm that the mutant receptor is more inclined to be desensitized than the native one, as previously demonstrated by others in desensitization

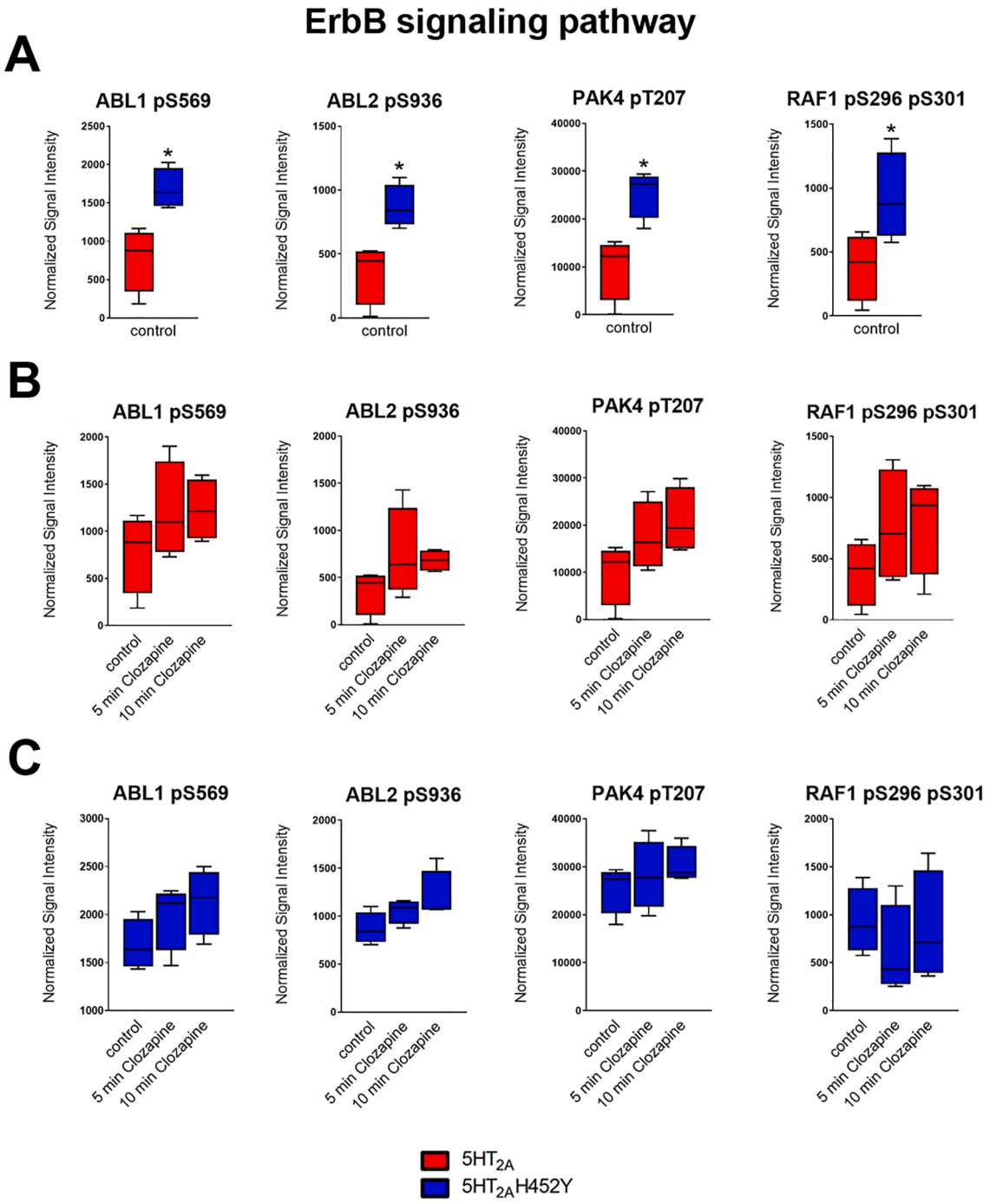

Fig. 11. Normalized signal intensity for the phosphopeptides included in the KEGG terms "ErbB signaling pathway" (Fig. 8C). (A) Normalized signal intensity for each phosphopeptide in both type of receptors under control conditions (vehicle-treated only). (B) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R after the treatment with clozapine for 5 and 10 min. (C) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R-H452Y after the treatment with clozapine for 5 and 10 min. In A, B and C the y axis represents the normalized signal intensity (median values ± SEM) for each indicated phosphopeptide in the condition and receptor indicated. The normalized signal intensity was obtained from four independent experiments (n = 4). Statistical significance was calculated using two tail unpaired *t*-test (*: $P \leq 0.05$ and **: $P \leq 0.01$ respect to 5-HT_{2A} receptor) in A, and one-way ANOVA test with Tukey post-test analysis in B and C. ABL1, tyrosine-protein kinase ABL1; ABL2, tyrosine-protein kinase ABL2; PAK4, serine/threonine-protein kinase PAK 4; RAF1, RAF proto-oncogene serine/threonine-protein kinase.

experiments based on agonist pretreatments before the functional assay [15].

Activation of MAPK signaling pathway, particularly phosphorylation of ERK1/2 (Extracellular Signal-Regulated Kinase), mediated by 5-HT_{2A}R in *in vitro* experimental models has been previously proved in different cell types such as cultured rat vascular smooth muscle cells [38], NIH3T3 expressing recombinant rat 5-HT_{2A} receptors [39] and cultured prefrontal cortex (PFC) rat neurons [40]. More recently, an extensive pharmacological characterization of MAPK activation determined in HEK293 cells expressing recombinant human 5-HT_{2A}R demonstrated that potencies and relative efficacies of some agonist compounds, including 5-HT, DOI and LSD, are comparable to those observed in inositol phosphates hydrolysis and subsequent intracellular calcium mobilization assays [41]. These results led the authors to exclude any evidence for functional selectivity when comparing DOI and other 5-HT_{2A}R agonists with respect to relative efficacies in activating

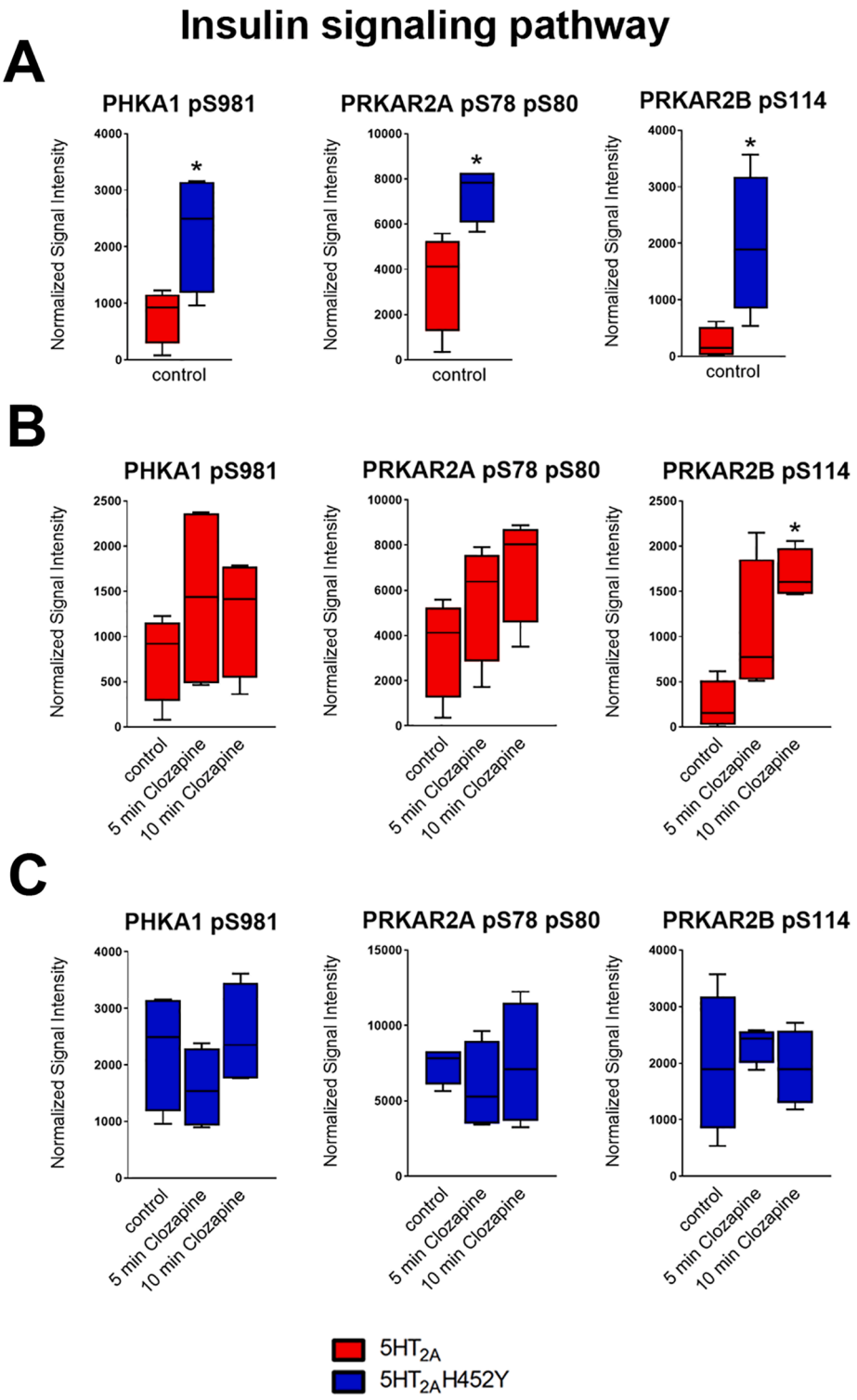

Fig. 12. Normalized signal intensity for the phosphopeptides included in the KEGG terms "Insulin signaling pathway" (Fig. 8C). (A) Normalized signal intensity for each phosphopeptide in both type of receptors under control conditions (vehicle-treated only). (B) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R after the treatment with clozapine for 5 and 10 min. (C) Normalized signal intensity for each phosphopeptide in 5-HT_{2A}R-H452Y after the treatment with clozapine for 5 and 10 min. In A, B and C the y axis represents the normalized signal intensity (median values ± SEM) for each indicated phosphopeptide in the condition and receptor indicated. The normalized signal intensity was obtained from four independent experiments (n = 4). Statistical significance was calculated using two tail unpaired *t*-test (*: $P \leq 0.05$ and **: $P \leq 0.01$ respect to 5-HT_{2A} receptor) in A, and one-way ANOVA test with Tukey post-test analysis (*: $P \leq 0.05$ respect to corresponding control) in B and C. PHKA1, phosphorylase b kinase regulatory subunit alpha (skeletal muscle isoform); PRKAR2A, cAMP-dependent protein kinase type II-alpha regulatory subunit; PRKAR2B, cAMP-dependent protein kinase type II-beta regulatory subunit.

MAPK and other canonical signaling pathways. Another approach to study the ERK1/2 activation mediated by a given GPCR is by analyzing the time course of this response. In this sense, and taking early results described with angiotensin AT1a receptor as a paradigm, G protein-mediated activation of ERK1/2 is rapid and transient in contrast with β-arrestin-mediated activity that is characterized by slower onset and greater persistence [42]. This disparity in β-arrestin versus G protein-mediated signaling indicates a potential for ligands to selectively activate one or the other pathway and, in turns, may imply distinct physiological consequences. In our present results, time course phosphoERK responses generated with 5-HT and DOI in cells expressing 5-HT_{2A}Rs reflect a definite difference between both agonists being the 5-HT response more sustained over time and therefore suggesting a major participation of β-arrestins in the response evoked by this agonist. Conversely, parallel experiments conducted with cells expressing mutant 5-HT_{2A}R-H452Y showed no differences in the time course curves obtained with both agonists. In this late case, both compounds display a curve profile akin to the one generated by 5-HT with the non-mutated receptor. Interestingly, these results strongly support a clear difference between 5-HT and DOI in terms of agonist-directed trafficking of the

ERK1/2 response mediated by 5-HT_{2A}Rs and, intriguingly, this difference in functional selectivity is lost when histidine at position 452 is substituted by tyrosine in the carboxy terminus of the receptor. Previous results from studies carried out using mice lacking β-arrestin-2 as well as MEF cells devoid of β-arrestin-1/2 expression are in line with a significant role of β-arrestins in ligand directed functional signaling of 5-HT_{2A}Rs [43]. In this respect, 5-HT induced a robust phosphoERK1/2 response in wild type MEF cells greater than the one obtained with DOI whereas equivalent experiments conducted with β-arrestin1/2-KO MEF cells resulted in a significant reduction of the stimulation generated by 5-HT and the absence of differences between both agonists.

A recent report describes the activation of ERK1/2 kinases by clozapine, and other atypical antipsychotics, via an agonistic mechanism mediated by 5-HT_{2A}Rs [27]. This response was observed in native HeLa cells, C6 glioma cells and Hela cells transiently expressing recombinant human 5-HT_{2A}Rs and was partially blocked by 5-HT_{2A}R antagonists. Contrarily, in our hands, clozapine failed to evoke any substantial phophoERK response when tested at 10^{-5} M for 5, 10 and 30 min in the cell lines expressing both receptor genotypes. The reason for this discrepancy may reside in the cellular context where 5-HT_{2A}Rs are expressed, that is, HEK293 versus HeLa and C6 glioma cells. Nevertheless, and in agreement with our results, other authors previously described the incapacity of clozapine to induce the activation of ERK in wild type MEF cells or in the frontal cortex of wild type mice [43].

Clozapine, the first atypical antipsychotic to be developed, constitutes currently the gold standard in the clinical practice for the treatment of schizophrenia due to its demonstrated superiority to other antipsychotic medications. From a pharmacodynamics perspective, the mechanism of action of clozapine resides in its antagonist action, that is, by blocking those binding sites where clozapine interacts with, essentially neurotransmitter receptors within the central nervous system. Despite the proven efficacy of clozapine to ameliorate positive and negative symptoms of schizophrenia devoid at the same time of extrapyramidal side effects compared to typical antipsychotics, some challenging issues still remain to be solved when used chronically such as severe neutropenia and an associated high risk of developing diabetes mellitus (for an extended review see [7]). For all these reasons there is a need to better understand its mechanism of action that would permit to develop new medications as effective as clozapine but with fewer side effects. One possible way to advance in the knowledge of the molecular mechanism of clozapine is to go in depth from receptor binding through down-stream cell signaling pathways with the objective to identify new and more specific pharmacological targets. With that aim, *in vitro* clozapine treatments of cells expressing either 5-HT_{2A}R or 5-HT_{2A}R-H452Y were carried out to subsequently proceed to the phosphoproteomics analysis of these samples. Phosphoproteomics is an analytical approach recently introduced in the study of GPCRs [44–48]. This technology facilitates to identify and quantify the phosphorylation of specific peptides in such a way that their inclusive evaluation permits to infer into the signaling pathways activated within cells in each moment. The first conclusion arising from the set of experiments described herein is that clozapine presents intrinsic activity in terms of modifying the phosphorylation status of several proteins which may have an impact on intracellular signaling pathways and this activity is at some extent dependent of 5-HT_{2A}R because substantial differences were found when comparing samples from cells expressing 5-HT_{2A}R with those ones expressing 5-HT_{2A}R-H452Y. This result exclude the traditional assumption that clozapine is a neutral antagonist in relation to 5-HT_{2A}Rs similarly as it was recently demonstrated for the Akt pathway [11]. On the other hand, the substitution His452Tyr at the C-terminus of 5-HT_{2A}Rs definitely changes the response elicited by clozapine being in this case less effective in terms of modification of phosphorylated peptides. This result may suggest a possible association with the poor response to clozapine described in 452Tyr carriers and points out future avenues of investigation in this respect [12,13]. Interestingly, the comparison of the phosphorylation status of cells expressing 5-HT_{2A}R with 5-HT_{2A}R-H452Y in the absence of clozapine treatment revealed a different basal proteome indicating an augmentation of phosphopeptides in the case of 5-HT_{2A}R-H452Y and, consequently, suggesting a possible constitutive activity of the signaling pathways associated to them. The ontology analysis of the data obtained either from the clozapine treatments or from the comparison between both basal phosphoproteomes indicated the enrichment of different signaling pathways, some of them related to neural development, regulation of energetic metabolism, endocytosis or viral infection among others. In several cases previous reports described the implication of clozapine with these signaling pathways. In this sense, it has been described that micromolar concentrations of clozapine can attenuate the activity of ErbB receptor kinases in cultured cell lines and cortical neurons [49]. In relation to endocytosis several works demonstrated the paradoxical internalization of 5-HT_{2A}Rs induced by clozapine in *in vitro* assays as well as the redistribution of these receptors in *in vivo* experimental models [50]. Similarly, a recent publication describes the inhibition caused by clozapine in a dose dependent manner on the induction of the Epstein-Barr virus lytic cycle gene expression in Burkitt lymphoma cells [51], in agreement with the identification in our analysis of both "Epstein-Barr virus infection" and "Herpes simplex infection" as enriched pathways in samples from cells expressing 5-HT_{2A}Rs and treated with clozapine. Regarding the participation of clozapine in signaling pathways related to energetic metabolism, essentially insulin, mTOR and AMPK signaling, previous observations pointed out in that direction. Actually, it is well accepted the connection of antipsychotics and glucose metabolism and, more particularly, the impairment of glucose metabolism by atypical antipsychotics supported by clinical and experimental evidences (for an extended review see [52]). In this sense, it has been demonstrated in *in vitro* models of neurons and skeletal muscle cells that clozapine reduces insulin-stimulated glucose intake through a mechanism mediated by the increment of Akt activity [53]. Interestingly, our phosphoproteomics data showed an increase in the phosphorylation of Akt2, an isoform which is specifically related to the maintenance of glucose homeostasis [54]. Another demonstration that clozapine can regulate insulin and energy sensing pathways was conducted in a rat hypothalamic neural cell model where clozapine promoted the inhibition of insulin-stimulated phophoAkt and increased AMPK phosphorylation [55], being the first evidence that antipsychotics can directly influence insulin signaling in hypothalamic neurons. Similar results were also reproduced in an equivalent mouse hypothalamic cell line [56]. With respect to clozapine and mTOR signaling pathway, a recent publication describes the quantification of phosphorylation status of mTOR signaling elements in the brain cortex from rats chronically treated with clozapine and other antipsychotic drugs [57], resulting in alterations of some of these elements such as GSK3β and phosphoAkt, and no changes in protein S6.

Our main goal in this study was to evaluate the effects of clozapine at two short time-points (5 and 10 min) as compared to vehicle in HEK293 cells stably expressing 5-HT_{2A}R or 5-HT_{2A}R-H452Y. Although our current data clearly support the hypothesis that the phosphoproteome response to clozapine is clearly affected by this SNP within the *5-HT_{2A}R* gene, additional investigation will be required to further define the effects of different antipsychotic compounds, such as olanzapine or risperidone, on these signaling cascades, as well as comparison with protein phosphorylation profiles in HEK293 cells that do not express either 5-HT_{2A}R with 5-HT_{2A}R-H452Y with the goal of defining which components of the basal phosphoproteomic profiles require constitutive serotonin receptor activity.

In summary, the results of phosphoproteomics analysis can be recapitulated in three points: 1) clozapine treatment in cells expressing 5-HT_{2A}Rs increases the phosphorylation of several proteins which may have an impact on intracellular signaling pathways (Fig. 13, upper panels); 2) cells expressing the 425Tyr allele have a different phosphoproteome with respect to cells expressing the non-mutated receptor in the absence of clozapine treatment and some of these changes include, for example, an increase in the phosphorylation of mTOR signaling

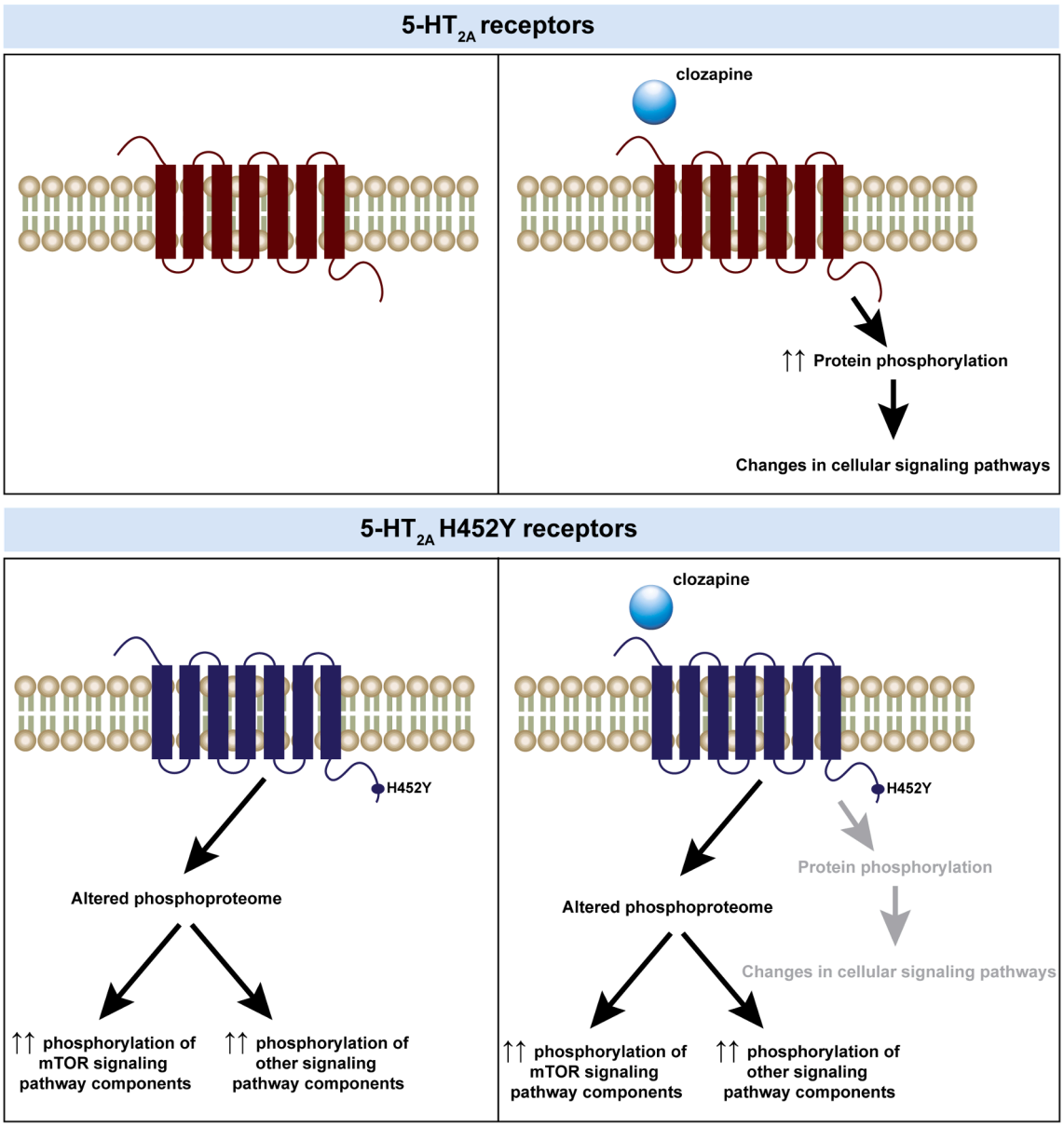

Fig. 13. Schematic overview of 5-HT$_{2A}$R and 5-HT2AR-H452Y in the presence or absence of clozapine treatment. The treatment of cells expressing 5-HT$_{2A}$R (referred as 5-HT$_{2A}$) with clozapine (upper panels) promotes the phosphorylation of several proteins, inducing changes in the phosphoproteome linked to multiple signaling pathways. In the case of cells expressing 5-HT$_{2A}$R-H452Y (referred as 5-HT$_{2A}$ H452Y), in the absence of clozapine treatment there is an increased phosphorylation of some components of mTOR signaling and other pathways relative to cells expressing 5-HT$_{2A}$R. Contrary to 5-HT$_{2A}$R, clozapine treatment has no substantial effect on protein phosphorylation and does not modify the phosphorylation of proteins linked to mTOR signaling and other signaling pathways that are already increased in basal conditions when compared to the 5-HT$_{2A}$R.

components (Fig. 13, left lower panel); and 3) clozapine treatment does not substantially modify the basal phosphorylation status of cells expressing the 452Tyr allele and does not show the same effect than in cells expressing 5-HT$_{2A}$R when clozapine is added, perhaps due to their different basal phosphoproteome (Fig. 13, right lower panel). Together, our data open a relatively new concept in molecular pharmacology that orthosteric ligands traditionally accepted as neutral antagonists and/or inverse agonists (this is, ligands that either do not affect signaling but instead block agonist-induced effects, and/or reduce a constitutive activity of the GPCR that they directly target) may also be able to recruit unique patterns of signaling pathway activation downstream of the GPCR. Additional work will be necessary to confirm whether this biased agonist-antagonist effect also occurs in animal models related to antipsychotic-mediated signaling and behaviour.

CRediT authorship contribution statement

Sandra M. Martín-Guerrero: Software, Validation, Formal analysis, Investigation, Visualization. **Paula Alonso:** Validation, Formal analysis, Investigation. **Alba Iglesias:** Validation, Formal analysis, Investigation. **Marta Cimadevila:** Validation, Formal analysis, Investigation. **José Brea:** Supervision, Visualization. **M. Isabel Loza:** Resources, Supervision, Visualization, Funding acquisition. **Pedro Casado:** Supervision, Software, Visualization. **David Martín-Oliva:** Supervision, Visualization. **Pedro R. Cutillas:** Resources, Supervision, Visualization. **Javier González-Maeso:** Conceptualization, Resources, Visualization, Funding acquisition. **Juan F. López-Giménez:** Conceptualization, Resources, Supervision, Formal analysis, Investigation, Visualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by the following grants and agencies: Proyecto RTI2018-079344-BI00 (FEDER/Minsterio de Ciencia e Innovación – Agencia Estatal de Investigación) to J.F.L-G. NIH R01 MH084894, NIH R01 MH111940, and NIH P30 DA033934 to J.G.-M. S.M.M-G was funded by a grant from Ministerio de Educación, Cultura y Deporte, Spain (FPU14/02219). Spanish Ministry of Economy and Competitiveness (SAF2017-85225-C3-1-R) and the European Regional Development Fund (ERDF) to M.I.L.

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