Quantitative Phosphoproteomics Unravels Biased Phosphorylation of Serotonin 2A Receptor at Ser 280 by Hallucinogenic versus Nonhallucinogenic Agonists

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Quantitative Phosphoproteomics Unravels Biased Phosphorylation of Serotonin 2A Receptor at Ser\textsuperscript{280} by Hallucinogenic versus Nonhallucinogenic Agonists*†§


The serotonin 5-HT\textsubscript{2A} receptor is a primary target of psychodelic hallucinogens such as lysergic acid diethylamide, mescaline, and psilocybin, which reproduce some of the core symptoms of schizophrenia. An incompletely resolved paradox is that only some 5-HT\textsubscript{2A} receptor agonists exhibit hallucinogenic activity, whereas structurally related agonists with comparable affinity and activity lack such a psychoactive activity. Using a strategy combining stable isotope labeling with amino acids in cell culture with enrichment in phosphorylated peptides by means of hydrophilic interaction liquid chromatography followed by immobilized metal affinity chromatography, we compared the phosphoproteome in HEK-293 cells transiently expressing the 5-HT\textsubscript{2A} receptor and exposed to either vehicle or the synthetic hallucinogen 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) or the nonhallucinogenic 5-HT\textsubscript{2A} agonist lisuride. Among the 5995 identified phosphorylated peptides, 16 sites were differentially phosphorylated upon exposure of cells to DOI versus lisuride. These include a serine (Ser\textsuperscript{280}) located in the third intracellular loop of the 5-HT\textsubscript{2A} receptor, a region important for its desensitization. The specific phosphorylation of Ser\textsuperscript{280} by hallucinogens was further validated by quantitative mass spectrometry analysis of immunopurified receptor digests and by Western blotting using a phosphosite-specific antibody. The administration of DOI, but not of lisuride, to mice, enhanced the phosphorylation of 5-HT\textsubscript{2A} receptors at Ser\textsuperscript{280} in the prefrontal cortex. Moreover, hallucinogens induced a less pronounced desensitization of receptor-operated signaling in HEK-293 cells and neurons than did nonhallucinogenic agonists. The mutation of Ser\textsuperscript{280} to aspartic acid (to mimic phosphorylation) reduced receptor desensitization by nonhallucinogenic agonists, whereas its mutation to alanine increased the ability of hallucinogens to desensitize the receptor. This study reveals a biased phosphorylation of the 5-HT\textsubscript{2A} receptor in response to hallucinogenic versus nonhallucinogenic agonists, which underlies their distinct capacity to desensitize the receptor. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.036558, 1273–1285, 2014.

Among the G Protein-Coupled Receptors (GPCRs)\textsuperscript{1} activated by serotonin (5-hydroxytryptamine, 5-HT), the 5-HT\textsubscript{2A} receptor continues to attract particular attention in view of its broad physiological role and implication in the actions of numerous psychotropic agents (1, 2). It is a primary target of widely used atypical antipsychotics such as clozapine, risperidone, and olanzapine, which act as antagonists or inverse agonists (1, 3). The activation of 5-HT\textsubscript{2A} receptors expressed in the prefrontal cortex has also been implicated in the psycho-mimetic effects of psychodelic hallucinogens, such as lysergic acid diethylamide (LSD), mescaline, and psilocybin, which are often used to model positive symptoms of schizophrenia (4–8). However, these psychoactive effects are not reproduced by structurally-related agonists, such as ergotamine and the anti-Parkinson agent lisuride, despite the fact that they exhibit comparable affinities and efficacies at 5-HT\textsubscript{2A} receptors (7, 9). This paradox was partially resolved by the demonstration that hallucinogens induce a specific transcript-
tomic signature because of the specific engagement of a Pertussis toxin-sensitive G₁₁/Src signaling pathway which is not activated by nonhallucinogenic agonists (7, 8). These findings suggest that hallucinogenic and nonhallucinogenic agonists induce different conformational states of the 5-HT₂ₐ receptor, and represent a striking example of functional selectivity that translates into contrasting patterns of mice behavior: induction of head-twitches by hallucinogenic but not by nonhallucinogenic agonists (9).

The differential influence of hallucinogenic versus nonhallucinogenic agonists on signaling pathways suggests that they trigger contrasting patterns of protein phosphorylation. To address this issue, we employed a quantitative phosphoproteomics strategy to directly compare the phosphoproteomes generated in HEK-293 cells by the synthetic hallucinogen 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) and the nonhallucinogenic 5-HT₂ₐ agonist lisuride. We found that DOI, but not lisuride, induced the phosphorylation of a serine residue (Ser²⁸₀) located in the third intracellular loop of the receptor itself. The hallucinogen-specific phosphorylation of this residue was further validated in vitro and in vivo by using a phosphosite-specific antibody. These findings were followed by a series of experiments to determine the impact of Ser²⁸₀ phosphorylation upon receptor desensitization and internalization.

**EXPERIMENTAL PROCEEDURES**

**Materials**—Human Embryonic Kidney-293 (HEK-293) cells were from the European Collection of Cell Cultures, culture media from Invitrogen (Carlsbad, CA). Lisuride maleate was from Santa Cruz Biotechnologies (Santa Cruz, CA). All other chemicals were from Sigma Aldrich. Isotope-labeled amino acids for SILAC experiments were from Euroisotop (Saint Aubin, France).

The rabbit anti-phospho-Thr²⁰²/Tyr²⁰⁴-Erk1,2, and anti-total Erk1,2 antibodies were from Cell Signaling Technology (Danvers, MA), the rabbit anti-phosphoSer²⁸₀-5-HT₂ₐ receptor antibody from Immunostar (Hudson, WI) and the mouse anti-HA antibody conjugated to agarose beads from Sigma Aldrich. The anti-phosphoSer²⁸₀-5-HT₂ₐ receptor antibody was generated by immunizing rabbits with the synthetic GTRAKLApSFSFL-C peptide coupled to Keyhole Limpet Hemocyanin (KLH, Eurogentec, Liege, Belgium).

The construct encoding the HA-tagged 5-HT₂ₐ receptor was described elsewhere (10). Following PCR amplification, the receptor cDNA was subcloned into the bicistronic plasmid pIRE2-EGFP (Clontech, Mountain View, CA) using the Xhol/BamHI restriction sites. This construct was transferred to pSinRep5 plasmid for Sindbis virus production (11). Plasmids encoding HA-tagged 5-HT₂ₐ receptor mutants (S²⁸₀A and S²⁸₀D) were generated using the Quick Change mutagenesis kit (Strategene, La Jolla, CA). All constructs were confirmed by DNA sequencing.

**Cell Cultures**—HEK-293 cells, grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% dialyzed, heat-inactivated fetal calf serum and antibiotics, were transfected at 40–50% confluence using polyethyleneimine (PEI, Sigma-Aldrich), as previously described (12), and used 48 h after transfection. For stable isotope labeling by amino acids in cell culture (SILAC) experiments (13), cells were maintained for 2 weeks in DMEM deficient in lysine and arginine, supplemented with 10% dialyzed serum and either L-lysine/L-arginine for light label (KDR, L) or L-Lysine-2HCl (²H₂, 96–98%)/L-Arginine-HCl (³¹C₆, 99%) for semi-heavy label (KAR6, M) or L-Lysine-2HCl (³¹C₆, 99%; ¹⁵N, 99%)/L-Arginine-HCl (³¹C₆, 99%; ¹⁵N, 99%) for heavy label (KBR10, H) (percentages represent the isotopic purity of the labeled amino acids). Under these conditions, analysis of semi-heavy amino acid incorporation at the protein level indicated a median ratio of 93% (first quartile at 88%, third quartile at 95%). A similar distribution was observed for the incorporation of the heavy amino acids.

Primary cultures of cortical neurons were prepared as described previously (14). Briefly, dissociated cells from the cerebral cortex of 17 day-old Swiss mice embryos were plated on 6- or 96-well plates coated successively with poly-l-ornithine (mol. Wt. 40,000; 15 μg/ml) and 10% fetal calf serum + 1 μg/ml laminin. The culture medium included a 1:1 mixture of DMEM and F-12 nutrient supplemented with 33 μM glucose, 2 μM glutamine, 13 mM NaHCO₃, 5 μM HEPES buffer, pH 7.4, 5 μl/ml (5 mg/ml) penicillin-streptomycin, and a mixture of salts and hormones containing 100 μg/ml transferrin, 25 μg/ml insulin, 20 ng/ml progesterone, 60 ng/ml putrescine, and 30 ng/ml Na₃SeO₃. Cultures were infected 5 days after seeding with the Sindbis virus expressing HA-tagged 5-HT₂ₐ receptor and were used 7 days after seeding. At this stage, they were shown to contain at least 95% of neurons (14).

**Global Quantitative Phosphoproteomics Analyses**—HEK-293 cells grown in SILAC media and transiently expressing 5-HT₂ₐ receptors were serum-starved for 4 h and challenged for 15 min with either vehicle (L), or lisuride (1 μM, M), or DOI (1 μM, H). Cells were lysed in 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM DTT). Cell lysates were clarified by centrifugation at 15,000 × g (20 min at 4 °C) and protein concentration was determined using the Bradford reagent. Equal amounts of proteins (3 mg) from each condition were mixed, reduced with 10 mM DTT, alkylated with 50 mM iodoacetamide and precipitated on ice with trichloroacetic acid (25%, 20 min) before their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8. Digests were acidified with 1% TFA, and their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8. Digests were acidified with 1% TFA, and their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8. Digests were acidified with 1% TFA, and their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8. Digests were acidified with 1% TFA, and their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8. Digests were acidified with 1% TFA, and their digestion with trypsin (1/200, w/w) in 2M urea, 25 mM triethylammonium bicarbonate pH 7.8.
Immunoprecipitated HA-5-HT2A receptors were resolved by SDS-PAGE. Gel bands containing the receptor were excised and digested on the Orbitrap and 10,000 MSn on the LTQ.

The raw MS data were analyzed using the MaxQuant/Andromeda software (v. 1.2.2.5) (16) with a false discovery rate of less than 0.01 for peptides and phosphosites and a minimum peptide length of six amino acids. The mass accuracy of the precursor ions was improved by retention time-dependent mass recalibration. Andromeda was used to search the top eight per 100 Da peak lists against the human complete proteome set database (http://www.uniprot.org/uniprot/). The phosphoSTY.txt file generated by MaxQuant was uploaded onto the R environment in order to plot log H/M ratios against log M/L ratios and color-display the B significance of phosphopeptide ratios in each of the three biological replicates (16). This version of the database contains both reviewed sequences from UniProtKB/Swiss-Prot and unreviewed sequences from UniProtKB/TrEMBL. Enzyme specificity was set to trypsin, additionally allowing cleavage N-terminal to proline and up to two missed cleavages. The search included cysteine carboxamidomethylation as a fixed modification, protein N-terminal acetylation, oxidation of methionine and phosphorylation of Ser, Thr, and Tyr as variable modifications. Peptide identification was based on a search with a mass deviation of the precursor ion up to 7 ppm after recalibration, and the allowed fragment mass deviation was set to 0.5 Da. Identifications across different replicates and adjacent fractions was performed using the “match between runs” MaxQuant option with a 3 min time window. Quantification of SILAC triplex signals was performed by MaxQuant with standard settings. The phosphoSTY.txt file generated by MaxQuant was uploaded onto Perseus software (v. 1.2.0.17) to calculate B significance of phosphopeptide ratios in each of the three biological replicates (16). This new table was then uploaded onto the R environment in order to plot log H/M ratios against log M/L ratios and color-display the B significance count ($p < 0.05$) for each quantified phosphopeptide.

**Targeted Analysis of 5-HT_{2A} Receptor Phosphorylation in HEK-293 Cells**—HEK-293 cells transiently expressing HA-tagged 5-HT_{2A} receptors were lysed in 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM DTT, and a protease inhibitor mixture (Roche). Samples were centrifuged at 15,000 X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM DTT, and a protease inhibitor mixture (Roche). Samples were centrifuged at 15,000 rpm for 30 min at 4 °C. Solubilized 5-HT_{2A} receptors were immunoprecipitated with the agarose-conjugated anti-HA antibody (Sigma Aldrich). Immunoprecipitated HA-5-HT_{2A} receptors were resolved by SDS-PAGE. Gel bands containing the receptor were excised and digested with trypsin (500 ng per condition). Peptides were analyzed by nano-LC-FT-MS/MS, top six per 30 Da windows peak lists were extracted using MSconvert 3.0 and searched with Mascot 2.4 against the same human Complete Proteome Set database, with phosphorylation of Ser, Thr, and Tyr as variable modifications, 7 ppm precursor mass tolerance, 0.5 Da fragment mass tolerance and trypsin/P digestion. MS2 spectra matching phosphorylated peptides with ion score over 15 were inspected using Prophossi software (17) for automatic annotation of 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 nonphosphorylated counterparts. Ser^{280} phosphorylation of immunoprecipitated receptors was also analyzed by Western blotting using the phosphosite specific antibody.

**Western Blotting**—Proteins, resolved onto 10% polyacrylamide gels, were transferred to Hybond C nitrocellulose membranes (GE Healthcare). Membranes were immunoblotted with primary antibodies (anti phospho-Ser^{280} 5-HT_{2A} receptor, 1:300; anti phospho-Thr^{202}/Ty^{204}-Erk1,2, 1:1000; anti Erk1,2, 1:1000; anti-HA, 1:1000; Anti-GFP, 1:1000; anti-RSK2, 1:1000) and then with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1: 3000, GE Healthcare). Immunoreactivity was detected with an enhanced chemiluminescence method (ECL™ plus detection reagent, GE Healthcare) and immunoreactive bands were quantified by densitometry using the ImageJ software. 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^{280} Phosphorylation in Mice Prefrontal Cortex**—Experiments were performed on wild type or 5-HT_{2A} receptor-deficient mice (8) and conformed to European ethics standards (66/609-EEC) and to decrees of the French National Ethics Committee (N° 87/848) for the care and use of laboratory animals. Mice (–30 g) were injected intraperitoneal with either vehicle (5% DMSO/5% Tween 80) or DOI or lisuride (10 mg/kg each). Thirty min after the onset of the treatment, mice were anesthetized with pentobarbital (100 mg/kg intraperitoneal, Ceva SA) and rapidly perfused transcardially with fixative solution containing 4% w/v paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5) containing NaF (100 mM) and sodium orthovanadate (1 mM). Brains were post-fixed for 48 h in the same solution and stored at 4 °C. Fifty micrometers-thick sections were cut with a vibratome (Leica), permeabilized with 0.2% Triton X-100 in Tris buffer saline (TBS) for 20 min, saturated for 1 h with 10% goat serum in TBS containing 0.03% Triton X-100 and incubated for 48 h at 4 °C with primary antibodies (anti phospho-Ser^{280} 5HT_{2A} receptor, 1:100 or anti 5-HT_{2A} receptor, 1:500) in TBS. After four washes, they were incubated for 1 h with an Alexa Fluor® 488-conjugated anti-rabbit antibody (1:1000, Invitrogen) in TBS. Immunofluorescent staining was observed with a Zeiss Axioimager Z1 microscope equipped with apotome. Images were acquired using the Axiovision 4.8 software driving an AxioCam MRm CCD camera (Carl Zeiss Microimaging).

**Inositol Phosphate Production**—Inositol phosphate production was analyzed as previously described (18).

**Immunocytochemistry and Fluorescent Microscopy**—HEK-293 cells transiently expressing HA-tagged 5-HT_{2A} receptors and grown on glass cover-slips were incubated with the rabbit anti HA antibody (1/500, 30 min at 10 °C) and then with drugs for 1 h at 37 °C. Cells were fixed in 4% (w/v) paraformaldehyde, 4% sucrose in PBS for 20 min, quenched 4 times 10 min with PBS containing 4% sucrose and 0.1% glycine, and incubated for 60 min at 4 °C with an Alexa Fluor® 594-coupled anti-rabbit antibody (1:1000 in PBS supplemented with 2% goat serum, Invitrogen) to label cell surface receptors. They were then rinsed three times PBS containing 2% goat serum, permeabilized with 0.2% (w/v) Triton X-100 in PBS containing 2% goat serum for 15 min and incubated for 30 min at 4 °C with the Alexa Fluor® 488-coupled anti-rabbit antibody (1:1000 in PBS supplemented with 2% goat serum, 0.05% Triton X-100) to label internalized receptors. After three washes, coverslips were mounted on glass slides in Mowiol® 4.88 (Calbiochem). Series of optical sections were collected with a Zeiss Axioimager Z1 microscope equipped with apotome. Images were acquired using the Axiovision 4.8 software driving an AxioCam MRm CCD camera (Carl Zeiss Microimaging).

**ELISA**—Quantification of receptor cell surface expression was performed by ELISA under nonpermeabilized conditions as previously described (18).
RESULTS

Phosphoproteome Changes Elicited by DOI and Lisuride in HEK-293 Cells—To directly compare the phosphorylation patterns generated upon 5-HT2A receptor stimulation by a hallucinogenic (DOI) and by a nonhallucinogenic (lisuride) agonist, we used the SILAC technology under three experimental conditions: light condition (vehicle-treated cells), semi-heavy label (lisuride-treated cells), and heavy label (DOI-treated cells). As cultured neurons do not divide in vitro, a complete SILAC labeling prerequisite for unbiased quantification could not be achieved in these cultures. Therefore, we performed our phosphoproteomics screen in HEK-293 cells transiently expressing 5-HT2A receptors. We first examined whether this model recapitulates the biased signaling at 5-HT2A receptors initially described in neurons, i.e., specific activation of G/i/o signaling by hallucinogens (7). Exposure of cells to DOI or LSD induced comparable stimulation of inositol phosphate production and Extracellular-regulated kinase (Erk)1,2 phosphorylation induced by LSD were slightly more pronounced (supplemental Figs. S1A and S2A). Pre-treating cells with Pertussis toxin (PTX) decreased PLC activation induced by DOI and LSD (supplemental Fig. S1B, S1C) and abolished Erk,1,2 phosphorylation induced by both hallucinogens (supplemental Fig. S2A), whereas PTX treatment did not affect the lisuride and ergotamine responses (Figs. S1D, S1E, and S2A). PTX likewise prevented Erk,1,2 phosphorylation induced by DOI and LSD without affecting lisuride and ergotamine responses in primary cultured cortical neurons (supplemental Fig. S2B). These observations indicate that, similarly to the observations in neurons, hallucinogens selectively engage G/i/o-operated signaling in HEK-293 cells, whereas nonhallucinogenic agonists do not.

After a 15-min stimulation of stable isotope-labeled HEK-293 cells with either vehicle or DOI or lisuride, cells were harvested and 1:1:1 mixtures of differentially labeled samples were digested with trypsin and phosphopeptides were enriched by HILIC followed by IMAC. Analysis of phosphopeptide-enriched fractions by nano-LC-FT-MS/MS identified 5995 phosphorylated peptides with a false discovery rate of 1%. A total of 3349 phosphopeptides were robustly quantified in at least two out of the three biological replicates. As shown in Fig. 1A, the majority of them did not exhibit significant changes in abundance, assessed by statistical significance B (16), upon 5-HT2A receptor stimulation by DOI or lisuride (agonist/vehicle ratios ~1). Only 30 phosphorylated peptides were significantly regulated by DOI versus vehicle and 24 following lisuride treatment (supplemental Table S1). Most importantly, 16 phosphopeptides were significantly different in abundance between DOI and lisuride-treated cells and 10 of them were significantly regulated by DOI, compared with vehicle (supplemental Table S1).

Hallucinogens but Not Nonhallucinogenic Agonists Induce 5-HT2A Receptor Phosphorylation at Ser280 In Vivo and In Vivo—Among the phosphopeptides exhibiting the highest differences in abundance between DOI and lisuride-treated cells, we identified a peptide located in the third intracellular (i3) loop of the receptor itself and phosphorylated on three Ser residues (Table I and supplemental Table S1) corresponding to Ser280, Ser288, and Ser291 in the entire receptor sequence. To further analyze 5-HT2A receptor phosphorylation pattern and to confirm its differential phosphorylation by hallucinogenic and nonhallucinogenic agonists, receptors originating from vehicle- or agonist-treated cells were purified by immunoprecipitation and digested with trypsin. LC-MS/MS analysis of receptor digests identified several phosphorylated forms of the same peptide located in the receptor i3 loop (LASFSFIPQSSISSEK) (Table I). MS2 spectra matching phosphorylated peptides were inspected using PhosphoSite software (17) for automatic annotation of unique transitions that pinpoint the position of phosphorylation sites (supplemental Fig. S3). In addition to a unique monophosphorylated form (phosphorylated at Ser280), various doubly and triply phosphorylated forms corresponding to phosphorylation on Ser280 and either on Ser283 or Ser287 or Ser288 or Ser290 or Ser291 or on two of these residues were also detected (Table I). Moreover, quantitative analysis of the corresponding ion signals from extracted ion chromatograms showed that the monophosphorylated peptide exhibited the highest relative abundance and that it was up-regulated by DOI or LSD exposure but not by lisuride or ergotamine (Table I, Fig. 1B, and supplemental Fig. S4). Though less abundant, the other multi-phosphorylated forms were likewise specifically up-regulated by hallucinogenic agonists (Table I and supplemental Fig. S4). In addition, these analyses identified another cluster of phosphorylated serines (Ser298 and Ser309) in a different receptor i3 loop peptide (LASFSFIPQSSISSEK) (Table I and supplemental Fig. S4). Both mono-phosphorylated (at Ser298) and doubly phosphorylated forms of this peptide were detected. However, the basal level of phosphorylation of these residues was weakly increased by both hallucinogens and nonhallucinogenic agonists (Table I and supplemental Fig. S4).

Given the apparent higher stoichiometry of Ser280 phosphorylation, compared with the phosphorylation of other residues, and the specific induction of its phosphorylation by hallucinogens, we produced a rabbit antibody against a phosphopeptide encompassing phosphorylated Ser280. We first validated the specificity of this antibody for the phosphorylated site by Western blotting using transfected HEK-293 cells. Although no immunoreactive signal was detected in blots from nontransfected cells, a clear signal was observed at the expected receptor size in blots obtained from cells expressing 5-HT2A receptor, and this immunoreactivity signal increased upon cell exposure to DOI or LSD, but not to ergotamine or lisuride (Fig. 2A). In contrast, DOI exposure did
not increase the immunoreactive signal in blots obtained from cells expressing 5-HT<sub>2A</sub> receptors mutated on Ser<sup>280</sup> (S<sup>280</sup>A, Fig. 2B and supplemental Fig. S5). Moreover, the signal observed in the absence of agonist treatment was lower in cells expressing mutant 5-HT<sub>2A</sub> receptors than in cells expressing wild type receptors (Fig. 2B). Collectively, these findings demonstrate a strong specificity of this antibody for phosphorylated Ser<sup>280</sup> and further confirm the unique capacity of hallu-
Bias 5-HT2A Receptor Phosphorylation

List of phosphorylated peptides identified from purified 5-HT2A receptors by nano-LC-MS/MS. HEK-293 cells transiently expressing HA-tagged 5-HT2A receptors were exposed to either Vehicle or DOI or LSD or lisuride or ergotamine (1 μM each, 15 min). Solubilized receptors were immunoprecipitated with the anti HA 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 nonphosphorylated peptide extracted ion chromatograms) are indicated. The data are representative of three independent experiments. ND: not determined

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Phosphorylation of 5-HT2A Receptors by Hallucinogens is Protein Kinase C-dependent and Gi/o-independent—We next searched for consensus motifs of phosphorylation by kinases in the Ser280 flanking sequence by using two different algorithms: (1) Scansite, which defines scores for phosphorylation sites according to a matrix based on an oriented peptide library to determine the optimal substrates of protein kinases (20), and (2) Group-based Prediction System (GPS, v2.1), which classifies protein kinases into a hierarchical structure with four levels and trains its algorithm against the PhosphoELM database (21), in order to determine individual false discovery rate for each kinase (22). GPS found a strong consensus for Akt/protein kinase B (PKB) (6.7/3.8) and ribosomal S6 kinases (RSKs, 2.0/1.9), whereas Scansite indicated a strong consensus for phosphorylation by protein kinase C delta (PKCδ, score 0.3926, percentile 0.143%) and PKB (0.5238, 0.341%). Thus, pharmacological inhibitors of PKC (NPC-15437, 20 μM) (23), PKB (GSK690693, 1 μM) (24), and RSKs (SL-0101–1, 10 μM) (25) were tested for a potential effect on DOI-elicited Ser280 phosphorylation. Neither GSK690693 nor SL-0101–1 had any effect on Ser280 phosphorylation in response to DOI, indicating that RSKs and PKB are not involved in this phosphorylation (Fig. 2C). In contrast, pretreatment of cells with NPC-15437 strongly decreased basal Ser280 phosphorylation and abolished the DOI-elicited response, indicating that Ser280 phosphorylation induced by hallucinogens was dependent on PKC activity (Fig. 2C). Moreover, treatment of cells with PTX did not affect Ser280 phosphorylation elicited by DOI (Fig. 2D).

Hallucinogenic and Nonhallucinogenic Agonists Differentially Desensitize and Internalize the 5-HT2A Receptor—Given the role of PKC in 5-HT2A Receptor desensitization and internalization and the importance of the 5-HT2A receptor i3 loop in the regulation of receptor responsiveness (26–29), we next examined whether hallucinogenic and nonhallucinogenic agonists differentially modulate receptor desensitization. Pretreatment of HEK-293 cells with either lisuride or ergotamine for 1 h, followed by extensive drug washout, inhibited inositol phosphate production induced by a further exposure of cells to 5-HT, whereas pretreating cells with DOI or LSD did not significantly affect the 5-HT response (Figs. 3A and 3B). The most pronounced difference in receptor desensitization (non-significant desensitization upon hallucinogen stimulation versus ~50% desensitization upon receptor stimulation by non hallucinogenic agonists) was observed after a 1 h treatment. After a 2 h treatment, both DOI and LSD desensitized the receptor, though to a differing extent (26 ± 4% desensitization) when compared with cells exposed for 2 h with lisuride or ergotamine (61 ± 5% desensitization). Therefore, 1 h pre-exposures to drugs were undertaken in further experiments. Treatment of HEK-293 cells with ergotamine and lisuride, but not with LSD and DOI, likewise induced strong desensitization of 5-HT2A receptor-operated Erk1,2 signaling (Fig. 3C). Hallucinogenic and nonhallucinogenic agonists produced a sim-
Fig. 2. Validation of the differential phosphorylation of Ser^{280} by hallucinogenic and nonhallucinogenic 5-HT_{2A} agonists using a phosphosite antibody. A-B. An antibody raised against the GTRAKLApSFSFL+C peptide was validated in HEK-293 cells transiently expressing HA-5-HT_{2A} receptors. Receptors were immunoprecipitated with the agarose bead-conjugated anti HA antibody. The generated antibody provided an immunoreactive signal in Western blots from cells expressing HA-5-HT_{2A} receptors at a molecular weight corresponding to the signal obtained with the anti-HA antibody. This immunoreactive signal increased when cells were treated for 15 min with LSD or DOI but not with lisuride or ergotamine (1 μM each). Moreover, the signal was strongly attenuated in cells expressing Ser^{280}A receptors (exposed or not to DOI), compared with cells expressing the wild type (WT) receptor. C-D. Impact of a 30-min cell pretreatment with NPC-15437 (20 μM, PKC inhibitor), or GSK690693 (1 μM, PKB inhibitor) or SL-0101–1 (10 μM, RSK inhibitor) and of a 18 h treatment with PTX (0.2 μg/ml) upon DOI-elicited Ser^{280} phosphorylation. Representative immunoblots of three independent experiments performed on different sets of cultured cells are illustrated. E. Immunofluorescence detection of cells positive for phospho-Ser^{280} 5-HT_{2A} receptor or 5-HT_{2A} receptor in the prefrontal cortex of wild type and 5-HT_{2A} receptor-deficient mice (four mice analyzed per condition) injected intraperitoneally with either vehicle or DOI or lisuride (10 mg/kg, intraperitoneal). Scale bar: 40 μm.

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amine or lisuride than in cells exposed to LSD or DOI (Fig. 5C), corroborating their differential efficacy to promote receptor internalization.

Ser280 Phosphorylation Underlies Differential Desensitization of the 5-HT<sub>2A</sub> Receptor by Hallucinogenic and Nonhallucinogenic Agonists—To directly determine the pertinence of Ser<sup>280</sup> phosphorylation upon 5-HT<sub>2A</sub> receptor desensitization, this residue was mutated into alanine or aspartate to inhibit or mimic its phosphorylation, respectively. Wild type, S<sup>280A</sup> and S<sup>280D</sup> 5-HT<sub>2A</sub> receptors displayed the same intrinsic efficacy in stimulating PLC and Erk1,2 upon activation by 5-HT (Figs. 5A and 5B). Nonetheless, the differential ability of hallucinogenic and nonhallucinogenic agonists to desensitize the receptor was not observed in cells expressing S<sup>280A</sup> or S<sup>280D</sup> 5-HT<sub>2A</sub> receptors: hallucinogenic as well as nonhallucinogenic agonists induced a strong desensitization of both PLC and Erk1,2 signaling in cells expressing the S<sup>280A</sup> receptor (to an extent comparable with that measured in cells expressing the wild type receptor after ergotamine or lisuride pretreatment), whereas all the four agonists tested induced a weak desensitization of receptor-operated signaling in cells expressing S<sup>280D</sup> 5-HT<sub>2A</sub> receptor, as observed in cells expressing wild type receptors following treatment with DOI or LSD (Figs. 5B and 5C).

**DISCUSSION**

The concept of functional selectivity or biased agonism was initially thought to reflect the ability of specific ligands of a
given GPCR to induce or stabilize different active conformations capable of activating distinct signaling pathways (31–33). The difference in 5-HT2A receptor-operated signaling upon activation by hallucinogenic and nonhallucinogenic agonists represents one of the most remarkable examples of functional selectivity so far characterized (9). More recently, phosphorylation of GPCRs at specific sites has emerged as one mechanisms contributing to functional selectivity (34) and several studies have revealed the capacity of different ligands of a given receptor to promote preferential receptor phosphorylation at distinct sites (35–38). The present report likewise demonstrated the ability of a subset of 5-HT2A receptor ago-

**Fig. 4.** Hallucinogenic and nonhallucinogenic agonists differentially internalize 5-HT2A receptors. A, HEK-293 cells transiently expressing HA-tagged 5-HT2A receptors were treated for 30 min at 10 °C with a rabbit anti HA antibody and then with either vehicle or DOI or LSD or lisuride or ergotamine (1 μM each) for 1 h at 37 °C. Cell surface receptors were labeled with the Alexa Fluor® 594-coupled anti-HA antibody (red channel) and internalized receptor with the Alexa Fluor® 488-coupled anti-HA antibody (green channel). Double immunofluorescence staining of receptors in single cells is shown. Representative images of three independent experiments are illustrated. B, Quantification of cell surface expression of receptors in cells exposed to the same treatments was performed by ELISA in nonpermeabilizing conditions. Data are means ± S.E. of quadruplicate determinations performed in a representative experiment. Two other independent experiments yielded similar results. * p < 0.05 versus vehicle-treated cells (ANOVA followed by Dunnett’s test). C, The data illustrated show the differential recruitment of β-arrestin2 by the 5-HT2A receptor (assessed by co-immunoprecipitation) in cells co-expressing HA-tagged 5-HT2A receptor and YFP-tagged β-arrestin2 and exposed to the same treatments. The Western blots illustrated are representative of three independent experiments.
nists to induce phosphorylation of one well-defined site, despite a similar intrinsic efficacy to transduce signals. Biased 5-HT2A receptor phosphorylation elicited by the different agonists tested correlated with their behavioral outcomes and might represent one critical step underlying functional selectivity at these receptors.

Mass spectrometry analyses identified several phosphorylated forms of the same peptide located in the receptor i3 loop (LASFSFIPQSSISSEK). These included a unique monophosphorylated form (phosphorylated at Ser280), and several less abundant doubly or triply phosphorylated forms systematically phosphorylated at Ser280. All these phosphorylated peptides were up-regulated by hallucinogens but not by nonhallucinogenic agonists. Collectively, these observations clearly identify Ser280 phosphorylation as the primary event governing further phosphorylation of downstream serine residues.

Differential phosphorylation of Ser280 upon receptor activation by hallucinogenic and nonhallucinogenic agonists was further established in vivo following systemic administration of these compounds to mice, by using a phosphosite specific antibody. Further supporting the relevance of Ser280 phosphorylation, the most prominent phospho-Ser280 immunoreactive signal in mice treated with a hallucinogenic agonist was detected in prefrontal cortex, the brain region involved in the psychomimetic effects of hallucinogens (8).

We identified another cluster of phosphorylated serines (Ser298 and Ser305) in a receptor i3 loop peptide (SIHREPGSYTGR). Both monophosphorylated (at Ser298) and doubly phosphorylated forms of this peptide were detected in the present study, in contrast with a previous large-scale analysis of synapse phosphoproteome in the mouse, which only identified phosphorylated Ser298 (39). The phosphorylation of Ser298 and Ser305 was weakly induced by both hallucinogens and nonhallucinogenic agonists. Moreover, our studies did not detect phosphorylation of Ser314, another serine located in the receptor i3 loop previously identified as a RSK2 substrate (26). Notably, phosphorylation of this residue was detected in vitro by incubating receptor i3 loop or the entire purified 5-HT2A receptor with recombinant RSK2, whereas the present study investigated the receptor phosphorylation state in HEK-293 cells. Further experiments suggested a role of Ser314 phosphorylation in attenuation of 5-HT2A receptor signaling induced by EGF and PDGF in a variety of cell types, including neurons (40). Our results suggest that in the absence of growth factors, Ser314 phosphorylation might occur at a lower stoichiometry than the other phosphorylated residues identified in the present study, even upon agonist stimulation of 5-HT2A receptors. Together with previous findings, they also identify the receptor i3 loop, which contains 18 potential phosphorylation sites for Ser/Thr kinases, as a hot
spot of phosphorylation potentially important for regulating receptor functional activity.

In an effort to identify protein kinase(s) contributing to hallucinogen-elicted Ser\textsuperscript{280} phosphorylation, we found that it was dependent on PKC activity, though we cannot conclude at this stage whether PKC directly phosphorylates Ser\textsuperscript{280} or whether the phosphorylation of this residue is elicited by a closely related kinase different from RSK and PKB and activated by PKC. This observation was quite unexpected as both hallucinogenic and nonhallucinogenic agonists activate the PLC pathway and therefore PKC. Moreover, PTX treatment did not affect DOI-elicited Ser\textsuperscript{280} phosphorylation, indicating that biased 5-HT\textsubscript{2A} receptor phosphorylation is not triggered by a pathway (Gi/o-dependent) selectively engaged by hallucinogens but rather by a common pathway activated by both hallucinogenic and hallucinogenic agonists. We thus hypothesize that Ser\textsuperscript{280} might be accessible for PKC phosphorylation only in a 5-HT\textsubscript{2A} receptor conformation that is specifically stabilized by hallucinogens.

Another important finding of the present study is the different ability of hallucinogenic and nonhallucinogenic agonists to induce 5-HT\textsubscript{2A} receptor desensitization and internalization, observed in both HEK-293 cells and cortical neurons. To our knowledge, no study has so far compared the ability of hallucinogenic and nonhallucinogenic compounds to desensitize 5-HT\textsubscript{2A} receptors. Nonetheless, the present findings are consistent with a recent study, which demonstrated that DOI was less efficient than 5-HT to internalize eGFP-tagged 5-HT\textsubscript{2A} receptors stably expressed in HEK-293 cells (27). They also provide convergent evidence indicating that the different effects of hallucinogenic versus non hallucinogenic agonists upon receptor desensitization reflect their differential capacity to promote Ser\textsuperscript{280} phosphorylation: (1) mutating Ser\textsuperscript{280} into alanine or aspartate abolished the difference in the agonist effects upon receptor desensitization; (2) hallucinogens were able to desensitize S\textsuperscript{280}A receptor to an extent comparable to that induced by nonhallucinogenic agonists in cells expressing wild type receptor and (3) nonhallucinogenic agonists did not promote desensitization of the Ser\textsuperscript{280}D receptor mutant. Collectively, these observations establish a direct link between Ser\textsuperscript{280} phosphorylation and the low capacity of hallucinogens to desensitize the receptor and suggest that Ser\textsuperscript{280} is phosphorylated at a high stoichiometry following hallucinogen treatment. These findings contrast with a previous study which showed that mutating into alanine two serine residues, one (Ser\textsuperscript{421}) located in the receptor C terminus and the other (Ser\textsuperscript{188}) in the 2 loop, strongly reduced quipazine-mediated receptor desensitization, whereas the deletion of residues 280–296 or residues 280–310 in the 3 loop (i.e., Ser\textsuperscript{280} and downstream residues phosphorylated upon hallucinogen treatment) had no effect on the time course and extent of 5-HT\textsubscript{2A} receptor desensitization (41). However, it is likely that the agonist used to induce receptor desensitization (quipazine), which is devoid of hallucinogenic activity in humans (42), does not induce S\textsuperscript{280} phosphorylation, like lisuride and ergotamine. Phosphorylation of other residues (e.g., Ser\textsuperscript{188} and/or Ser\textsuperscript{421}), though not detected in our MS/MS analyses, might thus underlie 5-HT\textsubscript{2A} receptor desensitization induced by any receptor agonist, whereas the specific phosphorylation of Ser\textsuperscript{280} (and/or of downstream serines in i3 loop) by hallucinogens might act as a brake limiting receptor desensitization. Alternatively, Ser\textsuperscript{280} phosphorylation might facilitate 5-HT\textsubscript{2A} receptor resensitization that occurs in the continuous presence of agonist, consistent with previous findings indicating that receptor resensitization, like Ser\textsuperscript{280} phosphorylation, is also dependent of PKC (28).

Initial studies on 5-HT\textsubscript{2A} receptor desensitization and internalization showed that they are both β-arrestin-independent (43), contrasting with what is more generally observed for numerous GPCRs. However, the situation is probably more complex than previously imagined, as a more recent study revealed a differential pattern of β-arrestin sensitivity for agonist-induced receptor internalization: though treatment with DOI or the 5-HT precursor L-5-hydroxy-tryptophan displayed similar efficacies to promote receptor internalization, DOI-induced receptor internalization was β-arrestin-independent, whereas 5-HT-induced internalization requires β-arrestins (44). β-arrestin-independent receptor internalization elicited by DOI treatment corroborates with the low ability of this compound to promote β-arrestin recruitment by the receptor (compared with nonhallucinogenic agonists), a property shared by LSD and likely reflecting the unique ability of hallucinogens to promote Ser\textsuperscript{280} phosphorylation. Whether β-arrestins contributes to receptor-internalization elicited by the nonhallucinogenic agonists remains to be elucidated.

In conclusion, our observations show that ligand identity not only determines the nature of 5-HT\textsubscript{2A} receptor-operated signaling but also the pattern of receptor phosphorylation at a site (Ser\textsuperscript{280}) involved in desensitization and internalization. They highlight the power of quantitative phosphoproteomics to identify mechanisms underlying functional selectivity and of potential relevance to the behavioral responses induced by biased ligands. The clinical significance of the biased 5-HT\textsubscript{2A} receptor phosphorylation remains to be established. In this regard, it would be of considerable interest to explore in future studies how Ser\textsuperscript{280} phosphorylation is affected by the different classes of antipsychotics and by 5-HT\textsubscript{2A} receptor heterodimerization with mGlu2 metabotropic glutamate receptor, a process critical for hallucinogen psychomimetic activity (45, 46).
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