

Regulation of Serotonin 1B Receptor by Glycogen Synthase Kinase-3

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ABSTRACT

In response to 5-hydroxytryptamine (5-HT), the type 1 serotonin receptors (5-HT1Rs) preferentially couple to the inhibitory G protein and elicit many physiological and behavioral processes. However, their regulation by intracellular protein kinases has not been fully investigated. In this study, we identified that glycogen synthase kinase-3 (GSK3) differentially regulates 5-HT1Rs. In receptor-expressing cells and brain slices, activation of both 5-HT1AR and 5-HT1BR reduced forskolin-stimulated cAMP production, but only the effect of 5-HT1BR was abolished by selective GSK3 inhibitors, deletion of GSK3 β by RNAi, or overexpression of impaired GSK3 β mutants (R96A and K85,86A). A consensus GSK3 phosphorylation sequence was identified between the serine-154 and threonine-158 in the second intracellular loop of 5-HT1BR. Mutation of either serine-

154 or threonine-158 to alanine significantly reduced response of 5-HT1BR to 5-HT. Active GSK3 β interacted with resting 5-HT1BR to form a protein complex. The interaction was enhanced by receptor activation, abolished by GSK3 inhibitors, and dependent on the phosphorylation state of serine-154. In addition, regulation of 5-HT1BR by GSK3 changed the dynamics of agonist-induced cell surface receptor internalization, in which lack of phosphorylation at Ser154 resulted in sustained reduction of 5-HT1BR at the cell surface. Although the physiological consequences of selective regulation of 5-HT1BR by GSK3 remain to be identified, findings in this study reveal a new function of GSK3 as a protein kinase that is able to selectively regulate G protein-coupled receptors.

Most serotonin (5-HT) receptors are G protein-coupled receptors (GPCRs) that are classified by their sequence homology and by the type of G proteins and signal transduction pathways with which they are associated (Hannon and Hoyer, 2008). The type 1 5-HT receptors (5-HT1Rs), including subtypes A (5-HT1AR) and B (5-HT1BR), are mainly coupled to the inhibitory G protein, which links the receptors to inhibition of adenylyl cyclase and regulation of several other signal pathways (Raymond et al., 2001). 5-HT1AR and 5-HT1BR are found in several brain areas, such as the dorsal

raphe nucleus, nucleus accumbens, cerebral cortex, hippocampus, and striatum. In the raphe nucleus, 5-HT1AR and 5-HT1BR are characterized as autoreceptors, with the former located at the somatodendritic area and the latter at the axon terminus. In many other brain regions and peripheral tissues, 5-HT1AR and 5-HT1BR also function as heteroreceptors in nonserotonergic neurons (Hannon and Hoyer, 2008). 5-HT1Rs are involved in many physiological processes in the brain, especially regulating turnover of 5-HT and other neurotransmitters in several brain regions (Ase et al., 2000). 5-HT1Rs are a major group of serotonin receptors that modulate behaviors, especially those relevant to neuropsychiatric diseases, such as anxiety, mood, and aggression (Gingrich and Hen, 2001). Therefore, proper regulation of activities of 5-HT1Rs is crucially important in maintaining normal brain function.

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; ANOVA, analysis of variance; BIO, 6-bromoindirubin-3'-oxime; BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; GSK3, glycogen synthase kinase-3; HEK, human embryonic kidney; 5-HT1R, type-1 serotonin receptor; 5-HT1AR, type-1 serotonin receptor subtype A; 5-HT1BR, type-1 serotonin receptor subtype B; K85,86A, mutation of lysine-85 and -86 to alanine; 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin; PBS, phosphate-buffered saline; Rluc, *Renilla reniformis* luciferase; SB216641, *N*-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1'-biphenyl]-4-carboxamide; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; SB224289, 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4'-piperidine] oxalate; shRNA, short hairpin RNA; YFP, yellow fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HA, hemagglutinin; Cdk, cyclin-dependent kinase; i2, second intracellular loop; WT, wild type.

Besides agonist-stimulated activation and coupling to G proteins, activities of GPCRs are regulated via dynamic interactions with intracellular proteins, such as phosphorylation by G protein-coupled receptor kinases and association with β -arrestin, which uncouples GPCRs from G proteins (Gainetdinov et al., 2004; Premont, 2005). Evidence of intracellular protein interaction with 5-HT1Rs has also emerged; particularly notable are the 5-HT1AR interaction with calmodulin (Turner et al., 2004) and the 5-HT1BR interaction with p11 protein (Svenningsson et al., 2006). Nevertheless, in lieu of the multiple physiological functions linked to 5-HT1Rs, regulation of their activities by intracellular proteins, such as protein kinases, has not been fully investigated.

Glycogen synthase kinase-3 (GSK3) (Embi et al., 1980) is a broadly influential enzyme that phosphorylates and modulates many protein substrates involved in gene expression, cell survival, and cytoskeletal structure (Jope and Johnson, 2004). Two major isoforms of GSK3, GSK3 α and GSK3 β , share 84% sequence homology, but they are encoded by different genes (Woodgett, 1990), and their functions are not always interchangeable when phosphorylating and regulating their substrate proteins (Wang et al., 1994; Liang and Chuang, 2006). To regulate a substrate by phosphorylation, GSK3 locates a serine or threonine residue that has been phosphorylated previously, or primed, by another protein kinase, and phosphorylates the serine or threonine four amino acids to the N-terminal side of the primed site (S/TXXXXS/T) (Fiol et al., 1987). Phosphorylation by GSK3 may activate or inhibit the activity of a substrate protein (Jope and Johnson, 2004).

GSK3 is constitutively active in all cells (Doble and Woodgett, 2003), but in accordance with its many important actions, the activity of this protein kinase is tightly regulated in an inhibitory manner. The mostly studied mechanisms of regulation include the N-terminal serine (serine-21 for GSK3 α and serine-9 for GSK3 β) phosphorylation by several protein kinases (Goode et al., 1992; Cross et al., 1995) and the canonical Wnt-regulated GSK3 protein interactions (Rubinfeld et al., 1996). Viewed from a pharmacological standpoint, the therapeutic agent lithium used in bipolar disorder is a selective inhibitor of GSK3 by directly competing for magnesium (Klein and Melton, 1996) and by indirectly increasing the inhibitory N-terminal serine phosphorylation (Chalecka-Franaszek and Chuang, 1999; De Sarno et al., 2002; Zhang et al., 2003). With its multiaxial functions, it has been recognized that abnormal regulation of GSK3 activity is involved in several human diseases, such as mood disorders (Jope and Roh, 2006), neurodegenerative diseases (Takashima, 2006), and diabetes (Woodgett, 2003). Therefore, GSK3 has been considered to be a promising molecular target in the treatment of these diseases (Meijer et al., 2004).

To date, there has been little investigation in a regulatory action of GSK3 on GPCRs. It is interesting that several early investigations observed that lithium had a noticeable effect on 5-HT1BR (Massot et al., 1999), but the mechanism of this action was not revealed. In this study, we used several experimental approaches to determine whether GSK3 has an impact in agonist-induced activation of 5-HT1Rs and whether subtypes of 5-HT1Rs are differentially regulated by GSK3.

Materials and Methods

Agonists and Inhibitors. Anpirtoline, kenpaullone, SB216641, SB216763, SB224289 (Tocris Bioscience, Ellisville, MO); 6-bromoindirubin-3'-oxime (BIO), cholera toxin, forskolin, roscovitine, 8-OH-DPAT, serotonin (5-HT) (Sigma-Aldrich, St. Louis, MO) are dissolved in distilled water or dimethyl sulfoxide in a concentrated solution before they were used for treatment.

DNA Constructs and Mutagenesis. Rat 5-HT1AR cDNA (provided by Dr. Paul Albert, University of Ottawa, Ottawa, ON, Canada) and mouse 5-HT1BR cDNA (provided by Dr. Per Svenningsson, The Rockefeller University, New York, NY) were subcloned into pcDNA6/myc-His (Invitrogen, Carlsbad, CA), pEGFP-C1 (Clontech, Mountain View, CA), or pRL-TK (Promega, Madison, WI) vectors. The cDNAs of mouse GSK3 β , R96A-GSK3 β , K85,86A-GSK3 β , and YFP-tagged GSK3 β were provided by Dr. Richard S. Jope (University of Alabama at Birmingham, Birmingham, AL). Mutagenesis of 5-HT1BR was performed using the Quik-Change site-directed mutagenesis protocol (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing before cellular transfection.

Cell Culture and Transfection. Chinese hamster ovary (CHO), human embryonic kidney-293 (HEK-293), and human SH-SY5Y neuroblastoma (SH-SY5Y) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (0.1 mg/ml). For transient transfection of DNA, cells were grown to 70% confluence, and DNAs were transfected into cells using the FuGeneHD transfection reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Stable-transfected cell lines were selected and maintained in media containing corresponding antibiotics. For experiments, cells were placed in serum-free media before treatment with inhibitors and/or activating agents.

RNAi of GSK3. SH-SY5Y cells were transduced with lentivirus particles containing short hairpin RNA (shRNA) sequences for human GSK3 α (Dr. Richard S. Jope) or GSK3 β (Sigma-Aldrich). Cells transduced with GFP-containing lentiviral GSK3 α shRNA (GAGT-TCAAGTTCCCTCAG) were identified using fluorescence-activated cell sorting (FACS) to select GFP-positive cells. The shRNA sequences for GSK3 β are as follows: 1, CCCAACTACACAGAATT-TAA; 2, GGTGTGGATCAGTTGGTAGAAA; 3, GACACTAAAGT-GATTGGAAAT; 4, GCAGGACAAGAGATTTAAGAA; and 5, CCACTGATTATACCTCTAGTA. GSK3 β shRNA-expressing cell lines were selected by the antibiotic puromycin (1 μ g/ml). All RNAi-induced GSK3 knockdowns were confirmed by immunoblots using the anti-GSK3 α/β antibody (Millipore, Billerica, MA).

Animals and Brain Slice Preparations. The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved animal use in this study. Twelve-week-old adult male C57BL/6 mice (Fredrick Cancer Research, Fredrick, MD) were housed 4 to 5 per cage with free access to food and water in a 12-h light/dark cycle animal facility for 1 week before they were used for experiments. Mice were sacrificed by decapitation, and brains were rapidly dissected in ice-cold saline for cerebral cortical slice preparation. Transverse cortical slices (300 μ m) were rinsed and preincubated in a HEPES-bicarbonate buffer (122 mM NaCl, 15 mM NaHCO₃, 11 mM glucose, 10 mM HEPES, 4.9 mM KCl, 1.2 mM MgSO₄, and 100 μ M CaCl₂) containing 5% CO₂/95% O₂ at 37°C for 30 min, followed by incubation with pharmacological agents for up to 2 h.

cAMP Assay. The level of cAMP was measured in cells and in cerebral cortical slices using an enzyme immunoassay kit (Direct BioTrak; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). To minimize the potential nonselective effects on GSK3 by other inhibitors, all experiments with cAMP measurement in this study were conducted in the absence of phosphodiesterase inhibitors. After pharmacological treatments, cells or cerebral cortical slices were lysed in a buffer containing 2.5% dodecyltrimethylammonium bromide. The cerebral cortical slices were thoroughly homogenized by

sonication and centrifuged at 9500 rpm to obtain tissue lysate from the supernatant. Duplicate lysates were transferred into individual wells of a 96-well plate precoated with donkey anti-rabbit IgG. After incubation with rabbit antiserum against cAMP and cAMP-horse-radish peroxidase conjugates, enzyme reaction was started by addition of peroxidase substrate, stopped with sulfuric acid, and color reaction was detected at 450 nm in the Synergy 2 spectrofluorometer (BioTek Instruments, Winooski, VT). Protein concentrations of cell lysate and solubilized cerebral cortical slices were measured using the Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). The level of cAMP was calculated against a protein standard and normalized by total protein content in each sample (in femtomoles per milligram).

Bioluminescence Resonance Energy Transfer Assay. 5-HT1BR-*Renilla reniformis* luciferase (Rluc) fusion protein, in which the Rluc was at the C-terminal end of the receptor, was stably expressed in HEK-293 cells. For experiments, cells expressing wild-type or mutant 5-HT1BR-Rluc were transfected with different amounts of DNA for YFP or YFP-GSK3 β . YFP expression was confirmed by measuring fluorescence signal acquired by excitation at 485 ± 10 nm and emission at 528 ± 10 nm using the Synergy 2 spectrofluorometer. GSK3 β expression was confirmed by immunoblotting GSK3 α/β . After treatment with pharmacological agents, cells were quickly washed with warm PBS, detached with EDTA (5 mM), and transferred into white opaque 96-well plates. Immediately after the addition of the Rluc substrate coelenterazine f (5 μ M; Invitrogen) into each well, emission was detected at the wavelengths of 485 ± 10 and 528 ± 10 nm, corresponding to the maxima of the emission spectra for Rluc and YFP, respectively. The bioluminescence resonance energy transfer (BRET) ratio was calculated using the equation [(emission at 528 ± 10 nm) – (emission at 485 ± 10 nm \times Cf)]/emission at 485 ± 10 nm (Angers et al., 2000), where Cf is (emission at 528 ± 10 nm)/emission at 485 ± 10 nm measured in cells transfected with 1 μ g of a DNA vector without YFP.

Immunoprecipitation and Immunoblotting. Fresh cells expressing GFP-5-HT1BR and HA-GSK3 β were lysed by rotating in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5% CHAPS, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 5 μ g/ml pepstatin, 0.1 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 100 mM okadaic acid. For coimmunoprecipitation, protein lysate (200 μ g) was incubated with anti-HA-conjugated agarose (Bethyl, Montgomery, TX) overnight. After washed for three times with a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.3% Triton X-100, and 10% glycerol, the immunocomplex was denatured at 67°C in SDS sample buffer, and proteins were subjected to electrophoresis in 10% polyacrylamide gels and immunoblotted with anti-GFP (Abcam Inc., Cambridge, UK) and anti-HA (Covance, Berkeley, CA) antibodies. Protein bands were detected with enhanced chemiluminescence (GE Healthcare).

Fluorescence-Activated Cell Sorting. CHO cells transfected with N-terminal GFP-tagged 5-HT1BR (wild-type and point mutants) were trypsinized and resuspended in 4% paraformaldehyde/PBS. After a wash in PBS, a mixture of GFP antibody (Abcam) and Zenon Alexa 647 (Invitrogen) was added to the cell slurry to label surface receptors. Cells were washed and resuspended in PBS and analyzed by FACS.

Immunocytochemistry of 5-HT1BR. HEK-293 cells were transfected with wild-type or mutant GFP-tagged 5-HT1BR and plated on poly(D-lysine) (Sigma)-coated coverslips (VWR, West Chester, PA). After being treated with 5-HT, cells were fixed with 4% paraformaldehyde/PBS and blocked with 1% bovine serum albumin/0.2% nonfat milk in PBS. Cell surface 5-HT1BR was labeled with rabbit anti-GFP antibody (Abcam), followed by Texas Red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescence signals were viewed with a confocal microscope (DM IREB; Leica, Wetzlar, Germany), and images of cells were acquired with photomultiplier tubes that are

internal parts of the confocal microscope. Fluorescent signals were acquired by excitation at wavelength of 561 nm for Texas Red and 488 nm for GFP, and signal intensities were quantified with the Metamorph software (Molecular Devices, Sunnyvale, CA). For each treatment condition, the average fluorescent intensity from 50 to 100 cells in 5 to 10 microscopic fields was used for data analysis.

Data Analysis and Statistics. All treatments were tested at least in duplicate for each experiment, and all experiments were repeated for statistical analysis. Data were organized in Microsoft Office Excel 2007 and analyzed by the analysis of variance (ANOVA) or Student's *t* test, where appropriate, using the SigmaStat 3.0 program (Systat Software, Inc., San Jose, CA). Values were expressed as mean \pm S.E.M. and were considered significant when $p < 0.05$.

Results

To measure agonist-stimulated activation of 5-HT1Rs, we used forskolin-stimulated cAMP production (Seamon et al., 1981) as a readout assay. Treatment with forskolin (10 μ M for 15 min) increased the level of cAMP in CHO cells (Fig. 1A). When these cells were transfected with either rat 5-HT1AR or mouse 5-HT1BR, pretreatment with 5-HT (10 μ M) 30 min before forskolin maximally reduced the level of forskolin-stimulated cAMP production.

To examine whether 5-HT1Rs can be regulated by GSK3, cells expressing 5-HT1AR or 5-HT1BR were pretreated with one of the three structurally diverse selective GSK3 inhibitors, BIO, SB216763, and kenpaullone, followed by examining 5-HT-induced inhibition of cAMP production (Fig. 1B). Each of the three GSK3 inhibitors concentration-dependently diminished the effect of 5-HT in 5-HT1BR-expressing cells, with BIO causing a maximal inhibitory effect at 1 μ M and SB216763 and kenpaullone at 10 μ M (96.6 ± 7.7 , 106.0 ± 10.8 , and 100.4 ± 5.7 of the forskolin-stimulated response, respectively). In contrast, neither of the GSK3 inhibitors affected the effect of 5-HT in 5-HT1AR-expressing cells. In addition, a 24-h treatment with the therapeutically used GSK3 inhibitor lithium concentration-dependently abolished the inhibitory effect of 5-HT in 5-HT1BR-expressing cells, reaching $101.9 \pm 10.9\%$ of the forskolin-stimulated response with 10 mM lithium chloride (Fig. 1C).

To examine whether GSK3 regulates 5-HT1Rs in a physiological preparation, cAMP production was measured in mouse cerebral cortical slices that express endogenous 5-HT1AR and 5-HT1BR (Bruinvels et al., 1994; Stenfors et al., 1998). Treatment with either the 5-HT1AR agonist 8-OH-DPAT (20 μ M) or the 5-HT1BR agonist anpirtoline (20 μ M) significantly reduced the level of forskolin-stimulated cAMP production to 47.1 ± 4.7 and $45.8 \pm 2.2\%$, respectively (Fig. 1D). Pretreatment of cortical slices with GSK3 inhibitors BIO and kenpaullone (10 μ M, 2 h) did not change the effect of 8-OH-DPAT but significantly diminished the effect of anpirtoline on cAMP production (82.6 ± 10.8 and $89.9 \pm 6.9\%$ of forskolin-stimulated response, respectively). Thus, results in both 5-HT1R-expressing cells and in mouse cerebral cortical slices show that GSK3 inhibitors reduce the response of 5-HT1BR to 5-HT.

The effect of GSK3 inhibitors on 5-HT1BR activity was selective because in CHO cells, inhibition of cyclin-dependent kinases (Cdk) with a selective Cdk inhibitor roscovitine (10 μ M) did not alter 5-HT-induced inhibition of cAMP production in 5-HT1BR-expressing cells (Fig. 1E), and pretreatment

of CHO cells with the GSK3 inhibitor SB216763 did not change cAMP production induced by the stimulatory G protein activator cholera toxin (1 μ g/ml) or the adenylyl cyclase activator forskolin (10 μ M) alone (Fig. 1F).

To test for a GSK3 isoform-specific effect, 5-HT_{1B}BR was expressed in human SH-SY5Y neuroblastoma cells, in which GSK3 α or GSK3 β was deleted by selective shRNAs. The forskolin-stimulated cAMP production was reduced by 5-HT (10 μ M) in control SH-SY5Y cells without GSK3 knockdown and in cells with GSK3 α knockdown (40.4 ± 5.0 and $30.4 \pm 13.6\%$ of forskolin-stimulated response, respectively). However, the inhibitory effect of 5-HT was completely abolished in GSK3 β knockdown cells ($100.8 \pm 5.4\%$ of forskolin-stimulated response) (Fig. 2A). This result was further tested in five different SH-SY5Y cell lines that express different se-

quences of GSK3 β shRNA. As observed by immunoblotting, two of the five GSK3 β shRNA-expressing cell lines had reduced levels of GSK3 β protein. After expression of 5-HT_{1B}BR in each of these cell lines, the effect of 5-HT in reducing cAMP production was only abolished in the two cell lines with low levels of GSK3 β (Fig. 2B).

The specific effect of GSK3 in regulating 5-HT_{1B}BR activity was also tested in cells overexpressing wild-type GSK3 β , R96A-GSK3 β (Frame et al., 2001), or K85,86A-GSK3 β (Eldar-Finkelman et al., 1996). The R96A mutation prevents the binding of GSK3 to a primed substrate (inactive), whereas the K85,86A mutation lacks kinase activity (Doble and Woodgett, 2003). 5-HT significantly reduced cAMP production ($37.2 \pm 4.2\%$ of forskolin-stimulated response) in wild-type GSK3 β -expressing cells similarly as in cells expressing

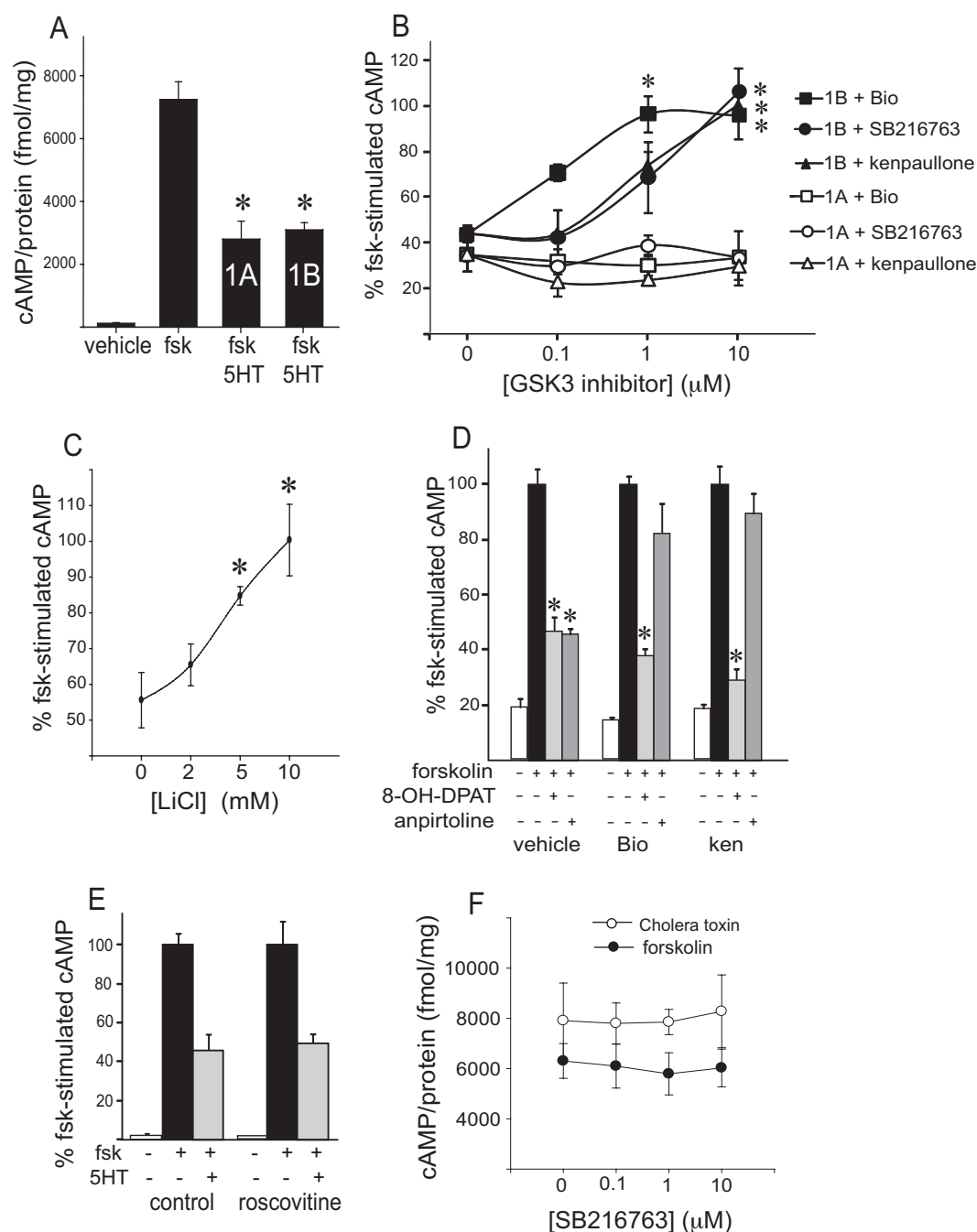


Fig. 1. GSK3 inhibitors selectively decrease 5-HT_{1B}BR activity. **A**, CHO cells were transiently transfected with 5-HT_{1A} or 5-HT_{1B} and treated with 5-HT (10 μ M) for 30 min before the addition of forskolin (fsk; 10 μ M) for 15 min to stimulate cAMP production. Forskolin-stimulated cAMP production was measured by enzyme immunoassay and the levels of cAMP are calculated as femtomoles per milligram of protein. **B**, CHO cells expressing 5-HT_{1A} (1A, open labels) or 5-HT_{1B} (1B, closed labels) were pretreated with the indicated concentrations of GSK3 inhibitors Bio (3 h), SB216763 (1 h), or kenpaullone (3 h), followed by 5-HT and forskolin treatment. **C**, 5-HT_{1B}-expressing cells were treated with indicated concentrations of LiCl for 24 h, followed by 5-HT and forskolin treatment. **D**, cerebral cortical slices were treated with a dimethyl sulfoxide vehicle, 10 μ M Bio, or 10 μ M kenpaullone for 2 h. 5-HT_{1A} and 5-HT_{1B} were activated with 8-OH-DPAT (20 μ M) and anpirtoline (20 μ M), respectively, for 30 min before the addition of forskolin (10 μ M, 30 min). **E**, CHO cells expressing 5-HT_{1B} were pretreated with the Cdk inhibitor roscovitine (10 μ M) for 3 h, followed by 5-HT and forskolin treatments. The levels of cAMP are calculated as a percentage of forskolin-stimulated response (B–E). **F**, levels of cholera toxin (1 μ g/ml)- and forskolin (10 μ M)-stimulated cAMP in the presence of indicated concentrations of the GSK3 inhibitor SB216763. *, $p < 0.01$ in one-way ANOVA when values are compared with forskolin treatment alone (A and D) or with no inhibitor treatment (B and C). Mean \pm S.E.M., $n = 6$ to 7.

endogenous GSK3 β (Fig. 2C), suggesting that endogenous active GSK3 β is sufficient for 5-HT-stimulated 5-HT1BR activity. In contrast, the effect of 5-HT was significantly impaired in cells expressing R96A-GSK3 β or K85,86A-GSK3 β (84.3 ± 6.8 and $92.4 \pm 6.6\%$ of forskolin-stimulated response, respectively). Thus, along with findings in GSK3 knockdown cells, these results demonstrate that active GSK3 β is necessary for 5-HT1BR-induced inhibition of cAMP production.

Because a major function of GSK3 is to phosphorylate primed substrate proteins, we searched protein sequences of human 5-HT1AR and 5-HT1BR for potential GSK3 phosphorylation sites. It is interesting that human 5-HT1AR only contains one consensus sequence for GSK3 phosphorylation (threonine-240) in the third intracellular loop, whereas human 5-HT1BR contains several consensus GSK3 phosphorylation sites distributed along all three intracellular loops of the receptor (Fig. 3A). These potential GSK3-regulating sequences are homologous in human and mouse 5-HT1BR.

To test whether phosphorylation states of these GSK3 consensus sites have any effect in agonist-stimulated 5-HT1BR activity, each of the five priming serine or threonine was replaced by alanine to eliminate primed phosphorylation (Fig. 3B, top). Expression of these 5-HT1BR mutants in CHO cells revealed that only the alanine mutant of threonine-158 (T158A) in the second intracellular (i2) loop had a diminished response to 5-HT ($87.3 \pm 6.9\%$ of forskolin-stimulated response), whereas mutation of all other potential GSK3 priming sites (T73A, T243A, S268A, and S279A) in the first and the third intracellular loops did not change the response to 5-HT (Fig. 3B, middle).

To test whether the putative GSK3 phosphorylation site at serine-154 (Ser154), a serine located 4 amino acids N-terminal of Thr158, is a determinant of 5-HT1BR activity, the Ser154 was replaced with alanine (S154A) to abolish phosphorylation by GSK3. The S154A mutant had minimal response to 5-HT in reducing forskolin-induced cAMP produc-

tion ($80.4 \pm 2.1\%$ of forskolin-stimulated response) (Fig. 3B, bottom). In contrast, the phospho-mimic aspartate (S154D) mutant responded to 5-HT in a similar manner as in cells expressing wild-type 5-HT1BR, causing significant reduction of forskolin-induced cAMP production. Furthermore, similar to the S154D mutant, mutation of Thr158 with the phospho-mimic aspartate (T158D) did not change the receptor response to 5-HT. Although the responses to 5-HT were different, in the absence of 5-HT, none of these mutants (S154A, S154D, T158A, and T158D) affected forskolin-induced cAMP production. The expression efficiency of each 5-HT1BR mutant on the cell surface was sufficient for agonist binding, because the N-terminal GFP-tagged wild-type and mutant 5-HT1BRs were each detected on the cell surface as determined using FACS analysis (Fig. 3C). Therefore, these results indicate that primed phosphorylation at the consensus GSK3 phosphorylation site in the i2 loop of 5-HT1BR is probably a determinant for 5-HT1BR activation.

In cells expressing wild-type 5-HT1BR, forskolin-stimulated cAMP production was dynamically regulated by 5-HT. Treatment with 5-HT for 5 to 45 min before forskolin gradually decreased cAMP production, with the effect peaked at 30 to 45 min and gradually diminished between 60 and 120 min (Fig. 4A). To examine the dynamic response of mutant 5-HT1BR to 5-HT, CHO cells were transfected with the S154A or the S154D mutant and treated with 5-HT for the indicated length of time. Somewhat unexpectedly, in cells expressing S154A mutant, 5-HT caused a rapid and marked reduction in cAMP production that reached a peak effect at 5-min treatment, but the response to 5-HT rapidly diminished at 15-min treatment and was nearly abolished at 30-min treatment. The overall activity of the S154A mutant after 5-HT stimulation was significantly different from the wild-type 5-HT1BR, with the difference more prominent at 5-, 30-, and 45-min treatment. On the other hand, the response of the S154D mutant to 5-HT was similar to that of

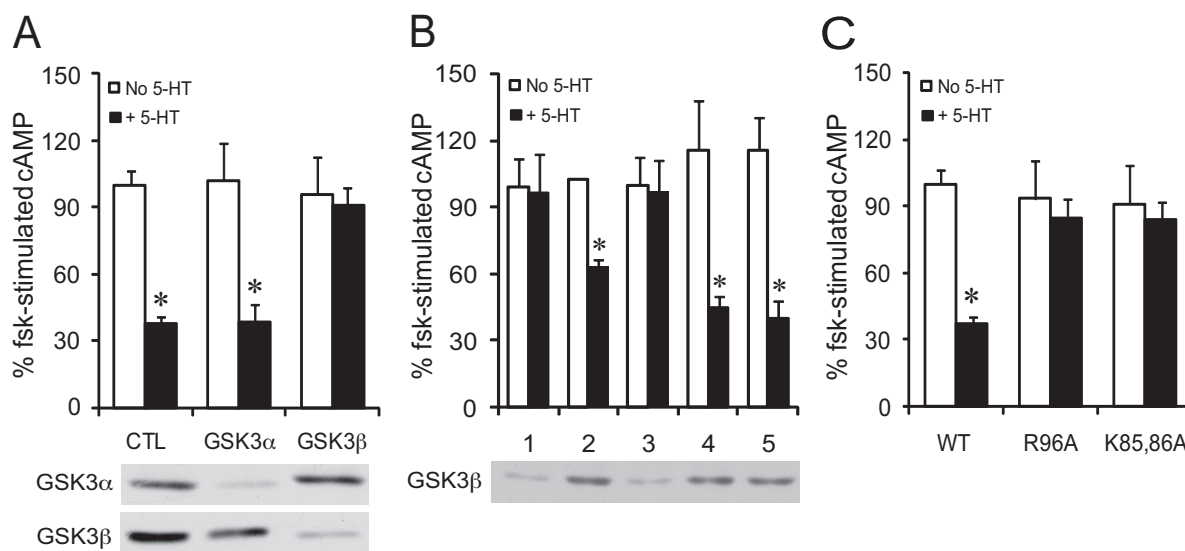


Fig. 2. GSK3 β , but not GSK3 α , is required for activation of 5-HT1BR. 5-HT1BR was expressed in SH-SY5Y cells that contain no shRNA (CTL), or stably express GSK3 α shRNA (GSK3 α) or GSK3 β shRNA (GSK3 β) (A), stably express different GSK3 β -targeting shRNAs (1–5) (B), and express wild-type GSK3 β (WT), R96A-GSK3 β , or K85,86A-GSK3 β (C). These cells were preincubated with or without 5-HT (10 μ M, 30 min) followed by forskolin treatment (10 μ M, 15 min), and the levels of cAMP were measured. Data are expressed as a percentage of forskolin-stimulated cAMP production in control (A and B) or in wild-type GSK3 β -expressing (C) cells. *, $p < 0.01$ in one-way ANOVA when values are compared with the same type of cells without 5-HT treatment. Mean \pm S.E.M., $n = 4$ to 6. Immunoblots below the graphs show the levels of total GSK3 α and GSK3 β in control and GSK3 shRNA-expressing cells.

the wild-type 5-HT_{1B}BR, except that a prolonged inhibition of cAMP production was significant at 120 min of 5-HT treatment. These observations suggest that the phosphorylation state of the Ser154 residue in the i2 loop is involved in both initiation and duration of agonist-induced receptor activation and properly transducing receptor signals to its downstream acceptors.

To confirm that the Ser154 residue is a GSK3-regulated site, cells expressing the S154A mutant were treated with the GSK3 inhibitor SB216763 before examining the time course of 5-HT in cAMP production. SB216763 had no effect on the S154A mutant, because the receptor exhibited the characteristic brief activation and rapid inactivation after 5-HT treatment either with or without SB216763 (Fig. 4B,

left). Furthermore, none of the GSK3 inhibitors—SB216763, kenpaullone, and BIO—changed the response of the S154D mutant to 5-HT (Fig. 4B, right). Therefore, the Ser154 residue is a selective GSK3-regulated site in 5-HT_{1B}BR.

For GSK3 to phosphorylate a substrate, it must interact with the substrate. We therefore used BRET (Angers et al., 2000) to examine whether GSK3 β interacts with 5-HT_{1B}BR. HEK-293 cells stably expressing 5-HT_{1B}BR-Rluc fusion protein were transfected with different concentrations of DNAs for YFP alone or YFP-GSK3 β . The expression of GSK3 β in these cells was confirmed by immunoblotting GSK3, and the expression of YFP was confirmed by measuring YFP fluorescence intensity (Fig. 5A). In YFP-GSK3 β expressing cells, the BRET ratio increased significantly in a fluorescence intensity-

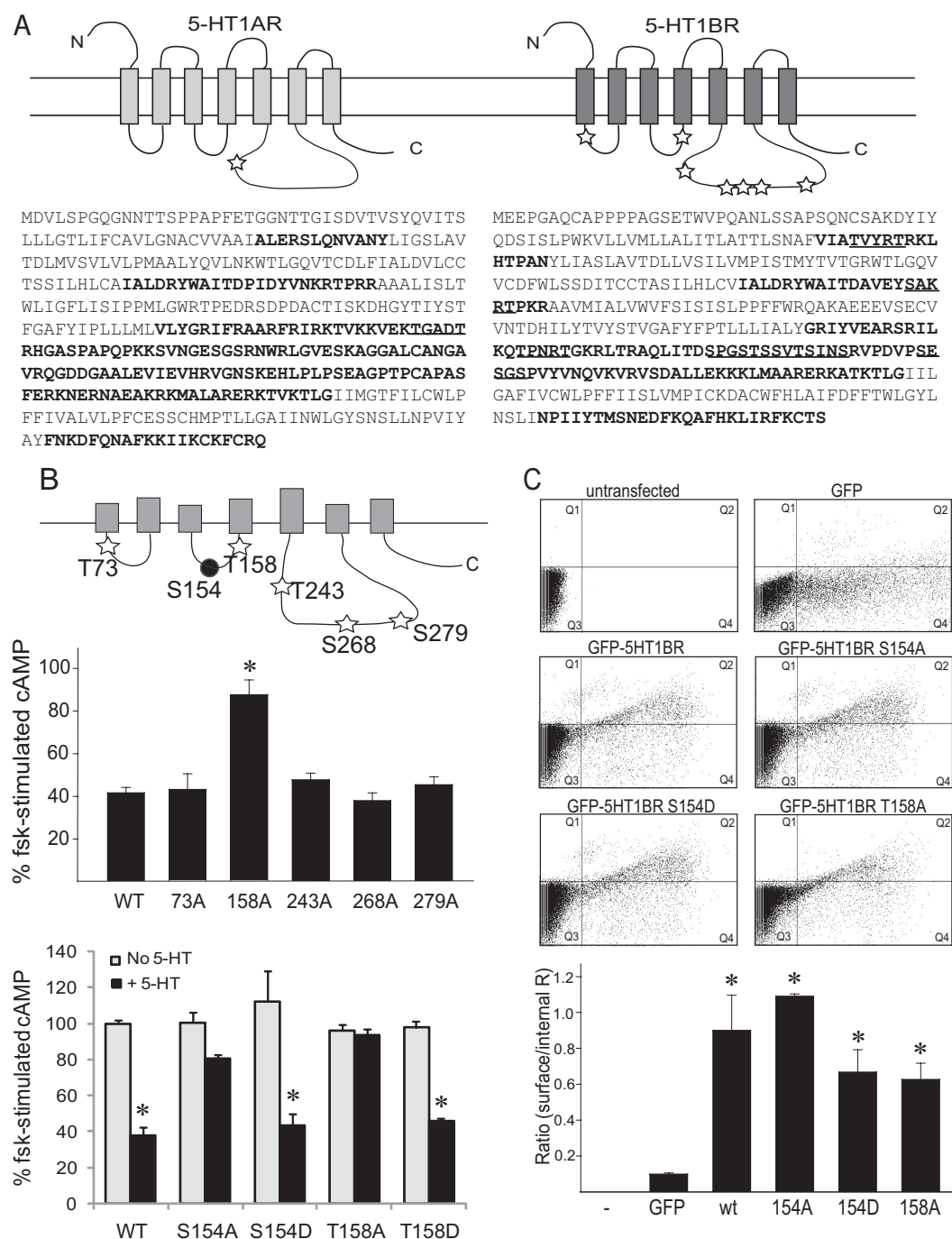


Fig. 3. Mutation of GSK3 consensus phosphorylation sites in the i2 loop of 5-HT_{1B}BR alters its response to 5-HT. **A**, schematic 5-HT_{1AR} (left) and 5-HT_{1BR} (right) with the consensus GSK3 phosphorylation sequences shown as stars. Protein sequence of receptors is demonstrated below the corresponding receptor with the four intracellular regions in boldface type and the consensus GSK3 phosphorylation sequences underlined. **B**, point mutations of mouse 5-HT_{1B}BR are shown in the schematic receptor (top), with stars for priming sites and a circle for putative GSK3 phosphorylation site. WT, alanine, or aspartate mutant 5-HT_{1B}BR was expressed in CHO cells, and the effect of 5-HT in forskolin-stimulated cAMP production was measured after cells were treated with 5-HT (10 μ M) for 30 min followed with forskolin (10 μ M) for 15 min. Data are expressed as the percentage of forskolin-stimulated cAMP production in cells expressing wild-type 5-HT_{1B}BR. *, $p < 0.01$ in one-way ANOVA when values are compared with 5-HT treatment (middle) or with forskolin treatment (bottom) in cells expressing wild-type 5-HT_{1B}BR. Mean \pm S.E.M., $n = 6$. **C**, CHO cells were transfected with wild-type, S154A, S154D, or T158A mutants of 5-HT_{1B}BR tagged with N-terminal GFP. Cell surface receptor expression was analyzed by FACS, in which the cell surface receptor signals are shown at the top, right (Q2). The bar graph at the bottom represents the ratio of surface to internal fluorescence signals as defined by Q2/Q4 of the FACS results. *, $p < 0.01$ in one-way ANOVA when values are compared with GFP control. Mean \pm S.E.M., $n = 5$.

dependent manner, reaching plateau at fluorescence intensity observed in cells transfected with 1 μ g of YFP-GSK3 β (Fig. 5B), whereas in YFP-expressing cells, the BRET ratio remained unchanged in a quasilinear manner even when the YFP expression increased 20 times with 1 to 2 μ g of transfected DNAs. The BRET ratio in YFP-GSK3 β -expressing cells was significantly higher than in YFP-expressing cells, indicating that 5-HT1BR and GSK3 β are proximal in cells. Furthermore, treatment of YFP-GSK3 β -expressing cells with 5-HT resulted in an additional significant increase in BRET ratio compared with no 5-HT treatment, suggesting that agonist stimulation enhances the interaction between 5-HT1BR and GSK3 β . The selective interaction between 5-HT1BR and GSK3 β was verified because no BRET signal was generated either between *R. reniformis* luciferase and YFP-GSK3 β or between 5-HT1AR-Rluc and YFP-GSK3 β (Fig. 5C). Similar to 5-HT, the selective 5-HT1BR agonist anpirtoline, but not the 5-HT1AR agonist 8-OH-DPAT, significantly increased BRET ratio between 5-HT1BR and GSK3 β (Fig. 5D), and the effect of 5-HT was blocked by the selective 5-HT1BR antagonists SB224289 and SB216641, which alone had no effect on the interaction.

To test whether the interaction requires active GSK3 β , cells expressing 5-HT1BR-Rluc and YFP-GSK3 β were treated with the GSK3 inhibitors SB216763 and lithium chloride. A significant reduction of BRET ratio in either the absence or the presence of 5-HT was observed at a concentration of SB216763 as low as 2 μ M (Fig. 5E). Lithium chloride also significantly reduced BRET ratio in a concentration-dependent manner, with an effective concentration as low as 1 mM in the presence of 5-HT. Together, these results

strongly suggest an interaction between 5-HT1BR and active GSK3 β .

To determine whether the phosphorylation state of Ser154 in 5-HT1BR affects interaction of the receptor with GSK3 β , cells stably expressing S154A or S154D mutant-Rluc were transfected with YFP-GSK3 β , and treated with 5-HT. Comparing with the wild-type 5-HT1BR, the S154A mutant had minimal interaction with GSK3 β , and 5-HT did not increase the interaction (Fig. 6A). S154D mutant 5-HT1BR, however, interacted with GSK3 β in a manner similar to wild-type 5-HT1BR, and responded to 5-HT with enhanced interaction. However, unlike the effect of GSK3 inhibitors in the S154D mutant-reduced cAMP, SB216763 significantly reduced the interaction of S154D mutant with GSK3 β (Fig. 6B). This result seems to indicate that interaction of 5-HT1BR with GSK3 β requires additional action of active GSK3 β besides phosphorylating Ser154 residue.

To test whether a protein complex between 5-HT1BR and GSK3 β is involved in the interaction of the two, cells were transfected with GFP-5-HT1BR and HA-GSK3 β and treated with 5-HT. Whole-cell lysates were immunoprecipitated using an anti-HA antibody to pull down HA-GSK3 β . When GFP was detected by immunoblotting, 5-HT1BR was detected in the immunocomplex (Fig. 7A). The protein complex was present even in the absence of 5-HT, whereas 5-HT treatment had a minimal effect on the amount of 5-HT1BR coimmunoprecipitated with GSK3 β . Comparing with wild-type 5-HT1BR, the S154A mutant did not form a protein complex with GSK3 β (Fig. 7B), indicating that phosphorylation of 5-HT1BR at the Ser154 residue is required for the protein complex formation. In contrast, the S154D mutant had

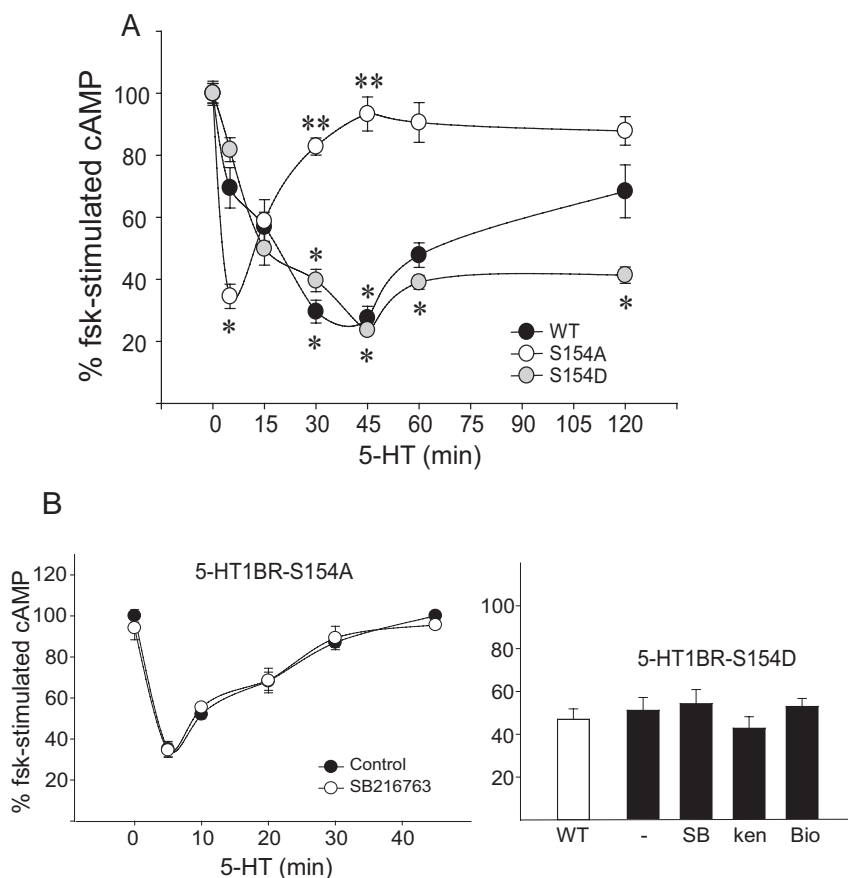


Fig. 4. Mutation of Ser154 of 5-HT1BR alters the dynamics of agonist-stimulated 5-HT1BR activity. **A**, CHO cells expressing the WT, S154A, or S154D mutants of 5-HT1BR were treated with 5-HT (10 μ M) for an indicated length of time before the addition of forskolin (10 μ M, 15 min) to stimulate cAMP production. **B**, cells expressing S154A mutant 5-HT1BR were pretreated with SB216763 (10 μ M) before the addition of 5-HT and forskolin (left). Cells expressing S154D mutant 5-HT1BR were pretreated with SB216763 (SB, 10 μ M), kenpaullone (Ken, 10 μ M), or Bio (10 μ M) for 1 h before the addition of 5-HT (30 min) followed by forskolin (15 min) (right). The effect of 5-HT on forskolin-stimulated cAMP production was measured, and values are expressed as a percentage of forskolin-stimulated cAMP production. *, $p < 0.05$ in one-way ANOVA when values are compared with control (no 5-HT) within each mutant group. **, $p < 0.05$ in two-way ANOVA with post hoc analysis comparing S154A mutant with wild-type and S154D mutant. Mean \pm S.E.M., $n = 4$ to 6.

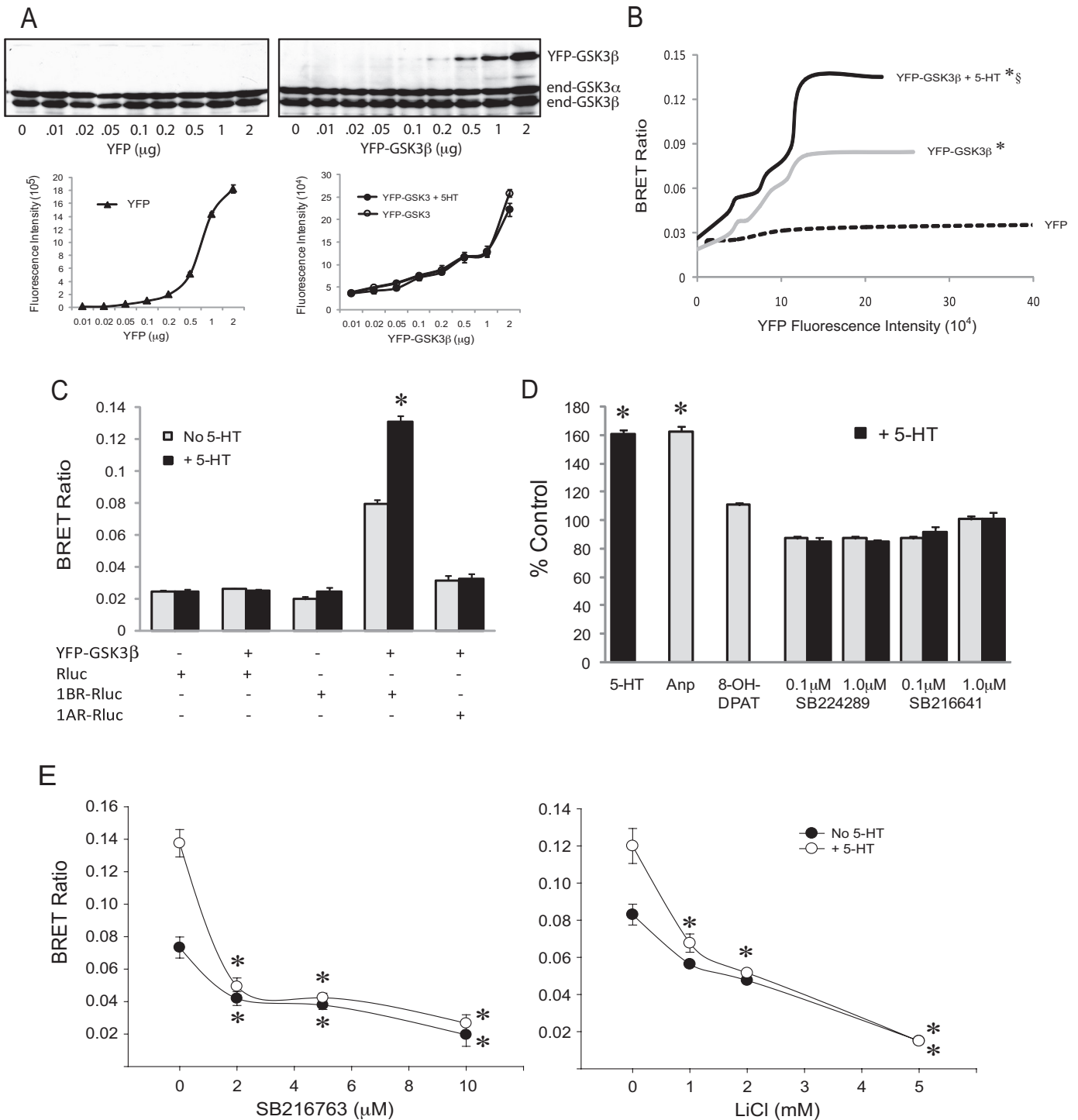


Fig. 5. Active GSK3 β interacts with 5-HT_{1B}R. **A**, HEK-293 cells stably expressing 5-HT_{1B}R-luc were transfected with the indicated amount of YFP alone (left) or YFP-GSK3 β (right) DNAs. The expression levels of endogenous GSK3 α/β (end-GSK3) and transfected YFP-GSK3 β in these cells were detected by immunoblots using an anti-GSK3 α/β antibody (top). The YFP expression is detected by fluorescence intensity at 528 \pm 10 nm after excitation at 485 \pm 10 nm (bottom). **B**, BRET was measured in cells expressing different amount of YFP or YFP-GSK3 β as shown in **A**. Some YFP-GSK3 β -expressing cells were treated with 5-HT (10 μ M) for 15 min before BRET measurement. BRET ratio is calculated as described under *Materials and Methods* and plotted against relative YFP fluorescence intensity. Data are analyzed by two-way ANOVA with post hoc analysis comparing YFP, YFP-GSK3 β , and YFP-GSK3 β plus 5-HT. *, $p < 0.01$ compared with YFP-expressing cells, §, $p < 0.01$ compared with YFP-GSK3 β -expressing cells without 5-HT treatment. Mean \pm S.E.M., $n = 6$. **C**, BRET ratio in cells expressing *R. reniformis* luciferase alone, *R. reniformis* luciferase and YFP-GSK3 β , 5-HT_{1B}R-luc alone, 5-HT_{1B}R-luc and YFP-GSK3 β , or 5-HT_{1A}R-luc and YFP-GSK3 β , with or without 5-HT. *, $p < 0.05$ when values are compared with no 5-HT treatment in the same cells. **D**, cells expressing 5-HT_{1B}R-luc and YFP-GSK3 β (1 μ M) were treated with 5-HT, anpirtoline (10 μ M), or 8-OH-DPAT (10 μ M) for 15 min or treated with the antagonists SB224289 or SB216641 for 2 h with or without 5-HT for 15 min. Data are expressed as the percentage of control (no treatment). *, $p < 0.05$ when values are compared with control. **E**, cells expressing 5-HT_{1B}R-luc and YFP-GSK3 β were treated with the indicated concentrations of GSK3 inhibitors SB216763 (2 h) (left) or lithium chloride (LiCl, 24 h) (right) before the addition of 5-HT for 15 min. *, $p < 0.05$ in one-way ANOVA when values are compared with control (no drug treatment in **D** or no GSK3 inhibitor in **E**). Mean \pm S.E.M., $n = 6$.

strong association with GSK3 β because it was coimmunoprecipitated with GSK3 β . Therefore, GSK3 β not only phosphorylates 5-HT1BR, but it associates with the receptor by forming a protein complex.

To elucidate whether regulation by GSK3 also changes the dynamics of 5-HT1BR internalization, a common response of GPCRs to receptor activation, we measured the immunofluorescent intensity of GFP-tagged 5-HT1BR on the cell surface before and after 5-HT treatment. In cells expressing wild-type, S154A mutant, and S154D mutant 5-HT1BR, the receptors were detectable on the cell surface (Figs. 3C and 8A). Upon 5-HT stimulation, wild-type 5-HT1BR on the cell surface transiently reduced but rapidly returned to near baseline level at and after 60-min treatment (Fig. 8B). Unlike wild-type 5-HT1BR, the S154A mutant 5-HT1BR responded to 5-HT with a gradual but persistent reduction at the cell surface, with only 50% of receptors remaining on the cell surface after a 120-min 5-HT treatment. The change of S154A mutant was significantly different from the wild-type receptor, with the difference more prominent at 90- and 120-min treatment. The surface distribution of S154D mutant 5-HT1BR responded to 5-HT in a manner similar to the wild-type 5-HT1BR, with a rapid reduction from the cell

surface followed by returning to the baseline level. During the 120-min 5-HT treatment, the observed change of surface 5-HT1BR was probably due to receptor internalization, because the total cellular GFP (representing all expressed 5-HT1BR) remained unchanged during treatment (data not shown), whereas the ratio of cell surface immunofluorescence to whole-cell GFP fluorescence exhibited similar patterns of changes as the surface receptor immunofluorescence (Fig. 8C). Therefore, phosphorylation at Ser154 by GSK3 may affect the availability of 5-HT1BR at the cell surface.

Discussion

This study provides the first evidence that GSK3 is able to regulate a GPCR. This effect of GSK3 is selective for the GPCR subtype and for the GSK3 isoform. Despite sharing 5-HT as an agonist and linking to similar downstream signal transduction pathways, the two major 5-HT1Rs (1A and 1B) are selectively regulated by GSK3. The exclusive association and regulation of 5-HT1BR, but not 5-HT1AR, by GSK3 may be a determinant of their differential neuronal localization, physiological function, and behavioral effects, which remain to be determined. However, our findings do not exclude that

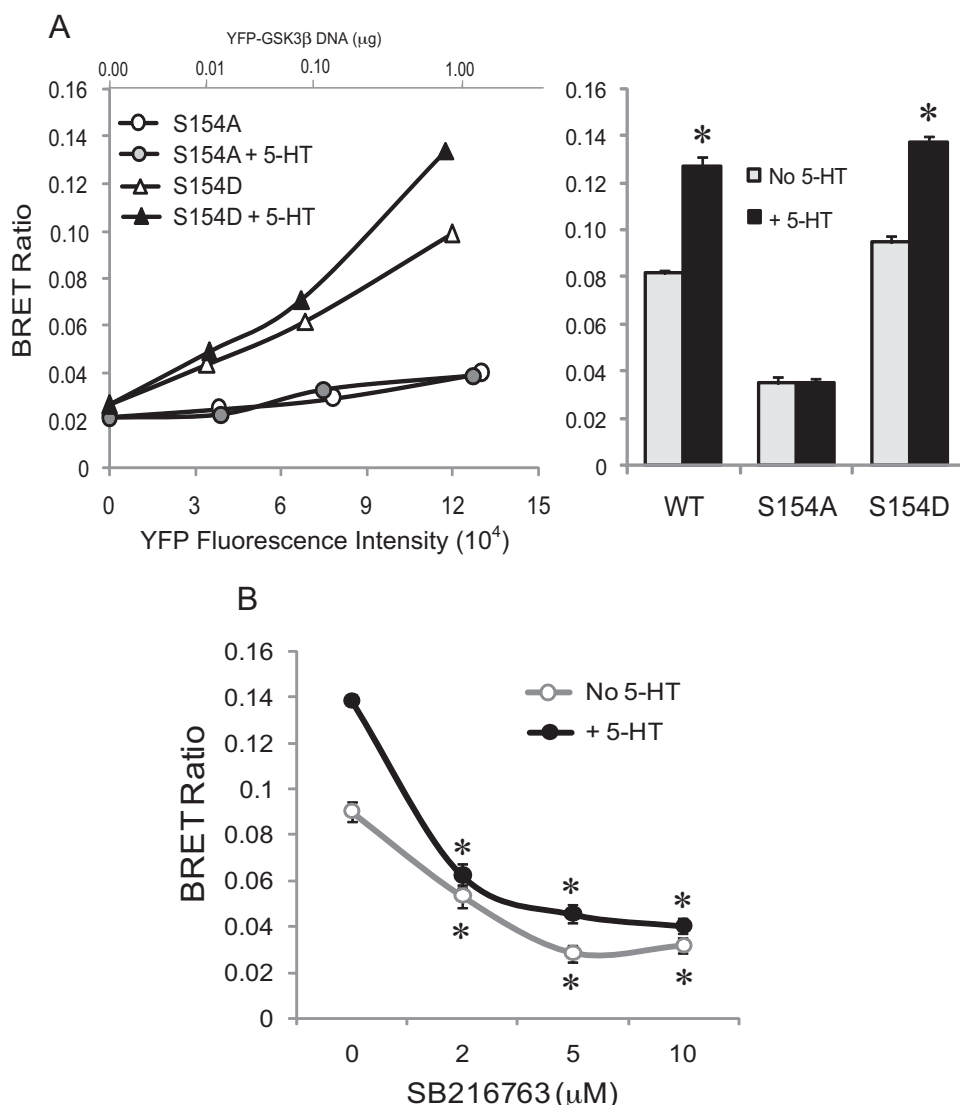


Fig. 6. Mutation of Ser154 affects 5-HT1BR interaction with GSK3 β . A, HEK-293 cells stably expressing S154A or S154D mutant 5-HT1BR were transfected with 0.01, 0.1, or 1 μ g of YFP-GSK3 β (left) and treated with or without 5-HT (10 μ M) for 15 min followed by measuring BRET. BRET ratio is plotted against relative YFP fluorescence intensity. BRET ratios of mutants with 1 μ g of YFP-GSK3 β are analyzed side by side with wild-type 5-HT1BR (right). *, $p < 0.05$ ($n = 6$) compared with no 5-HT treatment in corresponding cells with the same receptor. B, cells expressing S154D mutant 5-HT1BR and YFP-GSK3 β (1 μ g) were pretreated with the GSK3 inhibitor SB216763 for 2 h, followed by 5-HT for 15 min. BRET ratio is calculated; *, $p < 0.05$ in one-way ANOVA compared with no SB216763 treatment ($n = 4$).

GSK3 may regulate other GPCRs that are still to be identified. This study also identified GSK3 β as the preferred GSK3 isoform that regulates 5-HT1BR. This finding is in agreement with several previous studies showing that GSK3 α and GSK3 β phosphorylate and regulate different substrates (Wang et al., 1994; Liang and Chuang, 2006). Examining colocalization of 5-HT1BR and GSK3 β in specific brain regions and neurons may further determine their unique relationship.

Although the serine-rich third intracellular loop of GPCRs has been the focus of many studies, the short i2 loop may also play a role in receptor-mediated signaling. For example, early studies indicated that the hydrophobic residue near the highly conserved DRY motif in the N terminus of the i2 loop was involved in receptor-G protein coupling (Moro et al., 1993). This region was later identified as a common phosphorylation-independent β -arrestin binding region in several GPCRs (Marion et al., 2006). Phosphorylation of a serine in the midregion of the i2 loop was also found to be a determinant for agonist-mediated 5-HT2A receptor desensitization, but the responsible protein kinase was not identified (Gray et al., 2003). Our study provides new information that the phosphorylation states of both Ser154 and Thr158 play a pivotal role in agonist-induced signaling by the 5-HT1BR. Although we did not demonstrate direct *in vitro* phosphorylation of 5-HT1BR by recombinant GSK3, the positions of Ser154 and Thr158 suggest that Ser154 is a GSK3 phosphorylation site, whereas Thr158 is probably a priming site for docking GSK3 on the i2 loop of 5-HT1BR. The facts that several GSK3 inhibitors selectively inhibit signal transduction by wild-type 5-HT1BR but had no effect on alanine or aspartate receptor mutants and that 5-HT1AR does not contain a consensus GSK3 site in its i2 loop further provide evidence that the i2 loop of 5-HT1BR is regulated by GSK3. Unlike G protein-coupled receptor kinases that elicit agonist-dependent inactivation of GPCRs by phosphorylation (Gainetdinov et al.,

2004), phosphorylation of 5-HT1BR by GSK3 is unique in that it is required for agonist-induced receptor activation. However, phosphorylation by GSK3 alone is not sufficient for 5-HT1BR activation, because the phosphomimic S154D and T158D mutant receptors are not constitutively active. Instead, our results indicate that 5-HT1BR must be phosphorylated by GSK3 for 5-HT to signal to inhibition of adenylyl cyclase. Although this study did not examine the priming kinase that phosphorylates Thr158, this priming site is apparently a regulatory component of the i2 loop involved in engaging regulation by GSK3. Further identification of potential priming kinases for Thr158 is warranted to fully understand the regulatory mechanism of GSK3 on 5-HT1BR.

As shown by BRET and coimmunoprecipitation, the receptor-GSK3 interaction occurs in the absence of 5-HT and is not affected by 5-HT1BR antagonists, suggesting that active GSK3 interacts with resting 5-HT1BR, which is probably an important event to prepare 5-HT1BR for agonist-induced activation. The observation that binding of 5-HT to 5-HT1BR increases proximity of 5-HT1BR to GSK3 can be a result of agonist-induced receptor conformation change that facilitates energy transfer. Because the interaction cannot be observed in the S154A mutant, it demonstrates that phosphorylation of 5-HT1BR by GSK3 is necessary for 5-HT1BR to interact with GSK3. However, because the phosphomimic S154D mutant is able to interact with active GSK3, other binding sites, such as the priming Thr158 site, may also participate in forming the tight binding between 5-HT1BR and GSK3 β .

Besides preparing 5-HT1BR for agonist-induced activation, regulation by GSK3 may also have an effect in maintaining 5-HT1BR on the cell surface. Although the mechanism remains to be identified, our data suggest that phosphorylation of 5-HT1BR promotes its retention at the cell surface for agonist stimulation, because wild-type and S154D mutant receptors rapidly return to the cell surface

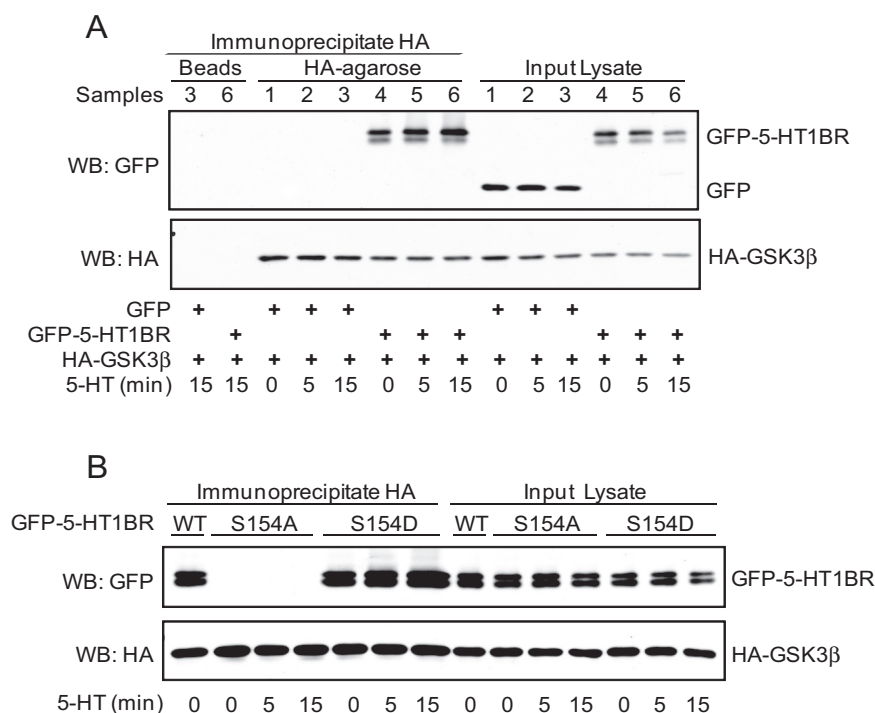


Fig. 7. Protein complex formation between 5-HT1BR and GSK3 β . **A**, HEK-293 cells were transfected with HA-GSK3 β along with either GFP or GFP-tagged wild-type 5-HT1BR (**A**), or GFP-5-HT1BR wild-type, S154A, or S154D mutant (**B**). Cells were treated without or with 5-HT for the indicated time, and whole-cell lysates were used for immunoprecipitation with anti-HA-conjugated agarose beads to pull down HA-GSK3 β . Denatured immunocomplex and cell lysate without immunoprecipitation (Input Lysate) were subjected to electrophoresis and immunoblotted for GFP and HA, respectively.

after 5-HT-induced receptor internalization, whereas the S154A mutant 5-HT₁BR at the cell surface progressively reduced. Although not studied here, we speculate that phosphorylation of the i2 loop of 5-HT₁BR and forming a protein complex with GSK3 may also have a role in facilitating functions of other receptor-associated proteins, such as G_iα, p11, or β-arrestin, which then synchronically transduce signals from 5-HT₁BR.

At physiological conditions, GSK3 activity is under strong inhibitory control by growth factor- and neurotransmitter-regulated signal transduction processes (Cross et al., 1994; Beaulieu, 2007). Because activation of 5-HT₁BR is GSK3-dependent, the level of GSK3 activity at physiological conditions may play an important role in normal receptor activation and function. Hsu et al. (2001) reported previously that activation of 5-HT₁BR can increase cellular Akt activity. Because Akt is a primary GSK3-regulating protein kinase (Cross et al., 1995), it would be interesting to identify whether this action by 5-HT₁BR results in inhibition of

GSK3, which may suggest the presence of a feedback regulatory loop between 5-HT₁BR and GSK3 to fine-tune the dynamic activity of the 5-HT₁BR. On the other hand, overactive GSK3 under pathological conditions, such as with neurotrophic deficiency, may cause abnormal 5-HT₁BR function.

Regulation of 5-HT₁BR by GSK3 may have clinical significance because GSK3 has been recognized as a crucial enzyme in several diseases, such as mood disorders, Alzheimer's disease, diabetes, and autoimmune diseases (Jope and Roh, 2006). It is interesting to note that several earlier studies reported that lithium selectively inhibited 5-HT binding to 5-HT₁BR, reduced 5-HT₁BR-induced GTPγS binding, and abolished 5-HT₁BR-reduced adenylyl cyclase activity (Mas-sot et al., 1999). Similar results were observed in human platelets from both healthy and depressed subjects where lithium dose-dependently reversed the inhibitory effect of a 5-HT₁BR agonist on adenylyl cyclase (Januel et al., 2002). With lithium being recognized as a selective GSK3 inhibitor

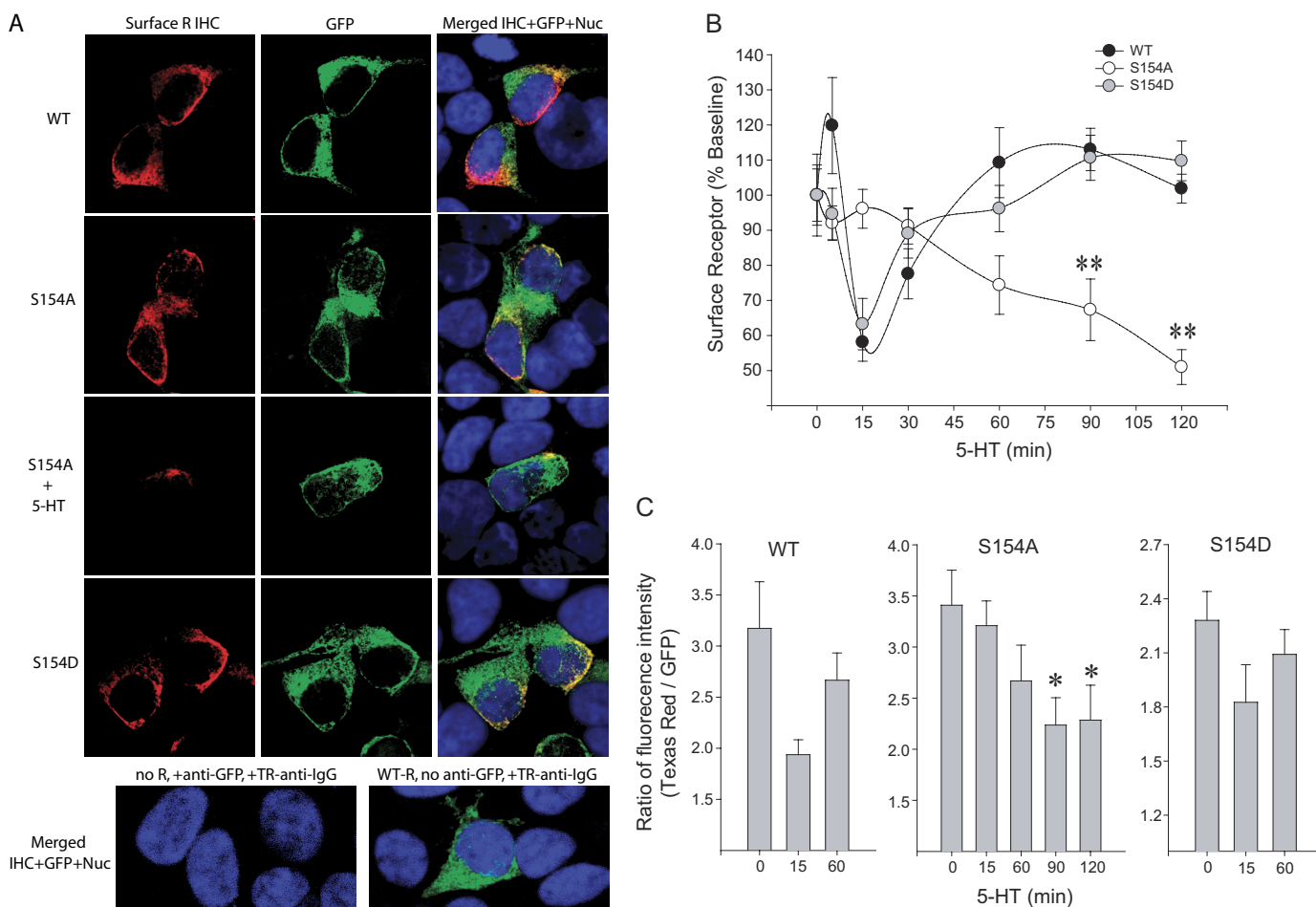


Fig. 8. Agonist-induced changes of wild-type and mutant 5-HT₁BR on the cell surface. HEK-293 cells were transfected with wild-type, S154A, or S154D mutant 5-HT₁BR tagged with N-terminal GFP, and treated with 5-HT (10 μ M) for the indicated length of time. A, representative immunofluorescent photographs of cells expressing wild-type, S154A, and S154D mutant 5-HT₁BR without 5-HT and S154A with 120 min 5-HT treatment (3rd row). Surface receptors are recognized by immunostaining nonpermeable cells with anti-GFP and Texas Red-conjugated anti-IgG (Surface R IHC), and total receptors are visualized by green fluorescence of GFP (without immunostain). The overlay includes immunostained surface receptor (red), GFP-tagged total receptors (green), and nuclei (blue). The bottom two figures are overlay images of cells without GFP-5-HT₁BR (left) and cells expressing GFP-5-HT₁BR immunostained with Texas Red-conjugated anti-IgG without anti-GFP (right). B, average fluorescence intensity of immunostained surface 5-HT₁BR from 50 to 100 cells at each 5-HT treatment interval was quantified, and data are expressed as the percentage of control (no 5-HT treatment). **, $p < 0.05$ in two-way ANOVA post hoc analysis comparing S154A mutant to wild-type and S154D mutant. C, the ratio of fluorescence intensities of Texas red (representing immunostained surface receptor) to green GFP (representing total GFP-tagged receptors) is calculated to represent a relative change of surface to total receptors in response to 5-HT. *, $p < 0.05$ in one-way ANOVA when values are compared with control (no 5-HT). Mean \pm S.E.M., $n = 50$ to 100 cells.

(Klein and Melton, 1996) and our new findings that GSK3 is a regulator of 5-HT1BR, further investigation of the function of GSK3 in 5-HT1BR-mediated neurotransmission may elucidate therapeutic implications of this regulatory mechanism.

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