Molecular Pharmacology Fast Forward. Published on September 9, 2009 as doi:10.1124/mol.109.056994
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MOL #56994

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

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Molecular Pharmacology Fast Forward. Published on September 9, 2009 as DOI: 10.1124/mol.109.056994
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Running Title: GSK3 and 5-HT1B Receptor

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Number of text pages	**20**
Number of tables	**0**
Number of figures	**8**
Number of references	**40**
Number of words in the Abstract	**225**
Number of words in the Introduction	**704**
Number of words in the Discussion	**1042**

Abbreviations

ANOVA, analysis of variance; BIO, 6-bromoindirubin-3′-oxime; BRET, Bioluminescence resonance energy transfer; cAMP, cyclic AMP; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FACS, Fluorescence-activated cell sorting; GFP, green fluorescent protein; G_i, inhibitory G protein; GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; G_s, stimulatory G protein; GSK3, glycogen synthase kinase-3; HEK-293, human embryonic kidney-293; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5-HT, serotonin; 5-HT1Rs, type-1 serotonin receptors; 5-HT1AR, subtype A 5-HT1R; 5-HT1BR, subtype B 5-HT1R; K85,86A, Mutation of lysine-85 and -86 to alanine; 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin; PBS, phosphate buffered saline; R96A, Mutation of arginine-96 to alanine; Rluc, Renilla Luciferase; RNAi, RNA interference; 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-1H-indol-3-yl)-1H-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; S154A, Mutation of serotonin-154 to alanine; S154D, Mutation of serotonin-154 to aspartate; SEM, standard error of the mean; shRNA, short hairpin RNA; SH-SY5Y, human SH-SY5Y neuroblastoma; YFP, yellow fluorescent protein

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Abstract

In response to 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 GSK3β by RNAi, or overexpression of impaired GSK3β 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 GSK3β 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 S154 resulted in sustained reduction of 5-HT1BR at 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.

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Introduction

Most serotonin (5-HT) receptors are G protein-coupled receptors (GPCRs) that are classified by their sequence homology, as well as 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 (G_i), 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 non-serotonergic neurons (Hannon and Hoyer, 2008). 5-HT1Rs are involved in many physiological processes in 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.

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 (GRKs) 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

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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, GSK3α and GSK3β, share 84% sequence homology, but they are encoded by different genes (Woodgett, 1990) and their function are not always interchangeable when phosphorylating and regulating their substrate proteins (Liang and Chuang, 2006; Wang et al., 1994). To regulate a substrate by phosphorylation, GSK3 locates a serine or threonine residue that has been previously phosphorylated, 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/TXXXS/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 GSK3α and serine-9 for GSK3β) phosphorylation by several protein kinases (Cross et al., 1995; Goode et al., 1992) and the canonical Wnt-regulated GSK3-protein interactions (Rubinfeld et al., 1996). Pharmacologically, 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 multi-axial functions, it has been

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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 as 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. Interestingly, several early investigations observed that lithium had noticeable effect on 5-HT1BR (Massot et al., 1999), but the mechanism of this action was not revealed. In this study, we utilized several experimental approaches to determine if GSK3 has an impact in agonist-induced activation of 5-HT1Rs and if subtypes of 5HT1Rs are differentially regulated by GSK3.

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Materials and Methods

Agonists and Inhibitors- anpirtoline, kenpaullone, SB216641, SB216763, SB224289 (Tocris, 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 (DMSO) 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) and mouse 5-HT1BR cDNA (provided by Dr. Per Sveningsson, The Rockefeller University) 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 GSK3β, R96A-GSK3β, K85,86A-GSK3β, and YFP-tagged GSK3β were provided by Dr. Richard S. Jope (University of Alabama at Birmingham). Mutagenesis of 5-HT1BR was performed using the Quick-Change site-directed mutagenesis protocol (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing prior to 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% confluency 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 prior to treatment with inhibitors and/or activating agents.

RNA interference (RNAi) of GSK3- SH-SY5Y cells were transduced with lentivirus particles containing short hairpin RNA (shRNA) sequences for human GSK3α (Dr. Richard S. Jope) or

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GSK3β (Sigma-Aldrich). Cells transduced with GFP-containing lentiviral GSK3α shRNA (GAGTTCAAGTTCCCTCAG) were identified using Fluorescence-activated cell sorting (FACS) to select GFP-positive cells. The shRNA sequences for GSK3β are: #1, CCCAAACTACACAGAATTTAA; #2, GGTGTGGATCAGTTGGTAGAAA; #3, GACACTAAAGTGATTGGAAAT; #4, GCAGGACAAGAGATTTAAGAA; #5, CCACTGATTATACCTCTAGTA. GSK3β shRNA-expressing cell lines were selected by the antibiotic puromycin (1 μg/ml). All RNAi-induced GSK3 knockdowns were confirmed by immunoblots using the anti-GSK3α/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 weeks old adult male C57BL/6 mice (Fredrick Cancer Research, Fredrick, Maryland) were housed 4-5 per cage with free access to food and water in a 12-hour light/dark cycle animal facility for one week before 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-μm) were rinsed and preincubated in a HEPES-bicarbonate buffer (122 mM NaCl, 15 mM $NaHCO_3$, 11 mM glucose, 10 mM HEPES, 4.9 mM KCl, 1.2 mM $MgSO_4$, 100 μM $CaCl_2$) containing 5% CO_2/95% O_2 at 37°C for 30 min, followed by incubation with pharmacological agents for up to 2 hr.

Cyclic AMP (cAMP) assay- The level of cAMP was measured in cells and in cerebral cortical slices using an enzyme immunoassay kit (Direct BioTrak, Amersham/GE). To minimize the potential non-selective 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

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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 pre-coated with donkey anti-rabbit IgG. After incubation with rabbit antiserum against cAMP and cAMP-horseradish 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 (Bio-Tek, Winooski, VA). Protein concentrations of cell lysate and solubilized cerebral cortical slices were measured using the Bradford Reagent (BioRad). The level of cAMP was calculated against a protein standard, and normalized by total protein content in each sample (fmol/mg).

Bioluminescence resonance energy transfer (BRET) assay- 5-HT1BR-Renilla 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 amount of DNA for YFP or YFP-GSK3β. 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. GSK3β expression was confirmed by immunoblotting GSK3α/β. After treatment with pharmacological agents, cells were quickly washed with warm PBS, detached with EDTA (5mM), and transferred into white opaque 96-well plate. Immediately after addition of the Rluc substrate Coelenterazine f (5 μM, Invitrogen) into each well, emission was detected at the wavelengths of 485 ± 10 nm and 528 ± 10 nm, corresponding to the maxima of the emission spectra for Rluc and YFP, respectively. The BRET ratio was calculated using the equation: [(emission at 528 ± 10 nm) – (emission at 485 ± 10 nm x

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Cf)]/emission at 485 ± 10 nm (Angers et al., 2000), where Cf is (emission at 528 ± 10)/emission at 485 ± 10 nm measured in cells transfected with 1 μg of a DNA vector without YFP.

Immunoprecipitation and immunobloting. Fresh cells expressing GFP-5-HT1BR and HA-GSK3β were lysed by rotating in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1mM EDTA, 1.5% CHAPS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 0.1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium vanadate, and 100 nM okadaic acid. For co-immunoprecipitation, protein lysate (200 μg) 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, Cambridge, United Kingdom) and anti-HA (Covance, Berkeley, CA) antibodies. Protein bands were detected with enhanced chemiluminescence (Amersham Biosciences).

Fluorescence-activated cell sorting (FACS)- 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 5HT1BR- 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 treated with 5-HT, cells were fixed with 4% paraformaldehyde/PBS and blocked with 1% BSA/0.2% nonfat milk in PBS. Cell surface 5-HT1BR was labeled with rabbit anti-GFP antibody (Abcam, Cambridge, MA), followed by Texas-Red-conjugated donkey anti-

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rabbit IgG (Jackson ImmunoResearch, 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, Downingtown, PA). For each treatment condition, the average fluorescent intensity from 50-100 cells in 5-10 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. Values were expressed as mean ± SEM and were considered significant when $p<0.05$.

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Results

To measure agonist-stimulated activation of 5-HT1Rs, we utilized forskolin-stimulated cAMP production (Seamon et al., 1981) as a read-out assay. Treatment with forskolin (10 μM 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 μM) 30 min prior to forskolin maximally reduced the level of forskolin-stimulated cAMP production.

To examine if 5-HT1Rs can be regulated by GSK3, cells expressing 5-HT1AR or 5-HT1BR were pre-treated 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 μM, and SB216763 and kenpaullone at 10 μM (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 5HT1AR-expressing cells. Additionally, a 24-hr 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±10.9% of the forskolin-stimulated response with 10 mM lithium chloride (Fig. 1C).

To examine if GSK3 regulates 5HT1Rs 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 μM) or the 5-HT1BR agonist anpirtoline (20 μM) significantly reduced the level of forskolin-stimulated cAMP production to 47.1±4.7% and 45.8±2.2%, respectively (Fig. 1D). Pre-treatment of cortical slices with GSK3 inhibitors BIO and kenpaullone (10 μM, 2 hr) did not

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change the effect of 8-OH-DPAT, but significantly diminished the effect of anpirtoline on cAMP production (82.6±10.8% and 89.9±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 since in CHO cells, inhibition of cyclin-dependent kinases (Cdk) with a selective cyclin-dependent kinase (Cdk) inhibitor roscovitine (10 μM) did not alter 5-HT-induced inhibition of cAMP production in 5-HT1BR-expressing cells (Fig. 1E), and pre-treatment of CHO cells with the GSK3 inhibitor SB216763 did not change cAMP production induced by the stimulatory G protein (Gs) activator cholera toxin (1 μg/ml) or the adenylyl cyclase activator forskolin (10 μM) alone (Fig. 1F).

To test for a GSK3 isoform-specific effect, 5-HT1BR was expressed in human SH-SY5Y neuroblastoma cells in which GSK3α or GSK3β was deleted by selective shRNAs. The forskolin-stimulated cAMP production was reduced by 5-HT (10 μM) in control SH-SY5Y cells without GSK3 knockdown and in cells with GSK3α knockdown (40.4±5.0% and 30.4±13.6% of forskolin-stimulated response, respectively). However, the inhibitory effect of 5-HT was completely abolished in GSK3β-knockdown cells (100.8±5.4% of forskolin-stimulated response) (Fig. 2A). This result was further tested in five different SH-SY5Y cell lines that express different sequences of GSK3β shRNA. As observed by imunobloting, two of the five GSK3β shRNA-expressing cell lines had reduced levels of GSK3β protein. Following expression of 5-HT1BR 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 GSK3β (Fig. 2B).

The specific effect of GSK3 in regulating 5-HT1BR activity was also tested in cells overexpressing wild-type GSK3β, R96A-GSK3β (Frame et al., 2001), or K85,86A-GSK3β

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(Eldar-Finkelman et al., 1996). The R96A mutation prevents the binding of GSK3 to a primed substrate (inactive), while the K85,86A mutation lacks kinase activity (Doble and Woodgett, 2003). 5-HT significantly reduced cAMP production (37.2±4.2% of forskolin-stimulated response) in wild-type GSK3β-expressing cells similarly as in cells expressing endogenous GSK3β (Fig. 2C), suggesting that endogenous active GSK3β is sufficient for 5-HT-stimulated 5-HT1BR activity. In contrast, the effect of 5-HT was significantly impaired in cells expressing R96A-GSK3β or K85,86A-GSK3β (84.3±6.8% and 92.4±6.6% of forskolin-stimulated response, respectively). Thus, along with findings in GSK3 knockdown cells, these results demonstrate that active GSK3β is necessary for 5-HT1BR-induced inhibition of cAMP production.

Since 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. Interestingly, 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 if 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, upper panel). 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±6.9% of forskolin-stimulated response), whereas mutation of all other potential GSK3 priming sites (T73A, T243A,

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S268A, and S279A) in the first and the third intracellular loops did not change the response to 5-HT (Fig. 3B, middle panel).

To test if the putative GSK3 phosphorylation site at serine-154 (S154), a serine located 4 amino acids N-terminal of T158, is a determinant of 5-HT1BR activity, the S154 was replaced with alanine (S154A) to abolish phosphorylation by GSK3. The S154A mutant had minimal response to 5-HT in reducing forskolin-induced cAMP production (80.4±2.1% of forskolin-stimulated response) (Fig. 3B, lower panel). In contrast, the phospho-mimic aspartate (S154D) mutant responded to 5-HT similarly as in cells expressing wild-type 5-HT1BR, causing significant reduction of forskolin-induced cAMP production. Furthermore, similar to S154D mutant, mutation of T158 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, as 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 likely 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 prior to forskolin gradually decreased cAMP production, with the effect peaked at 30-45 min, and gradually diminished between 60 to 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

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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 the wild type 5-HT1BR, 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 S154 residue in the i2 loop is involved in both initiation and duration of agonist-induced receptor activation and properly transducing receptor signals to its down-stream acceptors.

To confirm that the S154 residue is a GSK3-regulated site, cells expressing the S154A mutant were treated with the GSK3 inhibitor SB216763 prior to examining the time course of 5-HT in cAMP production. SB216763 had no effect on the S154A mutant, as the receptor exhibited the characteristic brief activation and rapid inactivation after 5-HT treatment either with or without SB216763 (Fig. 4B, left panel). Furthermore, none of the GSK3 inhibitors - SB216763, kenpaullone, and BIO changed the response of the S154D mutant to 5-HT (Fig. 4B, right panel). Therefore, the S154 residue is a selective GSK3-regulated site in 5-HT1BR.

For GSK3 to phosphorylate a substrate, it must interact with the substrate. We therefore used BRET (Angers et al., 2000) to examine if GSK3β interacts with 5-HT1BR. HEK-293 cells stably expressing 5-HT1BR-Rluc fusion protein were transfected with different concentrations of DNAs for YFP alone or YFP-GSK3β. The expression of GSK3β in these cells was confirmed by immunobloting GSK3, and the expression of YFP was confirmed by measuring YFP fluorescence intensity (Fig. 5A). In YFP-GSK3β expressing cells, the BRET ratio increased

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significantly in a fluorescence intensity-dependent manner, reaching plateau at fluorescence intensity observed in cells transfected with 1 μg YFP-GSK3β (Fig. 5B), whereas in YFP-expressing cells, the BRET ratio remained unchanged in a quasi-linear manner even when the YFP expression increased 20 times with 1-2 μg of transfected DNAs. The BRET ratio in YFP-GSK3β-expressing cells was significantly higher than in YFP-expressing cells, indicating that 5-HT1BR and GSK3β are proximal in cells. Furthermore, treatment of YFP-GSK3β-expressing cells with 5-HT resulted in an additional significant increase in BRET ratio when compared to without 5-HT treatment, suggesting that agonist stimulation enhances the interaction between 5-HT1BR and GSK3β. The selective interaction between 5-HT1BR and GSK3β was verified since no BRET signal was generated either between Renila luciferase and YFP-GSK3β or between 5-HT1AR-Rluc and YFP-GSK3β (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 GSK3β (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 if the interaction requires active GSK3β, cells expressing 5-HT1BR-Rluc and YFP-GSK3β 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 μM (Figure 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 GSK3β.

To determine if the phosphorylation state of S154 in 5-HT1BR affects interaction of the receptor with GSK3β, cells stably expressing S154A or S154D mutant-Rluc were transfected

Molecular Pharmacology Fast Forward. Published on September 9, 2009 as DOI: 10.1124/mol.109.056994
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with YFP-GSK3β, and treated with 5-HT. Comparing to wild type 5-HT1BR, the S154A mutant had minimal interaction with GSK3β, and 5-HT did not increase the interaction (Fig. 6A). S154D mutant 5-HT1BR, however, interacted with GSK3β similarly as 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 GSK3β (Figure 6B). This result appears to indicate that interaction of 5-HT1BR with GSK3β requires additional action of active GSK3β besides phosphorylating S154 residue.

To test if a protein complex between 5-HT1BR and GSK3β is involved in the interaction of the two, cells were transfected with GFP-5-HT1BR and HA-GSK3β and treated with 5-HT. Whole cell lysates were immunoprecipitated using an anti-HA antibody to pull down HA-GSK3β. When GFP was detected by immunobloting, 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 minimal effect on the amount of 5-HT1BR co-immunoprecipitated with GSK3β. Comparing to wild type 5-HT1BR, the S154A mutant did not form a protein complex with GSK3β (Fig. 7B), indicating that phosphorylation of 5-HT1BR at the S154 residue is required for the protein complex formation. In contrast, the S154D mutant had strong association with GSK3β since it was co-immunoprecipitated with GSK3β. Therefore, GSK3β not only phosphorylates 5-HT1BR, but it associates with the receptor by forming a protein complex.

To elucidate if 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

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on the cell surface (Fig. 3C, 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 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 similarly as 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 likely due to receptor internalization, since 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 S154 by GSK3 may affect the availability of 5-HT1BR at the cell surface.

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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 GSK3 may regulate other GPCRs that are still to be identified. This study also identified GSK3β as the preferred GSK3 isoform that regulates 5-HT1BR. This finding is in agreement with several previous studies showing that GSK3α and GSK3β phosphorylate and regulate different substrates (Liang and Chuang, 2006; Wang et al., 1994). Examining co-localization of 5-HT1BR and GSK3β 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 mid-region 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 S154 and T158 play a pivotal role in agonist-induced signaling by the 5-HT1BR. Although we did not demonstrate direct *in vitro*

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phosphorylation of 5-HT1BR by recombinant GSK3, the positions of S154 and T158 suggest that S154 is a GSK3 phosphorylation site, whereas T158 is likely 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 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 GRKs that elicit agonist-dependent inactivation of GPCRs by phosphorylation (Gainetdinov et al., 2004), phosphorylation of 5-HT1BR by GSK3 is quite unique in that it is required for agonist-induced receptor activation. However, phosphorylation by GSK3 alone is not sufficient for 5-HT1BR activation, as the phospho-mimic S154D and T158D mutant receptors are not constitutively active. Instead, our results indicate that 5-HT1BR must be phosphorylated by GSK3 in order for 5-HT to signal to inhibition of adenylyl cyclase. Although this study did not examine the priming kinase that phosphorylates T158, 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 T158 is warranted to fully understand the regulatory mechanism of GSK3 on 5-HT1BR.

As shown by BRET and co-immunoprecipitation, 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 likely 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. Since 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, since the phospho-mimic S154D mutant is able to interact with

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active GSK3, other binding sites, such as the priming T158 site, may also participate in forming the tight binding between 5-HT1BR and GSK3β.

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 suggests that phosphorylation of 5-HT1BR promotes its retention at the cell surface for agonist stimulation, since wild type and S154D mutant receptors rapidly return to the cell surface 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 Giα, 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 (Beaulieu, 2007; Cross et al., 1994). Since 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.* (Hsu et al., 2001) previously reported that activation of 5-HT1BR can increase cellular Akt activity. Since Akt is a primary GSK3-regulating protein kinase (Cross et al., 1995), it would be interesting to identify if 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 since GSK3 has been recognized as a crucial enzyme in several diseases, such as mood disorders, Alzheimer's disease,

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diabetes, and autoimmune diseases (Jope and Roh, 2006). Interestingly, 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 (Massot 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 (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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Acknowledgements

The authors thank Dr. Richard S. Jope for contributions of GSK3 DNA and shRNA constructs, as well as scientific advice, Drs. Per Svenningsson and Paul Albert for generously providing 5-HT receptor DNA constructs, Sufen Yang and Ling Song for technical support, UAB Center for AIDS Research Core facility for FACS assistance, and UAB High Resolution Imaging Facility for confocal imaging assistance.

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Footnotes

Drs. L. Chen and G.D.Salinas contributed equally to this project.

This research was supported by fundings from the National Institute of Health [MH064555] and [MH073723].

Molecular Pharmacology Fast Forward. Published on September 9, 2009 as DOI: 10.1124/mol.109.056994
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Figure Legends

Figure 1. GSK3 inhibitors selectively decrease 5-HT1BR activity. (A) CHO cells were transiently transfected with 5-HT1AR or 5-HT1BR and treated with 5-HT (10 μM) for 30 min prior to addition of forskolin (fsk; 10 μM) for 15 min to stimulate cAMP production. Forskolin-stimulated cAMP production was measured by enzyme immunoassay and the levels of cAMP are calculated as fmol/mg protein. (B) CHO cells expressing 5-HT1AR (1A, open labels) or 5-HT1BR (1B, closed labels) were pre-treated with the indicated concentrations of GSK3 inhibitors Bio (3 hr), SB216763 (1 hr), or kenpaullone (3 hr), followed by 5-HT and forskolin treatment. (C) 5-HT1BR-expressing cells were treated with indicated concentrations of lithium chloride (LiCl) for 24 hr, followed by 5-HT and forskolin treatment. (D) Cerebral cortical slices were treated with a DMSO vehicle, 10 μM Bio, or 10 μM kenpaullone for 2 hr. 5-HT1AR and 5-HT1BR were activated with 8-OH-DPAT (20 μM) and anpirtoline (20 μM), respectively, for 30 min prior to addition of forskolin (10 μM, 30 min). (E) CHO cells expressing 5-HT1BR were pre-treated with the Cdk inhibitor roscovitine (10 μM) for 3 hr, followed by 5-HT and forskolin treatments. The levels of cAMP are calculated as % of forskolin-stimulated response (B,C,D,E). (F) Levels of cholera toxin (1 μg/ml)- and forskolin (10 μM)-stimulated cAMP in the presence of indicated concentrations of the GSK3 inhibitor SB216763. *$p<0.01$ in one-way ANOVA when values are compared to forskolin treatment alone (A, D), or to no inhibitor treatment (B, C). Mean ± SEM, n=6-7.

Figure 2. GSK3β, but not GSK3α, is required for activation of 5-HT1BR. 5-HT1BR was expressed in SH-SY5Y cells that (A) contain no shRNA (CTL), or stably express GSK3α shRNA (GSK3α) or GSK3β shRNA (GSK3β), (B) stably express different GSK3β-targeting

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shRNAs (1 to 5), and (C) express wild type GSK3β (WT), R96A-GSK3β, or K85,86A-GSK3β. These cells were preincubated with or without 5-HT (10 μM, 30 min) followed by forskolin treatment (10 μM, 15 min), and the levels of cAMP were measured. Data is expressed as % of forskolin-stimulated cAMP production in control (A, B) or in wild type GSK3β-expressing (C) cells. *$p<0.01$ in one-way ANOVA when values are compared to the same type of cells without 5-HT treatment. Mean ± SEM, n=4-6. Immunoblots below the graphs show the levels of total GSK3α and GSK3β in control and GSK3 shRNA-expressing cells.

Figure 3. Mutation of GSK3 consensus phosphorylation sites in the i2 loop of 5-HT1BR alters its response to 5-HT. (A) Schematic 5-HT1AR (left) and 5-HT1BR (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 bolded and the consensus GSK3 phosphorylation sequences underlined. (B) Point mutations of mouse 5-HT1BR are shown in the schematic receptor (upper panel), with stars for priming sites and circle for putative GSK3 phosphorylation site. Wild type (WT), alanine or aspartate mutant 5-HT1BR were 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 μM) for 30 min followed with forskolin (10 μM) for 15 min. Data is expressed as % of forskolin-stimulated cAMP production in cells expressing wild type 5-HT1BR. *$p<0.01$ in one-way ANOVA when values are compared to 5-HT treatment (middle panel) or to forskolin treatment (lower panel) in cells expressing wild type 5-HT1BR. Mean ± SEM, n=6. (C) CHO cells were transfected with wild type, S154A, S154D, or T158A mutants of 5-HT1BR tagged with N-terminal GFP. Cell surface receptor expression was analyzed by FACS, in which the cell surface receptor signals were shown in the right upper area. The bar graph at

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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 to GFP control. Mean ± SEM, n=5.

Figure 4. Mutation of S154 of 5-HT1BR alters the dynamics of agonist-stimulated 5-HT1BR activity. (A) CHO cells expressing the wild type (WT), S154A or S154D mutants of 5-HT1BR were treated with 5-HT (10 μM) for indicated length of time prior to addition of forskolin (10 μM, 15 min) to stimulate cAMP production. (B) Cells expressing S154A mutant 5-HT1BR were pre-treated with SB216763 (10 μM) before addition of 5-HT and forskolin (left panel). Cells expressing S154D mutant 5-HT1BR were pre-treated with SB216763 (SB, 10 μM), kenpaullone (Ken, 10 μM), or Bio (10 μM) for 1 hr before addition of 5-HT (30 min) followed by forskolin (15 min) (right panel). The effect of 5-HT on forskolin-stimulated cAMP production was measured and values are expressed as % of forskolin-stimulated cAMP production. *p<0.05 in one-way ANOVA when values are compared to control (no 5-HT) within each mutant group. **p<0.05 in two-way ANOVA with post-hoc analysis comparing S154A mutant to WT and S154D mutant. Mean ± SEM, n=4-6.

Figure 5. Active GSK3β interacts with 5-HT1BR. (A) HEK-293 cells stably expressing 5-HT1BR-Rluc were transfected with indicated amount of YFP alone (left panel) or YFP-GSK3β (right panel) DNAs. The expression levels of endogenous GSK3α/β (end-GSK3) and transfected YFP-GSK3β in these cells were detected by immunoblots using an anti-GSK3α/β antibody (upper panel). The YFP expression is detected by fluorescence intensity at 528 ± 10 nm after excitation at 485 ± 10 nm (lower panel). (B) BRET was measured in cells expressing different

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amount of YFP or YFP-GSK3β as shown in (A). Some YFP-GSK3β-expressing cells were treated with 5-HT (10 μM) for 15 min before BRET measurement. BRET ratio is calculated as described in the Methods and plotted against relative YFP fluorescence intensity. Data is analyzed by two-way ANOVA with post-hoc analysis comparing YFP, YFP-GSK3β, and YFP-GSK3β plus 5-HT. *$p<0.01$ when compared to YFP-expressing cells, $^{\S}p<0.01$ when compared to YFP-GSK3β-expressing cells without 5-HT treatment. Mean ± SEM, n=6. (C) BRET ratio in cells expressing 1) renila luciferase alone, 2) renila luciferase and YFP-GSK3β, 3) 5-HT1BR-Rluc alone, 4) 5-HT1BR-Rluc and YFP-GSK3β, or 5) 5-HT1AR-Rluc and YFP-GSK3β, in the absence or the presence of 5-HT. *$p<0.05$ when values are compared to no 5-HT treatment in the same cells. (D) Cells expressing 5-HT1BR-Rluc and YFP-GSK3β (1 μg) were treated with 5-HT, anpirtoline (10 μM), or 8-OH-DPAT (10 μM) for 15 min, or treated with the antagonists SB224289 or SB216641 for 2 hr with or without 5-HT for 15 min. Data is expressed as % control (no treatment). *$p<0.05$ when values are compared to control. (E) Cells expressing 5-HT1BR-Rluc and YFP-GSK3β were treated with indicated concentrations of GSK3 inhibitors SB216763 (2 hr) (left panel) or lithium chloride (LiCl, 24 hr) (right panel) prior to addition of 5-HT for 15 min. *$p<0.05$ in one-way ANOVA when values are compared to control (no drug treatment in D or no GSK3 inhibitor in E). Mean ± SEM, n=6.

Figure 6. Mutation of S154 affects 5-HT1BR interaction with GSK3β. (A) HEK-293 cells stably expressing S154A or S154D mutant 5-HT1BR were transfected with 0.01, 0.1 or 1 μg of YFP-GSK3β (left panel) and treated with or without 5-HT (10 μM) for 15 min followed by measuring BRET. BRET ratio is plotted against relative YFP fluorescence intensity. BRET ratios of mutants with 1 μg YFP-GSK3β are analyzed side by side with wild type 5-HT1BR (right panel).

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*p<0.05 (n=6) when compared to no 5-HT treatment in corresponding cells with the same receptor. (B) Cells expressing S154D mutant 5-HT1BR and YFP-GSK3β (1 μg) were pre-treated with the GSK3 inhibitor SB216763 for 2 hr, followed by 5-HT for 15 min. BRET ratio was calculated, *p<0.05 in one way ANOVA when compared to no SB216763 treatment (n=4).

Figure 7. Protein complex formation between 5-HT1BR and GSK3β. (A) HEK-293 cells were transfected with HA-GSK3β along with (A) either GFP or GFP-tagged wild type 5-HT1BR, or (B) GFP-5-HT1BR wild type, S154A, or S154D mutant. Cells were treated without or with 5-HT for indicated time, and whole cell lysates were used for immunoprecipitation with anti-HA-conjugated agarose beads to pull down HA-GSK3β. Denatured immunocomplex and cell lysate without immunoprecipitation (Input Lysate) were subjected to electrophoresis and immunoblotted for GFP and HA, respectively.

Figure 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 μM) for 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 non-permeable 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 photos are overlay images of cells without GFP-5-HT1BR (left) and cells expressing GFP-5-HT1BR immunostained with Texas

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Red-conjugated anti-IgG without anti-GFP (right). (B) Average fluorescence intensity of immunostained surface 5-HT1BR from 50-100 cells at each 5-HT treatment interval was quantified and data are expressed as % of control (no 5-HT treatment). **$p<0.05$ in two-way ANOVA post-hoc analysis comparing S154A mutant to WT 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 to control (no 5-HT). Mean ± SEM, n=50-100 cells.

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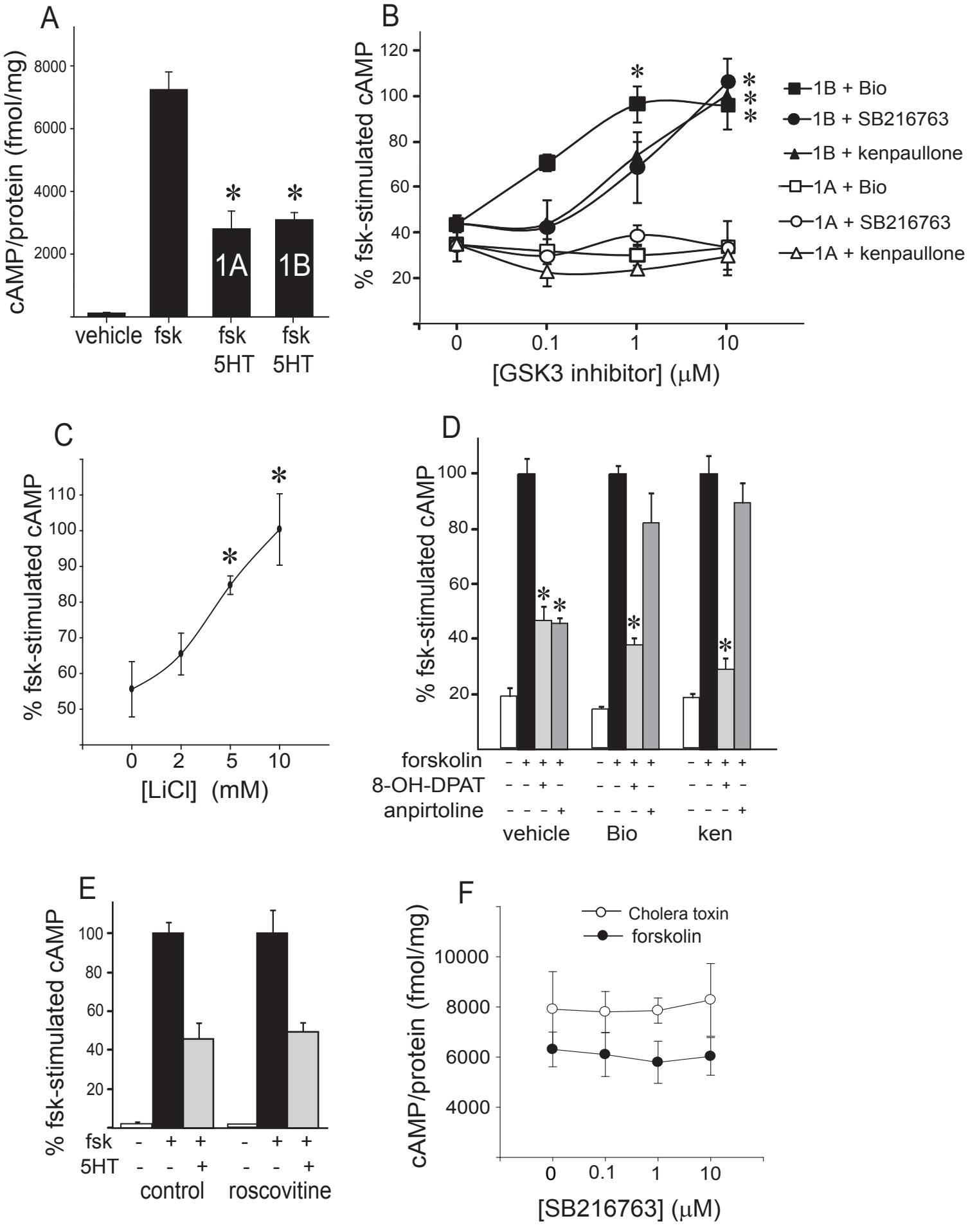

Figure 1

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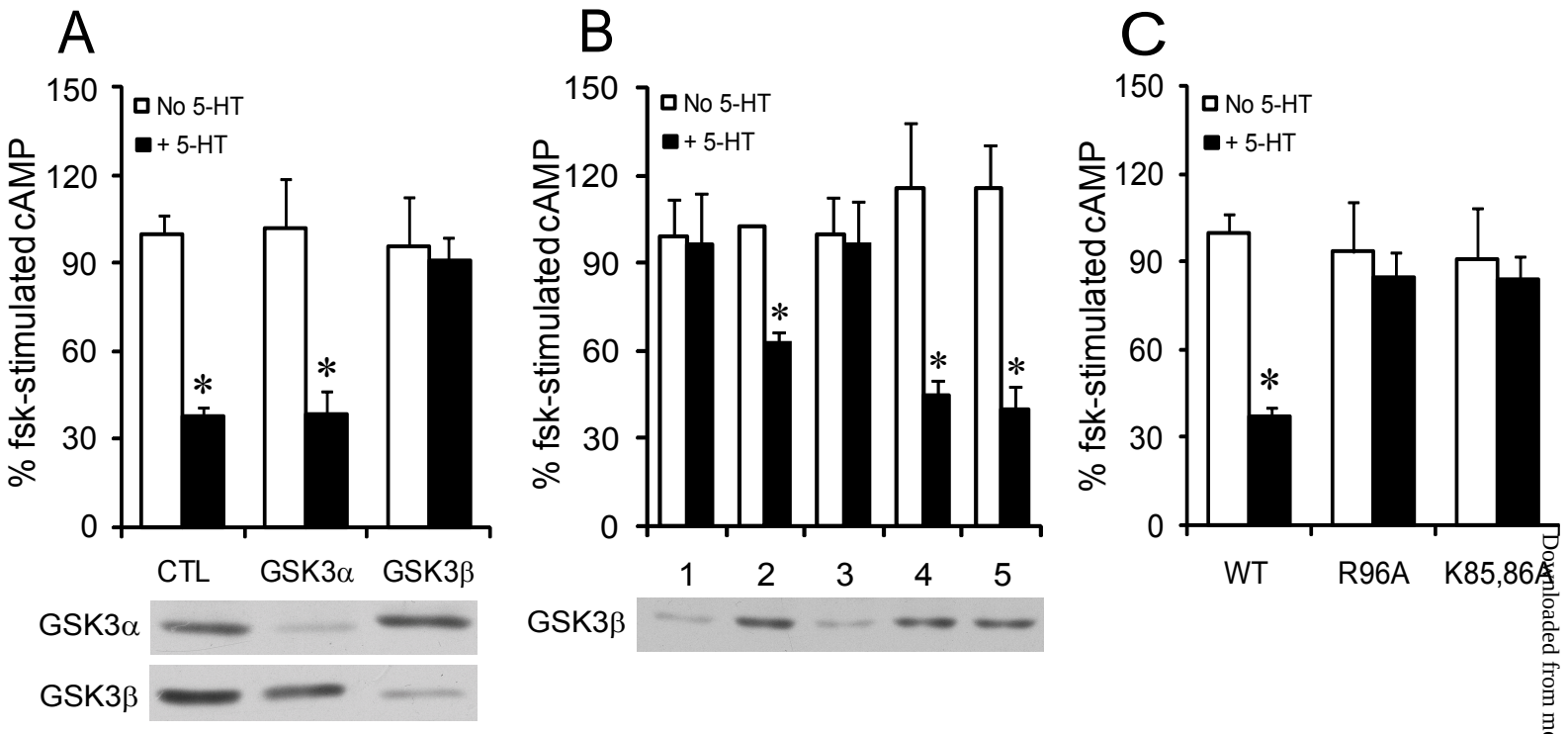

Figure 2

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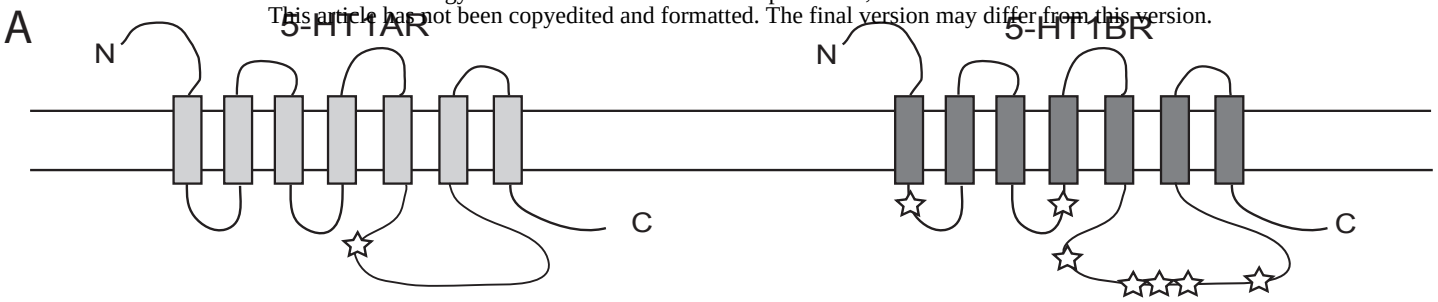

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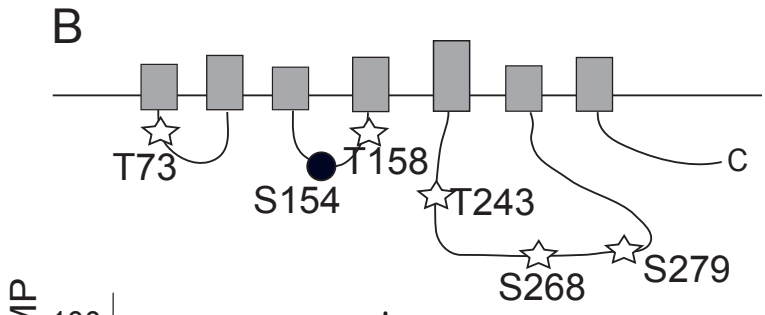

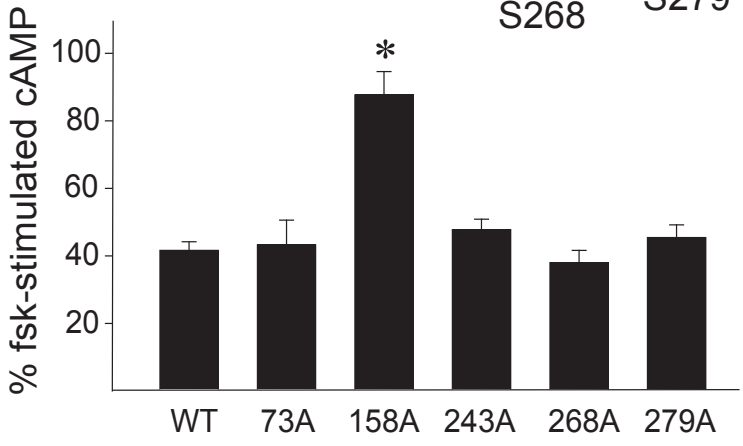

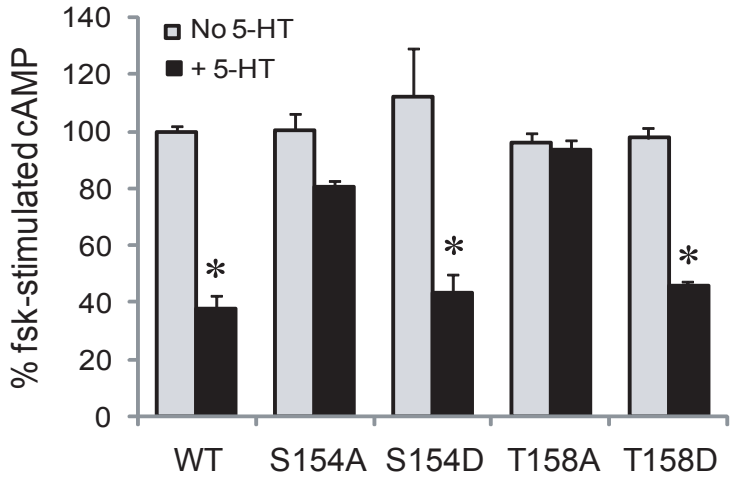

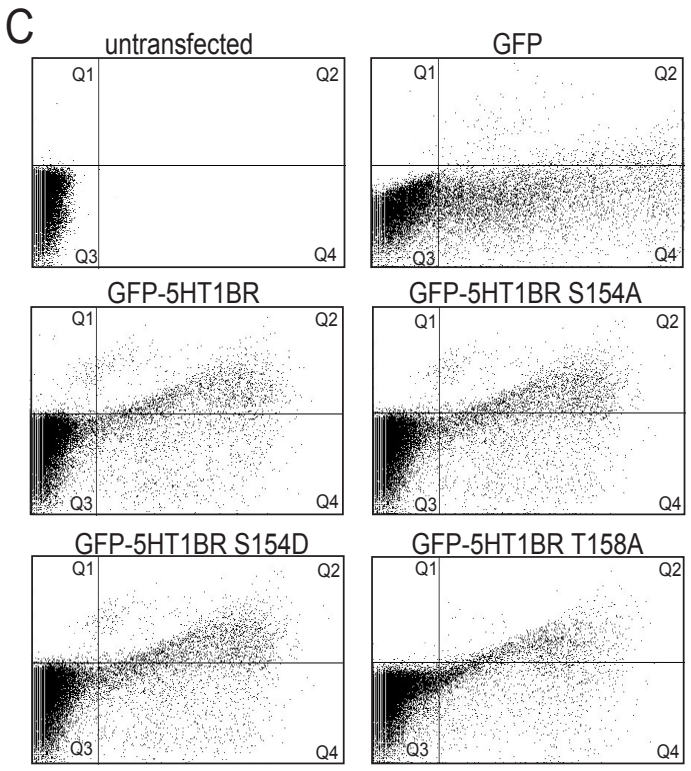

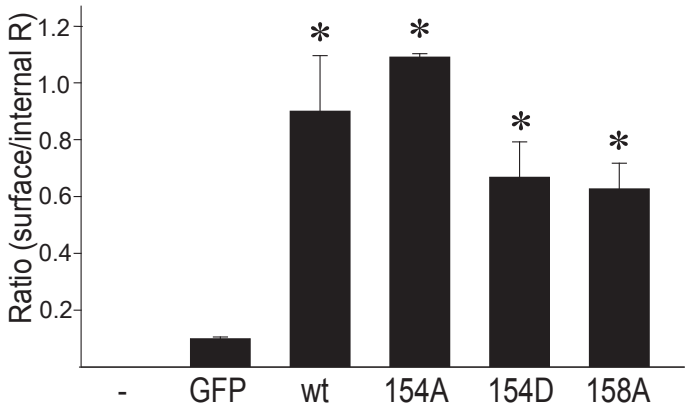

Figure 3

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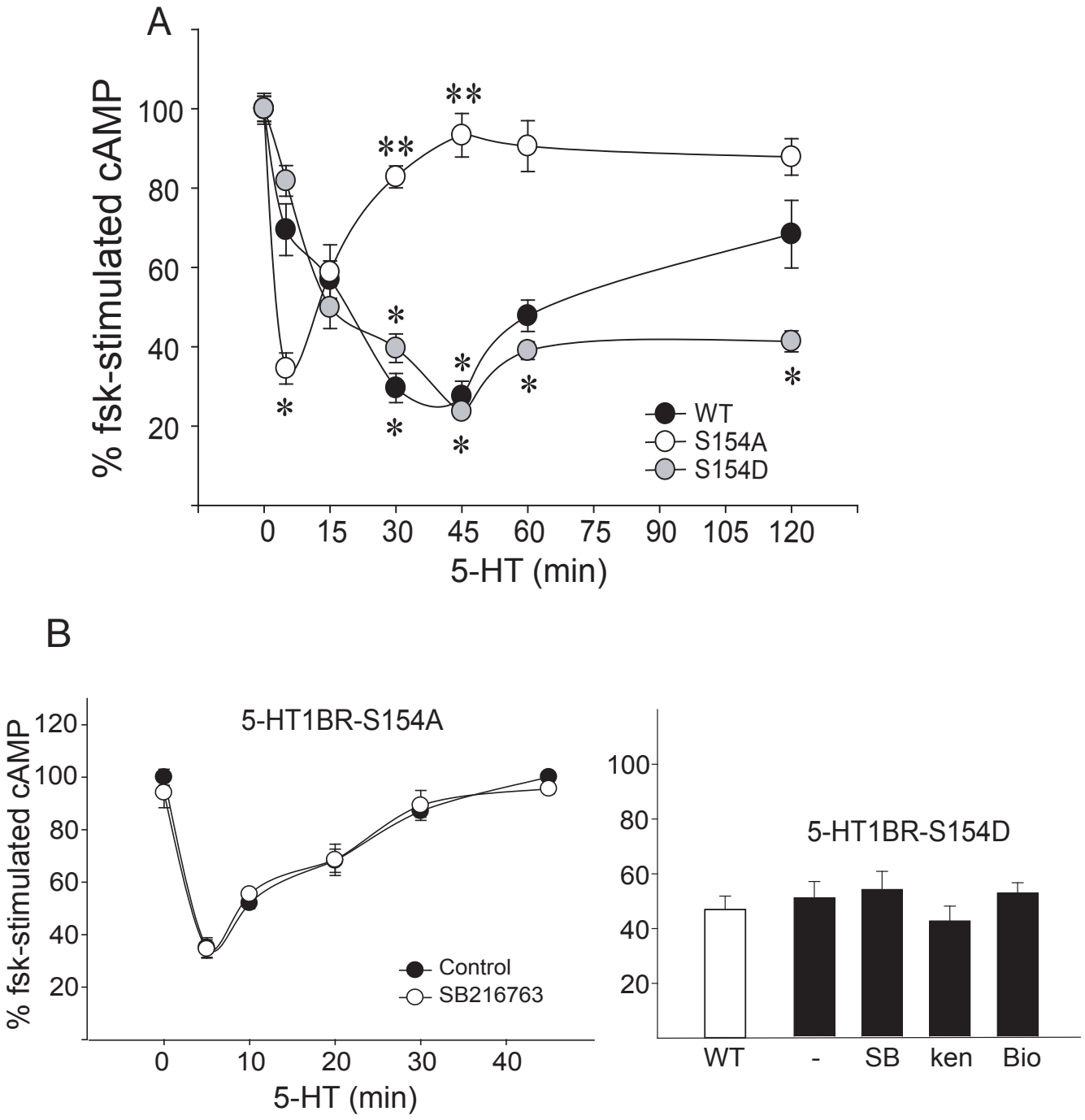

Figure 4

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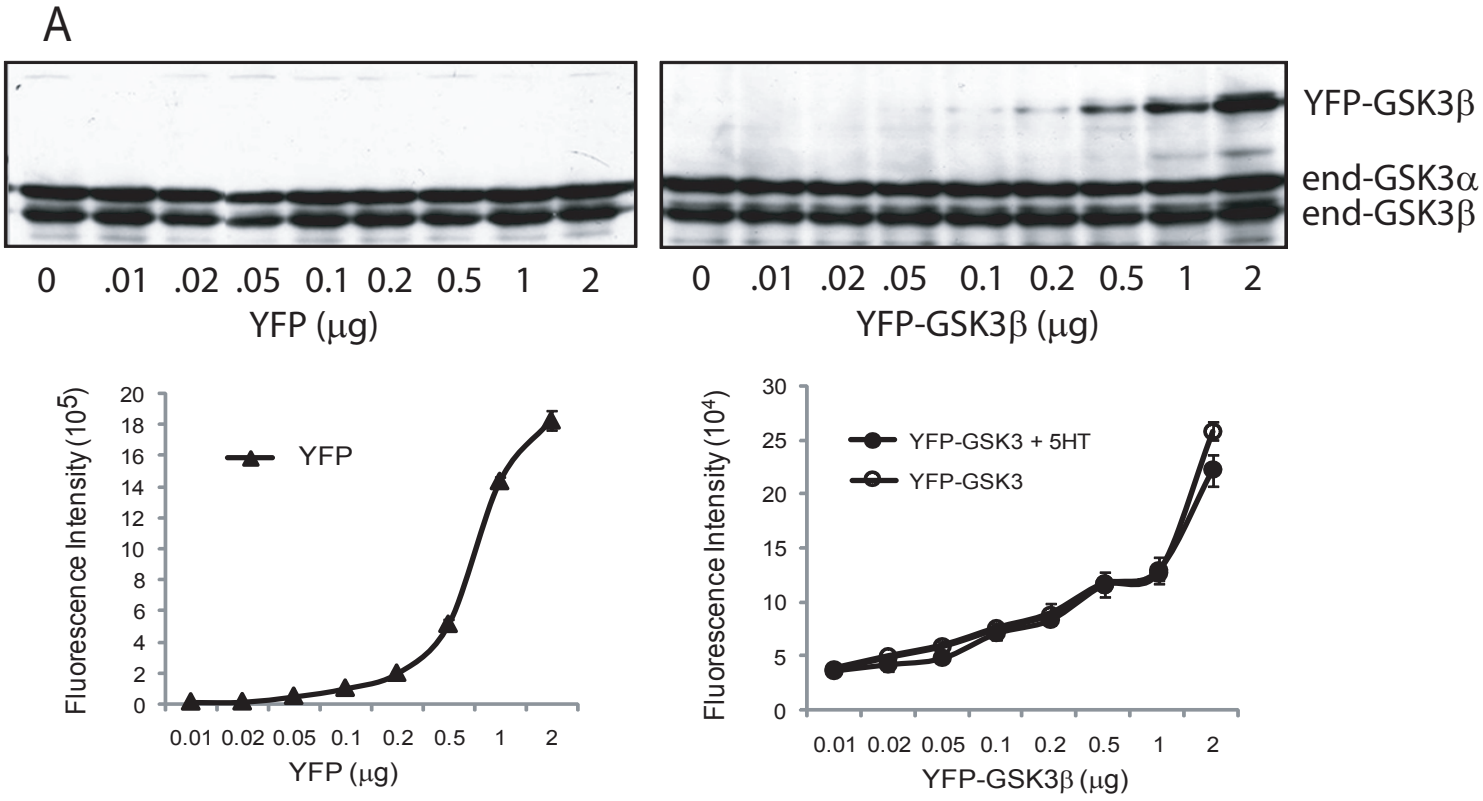

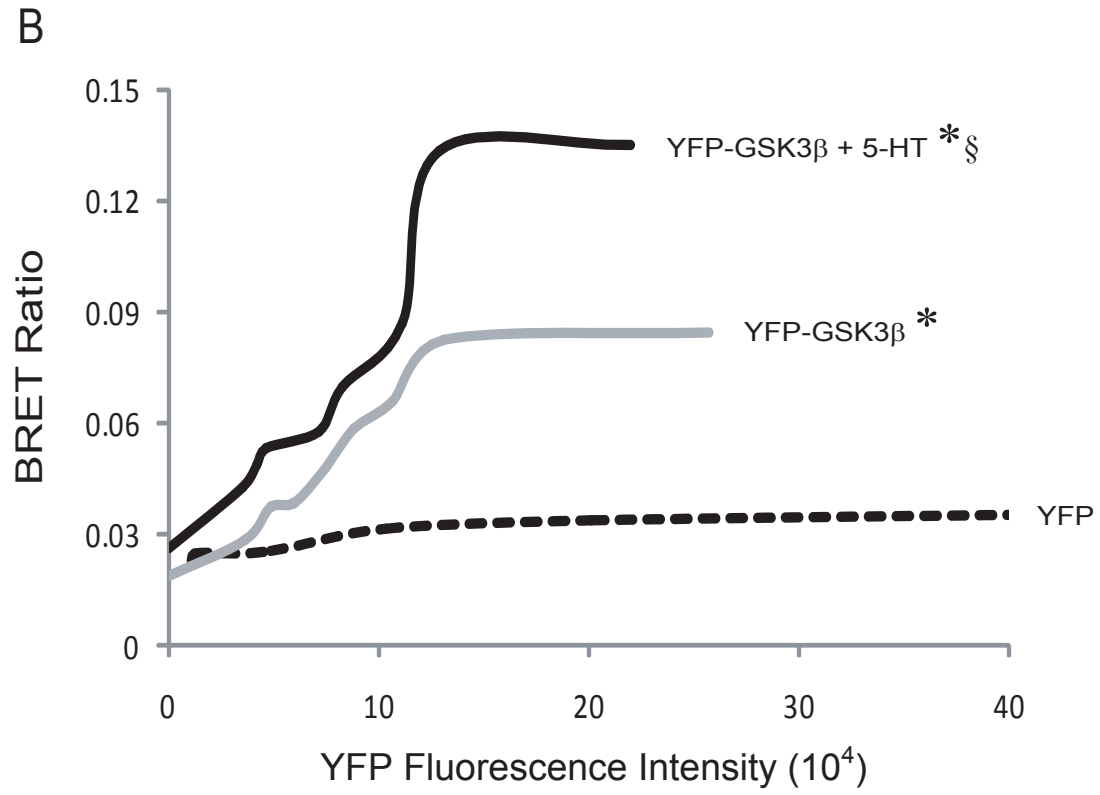

Figure 5

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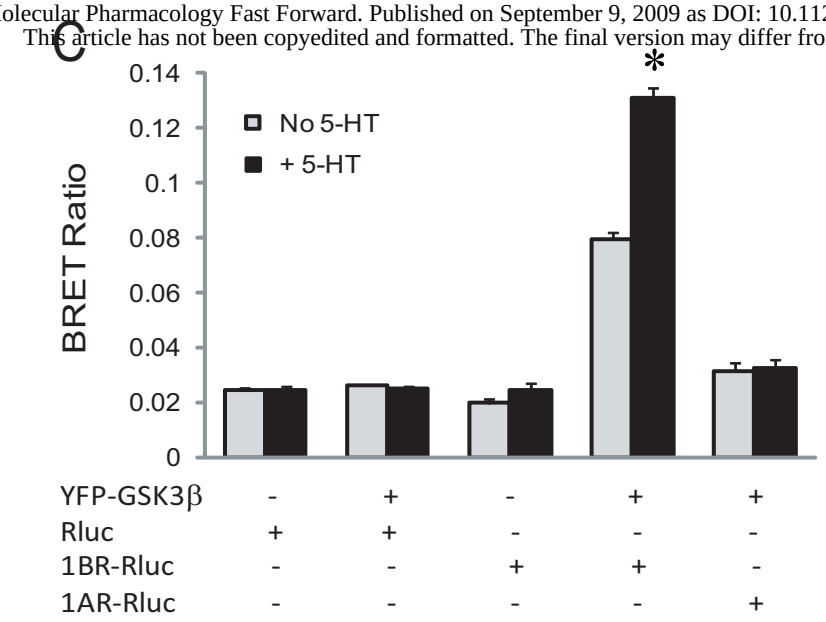

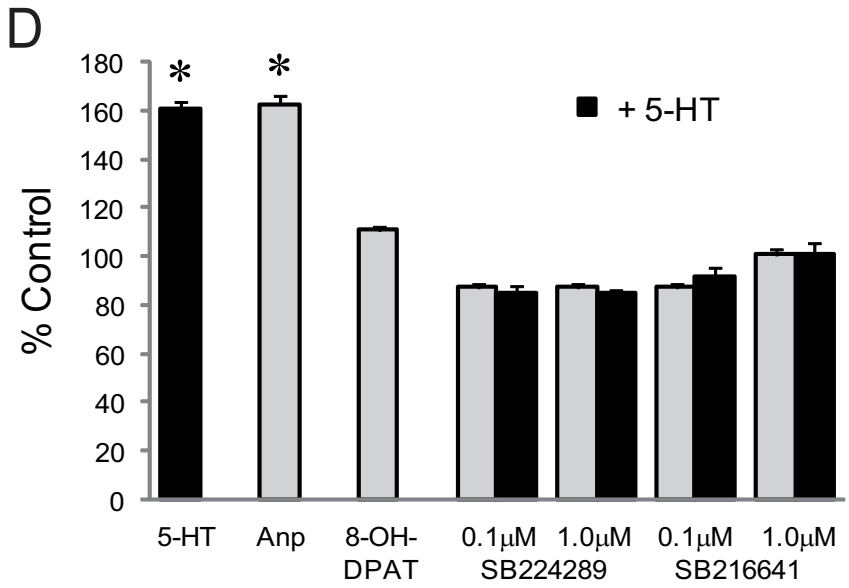

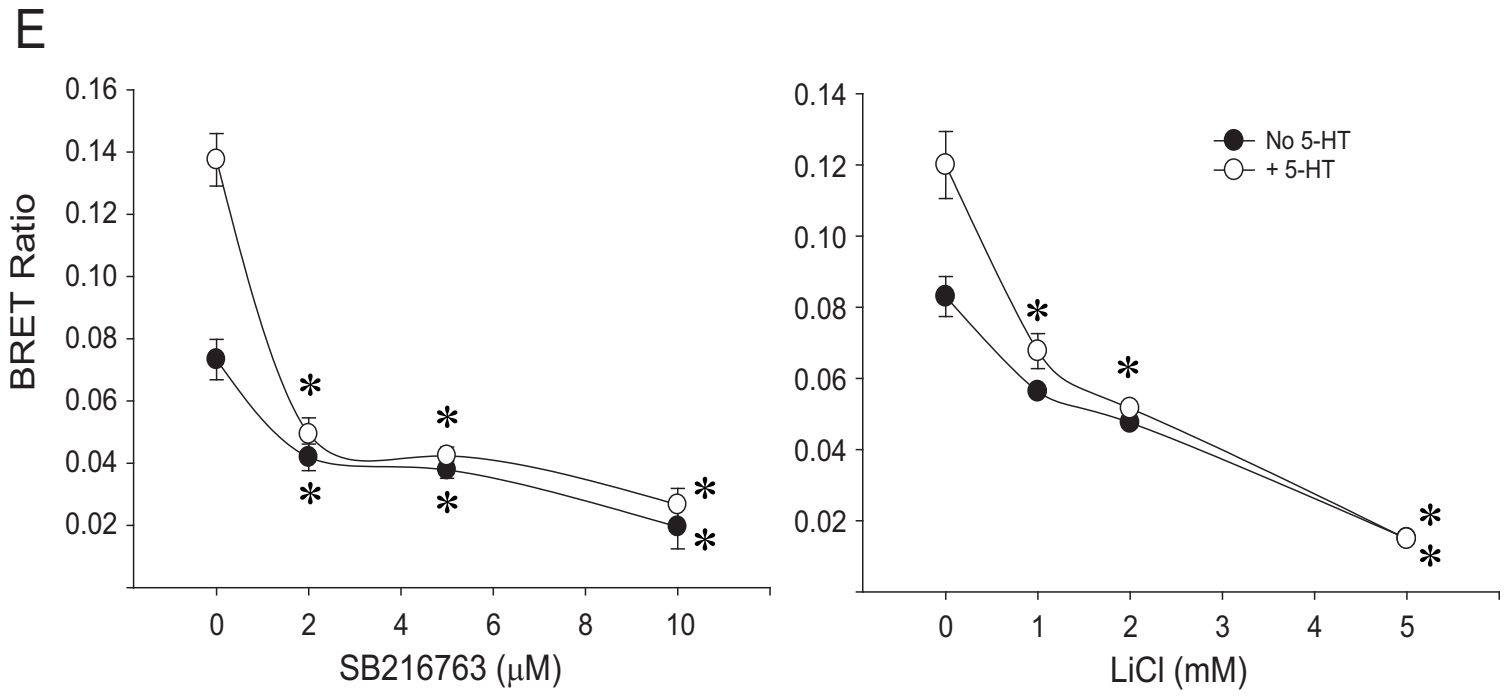

Figure 5

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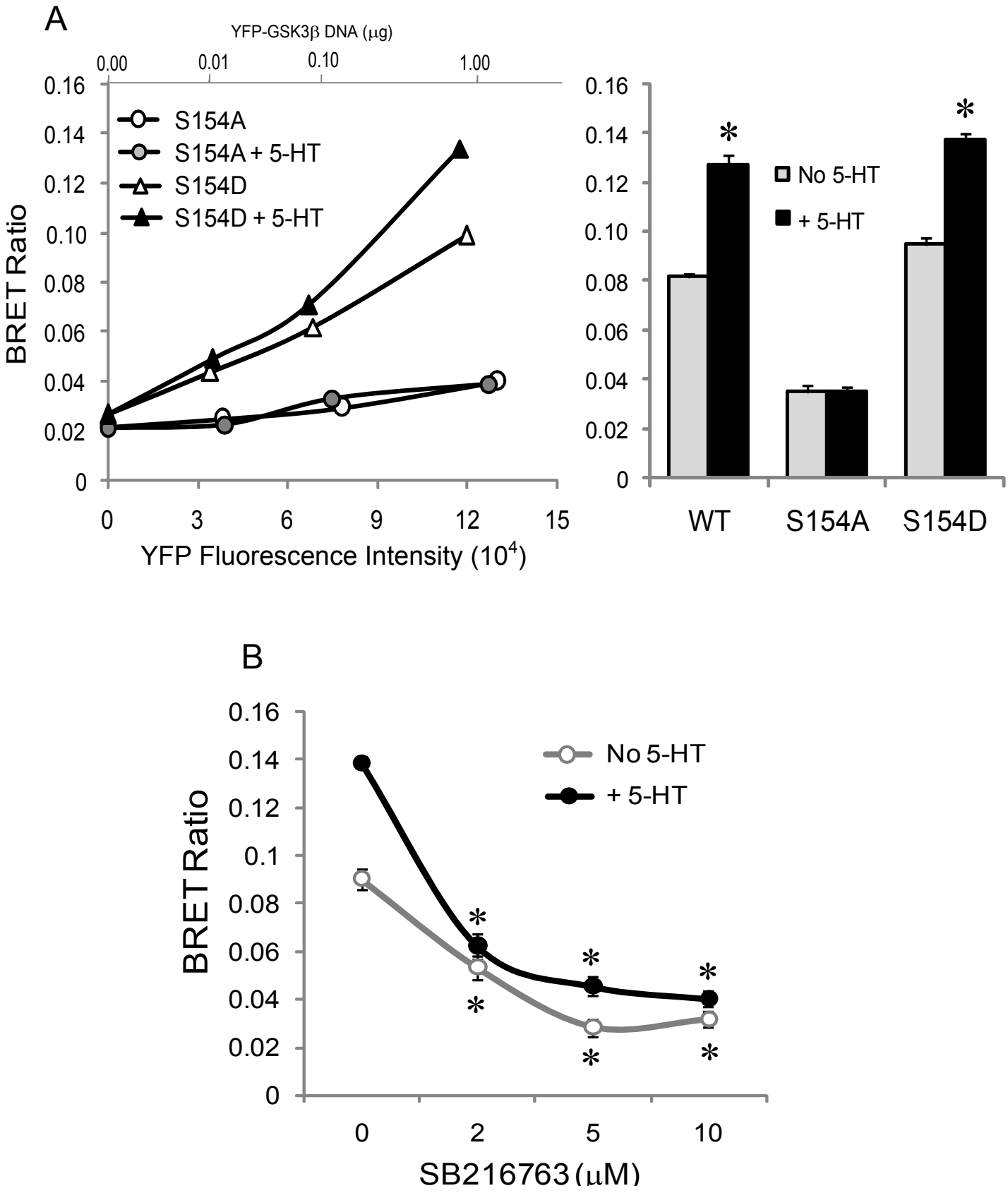

Figure 6

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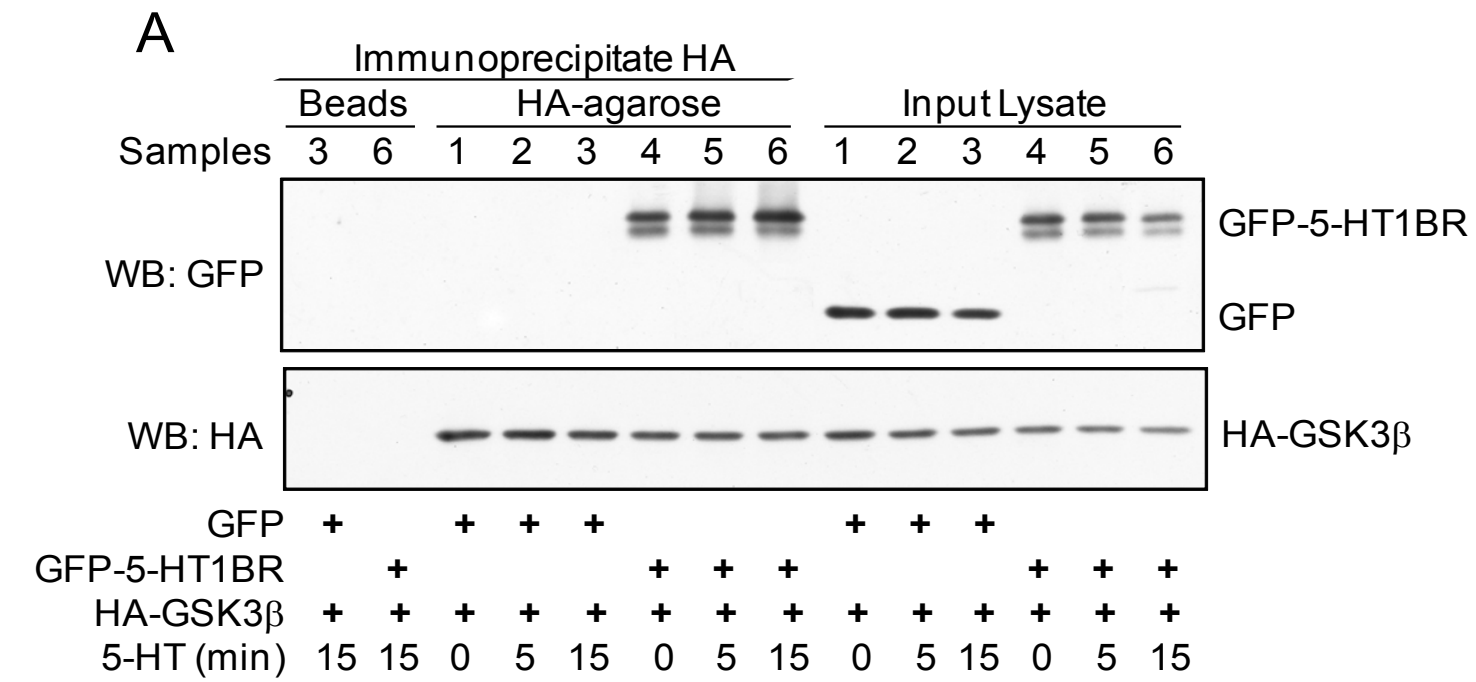

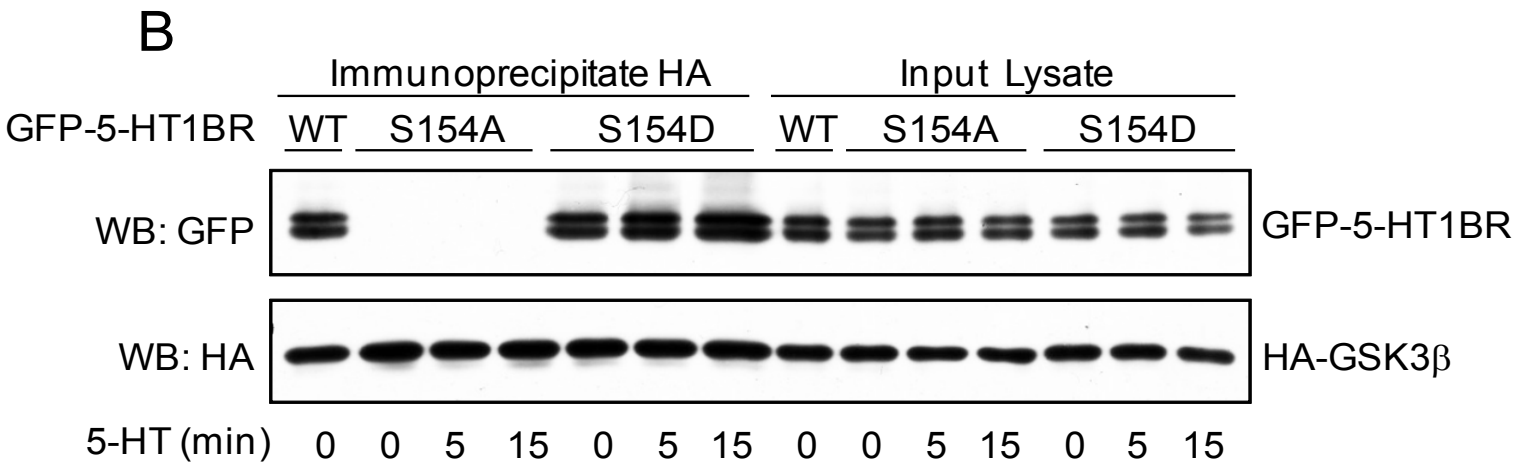

Figure 7

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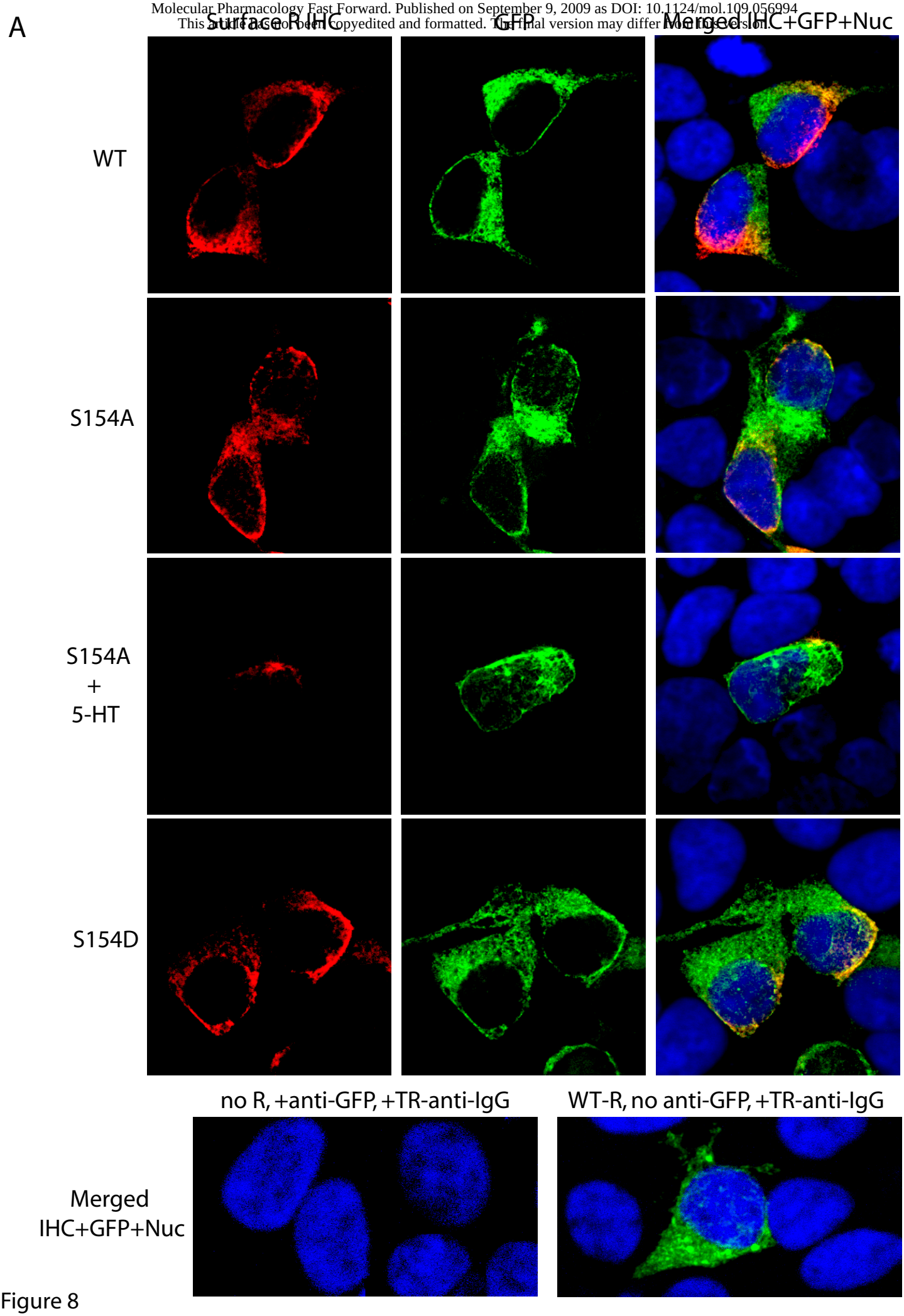

Figure 8

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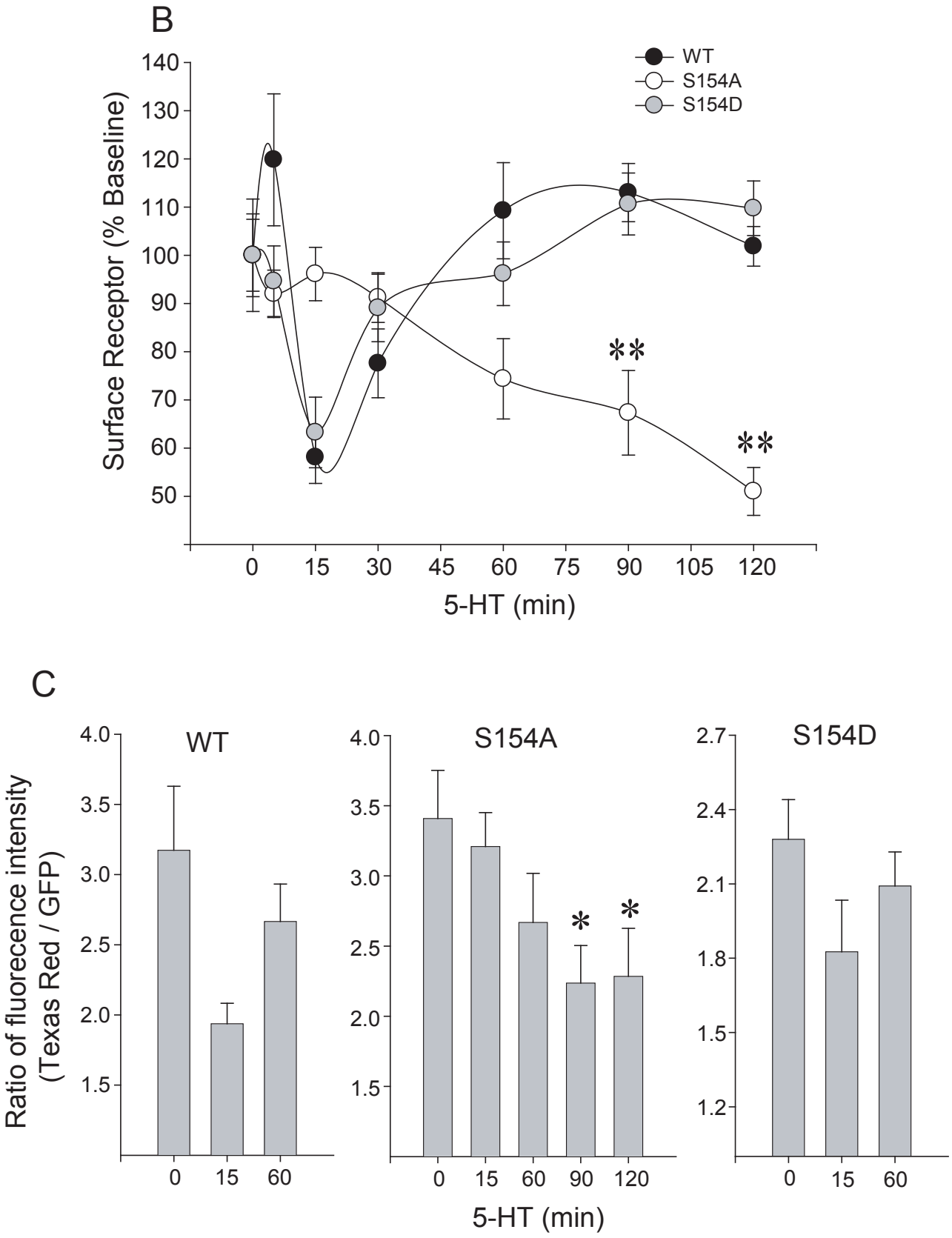

Figure 8