

Glycogen Synthase Kinase-3 β Is a Functional Modulator of Serotonin-1B Receptors^[S]

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ABSTRACT

Glycogen synthase kinase-3 (GSK3) is a constitutively active protein kinase that is involved in neuronal regulation and is a potential pharmacological target of neurological disorders. We found previously that GSK3 β selectively interacts with 5-hydroxytryptamine-1B receptors (5-HT1BR) that have important functions in serotonin neurotransmission and behavior. In this study, we provide new information supporting the importance of GSK3 β in 5-HT1BR-regulated signaling, physiological function, and behaviors. Using molecular, biochemical, pharmacological, and behavioral approaches, we tested 5-HT1BR's interaction with G α_2 and β -arrestin2 and 5-HT1BR-regulated signaling in cells, serotonin release in mouse cerebral cortical slices, and behaviors in wild-type and β -arrestin2 knockout mice. Molecular ablation of GSK3 β and GSK3 inhibitors abolished serotonin-induced change of 5-HT1BR coupling to G α_2 and associated signaling but had no effect on serotonin-induced recruitment of β -arrestin2 to 5-HT1BR. This effect is

specific for 5-HT1BR because GSK3 inhibitors did not change the interaction between serotonin 1A receptors and G α_2 . Two GSK3 inhibitors, *N*-(4-methoxybenzyl)-*N'*-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418) and 3-(5-bromo-1-methyl-1*H*-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione (BIP-135), efficiently abolished the inhibitory effect of the 5-HT1BR agonist anpirtoline on serotonin release in mouse cerebral cortical slices. GSK3 inhibitors also facilitated the 5-HT1BR agonist anpirtoline-induced behavioral effect in the tail suspension test but spared anpirtoline-induced locomotor activity. These results suggest that GSK3 β is a functional selective modulator of 5-HT1BR-regulated signaling, and GSK3 inhibitors fine-tune the physiological and behavioral actions of 5-HT1BR. Future studies may elucidate the significant roles of GSK3 in serotonin neurotransmission and implications of GSK3 inhibitors as functional selective modulators of 5-HT1BR.

Introduction

Glycogen synthase kinase-3 (GSK3) (Embi et al., 1980) is a multifunctional protein kinase that phosphorylates and modulates many protein substrates (Doble and Woodgett, 2003). GSK3 α and GSK3 β are paralogous proteins that share 84%

sequence homology (Woodgett, 1990) and possess common and distinguished protein substrates (Wang et al., 1994). To regulate a substrate by phosphorylation, GSK3 locates a serine or threonine residue that is four amino acids toward the N-terminal of a prime-phosphorylated residue (S/TXXXpS/T) (Doble and Woodgett, 2003), which led to the activation or inhibition of the substrate protein. The constitutively active enzyme is normally under inhibitory regulation by neuromodulators, such as neurotrophins and several neurotransmitters, whereas overactive GSK3 can be detrimental to neuronal function (Doble and Woodgett, 2007; Li and Jope, 2010). GSK3 is an attractive therapeutic target in several neurological disorders, such as mood disorders (Li and

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ABBREVIATIONS: GSK3, glycogen synthase kinase-3; BRET, bioluminescence resonance energy transfer; FST, forced swim test; GPCR, G protein-coupled receptor; 5-HT1BR, 5-hydroxytryptamine-1B receptor; 5-HT1AR, 5-hydroxytryptamine-1A receptor; 8-OH-DPAT, 8-hydroxy-*N,N*-dipropyl-2-aminotetralin; TST, tail suspension test; AR-A014418, *N*-(4-methoxybenzyl)-*N'*-(5-nitro-1,3-thiazol-2-yl)urea; GFP, green fluorescent protein; YFP, yellow fluorescent protein; Rluc, *Renilla reniformis* luciferase; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ANOVA, analysis of variance; HA, hemagglutinin; shRNA, short hairpin RNA; 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; WAY100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; BIP-135, 3-(5-bromo-1-methyl-1*H*-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione.

Joep, 2010) and neurodegenerative disorders (Muylle et al., 2008). However, to better develop disease-specific treatment by normalizing GSK3 activity, it would be important to identify selective GSK3 substrates that have specific neurological functions.

We found previously that GSK3 β selectively interacts with serotonin 1B receptors (5-HT1BR) (Chen et al., 2009). 5-HT1BR belong to the Class A G protein-coupled receptors (GPCRs) that activate the inhibitory G protein (G_i) to inhibit adenylyl cyclase and cAMP production (Raymond et al., 2001) and to activate trophic factor-regulated signaling, such as Akt (Leone et al., 2000). 5-HT1B autoreceptors in serotonin neurons are located at axon terminals (Sari, 2004) and function to inhibit serotonin release in serotonergic neuron-innervated brain regions (Riad et al., 2000). 5-HT1B heteroreceptors interact with other neurotransmitter systems, such as GABA, dopamine, glutamate, and acetylcholine (Sari, 2004). Upon a balanced activity between their autoreceptors and heteroreceptors, 5-HT1BR regulate various behaviors such as mood, anxiety, reward, activity, and aggression (Clark and Neumaier, 2001; Miczek et al., 2004; Sari, 2004). Although 5-HT1BR play a substantial role in regulating serotonin neurotransmission and related behaviors, pharmacological treatment targeting 5-HT1BR has not been fully investigated.

The selective interaction between GSK3 β and 5-HT1BR makes the molecular mechanism an attractive target for drug development once the physiological significance of this mechanism is fully elucidated. In this study, we examined the roles of GSK3 on 5-HT1BR coupling to $G_{i\alpha_2}$ and β -arrestin2, the two major intracellular protein mediators of GPCR signaling. We also investigated the effect of GSK3 in modulating 5-HT1BR-mediated cAMP and Akt signaling, serotonin release, and behaviors. The results show that active GSK3 β selectively modulates $G_{i\alpha}$ -coupled 5-HT1BR signaling, GSK3 inhibitors act to maintain serotonin output by abolishing the negative effect of 5-HT1B autoreceptors, and GSK3 inhibitors differentially affect 5-HT1BR-mediated behaviors.

Materials and Methods

Chemicals. Anpirtoline, *N*-[3-[3-(dimethylamino)ethoxy]-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1'-biphenyl]-4-carboxamide (SB216641), 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763), and 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 (SB224289) (Tocris Bioscience, Ellisville, MO); serotonin, 8-hydroxy-*N,N*-dipropyl-2-aminotetralin (8-OH-DPAT), *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide (WAY100635), and *N*-(4-methoxybenzyl)-*N'*-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418), (Sigma-Aldrich, St. Louis, MO); [3 H]serotonin (PerkinElmer Life and Analytical Sciences, Waltham, MA); and 3-(5-bromo-1-methyl-1*H*-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione (BIP-135) and (benzofuran-3-yl-indol-3-yl)maleimide compounds M1 to M13 (A. P. Kozikowski, University of Illinois, Chicago, IL) were obtained. Chemicals and drugs are dissolved in distilled water, dimethyl sulfoxide, or ethanol (EtOH) in a concentrated solution before they were used for experiments.

DNA Constructs and Mutagenesis. Mouse 5-HT1BR cDNA (Per Sveningsson, Karolinska Institute, Sweden); rat serotonin 1A receptor (5-HT1AR) cDNA (P. Albert, University of Ottawa, Ottawa, ON, Canada); mouse GSK3 β , R96A-GSK3 β , K85,86A-GSK3 β , and

S9A-GSK3 β (R. S. Joep, University of Alabama at Birmingham, Birmingham, AL); human $G_{i\alpha_2}$ and human β -arrestin2 (Missouri S&T cDNA Resource Center, Rolla, MO); pcDNA6 vector (Invitrogen, Carlsbad, CA); pEGFP-C1 and pEYFP-C1 (Clontech, Mountain View, CA); pRL-TK vector (Promega, Madison, WI). 5-HT1BR and 5-HT1AR cDNAs were subcloned into pEGFP-C1 vector with N-terminal GFP, or pRL-TK vector with C-terminal luciferase. Human $G_{i\alpha_2}$ and human β -arrestin2 cDNAs were subcloned into pEYFP-C1 with N-terminal YFP for $G_{i\alpha_2}$ and C-terminal YFP for β -arrestin2. Mutagenesis of mouse 5-HT1BR was performed as described previously (Chen et al., 2009). All constructs were verified by DNA sequencing before cellular transfection.

RNA Interference of GSK3. Short hairpin RNA (shRNA) sequences for human GSK3 β , GSK3 α , and nontargeting shRNA (Sigma-Aldrich) are constructed into lentivirus particles. The shRNA-targeting sequences are shown: GSK3 β : b1, CCCAACTACACAGAATTTAA; b2, GACACTAAAGTGATTGGAAAT; and b3, CCACTGATTATACCTCTAGTA; GSK3 α : a1, GACTAGAGGGCAGAGGTAAAT; a2, GGAGTTCAAGTTCCCTCAGATT; and a3, CTACATCTGTTCTCGCTACTAC; and nontargeting shRNA, CCGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCCTTCATCTTGTGTTTTT. Human embryonic kidney (HEK) 293 cells were transduced with shRNAs for human GSK3 β , GSK3 α , or nontargeting shRNA (control). Cell lines expressing shRNA clones were selected by the antibiotic puromycin (1 μ g/ml). All RNA interference-induced GSK3 knockdowns were confirmed by immunoblotting using the anti-GSK3 α/β antibody.

Cell Culture and Transfection. HEK293 cells and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 U/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 FuGene 6 transfection reagent (Roche Diagnostics, 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 activators. All experiments using transfected DNA constructs were performed in HEK293 cells except for Akt phosphorylation and cAMP assays that were performed in CHO cells in which the signaling cascades are well reserved.

Bioluminescence Resonance Energy Transfer Assay. HEK cells stably expressing 5-HT1BR-Rluc or 5-HT1AR-Rluc were transfected with YFP, YFP-GSK3 β , YFP- $G_{i\alpha_2}$, or β -arrestin2-YFP. Luciferase activity was measured using coelenterazine *f* or coelenterazine *h* (Invitrogen) as the substrate (5 μ M). YFP expression was confirmed by measuring fluorescence signal acquired by excitation at 485 \pm 10 nm and emission at 528 \pm 10 nm using the Synergy 2 spectrofluorometer. For real-time bioluminescence resonance energy transfer (BRET), cells were first detached with PBS-EDTA, washed, and suspended with 0.1% glucose in PBS containing 0.5 mM MgCl $_2$. Cells were transferred into white opaque 96-well plate and incubated at 37°C for 30 min before addition of coelenterazine *h*. Measurement of the dynamic BRET signal was initiated 1 min later. Serotonin was added 21 s after BRET measurement was initiated, and the measurement was continued for 10 min to assure that any change of dynamic protein interaction after treatment was observed for a sufficient length of time. For fixed-time BRET measurement, cells were lifted from culture dishes after they were treated with drugs, washed with PBS, and transferred into white opaque 96-well plate. BRET signal was detected 1 min after addition of coelenterazine *f* into each well. To measure BRET signals, emission was detected at the wavelengths of 485 \pm 10 and 528 \pm 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 \pm 10 nm) – (emission at 485 \pm 10 nm \times Cf)]/(emission at 485 \pm 10 nm) (Angers et al., 2000), where Cf is (emission at 528 \pm 10)/emission at 485 \pm 10 nm measured in cells transfected with 1 μ g of a DNA vector without YFP.

Immunoprecipitation and Immunoblotting. Cells coexpressing wild-type or mutant GFP-5-HT1BR and HA-GSK3 β , HA-G α_2 , or HA- β -arrestin2 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 nM okadaic acid. For coimmunoprecipitation, protein lysate (200 μ g) was incubated overnight with anti-HA-conjugated agarose (Bethyl, Montgomery, TX). After washing 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 for 5 min in SDS sample buffer, and proteins were subjected to electrophoresis in 10% polyacrylamide gels and immunoblotted with anti-GFP (Abcam Inc., Cambridge, MA) and anti-HA (Covance Research Products, Princeton, NJ) antibodies. Protein bands were detected with enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

For immunoblotting GSK3 and Akt, protein lysates from CHO cells were prepared in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% NP-40, 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 nM okadaic acid. Denatured proteins were subjected to electrophoresis in 10% polyacrylamide gels and immunoblotted with antibodies specific for total GSK3 α/β (Millipore, Billerica, MA), phospho-T308-Akt, phospho-S473-Akt, and total Akt (Cell Signaling Technology, Danvers, MA).

cAMP Assay. The level of cAMP was measured in CHO cells using an enzyme immunoassay kit (Direct BioTrak; Amersham/GE Healthcare) (Chen et al., 2009). 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 were lysed in a buffer containing 2.5% dodecyltrimethyl ammonium bromide. Duplicate lysates were transferred into individual wells of a 96-well plate precoated with donkey anti-rabbit IgG. After incubation with rabbit cAMP antiserum and cAMP-horseradish peroxidase conjugates, enzyme reaction was started by the 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 lysates 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 (measured in picomoles per milligram).

Animals. 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 four to five per cage with free access to food and water in a 12-h light/dark cycle animal facility for 1 week before used for experiments. Male homozygous β -arrestin2 knockout mice in C57BL/6 background (R. J. Lefkowitz, Duke University, Durham, NC) (Bohn et al., 1999) were continuously bred from homozygous breeders and were used along with strain-matched wild-type mice for behavior tests.

Serotonin Release. Male C57BL/6 wild-type mice were sacrificed by decapitation, and brains were rapidly dissected in ice-cold Krebs buffer [121 mM NaCl, 1.2 mM MgCl₂, 1.3 mM CaCl₂, 25 mM NaHCO₃, 1.0 mM NaH₂PO₄, 10 mM glucose, 13.5 mM KCl, 50 μ M *l*-tyrosine, 10 μ M nomifensine malate (dopamine reuptake inhibitor), 50 μ M *l*-tryptophan, and 10 μ M imipramine (serotonin reuptake inhibitor)]. Transverse cerebral cortical slices (300 μ m) were prepared by a tissue chopper (McILWAIN, Surrey, England) and rinsed in a cold Krebs buffer that was equilibrated with 95% O₂ and 5% CO₂. Brain slices were labeled with [³H]serotonin (1.3 μ Ci/ml) in Krebs buffer with 95% O₂ and 5% CO₂ at 37°C for 30 min, transferred into the perfusion chambers (10–11 slices/chamber), and superfused with aerated (95% O₂ and 5% CO₂) and preheated (37°C)

Krebs buffer. The flow rate was kept at 0.5 ml/min by a Bio-Rad multichannel peristaltic pump (Econo Pump; Bio-Rad). Beginning at 61 min of perfusion, the superfusion fluid was collected at 2 ml/min. For GSK3 inhibitor treatment, the Krebs buffer was supplemented with AR-A014418 (5 μ M), BIP-135 (1 μ M), or 0.1% EtOH (vehicle) at 20 min of perfusion and continued throughout. Serotonin release was induced by potassium chloride (KCl, 50 mM) at 63 min (P1) and at 83 min (P2), with the perfusion buffer replaced by a new Krebs buffer between P1 and P2 at 78 min when anpirtoline (5 μ M) was added. Collection of superfusion fluid continued until 98 min.

At the end of the experiment, the brain slices from each chamber were collected and homogenized in 200 μ l of 1 N HCl containing 0.1% Triton X-100. Superfusion fluid from each fraction and tissue homogenate (200 μ l) was mixed with scintillation liquid (5 ml of Univisor; MP Biomedicals, Solon, OH), and radioactivity was counted by a scintillation spectrometer (Multi-Purpose Scintillation Counter, LS-6500; Beckman Coulter, Fullerton, CA). To calculate [³H]serotonin release, the radioactivity from fractions collected at 61 to 63 min were averaged as a baseline for the first KCl stimulation, and fractions at 81 to 83 min were averaged for the second KCl stimulation. The radioactivity of all fractions was calculated as the following: counts per minute \times 100/baseline counts per minute. Data are processed by OriginPro 8.1 (OriginLab Corp., Northampton, MA), and potassium-evoked [³H]serotonin release was represented by the peak area surrounded by the fitting line and baseline. Effect of a drug was determined by the ratio of the second overflow (P2) to the first overflow (P1).

Drug Treatment. For intracerebroventricular infusion of experimental drugs, mice were anesthetized with ketamine and xylazine (100 mg/kg/10 mg/kg) to place a guide cannula stereotactically (posterior 0.8 mm and right 1.6 mm to the bregma; depth, 2.2 mm). Five days later, GSK3 inhibitors AR-A014418 (0.3 nmol), BIP-135 (0.08 nmol) or vehicle (10% dimethyl sulfoxide) was infused into the right ventricle via an internal cannula 90 min before anpirtoline (4 mg/kg) or saline injection intraperitoneally at a volume of 5 μ l/g body weight.

Behavior Tests. The tail suspension test (TST) was conducted using an automated testing system (Med Associates Inc., St. Albans, VT) (Polter et al., 2010). Movement is measured for 6 min, and the immobility time was recorded with the Med Associates software and calculated as the time the force of movement was below a preset threshold. Immobility was recorded as each 2-min bin and the last 4 min of testing. For drug treatment, anpirtoline or saline was given 30 min before testing. The locomotor activity was tested in a Plexiglas open field (Med Associates), and activity was monitored using the activity monitoring software (Med Associates) (Polter et al., 2010). Mice were allowed to habituate in the open field for 15 min before anpirtoline or saline injection, followed by an additional 30-min testing in the open field. Travel distance during each 5-min block and total distance traveled during the 15-min pretreatment and the 30-min post-treatment time periods were recorded.

Data Analysis and Statistics. All experiments and treatments were repeated for statistical analysis using SPSS (SPSS Inc., Chicago, IL). The analysis of variance (ANOVA) with post hoc or Student's *t* test was applied as appropriate for each set of experiments. Values are expressed as mean \pm S.E.M. and are considered significant when *p* < 0.05.

Results

As a G α -coupled GPCR, 5-HT1BR, coimmunoprecipitated with G α_2 in either the absence or the presence of serotonin (10 μ M), with serotonin treatment transiently increased the protein association (Fig. 1A). Because GSK3 β mainly interacts with the Ser154/Thr158 consensus GSK3 phosphorylation site located in the second intracellular loop of mouse 5-HT1BR (Chen et al., 2009), we coimmunoprecipitated the

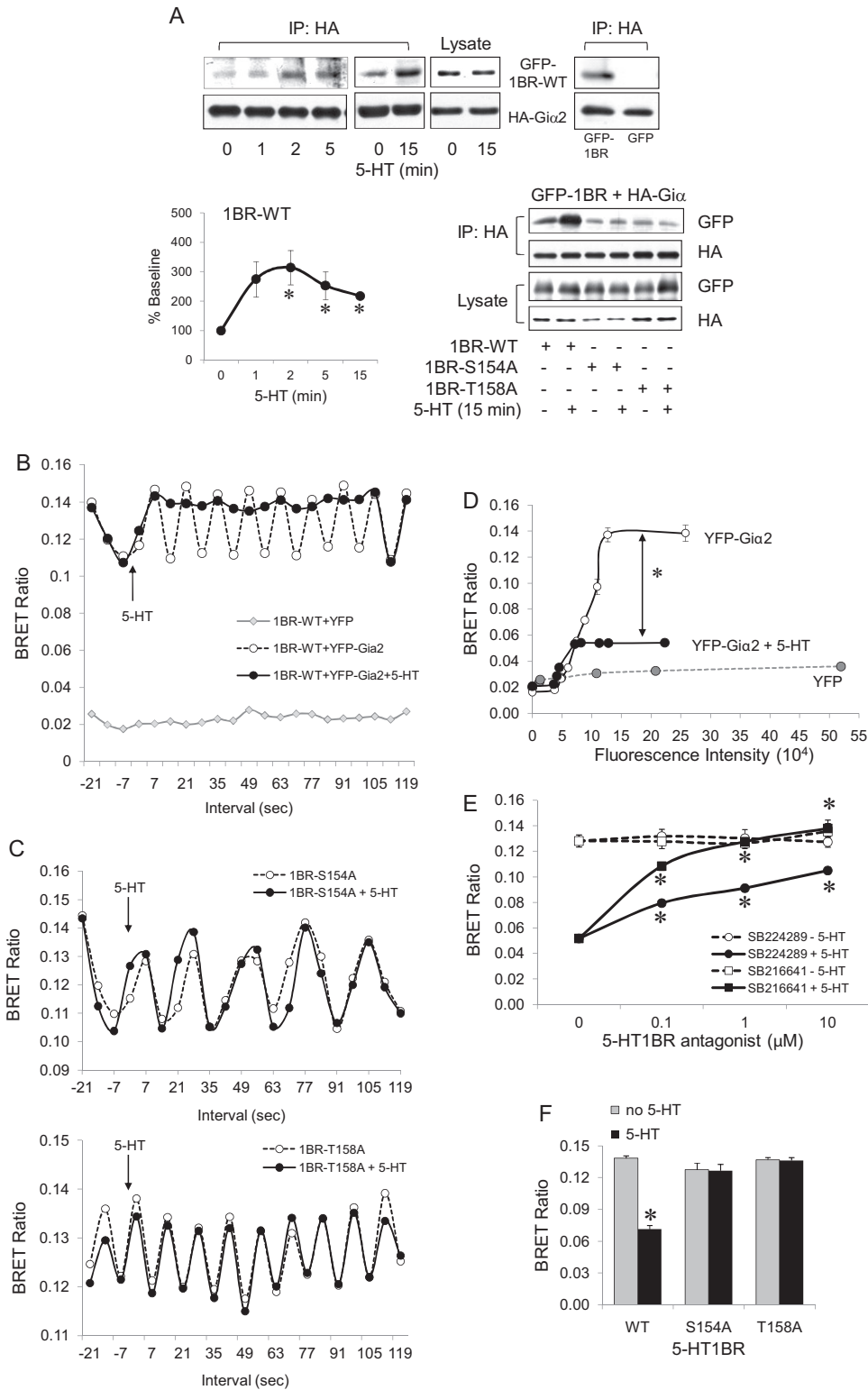


Fig. 1. Association and dynamic interaction between 5-HT_{1B} and G α_i . **A**, HEK293 cells were transfected with GFP-tagged 5-HT_{1B} wild-type (1BR-WT), S154A mutant (1BR-S154A), or T158A mutant (1BR-T158A), and cotransfected with HA-G α_i . Some of these cells were treated with serotonin (5-HT, 10 μ M) for the indicated time. Whole-cell lysates were immunoprecipitated with anti-HA-conjugated agarose beads. Both immunoprecipitate (IP: HA) and cell lysate (lysate) were immunoblotted for GFP and HA. Optimal density of immunoblotted GFP-5-HT_{1B}-WT was calculated as the percentage of baseline (0 min serotonin treatment). Mean \pm S.E.M., $n = 3$ to 5 at each time interval; *, $p < 0.05$ in ANOVA when treatment was compared with baseline (no serotonin). Cells cotransfected with a GFP vector (no 5-HT_{1B}) and HA-G α_i were also immunoprecipitated with anti-HA as negative control. **B**, wild-type 5-HT_{1B}-Rluc-expressing HEK293 cells were transfected with YFP or YFP-G α_i (1 μ g). Real-time BRET measurement was initiated by addition of coelenterazine *h* (5 μ M) and continued for 10 min. Serotonin (5-HT, 10 μ M) was added to the cells 21 s after the initiation of BRET measurement. Data are expressed as BRET ratio as described under *Materials and Methods*. **C**, cells stably expressing the S154A mutant (1BR-S154A) or T158A mutant (1BR-T158A) 5-HT_{1B}-Rluc were transfected with YFP-G α_i (1 μ g), and real-time BRET was measured in the absence or the presence of serotonin (10 μ M). **D**, in a different experiment, cells expressing wild-type 5-HT_{1B}-Rluc were transfected with

S154A and T158A mutant 5-HT1BR with $G_{i\alpha_2}$. Both mutant receptors associated with $G_{i\alpha_2}$, but in contrast to wild-type receptors, serotonin did not change the association between the mutant 5-HT1BR and $G_{i\alpha_2}$ (Fig. 1A), suggesting that only serotonin-induced, but not the resting state of 5-HT1BR- $G_{i\alpha_2}$ association is affected by GSK3.

To further determine the effect of GSK3 on the dynamic interaction between 5-HT1BR and $G_{i\alpha_2}$, we used the *BRET* assay that measures the proximity of two proteins in living cells (Angers et al., 2000). In intact cells, wild-type 5-HT1BR-Rluc and YFP- $G_{i\alpha_2}$ interacted with each other at the resting state with a small but reliably detectable oscillation (Fig. 1B). The oscillatory interaction was not detected between 5-HT1BR-Rluc and YFP. Treatment with serotonin (10 μ M) transiently stabilized the oscillation at the maximum level of interaction for approximately 100 s before it returned to oscillation. In the absence of serotonin, both the S154A and the T158A mutant 5-HT1BR also interacted with $G_{i\alpha_2}$ in an oscillatory pattern, but different from the wild-type 5-HT1BR, serotonin did not cause a transient stabilization of the interaction (Fig. 1C).

We also tested the interaction between 5-HT1BR and $G_{i\alpha_2}$ after termination of serotonin treatment. In this experiment, cells were preincubated with serotonin for 15 min and then were washed to remove serotonin, followed by measuring the 5-HT1BR- $G_{i\alpha_2}$ interaction using the fixed-time BRET. An YFP fluorescent intensity-dependent interaction between 5-HT1BR and $G_{i\alpha_2}$ (maximum at the fluorescent intensity of 12.8×10^4 with 1 μ g of YFP- $G_{i\alpha_2}$ DNA) was reduced significantly (Fig. 1D). This was not a resting state interaction, because it required preserotonin treatment and was concentration-dependently blocked by the 5-HT1BR antagonist SB224289 and SB216641 applied before serotonin treatment (Fig. 1E). Different from the wild-type 5-HT1BR, serotonin pretreatment did not cause a reduction in interaction between the mutant S154A or T158A mutant 5-HT1BR and $G_{i\alpha_2}$ (Fig. 1F). These results indicate that disruption of GSK3 β binding to the intracellular loop-2 of 5-HT1BR (Chen et al., 2009) drastically alters the activity-dependent conformational change between 5-HT1BR and $G_{i\alpha_2}$.

To further test whether the disrupted interaction between 5-HT1BR and $G_{i\alpha_2}$ observed with mutant 5-HT1BR is truly due to lacking modulation by GSK3, GSK3 β , and GSK3 α were eliminated from cells by GSK3 β - and GSK3 α -targeting shRNAs, respectively (Fig. 2A). Complete GSK3 β knockdown abolished both serotonin-induced transient stabilization (Fig. 2B) and serotonin pretreatment-induced reduction (Fig. 2C) of 5-HT1BR- $G_{i\alpha_2}$ interaction, and a partial GSK3 β knockdown sufficiently blocked the transient stabilization and moderately diminished the serotonin pretreatment-induced reduction of interaction. Cells with GSK3 α knockdown or expression of a nontargeting shRNA, however, responded to serotonin simi-

larly to cells expressing wild-type 5-HT1BR without shRNA (Fig. 2, B and C). Therefore, GSK3 β , but not GSK3 α , modulates 5-HT1BR- $G_{i\alpha_2}$ interaction. The effect of GSK3 β was also confirmed in cells expressing the inactive R96A-GSK3 β (Frame and Cohen, 2001) or K85,86A-GSK3 β (Eldar-Finkelman et al., 1996), in which serotonin pretreatment did not reduce the BRET ratio between 5-HT1BR and $G_{i\alpha_2}$, but cells expressing the constitutively active S9A-GSK3 β (Sutherland et al., 1993) responded to serotonin similarly to cells with wild-type GSK3 β (Fig. 2D). These data together suggest that serotonin-induced conformational change between 5-HT1BR and $G_{i\alpha_2}$ requires the presence of active GSK3 β .

Activation of G_i -protein links to both inhibition of cAMP (Raymond et al., 2001) and phosphorylation of Akt that represents activation of Akt (Hsu et al., 2001). We showed previously that inhibition of GSK3 abolishes 5-HT1BR-induced inhibition of cAMP production (Chen et al., 2009). Here, we further tested whether 5-HT1BR-induced phosphorylation of Akt also requires GSK3 interaction with 5-HT1BR. In wild-type 5-HT1BR-expressing cells, serotonin (1 μ M) and the 5-HT1BR agonist anpirtoline (1 μ M) significantly increased the levels of phospho-Thr308-Akt and phospho-Ser473-Akt without changing the level of total Akt (Fig. 3). The effect was blocked by the 5-HT1BR antagonist SB216641 but not by the 5-HT1AR antagonist WAY100635, and the 5-HT1AR agonist 8-OH-DPAT (1 μ M) had a minimal effect on Akt in these 5-HT1BR-expressing cells. In contrast, serotonin and anpirtoline had a minimal effect on phospho-Thr308-Akt and phospho-Ser473-Akt in S154A mutant 5-HT1BR-expressing cells, suggesting that GSK3 β interaction with 5-HT1BR is necessary for 5-HT1BR-induced phosphorylation of Akt.

Although coupling and activation of $G_{i\alpha}$ is the canonical signaling of 5-HT1BR, many GPCRs have been shown to interact with the adaptor protein β -arrestin2 (Lefkowitz and Shenoy, 2005). In cells expressing 5-HT1BR-Rluc and β -arrestin2-YFP, the two proteins exhibited a low level of interaction at resting state (Fig. 4A). Serotonin pretreatment significantly increased BRET ratio in an YFP fluorescent intensity-dependent manner, reaching maximum at 0.5 μ g of β -arrestin2 DNA. The serotonin-induced 5-HT1BR- β -arrestin2 interaction had a slow onset, reached maximal BRET ratio at 4 min of serotonin treatment, and lasted throughout the 10 min of real-time BRET recording (Fig. 4B). However, unlike the interaction with $G_{i\alpha_2}$, both S154A and T158A mutant 5-HT1BR responded to serotonin similarly to wild-type 5-HT1BR, with a time-dependent increase in BRET ratio (Fig. 4C). Therefore, lacking GSK3 β interaction with 5-HT1BR does not affect serotonin-induced recruitment of β -arrestin2 to 5-HT1BR.

Because GSK3 inhibitors have potential therapeutic implications (Cohen and Goedert, 2004; Li and Jope, 2010), the effect of GSK3 inhibitors on 5-HT1BR was further tested.

different amount of YFP or YFP- $G_{i\alpha_2}$ DNA (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 μ g) and treated with serotonin (10 μ M) for 15 min. The BRET signal was recorded immediately after serotonin was washed out, followed by the addition of the luciferase substrate coelenterazine *f* (5 μ M). BRET ratio is plotted against YFP fluorescent intensity recorded at 528 ± 10 nm. Mean \pm S.E.M., $n = 3$; *, $p < 0.001$ in ANOVA when serotonin treatment in YFP- $G_{i\alpha_2}$ -expressing cells was compared with no treatment. E, cells in D were transfected with 1 μ g of YFP- $G_{i\alpha_2}$ DNA, pretreated with the 5-HT1BR antagonist SB224289 or SB216641 at the indicated concentrations for 2 h before the addition of serotonin (10 μ M) for 15 min. BRET signal was measured immediately after serotonin was washed out. Mean \pm S.E.M., $n = 3$; *, $p < 0.05$ in ANOVA when antagonist treatment at each concentration in serotonin-treated cells was compared with no antagonist treatment (0 μ M). F, cells expressing wild-type, S154A mutant, or T158A mutant 5-HT1BR were transfected with $G_{i\alpha_2}$, pretreated with serotonin (10 μ M) for 15 min, and BRET signal was measured immediately after serotonin was washed out. Mean \pm S.E.M., $n = 3$ –6; *, $p < 0.05$ in *t* test when serotonin treatment in each mutant 5-HT1BR-expressing cells was compared with no serotonin treatment (no 5-HT).

The ATP-competitive GSK3 inhibitor SB216763 (10 μ M) itself had no effect on the resting state interaction between 5-HT1BR and G α_i , but it completely abolished serotonin-induced transient stabilization of the interaction (Fig. 5A)

and concentration-dependently abolished serotonin pretreatment-induced reduction in interaction (Fig. 5B). In contrast, SB216763 had no effect on serotonin-induced increase in 5-HT1BR and β -arrestin2 interaction (Fig. 5C).

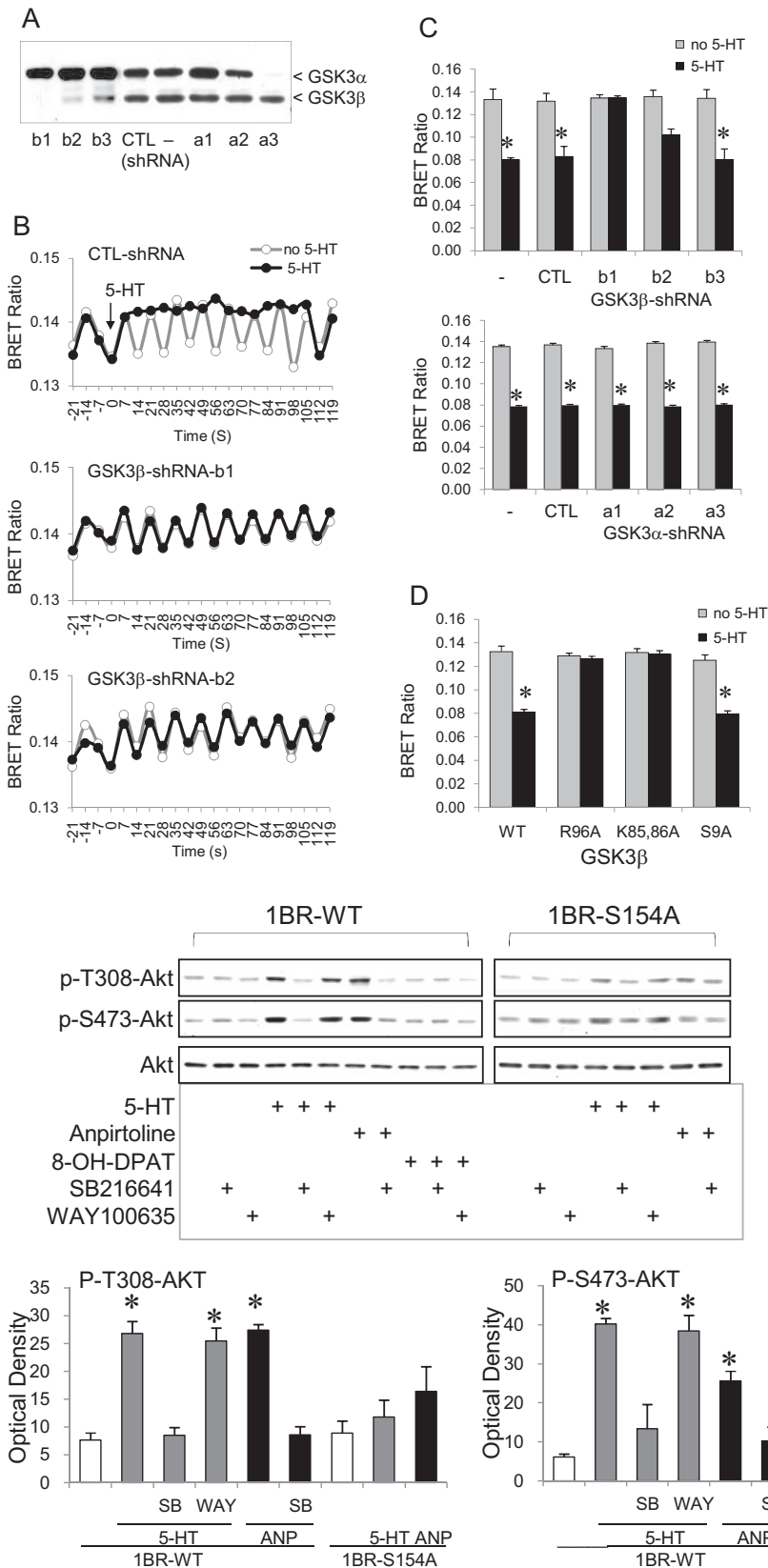


Fig. 2. Effects of GSK3 β -shRNA and inactive GSK3 β on 5-HT1BR-G α_i interaction. A, HEK293 cells stably expressing 5-HT1BR-Rluc were infected with lentivirus-carried shRNA for GSK3 β (b1, b2, b3), GSK3 α (a1, a2, a3), or a nontargeting shRNA (CTL). Cell lysates from these shRNA-expressing cells and noninfected cells (–) were immunoblotted for total GSK3 α and GSK3 β . B, cells in A with an indicated shRNA were transfected with YFP-G α_i (1 μ g), followed by measuring real-time BRET in the absence or the presence of serotonin (5-HT, 10 μ M). C, BRET was measured in these cells after serotonin pretreatment (10 μ M) for 15 min. Mean \pm S.E.M., $n = 3$ –6; *, $p < 0.05$ in t test when serotonin-treatment in each type of shRNA-expressing cells were compared with no serotonin treatment (no 5-HT). D, 5-HT1BR-Rluc-expressing cells were cotransfected with YFP-G α_i and a wild-type or a mutant GSK3 β (R96A, K85,86A, or S9A) and pretreated with serotonin (10 μ M) for 15 min before BRET measurement. Mean \pm S.E.M., $n = 3$; *, $p < 0.05$ in t test when serotonin treatment in each type of GSK3 β -expressing cells were compared with no serotonin treatment (no 5-HT).

Fig. 3. 5-HT1BR-induced Akt phosphorylation. CHO cells stably expressing wild-type (1BR-WT) or S154A-mutant (1BR-S154A) 5-HT1BR were treated with the 5-HT1BR antagonist SB216641 or the 5-HT1AR antagonist WAY100635 (1 μ M) for 30 min before serotonin (5-HT), anpirtoline, or 8-OH-DPAT (1 μ M) treatment for 5 min. Cell lysates were immunoblotted for phospho-Thr308-Akt, phospho-Ser473-Akt, and total Akt. Data is expressed as average optical density of immunoblots. Mean \pm S.E.M., $n = 3$ to 5; *, $p < 0.05$ in ANOVA compared with basal value (no drug treatment) in each experiment.

In 5-HT_{1A}-expressing cells, 5-HT_{1A} interacted with G_iα₂ in a manner similar to that of 5-HT_{1B} with an oscillation that was transiently stabilized by serotonin (Fig. 5D), and the interaction was reduced with serotonin pretreatment (Fig. 5E). However, the GSK3 inhibitor SB216763 did not alter either of the serotonin-dependent change of interaction.

To identify additional GSK3 inhibitors that have high potency and selectivity in modulating 5-HT_{1B} signaling, we screened a group of benzofuranyl-3-yl-(indol-3-yl)maleimides that are capable of inhibiting GSK3 at various concentrations (Supplemental Table 1) (Kozikowski et al., 2007; Gaisina et al., 2009) and compared their effects with a known GSK3 inhibitor, AR-A014418 (Bhat et al., 2003). Similar to SB216763 (Chen et al., 2009) (Fig. 5), AR-A014418 concentration-dependently abolished 5-HT_{1B} and GSK3β interaction (Fig. 6A), serotonin pretreatment-induced interaction between 5-HT_{1B} and G_iα₂ (Fig. 6B), and 5-HT_{1B}-mediated inhibition of cAMP production (Fig. 6C). Among the 14 benzofuranyl-3-yl-(indol-3-yl)maleimides, BIP-135 (Fig. 6A) and maleimide 6 (Supplemental Fig. 1A) had a potent effect in disrupting the interaction between 5-HT_{1B} and GSK3β either without or with serotonin treatment, and other maleimides had a moderate or biphasic effect (Supplemental

Fig. 1A). All tested maleimides concentration-dependently reversed serotonin pretreatment-induced reduction in interaction between 5-HT_{1B} and G_iα₂ but did not affect the interaction in the absence of serotonin (Fig. 6B; Supplemental Fig. 1B). However, among the four tested maleimides, only BIP-135 efficiently abolished the effect of serotonin in reducing cAMP production (Chen et al., 2009), reaching a maximal effect at 1 μM concentration (Fig. 6C). Maleimides 3 and 6 did not elicit a concentration-dependent effect, and maleimide 8 had a biphasic effect that did not agree with its effect on 5-HT_{1B}-GSK3β interaction (Supplemental Fig. 1A). Therefore, some but not all GSK3 inhibitors selectively modulate 5-HT_{1B}-regulated G_iα-cAMP signaling.

A major physiological function of active 5-HT_{1B} autoreceptors in brain is to negatively regulate serotonin release (Göthert et al., 1987), which has been reported as a G_i-dependent action (Ghavami et al., 1997). To test whether GSK3 inhibitors may affect this physiological function of 5-HT_{1B}, we measured 5-HT_{1B}-mediated inhibition of [³H]serotonin release in mouse cerebral cortical slices. Activation of 5-HT_{1B} by anpirtoline (5 μM) caused a 60% reduction in potassium chloride (50 mM)-evoked serotonin release (Fig. 7, A and B). Pretreatment of cortical slices with the GSK3 inhibitor AR-A014418 (5 μM) or the maleimide

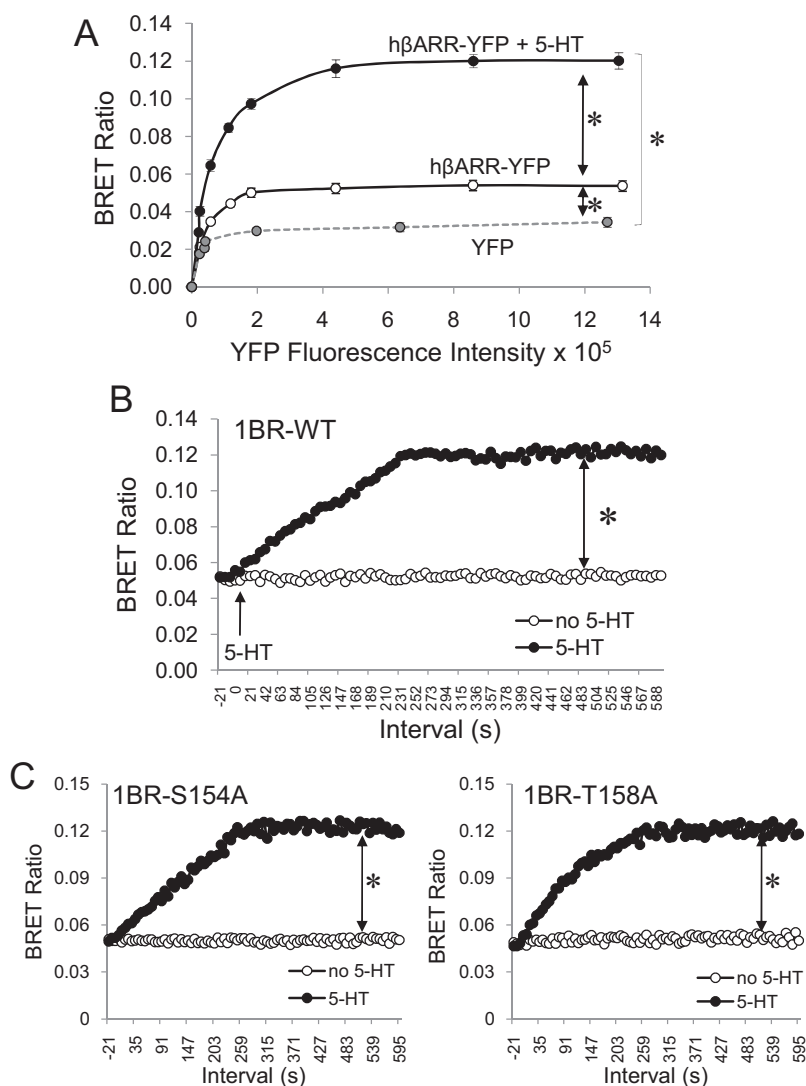


Fig. 4. Interaction between 5-HT_{1B} with β -arrestin2. **A**, HEK293 cells stably expressing wild-type 5-HT_{1B}-Rluc were transfected with different amount of YFP or human β -arrestin2 (hβARR)-YFP DNA (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 μg), and pretreated with serotonin (5-HT, 10 μM) for 15 min before BRET measurement. Mean \pm S.E.M., $n = 3$; *, $p < 0.001$ between the two groups in ANOVA. **B**, real-time BRET measurement was performed in cells expressing wild-type 5-HT_{1B}-Rluc and β -arrestin2-YFP (0.5 μg) in the absence or the presence of serotonin (10 μM). **C**, HEK293 cells stably expressing S154A or T158A mutant 5-HT_{1B}-Rluc (1BR-S154A or 1BR-T158A) were used for real-time BRET in the absence or the presence of serotonin. $n = 3$; *, $p < 0.001$ in ANOVA.

GSK3 inhibitor BIP-135 (1 μ M) completely abolished the inhibitory effect of anpirtoline on serotonin release (Fig. 7B).

5-HT1BRs participate in different serotonin-induced behaviors via both autoreceptors and heteroreceptors (Sari, 2004). Enhancing serotonin release by ablating 5-HT1B autoreceptor action reportedly facilitates 5-HT1BR agonist-induced reduction of immobility when tested in either the TST or forced swim test (FST) (O'Neill et al., 1996; Chenu et al., 2008). Because GSK3 inhibitors are able to maintain sufficient serotonin release by abolishing anpirtoline-induced activation of 5-HT1B autoreceptors, we tested whether GSK3 inhibitors may facilitate the effect of anpirtoline in the TST in mice. Systemic administration of anpirtoline (4 mg/kg i.p.) only mildly reduced the immobility in mice (Fig. 8A), which

was better observed at the first 4 min of a 6-min test but did not separate from control mice at the last 4 min of the test. Infusion of the GSK3 inhibitor AR-A014418 (0.3 nmol/mouse i.c.v.) itself had no effect on the TST, but when in combination with anpirtoline, the two drugs caused a significant reduction in immobility compared with control mice, and the combined effect seemed to be stronger at the last 4 min of the test. BIP-135 (0.08 nmol/mouse i.c.v.) itself also did not have an effect in the TST, but when combined with anpirtoline, the two drugs elicited a strong and significant effect in reducing immobility compared with either control or anpirtoline alone, and the effect sustained throughout the last 4 min of the 6-min test.

In contrast to the TST, AR-A014418 and BIP-135 had no

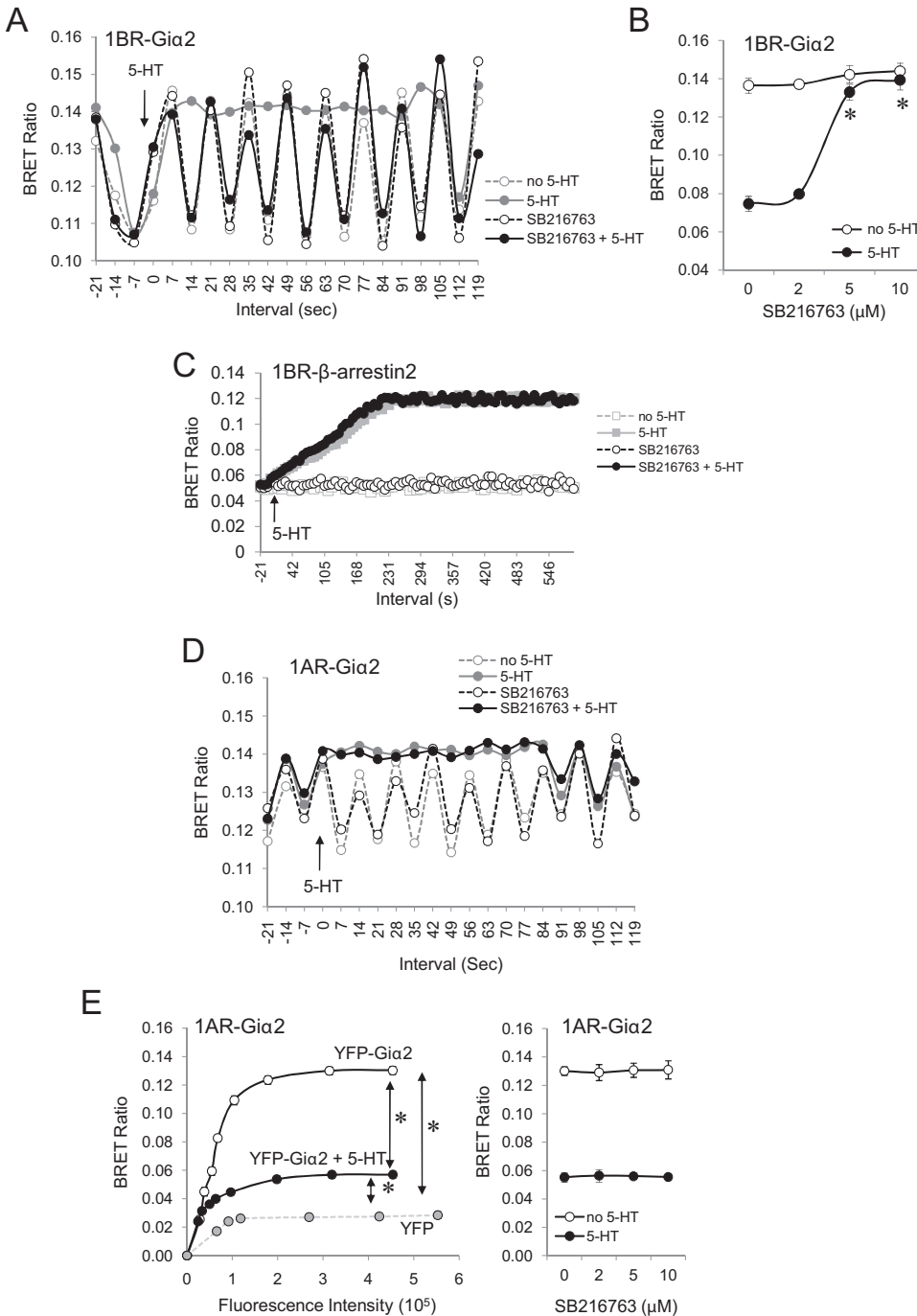


Fig. 5. The effect of GSK3 inhibitor on 5-HT1BR and 5-HT1AR interaction with $G_{i\alpha 2}$ and β -arrestin2. A, HEK293 cells expressing 5-HT1BR-Rluc and YFP- $G_{i\alpha 2}$ were pretreated with the GSK3 inhibitor SB216763 (10 μ M) for 2 h before used for real-time BRET measurement in the absence or the presence of serotonin (5-HT, 10 μ M). B, these cells were pretreated with indicated concentrations of SB216763 for 2 h before addition of serotonin (10 μ M) for 15 min, followed by BRET measurement. Mean \pm S.E.M., $n = 3-9$; *, $p < 0.05$ in ANOVA when inhibitor treatment at each concentration in serotonin-treated cells were compared with no inhibitor treatment. C, HEK293 cells expressing 5-HT1BR-Rluc were transfected with β -arrestin2-YFP (0.5 μ g), pretreated with SB216763 (10 μ M) for 2 h, followed by real-time BRET measurement in the absence or the presence of serotonin (10 μ M). D, HEK293 cells stably expressing 5-HT1AR-Rluc were transfected with YFP- $G_{i\alpha 2}$ (1 μ g) and pretreated with the GSK3 inhibitor SB216763 (10 μ M) for 2 h, followed by real-time BRET measurement in the absence or the presence of serotonin. E, cells in D were transfected with different amount of YFP or YFP- $G_{i\alpha 2}$ (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 μ g), pretreated with serotonin (5-HT, 10 μ M) for 15 min, followed by BRET measurement. Mean \pm S.E.M., $n = 3$, *, $p < 0.001$ between the two groups in ANOVA (left). For GSK3 inhibitor treatment, cells were transfected with 1 μ g of YFP- $G_{i\alpha 2}$, and pretreated with the GSK3 inhibitor SB216763 at indicated concentrations for 2 h before serotonin (10 μ M) for 15 min, followed by BRET measurement; mean \pm S.E.M., $n = 3$ (right).

effect in anpirtoline-induced increase in locomotor activity (Fig. 8B), which is mainly a 5-HT_{1B} heteroreceptor-mediated behavior (Pranzatelli et al., 1987; Cheetham and Heal, 1993). Because GSK3 inhibitors had no effect on 5-HT_{1B}- β -arrestin2 interaction (Fig. 5C), we tested whether the GSK3 inhibitor-insensitive locomotor activity is a β -arrestin2-dependent behavior. In β -arrestin2 knockout mice (Bohn et al., 1999), anpirtoline did not cause a detectable increase in locomotor activity at the time of treatment, and the total distance traveled after anpirtoline treatment was not significantly different from saline treatment in β -arrestin2 knockout mice (Fig. 8C). Therefore, 5-HT_{1B}-mediated locomotor activity at least partly depends on the presence of β -arrestin2, a signaling mechanism that is independent of 5-HT_{1B} interaction with GSK3 β .

Discussion

After our previous finding that GSK3 β selectively interacts with 5-HT_{1B} (Chen et al., 2009), this study further dis-

sected the functional aspects of this interaction on 5-HT_{1B}-regulated signaling, physiology, and behavior. The study results suggest that GSK3 β selectively modulates G α_2 -associated 5-HT_{1B} signaling pathways but not recruitment of β -arrestin2 to active 5-HT_{1B}; GSK3 β only affects the activation state of 5-HT_{1B}, with little effect on the resting state; GSK3 inhibitors modulate 5-HT_{1B} autoreceptor-regulated serotonin release; and GSK3 inhibitors differentially modulate 5-HT_{1B}-mediated behaviors.

Because G α_2 is a major mediator of 5-HT_{1B}-regulated signaling (Raymond et al., 2001), this study began by testing whether GSK3 affects 5-HT_{1B}-G α_2 interaction. The cell-based BRET assay is used because it allows studying dynamic protein interaction of GPCRs with G proteins (Angers et al., 2000). BRET assay clearly detected three interactive states of 5-HT_{1B} with G α_2 , the oscillatory interaction at resting state, the transient stabilization of interaction immediately after receptor activation by serotonin, and a drastic conformational change shortly after removing serotonin from

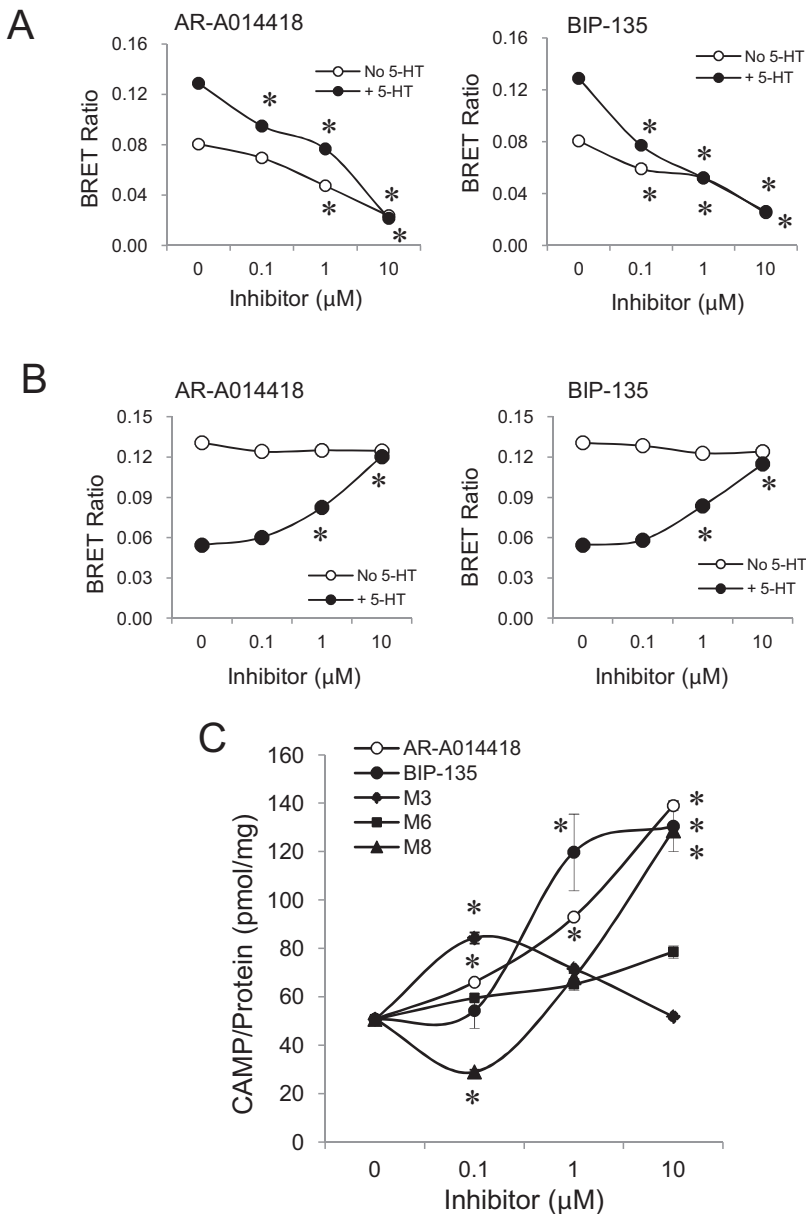


Fig. 6. Selection of GSK3 inhibitors for 5-HT_{1B} interaction with GSK3 β and G α_2 and regulation of cAMP. HEK293 cells expressing 5-HT_{1B}-RLuc and YFP-GSK3 β (A) or YFP-G α_2 (B) were pretreated with the GSK3 inhibitor AR-A014418 or a maleimide BIP-135 at indicated concentrations for 2 h. Some cells were pretreated with serotonin (5-HT, 10 μ M) for 15 min, followed by measuring BRET. $n = 3-6$; *, $p < 0.05$ in ANOVA when each concentration of an inhibitor treatment was compared with no inhibitor treatment. C, CHO cells transfected with 5-HT_{1B} were pretreated with the indicated concentrations of AR-A014418 or a maleimide for 2 h, followed by serotonin (10 μ M) for 30 min before stimulating cAMP production with forskolin (fsk; 10 μ M, 15 min). The levels of cAMP (in picomoles) are normalized by the protein concentration (in milligrams). $n = 3-4$; *, $p < 0.05$ in ANOVA when inhibitor treatment at each concentration was compared with no inhibitor treatment.

the receptor. The interaction pattern in the absence or the presence of serotonin also applies between 5-HT1AR and G α_2 , suggesting that this is a common dynamic interaction pattern between type I serotonin receptors and G α_2 . A small oscillatory interaction was observed by the real-time BRET during receptor resting state, which we confirm is not a baseline noise of the BRET assay because it was invariably detected in all experiments using different 5HT1BR and G α_2 DNA constructs. The oscillatory interaction between wild-type 5HT1BR and G α_2 can be invariably stabilized by serotonin treatment, whereas there is no oscillation between 5HT1BR and YFP or between 5HT1BR and β -arrestin2. Nevertheless, because the serotonin-induced change of oscillatory interaction is small and transient, we also used the fixed-time BRET to measure the 5-HT1BR-G α_2 interaction after serotonin treatment. This assay detected a nearly 60% reduction of BRET ratio only after serotonin was washed out. Because coimmunoprecipitation assay that measures a steady-state protein association did not detect a dissociation of 5-HT1BR and G α_2 , the serotonin-induced change of interaction seen in BRET assay is probably a dynamic protein conformational change between 5-HT1BR and G α_2 that represents G α_2 activation. However, to fully interpret the relationship of changes observed by real-time and fixed-time BRET, other protein structural studies are required that will not be further discussed here.

Because all GSK3 manipulations applied in the study resulted in change of agonist-induced 5-HT1BR-G α_2 interaction, it is confirming that GSK3 β is a modulator of 5-HT1BR-regulated G α_2 signaling, and it provides extended explanation of our previous finding that 5-HT1BR-induced inhibition of cAMP production depends on the presence of active GSK3 β (Chen et al., 2009). The selectivity of GSK3 β on 5-HT1BR-G α_2 interaction is likely by targeting 5-HT1BR, not G α_2 itself, because the similar 5-HT1AR-G α_2 interaction was not altered by GSK3 inhibitors.

Although we conclude that GSK3 β is required for activation of G α_2 -coupled signaling by 5-HT1BR, it should be acknowledged that the significant changes of 5-HT1BR response to agonists were mostly observed when GSK3 β was removed from 5-HT1BR by receptor mutation, GSK3 β knock-down, inactive GSK3 β , and GSK3 inhibitors, whereas overexpression of constitutively active S9A mutant GSK3 β had no additional effect on 5-HT1BR activity. This observation may suggest that the intrinsic active GSK3 β is an integrative component of the 5-HT1BR, which associates with the resting 5-HT1BR and is required for full activation of 5-HT1BR by serotonin. On the other hand, inhibition of GSK3 β may have significant implications in modulating 5-HT1BR-regulated signaling.

G α_2 -mediated cAMP production is not the only GSK3-dependent signaling pathway of 5-HT1BR because activation of Akt by serotonin was significantly diminished in S154A-mutant 5-HT1BR-expressing cells. The mechanisms of regulating Akt by 5-HT1BR are not fully understood, but it is believed that both α - and β -subunits of G-protein as well as β -arrestin are associated with GPCR-induced activation of Akt (DeWire et al., 2007; New et al., 2007; Yang et al., 2009). The finding that GSK3 influences 5-HT1BR-induced activation of Akt further supports the significant effect of GSK3 in 5-HT1BR-regulated signaling. Because Akt is one of the upstream GSK3-regulating protein kinases that phosphorylate GSK3 at the N-terminal serine to inactivate GSK3 activity (Cross et al., 1995), the GSK3-dependent activation of Akt by 5-HT1BR could function as a feedback regulation to prevent the prolonged effect of GSK3 on 5-HT1BR, but this postulation remains to be examined.

To our knowledge, 5-HT1BR interaction with β -arrestin2 has not been reported, but this interaction is expected because β -arrestin has been recognized as an adaptor and signal transducer of many GPCRs (Lefkowitz and Shenoy, 2005). In this study, recruitment of β -arrestin2 to 5-HT1BR

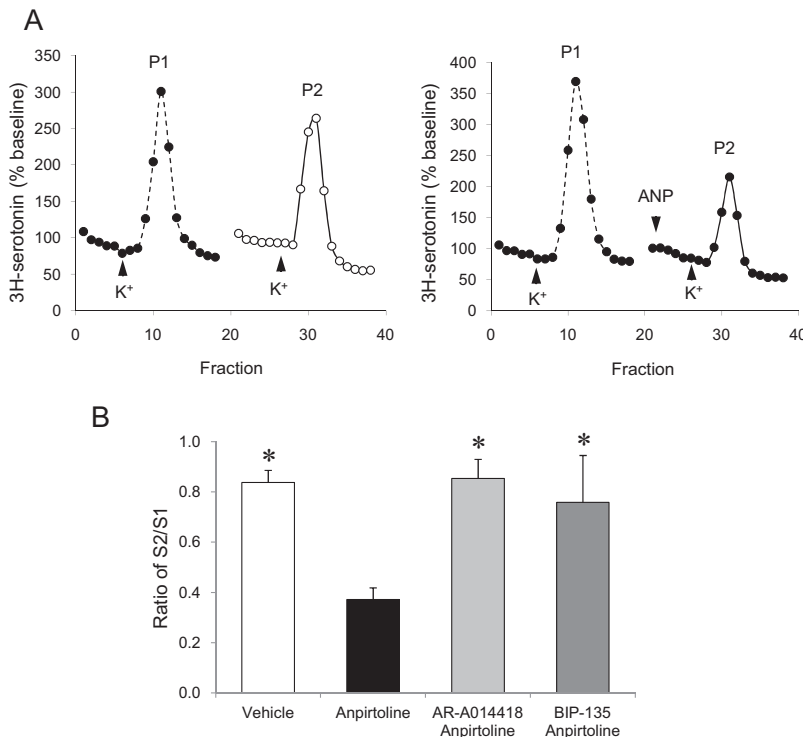


Fig. 7. The effect of GSK3 inhibitors on 5-HT1BR-mediated inhibition of serotonin release. Potassium-evoked [3 H]serotonin release was measured in freshly isolated mouse brain cerebral cortical slices. A, serotonin release was stimulated by potassium chloride (K⁺, 50 mM) twice. Before the second potassium stimulation, slices were treated with anpirtoline (ANP, 5 μ M) for 5 min (right). Data is expressed as the percentage of baseline [3 H]serotonin release. B, brain slices were pretreated with the GSK3 inhibitor AR-A014418 (5 μ M) or BIP-135 (1 μ M) for 58 min before addition of anpirtoline. Data are expressed as the ratio of second potassium-evoked peak (P2) to the first potassium-evoked peak (P1) of serotonin release. Mean \pm S.E.M., n = 3–5 in each treatment; *, p < 0.05 in ANOVA when values were compared with anpirtoline alone.

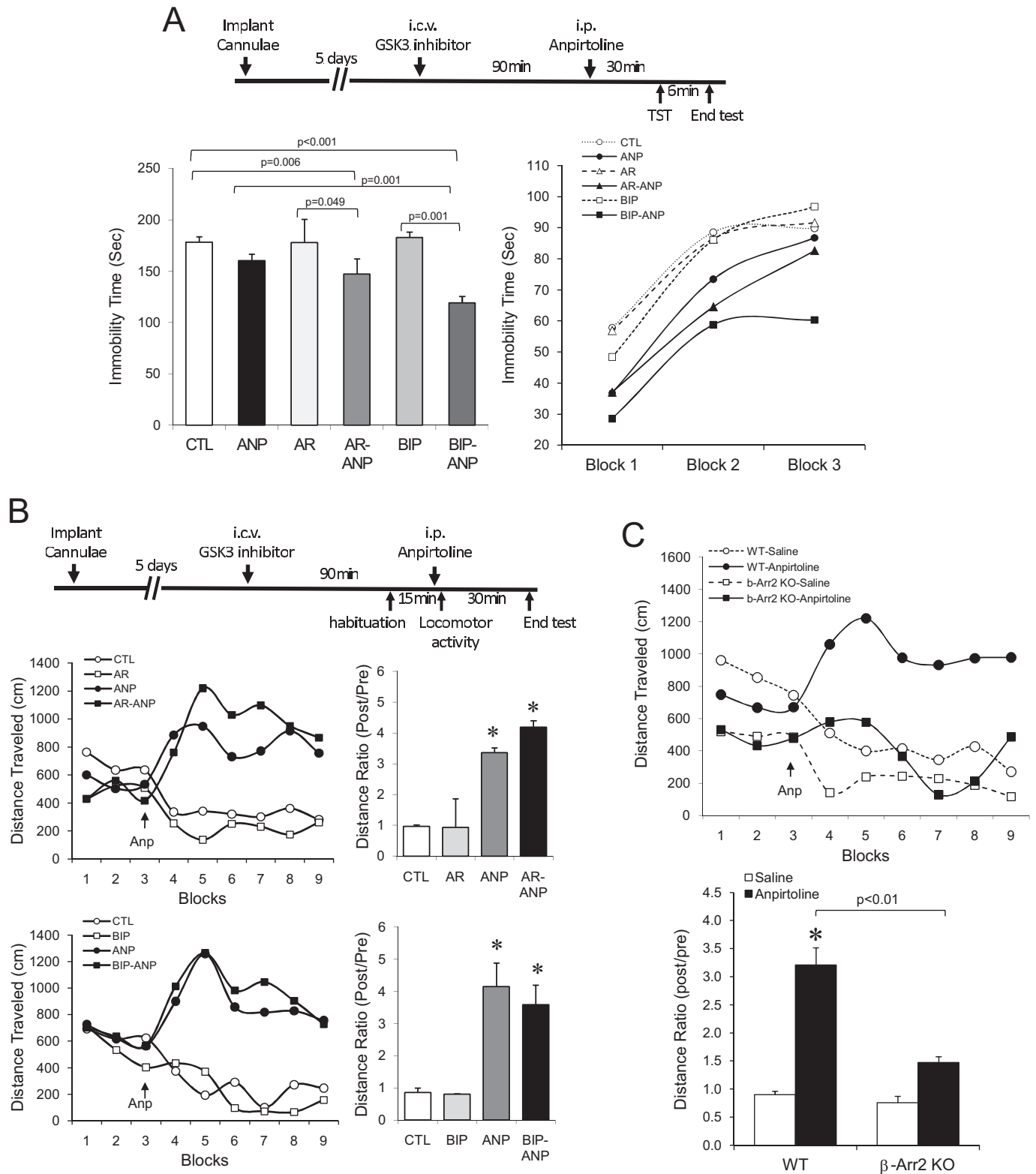


Fig. 8. Effects of GSK3 inhibitors on 5-HT_{1B}R-regulated behaviors. **A**, male C57BL/6 mice (8–12 weeks old) were pretreated with AR-A014418 (AR, 0.3 nmol), BIP-135 (BIP, 0.08 nmol), or vehicle (CTL) (intracerebroventricular administration, 90 min), followed by anpirtoline (ANP 4 mg/kg i.p.) or saline treatment for 30 min before they were subjected to the TST. Immobility time was recorded and expressed either as the last 4 min of the 6-min test (left) or by each 2-min block (right). Mean \pm S.E.M., $n = 6$ to 11 in each treatment group, p values indicate statistically significant difference in ANOVA. **B**, mice were treated with GSK3 inhibitors for 90 min and accommodated in the open field for 15 min before giving anpirtoline (4 mg/kg i.p.). Locomotor activity was represented by horizontal travel distance. Travel distance during each 5-min block was recorded (left), and post-treatment travel distance (30 min) is calculated as the ratio of pretreatment travel distance (15 min) (right). **C**, homozygous β -arrestin2 knockout mice with C57BL/6 background and strain-matched wild-type mice were accommodated in the open field for 15 min before giving anpirtoline (4 mg/kg i.p.). Horizontal travel distance was recorded as in **B**. Mean \pm S.E.M., $n = 6$ to 11 in each treatment group; *, $p < 0.05$ in ANOVA compared with control (saline treatment).

was examined as the measure of a G_i -independent signaling that allows to test the signal pathway-selective effect of GSK3 β . In strong contrast to 5-HT1BR agonist-induced activation of $G_{i\alpha_2}$ -cAMP signaling, removing or inhibiting GSK3 β did not affect β -arrestin2 recruitment to 5-HT1BR, which strongly suggest that removing GSK3 β does not make an inert 5-HT1BR; instead, GSK3 β probably has functional selective modulating effect on 5-HT1BR.

To select GSK3 inhibitors that are effective in 5-HT1BR-regulated $G_{i\alpha_2}$ -cAMP signaling, it is important to sequentially test the 5-HT1BR-GSK3 β interaction, 5-HT1BR- $G_{i\alpha_2}$ interaction, and cAMP production, because among all the GSK3-inhibiting agents tested in this study, only a few specifically abolish 5-HT1BR-mediated inhibition of $G_{i\alpha_2}$ -cAMP signaling. This could be due to the effect of some GSK3 inhibitors on other signaling mechanisms that regulate cAMP. The purpose of selecting GSK3 inhibitors with a specific effect on cellular action of 5-HT1BR is to determine whether these inhibitors may have potential for modulating 5-HT1BR functions in the brain. Although SB216763 (Smith et al., 2001), AR-A014418 (Bhat et al., 2003), and BIP-135 (Gaisina et al., 2009) have excellent GSK3 selectivity in cellular assays, SB216763 was not used for serotonin release and behavior tests because the effective concentration of this compound is higher, which limits its use in brain tissues.

We measured the effect of GSK3 inhibitors on serotonin release in mouse brain cerebral cortical slices because this is a representative function of 5-HT1B autoreceptors that distribute at the axon terminals of serotonin neurons reaching to the cerebral cortex and other brain regions (Riad et al., 2000). 5-HT1BR-regulated serotonin release is a pertussis toxin-dependent action mediated by the inwardly rectifying potassium channel (Innis et al., 1988; Ghavami et al., 1997; Trillat et al., 1997). Because activation of the pertussis toxin targets $G_i\alpha$, we hypothesized that the inhibitory effect of 5-HT1BR on serotonin release is a GSK3-dependent function. The finding that two highly effective GSK3 inhibitors blocked the inhibitory effect of anpirtoline on serotonin release suggests that GSK3 inhibitors are potential enhancers of serotonin release by minimizing the effect of 5-HT1B autoreceptors. This could potentially be an important modulatory mechanism to balance the counteractive effects of 5-HT1B autoreceptors and heteroreceptors.

Because behavior studies have been used to delineate the diverse functions of 5-HT1BR in brain (Clark and Neumaier, 2001; Miczek et al., 2004; Sari, 2004), we tested two well known 5-HT1BR-regulated behaviors, TST and locomotor activity, as another functional measure to configure the role of GSK3 in 5-HT1BR function and to provide evidence for the in vivo significance of this GSK3 action.

5-HT1BR can reduce immobility in TST and FST (O'Neill et al., 1996; Chenu et al., 2008). This behavioral effect per se is a function of 5-HT1B heteroreceptors located at nonserotonin neurons (Chenu et al., 2008), but the effect usually requires a high dose of 5-HT1BR agonist when administered via systemic route, because simultaneous activation of 5-HT1B autoreceptors could suppress serotonin release and dampen this heteroreceptor-mediated behavioral effect. In this study, the TST was applied because it is suitable for experiments involving intracerebroventricular drug administration with cannula placement. We used the dose of anpirtoline that by itself had no significant effect in the TST,

assuming that this treatment is at the interface in which the effect of 5-HT1B heteroreceptors is mostly masked by simultaneous autoreceptor activation. The results that anpirtoline, when combined with a GSK3 inhibitor, significantly reduced immobility highly suggests that GSK3 inhibitors selectively control 5-HT1B autoreceptor action but spare the actions of 5-HT1B heteroreceptors. It should be noted that several GSK3 inhibitors were reported to reduce immobility when tested in the TST or FST (Li and Jope, 2010), but the GSK3 target of the behavioral effect is unknown. Therefore, it is critical that we used intracerebroventricular administration of GSK3 inhibitors at low doses that alone had no effect on the TST because the aim of this study is to test the modulating effect of GSK3 inhibitors on 5-HT1BR function with minimal influence on the off-target or non-neuronal effects of GSK3.

The selectivity of GSK3 inhibitors on 5-HT1BR-regulated behaviors is further supported by their lack of action on anpirtoline-induced locomotor activity, which is primarily a function of 5-HT1B heteroreceptors at nonserotonin neurons (Pranzatelli et al., 1987; Cheetham and Heal, 1993) and by the diminished response to anpirtoline-induced locomotor activity in mice lacking β -arrestin2 that is independent of GSK3. However, the result of this study does not rule out the possibility that the dampened locomotor activity in β -arrestin2 knockout mice is a result of lacking dopamine D2 receptor activity, because D2 receptor-regulated locomotor activity is also a β -arrestin2-dependent action (Beaulieu et al., 2005).

Therefore, findings reported in this study provide new evidence that GSK3 is a functional selective modulator in 5-HT1BR-regulated $G_i\alpha$ signaling, serotonin release, and behavior. Future studies may further elucidate the function of GSK3 in serotonin neurotransmission and potential therapeutic applications of GSK3 inhibitors in neurological illnesses that are associated with altered 5-HT1BR activity.

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Authorship Contributions

Participated in research design: L. Chen, Zhou, P. Chen, and Li.
Conducted experiments: L. Chen, Zhou, Yang, and Li.
Contributed new reagents or analytic tools: P. Chen and Gaisina.
Performed data analysis: L. Chen, Zhou, and Li.
Wrote or contributed to the writing of the manuscript: L. Chen, Zhou, P. Chen, and Li.
Other: Li acquired funding for the research.

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