Glycogen Synthase Kinase-3beta is a Functional Modulator of Serotonin 1B Receptors

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Abbreviations
BRET, bioluminescence resonance energy transfer; DMSO, dimethyl sulfoxide; EtOH, ethanol; FST, forced swim test; Gi, inhibitory G protein; GPCR, G protein-coupled receptors; GSK3, Glycogen synthase kinase-3; 5-HT1BR, serotonin 1B receptors; 5-HT1AR, serotonin 1A receptors, 8-OH-DPAT, 8-hydroxy-N,N-dipropyl-2-aminotetralin; TST, tail suspension test.
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 previously found that GSK3β selectively interacts with 5-HT1B receptors (5-HT1BR) that have important functions in serotonin neurotransmission and behavior. In this study, we provide new information supporting the importance of GSK3β in 5-HT1BR-regulated signaling, physiological function, and behaviors. Using molecular, biochemical, pharmacological, and behavioral approaches, we tested 5-HT1BR interaction with Giα2 and β-arrestin2 and 5-HT1BR-regulated signalings in cells, serotonin release in mouse cerebral cortical slices, and behaviors in wild type and β-arrestin2 knockout mice. Molecular ablation of GSK3β and GSK3 inhibitors abolished serotonin-induced change of 5-HT1BR coupling to Giα2 and associated signaling, but had no effect on serotonin-induced recruitment of β-arrestin2 to 5-HT1BR. This effect is specific for 5HT1BR since GSK3 inhibitors did not change the interaction between serotonin 1A receptors (5-HT1AR) and Giα2. Two GSK3 inhibitors, AR-A014418 and 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 GSK3β 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). GSK3α and GSK3β are paralogous proteins that share 84% sequence homology (Woodgett, 1990), and possess common as well as distinguished protein substrates (Wang et al., 1994). To regulate a substrate by phosphorylation, GSK3 locates a serine or threonine residue that are four amino acids toward the N-terminal of a prime-phosphorylated residue (S/TXXXpS/T) (Doble and Woodgett, 2003), which led to 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 Jope, 2010) and neurodegenerative disorders (Muyllaert 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 previously found that GSK3β selectively interacts with serotonin 1B receptors (5-HT1BR) (Chen et al., 2009). 5-HT1BR belong to the Class A G protein-coupled receptors (GPCR) that activate the inhibitory G protein (G_i) to inhibit adenylyl cyclase and cAMP production (Raymond et al., 2001), as well as 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 GSK3β 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 Gia2 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 GSK3β selectively modulates Gia-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-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), 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, 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), N-(4-Methoxybenzyl)-N'- (5-nitro-1,3-thiazol-2-yl)urea (AR-A014418), (Sigma-Aldrich, St. Louis, MO); [3H]serotonin (PerkinElmer, Waltham, MA); benzofuranyl-3-yl-(indol-3-yl)maleimides BIP-135 and M1 to M13 (Kozikowski, A.P., University of Illinois at Chicago, IL). Chemicals and drugs are dissolved in distilled water, dimethyl sulfoxide (DMSO), 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 (Paul Albert, University of Ottawa); mouse GSK3β, R96A-GSK3β, K85,86A-GSK3β, and S9A-GSK3β (Richard S. Jope, University of Alabama at Birmingham); human Giα2 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 Giα2 and human β-arrestin2 cDNAs were subcloned into pEYFP-C1 with N-terminal YFP for Giα2 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 prior to cellular transfection.

**RNA interference (RNAi) of GSK3** Short hairpin RNA (shRNA) sequences for human GSK3β, GSK3α, and non-targeting shRNA (Sigma-Aldrich) are constructed into lentivirus particles. The shRNA-targeting sequences are shown below:

GSK3β: b1, CCCAAACTACACAGAATTTAA; b2, GACACTAAAGTGATTGGAAAT; b3, CCACTGATTATACCTCTAGTA.
GSK3α: a1, GACTAGAGGGCAGAGGTAAT; a2: GGAGTTCAAGTCCCTCAGATT; a3: CTACATCTGTTCGCCTACTAC.

non-targeting shRNA:
CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTTGGTTTTT.

Human embryonic kidney-293 (HEK293) cells were transduced with shRNAs for human GSK3β, GSK3α, or non-targeting shRNA (control). Cell lines expressing shRNA clones were selected by the antibiotic puromycin (1 μg/ml). All RNAi-induced GSK3 knockdowns were confirmed by immunoblotting using the anti-GSK3α/β antibody.

Cell Culture and Transfection
Human embryo kidney (HEK)-293 cells and Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (0.1 mg/ml). For transient transfection of DNA, cells were grown to 70% confluence and DNAs were transfected into cells using the FuGene 6 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 activators. All experiments using transfected DNA constructs were performed in HEK-293 cells except Akt phosphorylation and cAMP assays that were performed in CHO cells where the signaling cascades are well-reserved.

Bioluminescence resonance energy transfer (BRET) assay
HEK cells stably expressing 5-HT1BR-Rluc or 5-HT1AR-Rluc were transfected with YFP, YFP-GSK3β, YFP-Giα2, or β-arrestin2-YFP. Luciferase activity was measured using coelenterazine f or coelenterazine h (Invitrogen) as the substrate (5 μM). 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. For real-time BRET, cells were first detached with PBS-EDTA, washed and suspended with 0.1% glucose in PBS containing 0.5 mM MgCl₂. 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 sec 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 ± 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: \[
\frac{(\text{emission at } 528 \pm 10 \text{ nm}) - (\text{emission at } 485 \pm 10 \text{ nm} \times C_f)}{(\text{emission at } 485 \pm 10 \text{ nm})}
\] (Angers et al., 2000), where \(C_f\) 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** Cells co-expressing wild type or mutant GFP-5-HT1BR and HA-GSK3β, HA-Giα2, or HA-β-arrestin2 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 overnight with anti-HA-conjugated agarose (Bethyl, Montgomery, TX). 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 for 5 min 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).

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% NP40, 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. Denatured proteins were subjected to electrophoresis in 10% polyacrylamide gels and immunoblotted with antibodies specific for total GSK3α/β (Upstate Biotech, Lake Placid, NY), phospho-T308-Akt, phospho-S473-Akt, and total Akt (Cell Signaling Technologies, Danvers, MA).

Cyclic AMP (cAMP) assay The level of cAMP was measured in CHO cells using an enzyme immunoassay kit (Direct BioTrak, Amersham/GE) (Chen et al., 2009). 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 were lysed in a buffer containing 2.5% dodecyltrimethylammonium bromide. Duplicate lysates were transferred into individual wells of a 96-well plate pre-coated with donkey anti-rabbit IgG. After incubation with rabbit cAMP antiserum 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 (pmol/mg).

**Animals** 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. Male homozygous β-arrestin2 knockout mice in C57BL/6 background (RJ Lefkovitz, Duke University) (Bohn et al., 1999) were continuously bred from homozygous breeders and 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 μM l-tyrosine, 10 μM nomifensine malate (dopamine reuptake inhibitor), 50 μM l-tryptophan, and 10 μM imipramine (serotonin reuptake inhibitor)]. Transverse cerebral cortical slices (300-μm) 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 μCi/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 per minute by a BIO-RAD multichannel peristaltic pump (Econo Pump, USA). 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 μM), BIP-135 (1 μM), 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 µM) 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 µl of 1N HCl containing 0.1% Triton X-100. Superfusion fluid from each fraction and tissue homogenate (200 µl) was mixed with scintillation liquid (5 ml, Universol, MP) and radioactivity was counted by a scintillation spectrometry (Multi-Purpose Scintillation Counter, LS-6500, Beckman). To calculate [3H]-serotonin release, the radioactivity from fractions collected at 61-63 min were averaged as a baseline for the first KCl stimulation, and fractions at 81-83 min were averaged for the second KCl stimulation. The radioactivity of all fractions was calculated as: CPM x 100/baseline CPM. Data are processed by OringPro 8.1, 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 (i.c.v.) 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% DMSO) was infused into the right ventricle via an internal cannula 90 min before anpirtoline (4 mg/kg) or saline injection intraperitoneally (i.p.) at a volume of 5 µl/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, St. Albans, VT), and activity was monitored using the activity monitoring software (Med Associates, St. Albans VT) (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 travelled during the 15 min pre-treatment 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. 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 ± SEM and are considered significant when p<0.05.

Results

As a Gi-coupled GPCR, 5-HT1BR and Giα2 co-immunoprecipitated either in the absence or the presence of serotonin (10 μM), with serotonin treatment transiently increased the protein association (Fig. 1A). Since GSK3β mainly interacts with the S154/T158 consensus GSK3 phosphorylation site located in the second intracellular loop of mouse 5-HT1BR (Chen et al., 2009), we co-immunoprecipitated the S154A- and T158A-mutant 5-HT1BR with Giα2. Both mutant receptors associated with Giα2, but in contrast to wild type receptors, serotonin did not change the association between the mutant 5-HT1BR and Giα2 (Figure 1A), suggesting that only serotonin-induced, but not the resting state of 5-HT1BR-Giα2 association is affected by GSK3.
To further determine the effect of GSK3 on the dynamic interaction between 5-HT1BR and Giα2, 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-Giα2 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 μM) transiently stabilized the oscillation at the maximum level of interaction for about 100 sec before it returned to oscillation. In the absence of serotonin, both the S154A- and the T158A-mutant 5-HT1BR also interacted with Giα2 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 Giα2 after termination of serotonin treatment. In this experiment, cells were pre-incubated with serotonin for 15 min, then were washed to remove serotonin, followed by measuring 5-HT1BR-Giα2 interaction using the fixed-time BRET. An YFP fluorescent intensity-dependent interaction between 5-HT1BR and Giα2 (maximum at the fluorescent intensity of 12.8 x 10^4 with 1 μg of YFP-Giα2 DNA) was significantly reduced (Fig. 1D). This was not a resting state interaction, since it required pre-serotonin treatment, and was concentration-dependently blocked by the 5-HT1BR antagonist SB224289 and SB216641 applied prior to serotonin treatment (Fig. 1E). Different from the wild type 5-HT1BR, serotonin pre-treatment did not cause a reduction in interaction between the mutant S154A- or T158A-mutant 5-HT1BR and Giα2 (Fig. 1F). These results indicate that disruption of GSK3β binding to the intracellular loop-2 (i2-loop) of 5-HT1BR (Chen et al., 2009) drastically alters the activity-dependent conformational change between 5-HT1BR and Giα2.
To further test if the disrupted interaction between 5-HT1BR and Giα2 observed with mutant 5-HT1BR is truly due to lacking modulation by GSK3, GSK3β and GSK3α were eliminated from cells by GSK3β- and GSK3α-targeting shRNAs, respectively (Fig. 2A). Complete GSK3β knockdown abolished both serotonin-induced transient stabilization (Fig. 2B) and serotonin pre-treatment-induced reduction (Fig. 2C) of 5-HT1BR-Giα2 interaction, and a partial GSK3β knockdown sufficiently blocked the transient stabilization, and moderately diminished the serotonin pre-treatment-induced reduction of interaction. Cells with GSK3α knockdown or expression of a non-targeting shRNA, however, responded to serotonin similarly as cells expressing wild type 5-HT1BR without shRNA (Figs. 2B and 2C). Therefore, GSK3β, but not GSK3α, modulates 5-HT1BR-Giα2 interaction. The effect of GSK3β was also confirmed in cells expressing the inactive R96A-GSK3β (Frame and Cohen, 2001) or K85,86A-GSK3β (Eldar-Finkelman et al., 1996), in which serotonin pre-treatment did not reduce the BRET ratio between 5-HT1BR and Giα2, but cells expressing the constitutively active S9A-GSK3β (Sutherland et al., 1993) responded to serotonin similarly as cells with wild type GSK3β (Fig. 2D). These data together suggest that serotonin-induced conformational change between 5-HT1BR and Giα2 requires the presence of active GSK3β.

Activation of Gi-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 previously showed that inhibition of GSK3 abolishes 5-HT1BR-induced inhibition of cAMP production (Chen et al., 2009). Here we further tested if 5-HT1BR-induced phosphorylation of Akt also requires GSK3 interaction with 5-HT1BR. In wild type 5-HT1BR-expressing cells, serotonin (1 μM) and the 5-HT1BR agonist anpirtoline (1 μM) significantly increased the levels of phospho-T308-Akt and phospho-S473-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 μM) had minimal effect on Akt in these 5-HT1BR expressing cells. In contrast, serotonin and anpirtoline had minimal effect on phospho-T308-Akt and phospho-S473-Akt in S154A-mutant 5-HT1BR-expressing cells, suggesting that GSK3β interaction with 5-HT1BR is necessary for 5-HT1BR-induced phosphorylation of Akt.

While coupling and activation of Giα 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 pre-treatment significantly increased BRET ratio in an YFP fluorescent intensity-dependent manner, reaching maximum at 0.5 μg 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 Giα2, both S154A- and T158A-mutant 5-HT1BR responded to serotonin similarly as wild type 5-HT1BR, with a time-dependent increase in BRET ratio (Fig. 4C). Therefore, lacking GSK3β interaction with 5-HT1BR does not affect serotonin-induced recruitment of β-arrestin2 to 5-HT1BR.

Since 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. The ATP competitive GSK3 inhibitor SB216763 (10 μM) itself had no effect on the resting state interaction between 5-HT1BR and Giα2, but it completely abolished serotonin-induced transient stabilization of the interaction (Fig. 5A), and concentration-dependently abolished serotonin pre-treatment-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).
In 5-HT1AR-expressing cells, 5-HT1AR interacted with Giα2 in a similar manner as 5-HT1BR with an oscillation that was transiently stabilized by serotonin (Fig. 5D), and the interaction was reduced with serotonin pre-treatment (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-HT1BR 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) (Gaisina et al., 2009; Kozikowski et al., 2007), and compared their effects with a known GSK3 inhibitor AR-A014418 (Bhat et al., 2003). Similar to SB216763 ((Chen et al., 2009) and (Fig. 5)), AR-A014418 concentration-dependently abolished 5-HT1BR and GSK3β interaction (Fig. 6A), serotonin pre-treatment-induced interaction between 5-HT1BR and Giα2 (Fig. 6B), and 5-HT1BR-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 potent effect in disrupting the interaction between 5-HT1BR and GSK3β either without or with serotonin treatment, and other maleimides had moderate or biphasic effect (Supplemental Fig. 1A). All tested maleimides concentration-dependently reversed serotonin pre-treatment-induced reduction in interaction between 5-HT1BR and Giα2, but did not affect the interaction in the absence of serotonin (Fig. 6B and 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-HT1BR-GSK3β interaction (Supplemental
Fig. 1A). Therefore, some, but not all GSK3 inhibitors selectively modulate 5-HT1BR-regulated Giα-cAMP signaling.

A major physiological function of active 5-HT1B autoreceptors in brain is to negatively regulate serotonin release (Gothert et al., 1987), which has been reported as a Gi-dependent action (Ghavami et al., 1997). To test if GSK3 inhibitors may affect this physiological function of 5-HT1BR, we measured 5-HT1BR-mediated inhibition of [³H]-serotonin release in mouse cerebral cortical slices. Activation of 5-HT1BR by anpirtoline (5 µM) caused a 60% reduction in potassium chloride (50 mM)-evoked serotonin release (Fig. 7A, 7B). Pre-treatment of cortical slices with the GSK3 inhibitor AR-A014418 (5 µM) or the maleimide GSK3 inhibitor BIP-135 (1 µM) completely abolished the inhibitory effect of anpirtoline on serotonin release (Fig. 7B).

5-HT1BR 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) (Chenu et al., 2008; O'Neill et al., 1996). Since GSK3 inhibitors are able to maintain sufficient serotonin release by abolishing anpirtoline-induced activation of 5-HT1B autoreceptors, we tested if 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 when compared to control mice, and the combined effect appeared 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 when compare to 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 effect in anpirtoline-induced increase in locomotor activity (Fig. 8B), which is mainly a 5-HT1B heteroreceptor-mediated behavior (Cheetham and Heal, 1993; Pranzatelli et al., 1987). Since GSK3 inhibitors had no effect on 5-HT1BR-β-arrestin2 interaction (Fig. 5C), we tested if 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-HT1BR-mediated locomotor activity at least partly depends on the presence of β-arrestin2, a signaling mechanism that is independent of 5-HT1BR interaction with GSK3β.

Discussion

Following our previous finding that GSK3β selectively interacts with 5-HT1BR (Chen et al., 2009), this study further dissected the functional aspects of this interaction on 5-HT1BR-regulated signaling, physiology, and behavior. The study results suggest that GSK3β selectively modulates Giα2-associated 5-HT1BR signaling pathways, but not recruitment of β-arrestin2 to active 5-HT1BR; GSK3β only affects the activation state of 5-HT1BR, with little effect on the resting state; GSK3 inhibitors modulate 5-HT1B autoreceptor-regulated serotonin release; and GSK3 inhibitors differentially modulate 5-HT1BR-mediated behaviors.
Since Giα is a major mediator of 5-HT1BR-regulated signaling (Raymond et al., 2001), this study began by testing if GSK3 affects 5-HT1BR-Giα2 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-HT1BR with Giα2, 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 the receptor. The interaction pattern in the absence or the presence of serotonin also applies between 5-HT1AR and Giα2, suggesting that this is a common dynamic interaction pattern between type I serotonin receptors and Giα2. 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 Giα2 DNA constructs. The oscillatory interaction between wild type 5HT1BR and Giα2 can be invariable stabilized by serotonin treatment, whereas there is no oscillation between 5HT1BR and YFP, or between 5HT1BR and β-arrestin2. Nevertheless, since the serotonin-induced change of oscillatory interaction is small and transient, we also used the fixed-time BRET to measure the 5-HT1BR-Giα2 interaction after serotonin treatment. This assay detected a nearly 60% reduction of BRET ratio only after serotonin was washed out. Since co-immunoprecipitation assay that measures a steady state protein association did not detect a dissociation of 5-HT1BR and Giα2, the serotonin-induced change of interaction seen in BRET assay is likely a dynamic protein conformational change between 5-HT1BR and Giα2 that represents Giα2 activation. However, to fully interpret the relationship of changes observed by real-time and fixed-time BRET assays would require other protein structural studies, which will not be further discussed here.
As all GSK3 manipulations applied in the study resulted in change of agonist-induced 5-HT1BR-Giα2 interaction, it is confirming that GSK3β is a modulator of 5-HT1BR-regulated Gi signaling, and it provides extended explanation of our previous finding that 5-HT1BR-induced inhibition of cAMP production depends on the presence of active GSK3β (Chen et al., 2009). The selectivity of GSK3β on 5-HT1BR-Giα2 interaction is likely by targeting 5-HT1BR, not Giα2 itself, because the similar 5-HT1AR-Giα2 interaction was not altered by GSK3 inhibitors.

Although we conclude that GSK3β is required for activation of Giα2-coupled signaling by 5-HT1BR, it should be acknowledged that the significant changes of 5-HT1BR response to agonists were mostly observed when GSK3β was removed from 5-HT1BR by receptor mutation, GSK3β knockdown, inactive GSK3β, and GSK3 inhibitors, whereas overexpression of constitutively active S9A-mutant GSK3β had no additional effect on 5-HT1BR activity. This observation may suggest that the intrinsic active GSK3β is an integrative component of the 5-HT1BR, which associates with the resting 5-HT1BR and is required for fully activation of 5-HT1BR by serotonin. On the other hand, inhibition of GSK3β may have significant implications in modulating 5-HT1BR-regulated signalings.

Giα-mediated cAMP production is not the only GSK3-dependent signaling pathway of 5-HT1BR since 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 thought that both α- and βγ-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 signalings. As 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 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 since 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 was examined as the readout of a Gi-independent signaling that allows to test the signal pathway-selective effect of GSK3β. In strong contrast to 5-HT1BR agonist-induced activation of Gia2-cAMP signaling, removing or inhibiting GSK3β did not affect β-arrestin2 recruitment to 5-HT1BR, which strongly suggest that removing GSK3β does not make an inert 5-HT1BR, instead, GSK3β likely has functional selective modulating effect on 5-HT1BR.

To select GSK3 inhibitors that are effective in 5-HT1BR-regulated Gia2-cAMP signaling, it is important to sequentially test the 5-HT1BR-GSK3β interaction, 5-HT1BR-Gia2 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 Gia2-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 specific effect on cellular action of 5-HT1BR is to determine if these inhibitors may have potential for modulating 5-HT1BR functions in 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 (Ghavami et al., 1997; Innis et al., 1988; Trillat et al., 1997). Since activation of the pertussis toxin targets Giα, 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.

Since 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 readout 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 (Chenu et al., 2008; O'Neill et al., 1996). This behavioral effect per se is a function of 5-HT1B heteroreceptors located at non-serotonin neurons (Chenu et al., 2008), but the effect usually requires 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 i.c.v. 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 where 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 i.c.v 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 non-serotonin neurons (Cheetham and Heal, 1993; Pranzatelli et al., 1987), and by the diminished response to anpirtoline-induced locomotor activity in mice lacking β-arrestin2 that is independent of GSK3. However, 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, since D2 receptor-regulated locomotor activity is also a β-arrestin2-dependent action (Beaulieu et al., 2005).

Therefore, findings reported in this study provided new evidence that GSK3 is a functional selective modulator in 5-HT1BR-regulated Giα 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. C. Chen, and Li

Conducted experiments: L. Chen, Zhou, Yang, and Li

Contributed new reagents or analytic tools: P.C. Chen and Gaisina

Performed data analysis: L. Chen, Zhou, and Li

Wrote or contributed to the writing of the manuscript: L. Chen, Zhou, P. C. Chen, and Li

Others: Li acquired funding for the research
References


Gaisina IN, Gallier F, Ougolkov AV, Kim KH, Kurome T, Guo S, Holzle D, Luchini DN, Blond SY, Billadeau DD and Kozikowski AP (2009) From a natural product lead to the


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Conflict of Interest: The authors declare that they have no conflict of interest.
Figure Legends

Figure 1. Association and dynamic interaction between 5-HT1BR and Giα2. (A) HEK-293 cells were transfected with GFP-tagged 5-HT1BR wild type (1BR-WT), S154A-mutant (1BR-S154A), or T158A-mutant (1BR-T158A), and co-transfected with HA-Giα2. Some of these cells were treated with serotonin (5-HT, 10 μM) for indicated time. Whole cell lysates were immunoprecipitated with anti-HA-conjugated agarose beads. Both immunocomplex (IP: HA) and cell lysate (Lysate) were immunoblotted for GFP and HA. Optimal density of immunoblotted GFP-5-HT1BR-WT was calculated as % of baseline (0 min serotonin treatment). Mean ± SEM, n=3-5 at each time interval, *p<0.05 in ANOVA when treatment was compared to baseline (no serotonin). Cells co-transfected with a GFP vector (no 5-HT1BR) and HA-Giα2 were also immunoprecipitated with anti-HA as negative control. (B) Wild type 5-HT1BR-Rluc-expressing HEK-293 cells were transfected with YFP or YFP-Giα2 (1 μg). Real-time BRET measurement was initiated by addition of coelenterazine h (5 μM) and continued for 10 min. Serotonin (5-HT, 10 μM) was added to the cells 21 seconds after the initiation of BRET measurement. Data is expressed as BRET ratio as described in the Methods. (C) Cells stably expressing the S154A-mutant (1BR-S154A) or T158A-mutant (1BR-T158A) 5-HT1BR-Rluc were transfected with YFP-Giα2 (1 μg), and real-time BRET was measured in the absence or the presence of serotonin (10 μM). (D) In a different experiment, cells expressing wild type 5-HT1BR-Rluc were transfected with different amount of YFP or YFP-Giα2 DNA (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 μg) and treated with serotonin (10 μM) for 15 min. The BRET signal was recorded immediately after serotonin was washed out, followed by addition of the luciferase substrate coelenterazine f (5 μM). BRET ratio is plotted against YFP fluorescent intensity recorded at
528±10 nm. Mean ± SEM, n=3, *p<0.001 in ANOVA when serotonin treatment in YFP-Giα2-expressing cells was compared to no treatment. (E) Cells in (D) were transfected with 1 μg YFP-Giα2 DNA, pre-treated with the 5-HT1BR antagonist SB224289 or SB216641 at the indicated concentrations for 2 hr before addition of serotonin (10 μM) for 15 min. BRET signal was measured immediately after serotonin was washed out. Mean ± SEM, n=3, *p<0.05 in ANOVA when antagonist treatment at each concentration in serotonin-treated cells was compared with no antagonist treatment (0 μM). (F) Cells expressing wild type, S154A-mutant, or T158A-mutant 5-HT1BR were transfected with Giα2, pre-treated with serotonin (10 μM) for 15 min, and BRET signal was measured immediately after serotonin was washed out. Mean ± SEM, n=3-6, *p<0.05 in t-test when serotonin-treatment in each mutant 5-HT1BR-expressing cells were compared to no serotonin treatment (no 5-HT).

Figure 2. Effects of GSK3β-shRNA and inactive GSK3β on 5-HT1BR-Giα2 interaction. (A) HEK-293 cells stably expressing 5-HT1BR-Rluc were infected with lentivirus-carried shRNA for GSK3β (b1, b2, b3), GSK3α (a1, a2, a3), or a non-targeting shRNA (CTL). Cell lysates from these shRNA-expressing cells and non-infected cells (-) were immunoblotted for total GSK3α and GSK3β. (B) Cells in (A) with an indicated shRNA were transfected with YFP-Giα2 (1 μg), followed by measuring real-time BRET in the absence or the presence of serotonin (5-HT, 10 μM). (C) BRET was measured in these cells after serotonin pre-treatment (10 μM) for 15 min. Mean ± SEM, 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 co-transfected with YFP-Giα2 and a wild type or a mutant GSK3β (R96A, K85,86A, or S9A), and pre-treated with serotonin (10 μM) for 15 min before BRET
measurement. Mean ± SEM, n=3, *p<0.05 in t-test when serotonin-treatment in each type of GSK3β-expressing cells were compared with no serotonin treatment (no 5-HT).

Figure 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 µM) for 30 min before serotonin (5-HT), anpirtoline, or 8-OH-DPAT (1 µM) treatment for 5 min. Cell lysates were immunoblotted for phospho-T308-Akt, phospho-S473-Akt, and total Akt. Data is expressed as average optical density of immunoblots. Mean ± SEM, n=3-5, *p<0.05 in ANOVA when compared with basal value (no drug treatment) in each experiment.

Figure 4. Interaction between 5-HT1BR with β-arrestin2. (A) HEK-293 cells stably expressing wild type 5-HT1BR-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, 2 µg), and pre-treated with serotonin (5-HT, 10 µM) for 15 min before BRET measurement. Mean ± SEM, n=3, *p<0.001 between the two groups in ANOVA. (B) Real-time BRET measurement was performed in cells expressing wild type 5-HT1BR-Rluc and β-arrestin2-YFP (0.5 µg) in the absence or the presence of serotonin (10 µM). (C) HEK-293 cells stably expressing S154A- or T158A-mutant 5-HT1BR-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.

Figure 5. The effect of GSK3 inhibitor on 5-HT1BR and 5-HT1AR interaction with Gια2 and β-arrestin2. (A) HEK293 cells expressing 5-HT1BR-Rluc and YFP-Gια2 were pre-treated with the
GSK3 inhibitor SB216763 (10 µM) for 2 hr before used for real-time BRET measurement in the absence or the presence of serotonin (5-HT, 10 µM). (B) These cells were pre-treated with indicated concentrations of SB216763 for 2 hr before addition of serotonin (10 µM) for 15 min, followed by BRET measurement. Mean ± SEM, 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) HEK-293 cells expressing 5-HT1BR-Rluc were transfected with β-arrestin2-YFP (0.5 µg), pre-treated with SB216763 (10 µM) for 2 hr, followed by real-time BRET measurement in the absence or the presence of serotonin (10 µM). (D) HEK-293 cells stably expressing 5-HT1AR-Rluc were transfected with YFP-Giα2 (1 µg) and pre-treated with the GSK3 inhibitor SB216763 (10 µM) for 2 hr, 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-Giα2 (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 µg), pre-treated with serotonin (5-HT, 10 µM) for 15 min, followed by BRET measurement. Mean ± SEM, n=3, *p<0.001 between the two groups in ANOVA (left panel). For GSK3 inhibitor treatment, cells were transfected with 1 µg YFP-Giα2, and pre-treated with the GSK3 inhibitor SB216763 at indicated concentrations for 2 hr before serotonin (10 µM) for 15 min, followed by BRET measurement, Mean ± SEM, n=3 (right panel).

Figure 6. Selection of GSK3 inhibitors for 5-HT1BR interaction with GSK3β and Giα2, and regulation of cAMP. HEK293 cells expressing 5-HT1BR-Rluc and YFP-GSK3β (A) or YFP-Giα2 (B) were pre-treated with the GSK3 inhibitor AR-A014418 or a maleimide BIP-135 at indicated concentrations for 2 hr. Some cells were pre-treated with serotonin (5-HT, 10 µM) 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-HT1BR were pre-treated with the indicated concentrations of AR-A014418 or a maleimide for 2 hr, followed by serotonin (10 μM) for 30 min prior to stimulating cAMP production with forskolin (fsk; 10 μM, 15 min). The levels of cAMP (pmole) are normalized by the protein concentration (mg). n=3-4, *p<0.05 in ANOVA when inhibitor treatment at each concentration was compared with no inhibitor treatment.

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

Figure 8. Effects of GSK3 inhibitors on 5-HT1BR-regulated behaviors. (A) Male C57BL/6 mice (8-12 weeks old) were pre-treated with AR-A014418 (AR, 0.3 nmol), BIP-135 (BIP, 0.08 nmol), or vehicle (CTL) (i.c.v., 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 panel) or by each 2 min blocks (right panel). Mean ± SEM, n=6-11 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 prior to 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 panel), and post-treatment travel distance (30 min) is calculated as the ratio of pre-treatment travel distance (15 min) (right panel). (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 prior to giving anpirtoline (4 mg/kg, i.p.). Horizontal travel distance was recorded as in (B). Mean ± SEM, n=6-11 in each treatment group, *p<0.05 in ANOVA when compared with control (saline treatment).
Figure 1
Figure 2

A) Western blot showing GSK3α and GSK3β. Lower panel is antibody controls (CTL - a1 a2 a3 (shRNA)).

B) BRET ratio time course with GSK3β-shRNA-b1 and -shRNA-b2. Open circles are no 5-HT and filled circles are 5-HT.

C) Bar graph showing BRET ratio for GSK3β-shRNA with no 5-HT and 5-HT. Asterisks indicate statistical significance.

D) Bar graph showing BRET ratio for GSK3α-shRNA with no 5-HT and 5-HT. Asterisks indicate statistical significance.

Figure 2
Figure 3
Figure 4
Figure 6
**Figure 7**

**A**

Graphs showing the release of $^3$H-serotonin in response to potassium ($K^+$) stimulation. Two peaks are observed, designated as P1 and P2. The X-axis represents the fraction, and the Y-axis shows the percentage of $^3$H-serotonin baseline.

**B**

Bar graph comparing the ratio of $^3$H-serotonin release (S2/S1) under different conditions:
- Vehicle
- Anpirtoline
- AR-A014418
- BIP-135

Significance marked with asterisks (*) indicates statistical differences.
Figure 8
Glycogen Synthase Kinase-3beta is a Physiological and Behavioral Modulator of Serotonin 1B Receptors

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In Molecular Pharmacology

Supplemental Materials

Supplemental Table 1
Supplemental Figure 1
### Supplemental Table 1. GSK-3β Inhibition by Benzofuranyl-3-yl-(indol-3-yl)maleimides

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<th>Maleimide&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5,6-difluoro</td>
<td>H</td>
<td>CH₃</td>
<td>438</td>
</tr>
</tbody>
</table>

<sup>a</sup> Structure of Benzofuranyl-3-yl-(indol-3-yl)maleimides

<sup>b</sup> Published in (Gaisina et al., 2009; Kozikowski et al., 2007).

<sup>c</sup> Synthesized according to (Gaisina et al., 2009).

**References**


Supplemental Figure 1
Supplemental Figure 1. Selection of GSK3 inhibitors for 5-HT1BR interaction with GSK3β and Giα2, and regulation of cAMP. HEK293 cells expressing 5-HT1BR-Rluc and YFP-GSK3β (A) or YFP-Giα2 (B) were pre-treated with the GSK3 inhibitor AR-A014418 or a maleimide (BIP-135, M1-M13) at indicated concentrations for 2 hr. Some cells were pre-treated with serotonin (5-HT, 10 µM) 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.