Disruption of 5-hydroxytryptamine 1A receptor and orexin receptor 1 heterodimer formation affects novel G protein-dependent signaling pathways and has antidepressant effects in vivo

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G protein-coupled receptor (GPCR) heterodimers are new targets for the treatment of depression. Increasing evidence supports the importance of serotonergic and orexin-producing neurons in numerous physiological processes, possibly via a crucial interaction between 5-hydroxytryptamine 1A receptor (5-HT1AR) and orexin receptor 1 (OX1R). However, little is known about the function of 5-HT1AR/OX1R heterodimers. It is unclear how the transmembrane domains (TMs) of the dimer affect its function and whether its modulation mediates antidepressant-like effects. Here, we examined the mechanism of 5-HT1AR/OX1R dimerization and downstream G protein-dependent signaling. We found that 5-HT1AR and OX1R form constitutive heterodimers that induce novel G protein-dependent signaling, and that this heterodimerization does not affect recruitment of β-arrestins to the complex. In addition, we found that the structural interface of the active 5-HT1AR/OX1R dimer transforms from TM4/TM5 in the basal state to TM6 in the active conformation. We also used mutation analyses to identify key residues at the interface (5-HT1AR R1514.40, 5-HT1AR Y1985.41, and OX1R L2305.54). Injection of chronic unpredictable mild stress (CUMS) rats with TM4/TM5 peptides improved their depression-like emotional status and decreased the number of endogenous 5-HT1AR/OX1R heterodimers in the rat brain. These antidepressant effects may be mediated by upregulation of BDNF levels and enhanced phosphorylation and activation of CREB in the hippocampus and medial prefrontal cortex. This study provides evidence that 5-HT1AR/OX1R heterodimers are involved in the pathological process of depression. Peptides including TMs of the 5-HT1AR/OX1R heterodimer interface are candidates for the development of compounds with fast-acting antidepressant-like effects.

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INTRODUCTION

G protein-coupled receptor (GPCR) dimerization is crucial for various receptor functions, including agonist affinity, efficacy, trafficking, and specificity of signal transduction. GPCRs can form heterodimers and homodimers; the former is exemplified by orexin receptor 1 (OX1R) and kappa opioid receptor heterodimers, as well as by apelin receptor (APJ) and bradykinin 1 receptor heterodimers [1, 2]. In addition, APJ can also form homodimers [3]. GPCR dimers have specific functional characteristics that differ from those of the monomers. Indeed, the dimerization of GPCRs is an important way to regulate receptor function. For example, hetero-oligomerization of dopamine D2 receptors and somatostatin receptor type 5 increases their functional responses to the agonists dopamine and somatostatin [4]. In addition, heterodimerization of OX1R and cholecystokinin A receptor inhibits the migration of colon cancer cells in humans [5]. Many GPCR dimers are associated with pathological conditions such as schizophrenia, Parkinson’s disease, and drug addiction [6]. Consequently, GPCR homo and heterodimers are often preferentially targeted over the monomers when designing novel drugs [7–10].

5-Hydroxytryptamine (5-HT, serotonin) is an important signaling molecule that regulates and modulates several physiological and behavioral processes in the human body. In the central nervous system (CNS), 5-HT modulates processes associated with mood, perception, reward, anger, aggression, appetite, memory, sexual behavior, and attention [11]. Furthermore, 5-HT is involved in the pathogenesis of various psychiatric diseases, including depression,
schizophrenia, and anxiety. There are 14 subtypes of the 5-HT receptor in humans, of which 13 are GPCRs, and five different genes encode the 5-HT1A, 1B, 1D, 1E, and 1F receptor subtypes. In general, the 5-HT1A receptor (5-HT1AR) couples with Gα/0 proteins and inhibits adenylate cyclase activity [12]. This receptor subtype has been well-studied, and its dysregulation is involved in disease states such as depression and anxiety [13].

In the CNS, orexins and orexin receptors regulate various behavioral and physiological responses, including arousal and narcolepsy, control of energy metabolism, food intake, drug reward, anxiety, and depression-related responses [14, 15]. Orexin-A and -B orchestrate their diverse effects by binding to and activating two GPCRs, orexin receptor 1 (OX1R) and OX2R, both of which are expressed widely in the CNS and can couple with Gα, Gβ, and Gδ [16, 17]. Although orexin-A binds preferentially to OX1R, it can also bind to OX2R. Human OX1R forms both homodimers and heterodimers, the latter with, for example, growth hormone secretagogue receptor 1α or cannabinoid receptor type 1 [18, 19].

There are some overlaps between the functions and distributions of 5-HT1AR and OX1R in the CNS. For example, 5-HT inhibits orexin neurons directly by binding to 5-HT1AR and indirectly by facilitating GABAergic inhibitory inputs without affecting glutamatergic inputs [20]. In addition, expression of both 5-HT1AR and OX1R is associated with depression and anxiety. Recently, homodimerization has been reported for 5-HT1AR [21]. In addition, 5-HT1AR can interact with other GPCRs to form heterodimers, which may selectively modulate functions, remain controversial.

Here, we investigated whether 5-HT1AR and OX1R can heterodimerize in vitro and in vivo. Our findings suggest novel signal transduction upon heterodimerization of these receptors. Mass spectrometry and peptides, in which specific transmembrane domains (TMs) of 5-HT1AR and OX1R were fused with the human immunodeficiency virus trans-acting transcriptional activator (HIV TAT), were used to examine the formation of 5-HT1AR/OX1R heterodimers. We also examined the 5-HT1AR/OX1R dimer interface and its dynamics during receptor activation. Further mutational analyses confirmed the key residues important for the interaction interface. In vivo experiments, we found that increased expression of the 5-HT1AR/OX1R heterodimer in the hippocampus and medial prefrontal cortex (mPFC) was related to depression and anxiety-like behaviors in rats. We then examined the effects of injecting specific 5-HT1AR and OX1R-TM peptides into the lateral ventricle of rats exposed to chronic unpredictable mild stress (CUMS). Our findings suggest that TM peptides provide an experimental basis for further research into the etiology of depression and represent new candidate small molecules that could be used to interfere with 5-HT1AR/OX1R dimerization for the treatment of depression.

**MATERIALS AND METHODS**

Full details of the materials and methods are provided in the Supplementary Information.

**Animals**

CUMS rat model procedures were performed as described previously [26]. To validate the efficacy of the CUMS protocol, weight gain was determined, and forced swim test (FST) and sucrose preference test (SPT) assessments were performed as described in previous reports [27, 28]. Rats in the CUMS plus TM peptide treatment group were injected with the corresponding TM peptide for 6 days (100 μg/8 μl). Rats in the CUMS group were injected with 8 μl normal saline on the same days, and the control group was left untreated. The study was approved by the local ethics board of Jining Medical University and met the standards of the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People’s Republic of China in 2006.

**Co-immunoprecipitation**

HEK293 cells were co-transfected with Myc-5-HT1AR and 3HA-OX1R or a vector control and lysed 48 h later. After centrifuging for 15 min at 4°C and 16,000 x g, the whole cell lysates were incubated with an anti-HA antibody and Protein G-sepharose beads for 4 h with gentle rotation at 4°C. The beads were then washed four times with cell lysis buffer. Precipitates were eluted with 4x SDS-PAGE sample buffer and analyzed by western blotting for anti-Myc immunoreactivity.

**Double-immunofluorescence staining**

For 5-HT1AR and OX1R localization experiments, rat brains were fixed in 4% formaldehyde overnight, and then embedded in paraffin blocks and sectioned using a microtome. The hippocampal sections were incubated with goat anti-5-HT1AR (1:100; Abcam, Cambridge, UK) and rabbit anti-orexin receptor 1-ATTO-488 (1:60; Alomone Labs, Jerusalem, Israel) antibodies overnight at 4°C. After washing with PBS, each section was incubated with a Cy3-conjugated goat anti-rabbit IgG antibody (1:100; Boster Biological Technology, Pleasanton, US) at 25°C for 2 h. After a further wash with PBS, each section was incubated with DAPI (1:100,000; Invitrogen, California, US) at 25°C for 10 min. The stained sections were examined under a Leica DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK).

**In situ proximity ligation assay (PLA)**

Interactions of 5-HT1AR and OX1R were detected in the native tissue using the Duolink II in situ PLA detection kit (Sigma-Aldrich, St. Louis, MO, USA), following the supplier’s instructions. Rat brain (hippocampus and mPFC) sections of 5 μm thickness were used in the PLA experiments. A mixture of the primary antibodies (goat polyclonal anti-5-HT1AR (1:100; Sigma-Aldrich Gillingham, UK) and rabbit polyclonal anti-OX1R (1:100; Abcam)) was used to detect 5-HT1AR/OX1R heterodimers, and PLA probes were used to detect the goat and rabbit antibodies. Punctate fluorescent signals were indicative of proximity (~10 nm) of the 5-HT1AR and OX1R promoters. The formation of heterodimers was then confirmed by laser scanning confocal microscopy with an apochromatic 63× oil-immersion objective, and 405 and 561 nm laser lines. Similar methods were used to identify 5-HT1AR/OX1R heterodimers in HEK293 cells transfected with vectors expressing each promoter. HEK293 cells expressing OX1R alone were used as negative controls.

To examine the effects of TM peptides on 5-HT1AR/OX1R dimerization, HEK293 cells were transfected with the pcDNA3.1-5-HT1AR and pcDNA3.1-OX1R plasmids in 12-well plates. After 24 h, the cells were distributed into 6-well plates and treated for 6 h with HIV TAT-fused TM peptides (10 μM) corresponding to TM4 or TM5 of 5-HT1AR, or TM1 or TM5 of OX1R, and then assayed for dimerization as described above.

**Bioluminescence resonance energy transfer (BRET) saturation assay**

To monitor constitutive 5-HT1AR-OX1R interactions, HEK293 cells were transfected with 5-HT1AR-RLuc and OX1R-EGFP or OX1R-RLuc and 5-HT1AR-EGFP plasmids at ratios of 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6. Coelenterazine h was then added, and BRET measurements were taken using a Tristar LB941 plate reader with RLuc (400–475 nm) and EGFP (500–550 nm) filters.

**Fluorescence resonance energy transfer (FRET)**

To obtain calibration coefficients and eliminate excitation and emission crosstalk, 5-HT1AR-ECFP and OX1R-EYFP were transfected into HEK293 cells as donor and acceptor channels, respectively. After 24 h, FRET signals were detected with a FRET kit using a Leica AM TIRF MC System. FRET efficiency (EA) was calculated as shown in the equation below: A, B, and C correspond to the intensities of the three signals (donor, FRET, and acceptor, respectively), and α, β, γ, and δ are the calibration factors generated by the acceptor-only and donor-only references:

\[
EA(i) = \frac{B - A \times \beta - C \times (y - \alpha \times \beta)}{C \times (1 - \beta \times \delta)}
\]
Design and synthesis of TM peptides
Wild-type and mutant human 5-HT1AR and OX1R-TM peptides were custom-synthesized; their primary sequences are shown in Table 1. Because HIV TAT is an excellent transporter for delivery purposes, peptides were fused to TAT to mediate the translocation of peptides into target cells. HIV TAT was fused at the N-terminus of even-numbered TMs and at the C-terminus of odd-numbered TMs to ensure proper orientation of the inserted peptide.

Mass spectrometry
Mass spectrometry was performed to identify 5-HT1AR/OX1R dimer interfaces in samples treated with TM peptides. HEK293 cells were transfected with 5-HT1AR or OX1R, and 48 h later, were treated with or without the indicated HIV TAT-TM fused peptides (4 μM) for 60 min at 37°C. The 5-HT1AR or OX1R complex was analyzed using a matrix-assisted laser desorption/ionization-time of flight spectrometer [29].

Statistical analysis
All data are shown as means ± SEM. Data are presented and analyzed using GraphPad Prism 5.0 software. Sigmoidal curves were fitted to the dose–response data using nonlinear regression. Statistical analysis was performed using one-way analysis of variance followed by Tukey's multiple comparison post-test. Differences between the means were considered statistically significant at P < 0.05.

RESULTS
5-HT1AR and OX1R form heterodimers in vitro and in vivo
Cell surface localization of 5-HT1AR and OX1R was unaffected by their co-expression in HEK293 cells. Immunofluorescence and total internal reflection fluorescence microscopy analyses of HEK293 cells co-expressing Myc-tagged 5-HT1AR and EGFP-tagged OX1R confirmed that both proteins were co-localized on the cell membrane (Fig. 1A, B). This figure showed that labeling is observed in neurons in the rat hippocampus. In vivo, HEK293 cells co-expressing α1b-adrenoceptor-EGFP and OX1R-Rluc in cells resulted in a low BRET ratio (Fig. 2D), which is consistent with a previous report that BRET signals are low in cells co-expressing 5-HT1AR-Rluc and α1b-adrenoceptor-EYFP [31]. In addition, a BRET saturation assay of HEK293 cells co-transfected with 5-HT1AR-EGFP and OX1R-Rluc produced a saturation curve, confirming the interaction of 5-HT1AR and OX1R in vitro (Fig. 2E). BRET saturation assays also confirmed that constructs with labels in the “opposite” configuration (i.e., 5-HT1AR-Rluc and OX1R-EGFP) were able to dimerize when expressed in HEK293 cells (Fig. 2F).

Next, we transfected HEK293 cells with 5-HT1AR-ECFP alone, OX1R-EYFP alone, or 5-HT1AR-ECFP and OX1R-EYFP (Fig. 2G, interaction sites are shown in yellow). Low fluorescence resonance energy transfer (FRET) signals were observed in cells transfected with either construct alone. However, an enhanced FRET signal

Table 1. Amino acid sequences of synthetic peptides derived from the transmembrane domains of OX1R and 5-HT1AR.

| OX1R-TM          | Molecular weight (Da) |
|------------------|-----------------------|
| TM1              | WWLIAAYAVFVIALGVTNLYGRKKRRQRRR 3787.61 |
| TM4              | YGRKKRRQRRRARGSGLGWLASMAMYPQAAV 3765.54 |
| TM5              | IYHSCFITLYALPGMLAMAYYGRKKRRQRRR 4066.88 |
| TM6              | YGRKKRRQRRRMLMVLLFACLYPSLVNLVK 4134.23 |
| TM6-L309A        | YGRKKRRQRRRMLMVLLPAACLYPSLVNLVK 4092.15 |
| 5-HT1AR-TM       | Molecular weight (Da) |
| TM3              | LFIALDVCLCTTTLHCALAIYGRKKRRQRRR 3874.75 |
| TM4              | YGRKKRRQRRRRAALSLTLWGLFISIPMLGWTP 4692.70 |
| TM5              | DHGYTIYSTFGAYFILLMLMLVYGRKKRRQRRR 4566.41 |
| TM6              | YGRKKRRQRRRVTLLGIMGTFLCWLFFIVAL 4138.16 |
| TM4-R151A        | YGRKKRRQRRRRAAAALSLTLWGLFISIPMLGWTP 4670.40 |
| TM6-M351A        | YGRKKRRQRRRVTLLGIAATFLCWLFFIVAL 4078.04 |
| TM6-L356A        | YGRKKRRQRRRVTLLGIMGTFCWNLFFIVAL 4096.08 |

Italic letter indicates replacement of the original amino acid by alanine.
was seen in cells expressing 5-HT1AR-ECFP and OX1R-EYFP, providing further evidence that 5-HT1AR and OX1R can form dimers (Fig. 2G). Normalized FRET values were calculated as described in the Materials and methods section, and the FRET efficiency of the 5-HT1AR/OX1R heterodimer is shown in Fig. 2H. Heterodimerization of 5-HT1AR and OX1R affects downstream signaling pathways

Heterodimerization of 5-HT1AR and OX1R increases intracellular cAMP levels. The effects of 5-HT1AR/OX1R dimerization on intracellular cAMP concentrations were measured using an ELISA assay. Specifically, cAMP accumulation was compared in HEK293 cells expressing 5-HT1AR alone, OX1R alone, and 5-HT1AR/OX1R. The cells were stimulated with forskolin (10 μM) in the absence or presence of various concentrations of 8-OH-DPAT (0.001–1000 nM) and/or orexin-A (0.001–1000 nM). The cAMP level in cells expressing 5-HT1AR/OX1R and treated with both ligands was markedly higher than that in the same cells treated with either ligand individually (Fig. 3A). A cAMP BRET biosensor analysis confirmed that co-treatment of HEK293 cells expressing 5-HT1AR/OX1R with 8-OH-DPAT and/or orexin-A can reduce BRET ratio and increased the intracellular cAMP level (Fig. 3B). We measured cAMP levels after stimulation with 8-OH-DPAT or 8-OH-DPAT and orexin-A, and the results were high with orexin-A in the 5-HT1AR/OX1R co-expression group.

Next, HEK293 cells were co-transfected with 5-HT1AR/OX1R and the BRET EPAC biosensor for cAMP monitoring, and the transfected cells were incubated at 37 °C for 2 h with HIV Tat-fused peptides (10 μM) corresponding to TM5 of OX1R or TM4 or TM5 of 5-HT1AR. Subsequently, the cells were stimulated with 8-OH-DPAT alone, orexin-A alone, or both agonists. The increased intracellular cAMP levels seen in cells expressing 5-HT1AR and OX1R were reduced following treatment with the 5-HT1AR TM4 or TM5 or OX1R-TM5 peptides respectively, suggesting disruption of
cells were co-transfected with a constant amount of the OX1R-Rluc (control group (mOX2 in independent experiments were performed with duplicate samples and the results were expressed as the mean ± SEM of four experiments (n = 4)) (**p < 0.01 vs. other groups). The data represent means ± SEM of four independent experiments (n = 4). Heterodimerization of 5-HT1AR/OX1R was measured by BRET (B). Effects of 8-OH-DPAT or orexin-A on the BRET ratio. HEK293 cells were co-transfected with OX1R-Rluc and 5-HT1AR-EGFP plasmids (1:3). After 24 h of transfection, the Rluc substrate Coelenterazine h was added for 5 min, and the cells were treated with 8-OH-DPAT (100 nM) or orexin-A (100 nM) or vehicle for 10 min, BRET ratios were analyzed and are expressed as the mean ± SEM of four experiments (n = 4) (B). C BRET ratios were analyzed and are expressed as the means ± SEM of four experiments (n = 4). ***p < 0.001, OX1R-Rluc + 5-HT1AR-EGFP versus a negative control group (mOX2xα-Rluc + mOX2xα-EGFP), as a positive control group (sOR-Rluc + APJ-EGFP) (C). D, E, F BRET saturation assay HEK293 cells were co-transfected with a constant amount of the OX1R-Rluc (D, E) or 5-HT1AR-Rluc (F) construct, each at 0.15 μg/well, and increasing amounts of the EGFP construct (0.15–0.9 μg/well). Calculated BRET ratios were plotted relative to total fluorescence/luminescence ratios and data were analyzed by nonlinear regression curve fitting (one site-specific binding) using GraphPad Prism. BRET ratios were analyzed and expressed as means ± SEM of four experiments (n = 4). G FRET imaging of constitutive 5-HT1AR/OX1R heteromeric interactions in living cells. HEK293 cells were transiently transfected with plasmids encoding (a) 5-HT1AR-ECFP (donor), (b) OX1R-EYFP (acceptor), (c) 5-HT1AR-ECFP and OX1R-EYFP. Left panels: ECFP center panels, EYFP right panels, corrected FRET. H 5-HT1AR/OX1RAPJ heterodimer FRET efficiency. Four independent experiments were performed with duplicate samples and the results were expressed as the mean ± SEM of four experiments (n = 4) (**p < 0.01 vs. other groups).

the heterodimers (Fig. 3C). Notably, treatment with TM peptide did not eliminate cAMP in transfected cells, suggesting that 5-HT1AR and OX1R have specific functional effects on cAMP levels, even in the absence of dimerization. Overall, these results suggest that TM peptide affects the formation of heterodimerization of 5-HT1AR and OX1R in vitro, rather than changing the cAMP of 5-HT1AR and OX1R monomers (Fig. 3C).

Heterodimerization of 5-HT1AR and OX1R increases the activities of CRE and SRF-RE. Reporter gene assays offer a simple solution for the study and characterization of receptor/G protein coupling. The activities of cAMP response element (CRE), nuclear factor of activated T-cells response element (NFAT-RE), serum response element (SRE), and serum response factor response element (SRF) were assessed in HEK293 cells expressing 5-HT1AR alone, OX1R alone, or 5-HT1AR/OX1R (Supplementary Fig. 1A–D). Following stimulation with 8-OH-DPAT alone or 8-OH-DPAT and orexin-A, cells expressing 5-HT1AR/OX1R heterodimers displayed enhanced specific response elements (CRE and SRF, Supplementary Fig. 1A, B) causing Gαs- and Gα12/13-G-dependent downstream signal transduction pathways. The downstream signaling activities of most GPCRs are terminated by phosphorylation and subsequent binding of β-arrestin proteins; thus, we examined whether heterodimerization of 5-HT1AR and OX1R would affect the recruitment of β-arrestins. Stimulation of HEK293 cells expressing 5-HT1AR-Rluc, untagged OX1R, and β-arrestin1-EGFP or β-arrestin2-EGFP with 100 nM 8-OH-DPAT or 100 nM orexin-A resulted in a robust and continuous increase in the ligand-induced BRET signal (Supplementary Fig. 1E, F), indicating that 5-HT1AR/OX1R heterodimerization does not alter the recruitment of β-arrestins to the complex.

TM4/TM5 and TM6/TM6 form the interface for 5-HT1AR/OX1R dimerization

Mass spectrometry analyses. TMs are crucial for creating head-to-head interfaces in class A GPCR dimers [32, 33]. The sequences and molecular weights of the seven TMs in 5-HT1AR and OX1R are shown in Table 1. To identify dimerization interfaces in the TMs of 5-HT1AR and OX1R, the effects of cell-penetrating interference peptides containing the sequences of the hydrophobic transmembrane helices on 5-HT1AR/OX1R heterodimer formation were examined by MALDI-TOF mass spectrometry.

These analyses revealed that 5-HT1AR + OX1R-TM4, 5-HT1AR + OX1R-TM6, and OX1R + 5-HT1AR-TM6 dimers were not (Supplementary Figs. 2, 1A, C, F), Whereas 5-HT1AR + OX1R-TM5, OX1R + 5-HT1AR-TM4, and OX1R + 5-HT1AR-TM5 dimers were formed (Supplementary Figs. 2, 1B, D, E) in the inactive state (absence of...
These findings indicate that the structural interface of the 5-HT1AR/OX1R dimer consists of a combination of TM4 and TM5 in 5-HT1AR and TM5 in OX1R, which are likely bound within themselves in the inactive state.

We then examined the dynamics of this structural interface following stimulation of the heterodimer with 8-OD-DPAT or orexin-A. In this case, the OX1R + 5-HT1AR-TM6 and 5-HT1AR + OX1R-TM6 dimers were detected by mass spectrometry (Supplementary Fig. 2B, C, F), suggesting that the structural interface of the 5-HT1AR/OX1R heterodimer changes from TM4/TM5 to TM6 in the active conformation.

Next, mass spectrometry analyses of 5-HT1AR/OX1R heterodimers containing various point mutations in the TMs were performed to identify the specific residues involved in dimer formation. The R151A mutation in 5-HT1AR-TM6 and the M351A mutation in 5-HT1AR-TM6 abolished the formation of a heterodimer with wild-type OX1R. By contrast, the L356A mutation in 5-HT1AR-TM6 did not affect the formation of a heterodimer with wild-type OX1R after agonist stimulation (Supplementary Figs. 2C, 3E). In addition, the L309A mutation in OX1R-TM6 abolished the interaction with wild-type 5-HT1AR (Supplementary Figs. 2, 3F).

**BRET assays.** The BRET ratios of HEK293 cells expressing 5-HT1AR and OX1R were reduced significantly after incubation with HIV TAT-fused 5-HT1AR-TM4, 5-HT1AR-TM5, and OX1R-TM5 peptides, but were not affected by incubation with the OX1R-TM1 peptide. These findings suggest that the 5-HT1AR-TM4, 5-HT1AR-TM5, and OX1R-TM5 peptides impaired the heterodimerization of 5-HT1AR and OX1R in vitro (Supplementary Fig. 3A, B). In addition, in agonist-stimulated cells, the BRET ratios were reduced significantly by incubating with the HIV TAT-fused 5-HT1AR-TM6 peptide (Supplementary Fig. 3C).

The importance of TM4, TM5, and TM6 to formation of the 5-HT1AR/OX1R heterodimer interface was confirmed by point mutation of specific residues that have been reported previously to mediate receptor dimerization [34]. Briefly, we generated 31 mutant receptors that harbored mutations of various outward-facing and hydrophobic residues (Table 2). Proper membrane localization is a prerequisite for BRET, so the possibility of a decreased BRET ratio caused by incorrect 5-HT1AR/OX1R localization was excluded by observing the immunofluorescence of all mutants prior to BRET measurements. Notably, the 5-HT1AR R151A and OX1R M176A TM4 mutants and the 5-HT1AR Y198A and OX1R L230A TM5 mutants exhibited markedly...
lower BRET signals than the wild-type proteins, highlighting the significance of TM4 and TM5 to the formation of the dimer interface in the basal or inactive state (Fig. 3D, E). In particular, we found that the 5-HT1AR R151A4.40 and OX1R M176A4.57 mutations had the greatest disruptive effect on the interaction and heterodimerization of 5-HT1AR and OX1R. Following agonist stimulation, the BRET signals of the dimers containing the 5-HT1AR M351A6.41 and OX1R L309A6.49 TM6 mutants were 50% lower than those of the wild-type dimer (Supplementary Fig. 3D, E). Notably, agonist stimulation of the dimer harboring the 5-HT1AR L356A 6.46 TM6 mutant increased the BRET signal (Supplementary Fig. 3D).

**Proximity ligation assay (PLA).** When the cells expressing 5-HT1AR alone were used as the negative control group in the PLA experiment, no red fluorescent spots were found in the measurement (Fig. 4A). Red fluorescence spots were detected in HEK293 cells co-expressing 5-HT1AR and OX1R, indicating successful formation of the heterodimer (Fig. 4B). Pre-incubation of HEK293 cells co-expressing 5-HT1AR/OX1R with an OX1R-TM1 peptide (Fig. 4C) or OX1R-TM5 peptide (Fig. 4D), the latter significantly reduces the amount of PLA product. However, pre-incubation with a 5-HT1AR-TM3 peptide (Fig. 4E) did not change the number of PLA products. The pre-incubation with a 5-HT1AR-TM4 or -TM5 peptide (Fig. 4F, G) reduced the number of PLA products markedly. Quantification of the PLA data is shown in Fig. 4H, I.

**Injection of 5-HT1AR/OX1R-TM peptides increases the amounts of proteins associated with antidepressant effects in the rat mPFC and hippocampus.**

Next, we investigated the role of the 5-HT1AR/OX1R heterodimer in the onset of depression in vivo. To this end, we used the CUMS method to model depression in rats and injected the rats with 5-HT1AR/OX1R-TM peptides to disrupt formation of the heterodimer (Fig. 5A).

The experimental groups included an untreated control group (n = 10), a CUMS control group in which rats were injected with saline (n = 10), and a CUMS treatment group in which rats were injected with 5-HT1AR/OX1R-TM peptides were divided into three groups, each group (N = 10). After 28 days of the CUMS protocol, we measured the weight gain of the rats and performed SPT and FST to determine whether the model was successful. Ten rats that were successfully modeled were selected for subsequent experiments. Subsequently, the rats received a single injection of saline or TM peptides into the lateral ventricle, and weight gain, SPT, and FST assessments were performed 6 days later.

Weight gain of the CUMS group was significantly lower than that of the control group (**P < 0.01); however, treatment of the CUMS model rats with TM peptides increased weight gain significantly (P < 0.05) (Fig. 5B). Similarly, the sucrose preference of the CUMS group was significantly lower than that of the control group (**P < 0.01) but was increased significantly following TM peptide injection (P < 0.05; $P < 0.01) (Fig. 5C). In addition, injection of the TM peptides reversed the CUMS-induced increase
in the immobility time in the FST (Fig. 5D). Taken together, these results suggest that the 5-HT1AR/OX1R dimer may be involved in the occurrence and development of depression. Six days of treatment with TM peptides increased proteins associated with antidepressant onset. These antidepressant effects may be mediated by upregulation of BDNF levels (Fig. 5E) and enhanced phosphorylation and activation of CREB (Fig. 5F) in the mPFC.

To examine the role of the 5-HT1AR/OX1R heterodimer in the onset of depression further, we used a PLA to assess the amount of dimer formed in the rat hippocampus. The PLA signal representing the 5-HT1AR/OX1R dimer in the hippocampus was markedly higher in the CUMS group than in the control group (Fig. 6A, B). Next, in situ PLA signals were quantified as the number of blobs per cell in CUMS rats injected with the 5-HT1AR-TM4, 5-HT1AR-TM5, or OX1R-TM5 peptide compared with that in the control and CUMS groups (Fig. 6C). Compared with CUMS rats injected with 5-HT1AR-TM4 (Fig. 6D), 5-HT1AR-TM5 (Fig. 6E), or OX1R-TM5 (Fig. 6F), the PLA signal number of 5-HT1AR/OX1R heterodimer was lower.

Increased amounts of BDNF protein associated with antidepressant-like effects were seen in the hippocampus after rats were treated with 5-HT1AR-TM4 (2.66 mM), 5-HT1AR-TM5 (2.74 mM), or OX1R-TM5 (3.07 mM) respectively for 6 days (Fig. 6G). In addition, the level of BDNF and the phosphorylation and activation of CREB were upregulated after treatment with the TM peptides (Fig. 6H).

**DISCUSSION**

Although the formation of a 5-HT1AR/OX2R heterodimer has been reported previously, no study has revealed the characteristics of human 5-HT1AR/OX1R heterodimers. Here, we have demonstrated the formation of constitutive and functional 5-HT1AR/OX1R heterodimers in vitro and in vivo. Our results demonstrate that 5-HT1AR/OX1R heterodimers are formed in recombinant cell systems and neuronal cells of the rat mPFC (Supplementary Fig. 4) and hippocampus.

GPCR dimerization or multimerization is physiologically and functionally relevant, and often acts as the first step in the induction of intracellular signals following ligand binding [35, 36]. For example, heteromultimerization of cannabinoid receptor type 1 and OX1R generates a unique complex in which both protomers are regulated by orexin-A [19]. 5-HT1AR and 5-HT7R can form heterodimers in vitro and in vivo, and this heterodimerization reduces Gαi protein coupling of 5-HT1AR without affecting coupling of 5-HT7R to the Gαs protein [22]. 5-HT1AR and OX1R can also interact with other GPCRs to form heterodimers; thus, heterodimerization provides an additional mechanism to regulate cellular processes through the fine tuning of receptor-mediated signaling. Although 5-HT1AR and OX1R reportedly couple with Gαi and Gαq, respectively, we found that stimulation of 5-HT1AR/OX1R in HEK293 cells increased the coupling of the Gas proteins [22]. 5-HT1AR and OX1R can interact with other GPCRs to form heterodimers; thus, heterodimerization provides an additional mechanism to regulate cellular processes through the fine tuning of receptor-mediated signaling. Although 5-HT1AR and OX1R reportedly couple with Gai and Gq, respectively, we found that stimulation of 5-HT1AR/OX1R in HEK293 cells increased the coupling of the Gas and Gai12/13 subunits, indicating that these G protein subunits may be activated by binding of ligands to the 5-HT1AR/OX1R heterodimer. In line with this finding, the intracellular cAMP level was increased by double ligand stimulation of HEK293 cells expressing the 5-HT1AR/OX1R heterodimer. Conversely, incubation of transfected HEK293 cells with 5-HT1AR-TM4, 5-HT1AR-TM5, or OX1R-TM5 reduced the ligand-induced increase in the cAMP level, which is consistent with our observation that 5-HT1AR-TM4, 5-HT1AR-TM5,
and OX1R-TM5 impaired formation of the 5-HT1AR/OX1R heterodimer. Overall, these results suggest that binding of a ligand to the 5-HT1AR/OX1R heterodimer increases the activation of $G_\alpha_s$ and $G_\alpha_{12}/G_\alpha_{13}$. 5-HT1AR/OX1R is recruited into these heteropolymer complexes in novel G protein-dependent signaling pathways.

We used TM peptide and MALDI-TOF mass spectrometry as methods to examine the function of regulating 5-HT1AR/OX1R heterodimer. The results revealed that peptides encoding 5-HT1AR-TM4 and 5-HT1AR-TM5 could bind to OX1R, and a peptide encoding OX1R-TM5 could bind to 5-HT1AR, suggesting that these specific domains are involved in interface formation. GPCR dimers are a dynamic species with multiple forms and a dimerization interface that shifts during receptor activation and inactivation [37]. Here, we found that the structural interface of the active 5-HT1AR/OX1R dimer transforms from TM4 (5-HT1AR only) and TM5 (both protomers) in the basic or inactive state to mainly TM6 in the active conformation (Supplementary Fig. 5). Understanding that the 5-HT1AR/OX1R heterodimers are activated in this manner will aid the identification of new drug targets for small molecule interference in different dimer states.

In our previous study, we demonstrated that TM1, 2, 3, and 4 of APJ form the homodimer interface [3]. We propose that 5-HT1AR/OX1R protomers form type II dimers involving TM4 (5-HT1AR only), 5, and 6, and that TM6 plays a role in modulating 5-HT1AR/
OX1R function. Although OX1R-TM4 did not bind to 5-HT1AR in our experiments (four replicates), we cannot rule out the possibility that this domain is involved in dimer formation, or that 5-HT1AR/OX1R may form asymmetric heterodimers. Similar to our findings presented here, the constitutive metabotropic glutamate receptor 2 homodimer undergoes a 45° rotation of each domain upon ligand binding, leading to a major change of the dimer interface from TM4 and TM5 in the inactive state to TM6–TM6 in the active conformation, revealing a key step in class C GPCR activation [38]. In addition, the structure of the chemokine receptor CXCR4 homodimer is based on a TM4/S interface localized to the upper regions of the helices [39].

Our findings suggest that TM peptides could have therapeutic potential, as they can disrupt dimerization and influence receptor function. Bulenger et al. [35] showed that a peptide derived from TM6 of the β2-adrenergic receptor (β2-AR) can disrupt the dimer and decrease receptor function [40] and we have shown previously that disruption of TM1, 2, 3, and 4 in APJ impairs homodimer formation [3]. Due to the high sensitivity and accuracy of mass spectrometry, this method provides useful information for understanding GPCR structure and conformational changes during dimer formation. Point mutation experiments revealed that the 5-HT1AR R151A [40] and OX1R M176A [47] TM4 mutants (Supplementary Fig. 3) and the 5-HT1AR Y198A [41] and OX1R L230A [49] TM5 mutants exhibited a reduced ability to dimerize, further supporting the involvement of TM4 and TM5 in the dimer 5-HT1AR/OX1R interface. In addition, the 5-HT1AR M351A [41] and OX1R L309A [49] TM6 mutants inhibited agonist-stimulated 5-HT1AR/OX1R dimer formation, suggesting that these residues are involved in dimer formation in the active state. Interestingly, the 5-HT1AR L356A [46] TM6 mutation increased 5-HT1AR/OX1R dimerization following agonist stimulation. In addition, a mass spectrometry analysis revealed that the 5-HT1AR L356A mutant peptide was able to bind to OX1R following agonist stimulation. To our knowledge, this is the first report that the 5-HT1AR-L356A [46] mutation promotes 5-HT1AR/OX1R dimer formation. Further in vivo studies are required to clarify the specific role of this point mutation in increasing dimer formation.

The CUMS model used here is one of the most widely recognized animal models of depression [41]. Several behavioral effects that parallel symptoms of depression have been described in rats exposed to CUMS. Here, the FST and SPT were chosen as reliable methods for examining CUMS-induced behavioral effects. Using these parameters, as well as weight gain measurements, we examined the antidepressant-like effects of TM peptides in vivo. Six days after injection of one of three TM peptides (5-HT1AR-TM4, 5-HT1AR-TM5, and OX1R-TM5) into the lateral ventricle of CUMS rats, the CUMS-induced deficits in the SPT and increased immobility time in the FST were reversed. These TM peptides were designed to destroy the interface of the 5-HT1AR/OX1R heterodimer, suggesting that they inhibited the specific depression-related function of the GPCR dimer.

The hippocampus-PFC circuit plays a major role in stress and the neurobiology of depression. Accumulating evidence suggests dysregulation of synaptic plasticity in the etiology of depression, leading to synaptic weakening and neuronal atrophy in vulnerable brain regions such as the hippocampus and mPFC [42]. In addition, changes in the levels of neuropeptides and neurotrophic factors also play an important role in depression. Increased BDNF levels in the hippocampus and mPFC are associated with an antidepressant-like response in behavioral models of depression, providing a biomarker that corroborates the onset of these behavioral effects [43]. CREB impinges on the regulation of BDNF signaling, which exerts a highly circuit-specific influence on mood-related behaviors [44]. A leading hypothesis for the pathogenesis of depression is that CREB and BDNF play an important role in adaptation of the hippocampus to chronic stress and antidepressants [45]. Here, we found that treatment of CUMS rats with the 5-HT1AR-TM4, 5-HT1AR-TM5, and OX1R-TM5 peptides upregulated the BDNF level as well as phosphorylation and activation of CREB in the mPFC and hippocampus, in line with the observed alleviation of the CUMS-induced behavioral effects by these peptides. Notably, BDNF and pCREB/CREB were upregulated in the hippocampus and mPFC after just 6 days of TM peptide treatment; this rapid increase in the levels of these proteins is consistent with the effects of other putative fast-onset antidepressant treatments, such as short-term treatment with 5-HT2C receptor antagonists [46] and 5-HT4 receptor agonists [47]. Further research is needed to determine the correlation between the upregulation of BDNF and pCREB/CREB levels and the rapid initiation of antidepressant-like activities of the TM peptides used here.

Using the PLA method, we demonstrated that the number of endogenous 5-HT1AR/OX1R heterodimers in the rat hippocampus was markedly higher in the depression model group than in the control group. Alongside the improvement in depression-related effects, 6 days of treatment with the three TM peptides (5-HT1AR-TM4, 5-HT1AR-TM5, and OX1R-TM5) reduced the amount of 5-HT1AR/OX1R heterodimers in the hippocampus. Taken together, these findings provide direct evidence that 5-HT1AR/OX1R heterodimers are involved in the pathological process of depression. We suggest that TM peptides targeting the 5-HT1AR/OX1R heterodimer interface could be lead compounds for the development of fast-acting antidepressants.

In conclusion, this study advances current knowledge of the structure and function of 5-HT1AR/OX1R heterodimers. We have demonstrated that 5-HT1AR/OX1R can induce novel G protein-dependent signaling, and that heterodimerization of the protomers does not affect the recruitment of β-arrestins to the complex. We have also revealed the dynamics of the TM interface of the 5-HT1AR/OX1R heterodimer during receptor activation in vitro and in vivo. The antidepressant effects of TM peptides targeting the 5-HT1AR/OX1R dimer might be mediated by upregulation of BDNF levels in the mPFC and hippocampus, as well as by enhancement of CREB phosphorylation and activation. Our findings could aid the development of novel therapeutic drugs and small molecules that interfere with the interface of the 5-HT1AR/OX1R dimer in different states.

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AUTHOR CONTRIBUTIONS
JC conceived and designed the experiments; RZ, DL, XW, HM, MX, QX, and YLJ performed the experiments. SZ, CW, XC, BJ, MY, XC, BD, and YLJ performed analyzed the data the data. JC, and CL, supervised the project. JC, HR, and GL wrote the paper.

COMPETING INTERESTS
The authors declare no competing interests.

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