



A Novel 5-HT_{1B} Receptor Agonist of Herbal Compounds and One of the Therapeutic Uses for Alzheimer's Disease

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Specialty section:

This article was submitted to
Experimental Pharmacology and
Drug Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 18 July 2021

Accepted: 23 August 2021

Published: 06 September 2021

Citation:

Yang Y, Zhang L, Yu J, Ma Z, Li M,
Wang J, Hu P, Zou J, Liu X, Liu Y, An S,
Xiang C, Guo X, Hao Q and Xu T-R
(2021) A Novel 5-HT_{1B} Receptor
Agonist of Herbal Compounds and
One of the Therapeutic Uses for
Alzheimer's Disease.
Front. Pharmacol. 12:735876.
doi: 10.3389/fphar.2021.735876

The serotonin receptor 5-HT_{1B} is widely expressed in the central nervous system and has been considered a drug target in a variety of cognitive and psychiatric disorders. The anti-inflammatory effects of 5-HT_{1B} agonists may present a promising approach for Alzheimer's disease (AD) treatment. Herbal antidepressants used in the treatment of AD have shown functional overlap between the active compounds and 5-HT_{1B} receptor stimulation. Therefore, compounds in these medicinal plants that target and stimulate 5-HT_{1B} deserve careful study. Molecular docking, drug affinity responsive target stability, cellular thermal shift assay, fluorescence resonance energy transfer (FRET), and extracellular regulated protein kinases (ERK) 1/2 phosphorylation tests were used to identify emodin-8-O-β-D-glucopyranoside (EG), a compound from Chinese medicinal plants with cognitive deficit attenuating and antidepressant effects, as an agonist of 5-HT_{1B}. EG selectively targeted 5-HT_{1B} and activated the 5-HT_{1B}-induced signaling pathway. The activated 5-HT_{1B} pathway suppressed tumor necrosis factor (TNF)-α levels, thereby protecting neural cells against beta-amyloid (Aβ)-induced death. Moreover, the agonist activity of EG towards 5-HT_{1B} receptor, in FRET and ERK1/2 phosphorylation, was antagonized by SB 224289, a 5-HT_{1B} antagonist. In addition, EG relieved AD symptoms in transgenic worm models. These results suggested that 5-HT_{1B} receptor activation by EG positively affected Aβ-related inflammatory process regulation and neural death resistance, which were reversed by antagonist SB 224289. The active compounds such as EG might act as potential therapeutic agents through targeting and stimulating 5-HT_{1B} receptor for AD and other serotonin-related disorders. This study describes methods for identification of 5-HT_{1B} agonists from herbal compounds and for evaluating agonists with biological functions, providing preliminary information on medicinal herbal pharmacology.

Keywords: 5-HT_{1B} receptor, molecular docking, drug affinity responsive target stability, fluorescence resonance energy transfer, alzheimer's disease, emodin-8-O-β-D-glucopyranoside

Abbreviations: CNS, central nervous system; AD, Alzheimer's disease; EG, emodin-8-O-β-D-glucopyranoside; DARTS, drug affinity responsive target stability; CETSA, cellular thermal shift assays; FRET, fluorescence resonance energy transfer.

INTRODUCTION

The serotonergic system is important in regulating crucial processes in the central nervous system (CNS). These effects are mediated by serotonin receptors, such as the serotonin receptor subtype 1B (5-HT_{1B}). These receptors, belonging to the G protein-coupled receptors (GPCRs) superfamily, are abundantly expressed in the CNS and constitute validated as well as putative drug targets in a variety of cognitive and psychiatric disorders, including depression and Alzheimer's disease (AD), the most common incurable neurodegenerative disease (Monti and Jantos, 2008; Tiger et al., 2018; Gadgaard and Jensen, 2020).

Activation of the serotonergic system blocks the beta-amyloid (A β) oligomer-induced inflammatory response in AD (Ledo et al., 2016). A β -induced inflammatory response and neuronal death play important roles in AD pathology. Recent studies have identified inflammatory pathways as potential new drug targets for treating AD (Unzeta et al., 2016). A β oligomer-mediated activation of pro-inflammatory tumor necrosis factor (TNF)- α signaling in the brain has been reported as a cause of memory loss in mice (Lourenco et al., 2013). Activation of 5-HT_{1B} by its agonist sumatriptan, an anti-migraine agent, significantly diminishes the mRNA levels of TNF- α in rat nerve cells (Khalilzadeh et al., 2018). Moreover, it has been reported that the frontal and temporal cortices of AD patients show a reduction in 5-HT_{1B/1D} receptor levels (Garcia-Alloza et al., 2004). Therefore, the anti-inflammatory effects of 5-HT_{1B} agonists may present a promising approach in AD treatment.

Overall activation of 5-HT_{1B} in the CNS may have antidepressant effects (Sanchez et al., 2015). Several lines of evidence support the therapeutic use of herbal antidepressants in the treatment of AD (Jeon et al., 2019; Chen et al., 2020; Li et al., 2020), wherein various compounds present in the ingredients reduce neuronal inflammation and apoptosis. However, the targets of the herbal antidepressants and the interactions between the active compounds and their targets, which constitute the basic issue of medicinal herbal pharmacology, remain to be elucidated. The functional overlap between herbal antidepressants of traditional Chinese medicine and 5-HT_{1B} stimulation suggests the therapeutic potential of compounds from the ingredients of these medicinal plants, as they can target and stimulate 5-HT_{1B} for AD treatment.

In this study, the active compound emodin-8-O- β -D-glucopyranoside (EG), a component of several Chinese medicinal plants such as *Polygonum multiflorum* and *Rheum officinale* that contain herbal antidepressants that could be used in AD treatment (Lin et al., 2015; Jeon et al., 2019; Chen et al., 2020; Li et al., 2020), was identified as a ligand targeting the 5-HT_{1B} receptor. The role of serotonin-system-mediated neural protection was also investigated. The results support the importance of 5-HT_{1B} receptor as a therapeutic target and validate the potential use of 5-HT_{1B} agonists as therapeutic agents for serotonin system-related diseases such as AD.

MATERIALS AND METHODS

Drugs, Chemicals, Reagents, and Other Materials

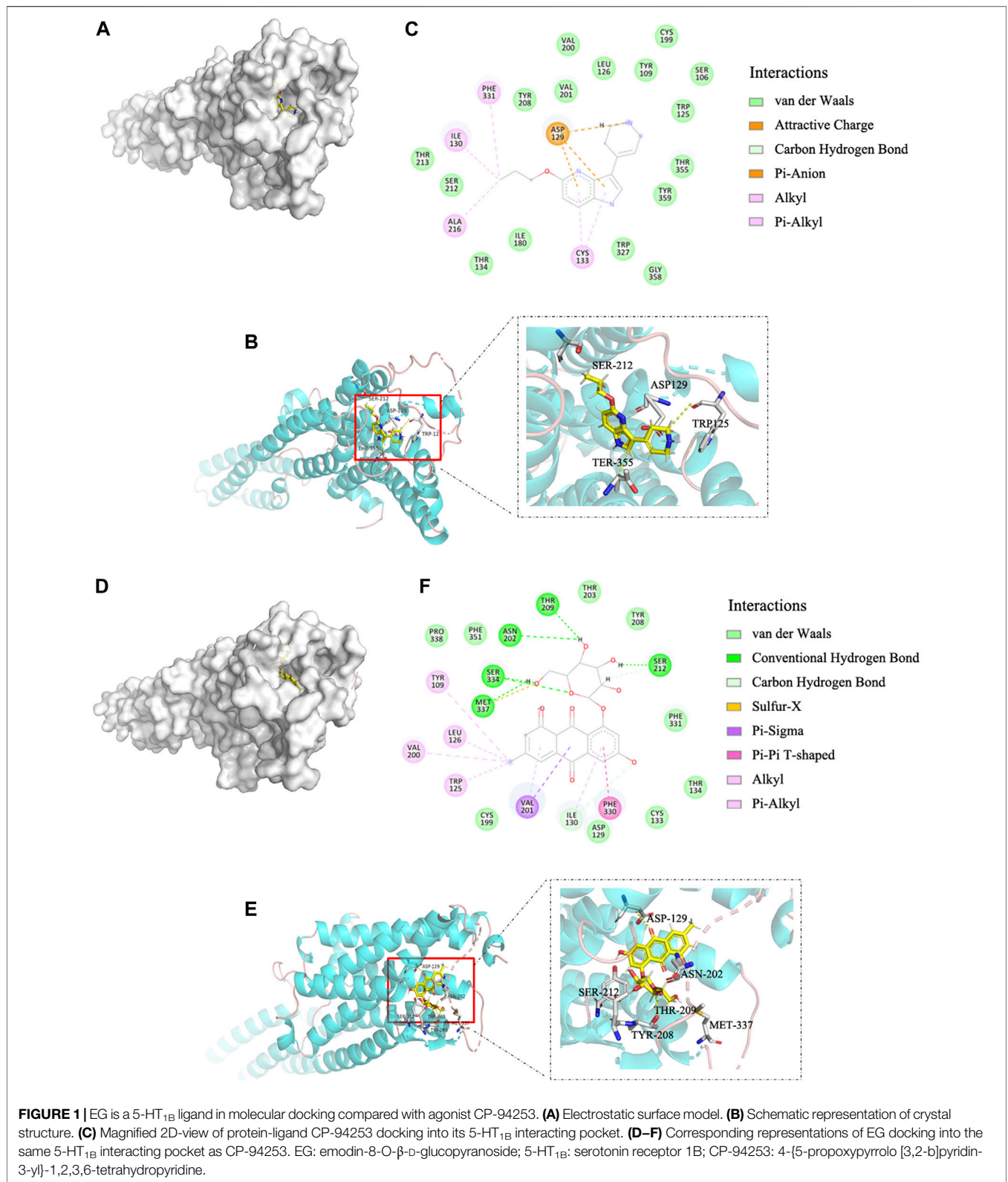
EG (cas 23,313–21-5) was obtained from Weikeqi (Sichuan, China), CP-94253 (4-{5-propoxyppyrrrolo [3,2-b]pyridin-3-yl}-1,2,3,6-tetrahydropyridine, catalog # SML0588), DOI (1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine, catalog #D101), dopamine (4-(2-Aminoethyl)benzene-1,2-diol, catalog #H8502), and caffeine from Sigma-Aldrich, ramelteon (N-[2-[(8S)-2,6,7,8-tetrahydro-1H-cyclopenta [e][1]benzoxol-8-yl]ethyl]propanamide) from MedChemExpress (catalog # HYA0014), and dihydroergotamine ((2,4, 7R)-N-[(1S,2S,4R, 7S)-7-benzyl-2-hydroxy-4-methyl-5,8-dioxo-3-oxa-6,9-diazatetracyclo [7.3.0.0[^]{2.6}]dodecan-4-yl]-6-methyl-6,11-diazatetracyclo [7.6.1.0[^]{2.7}.0[^]{12.16}]hexadeca-1 (16),9,12,14-tetraene-4-carboxamide) and SB 224289 ([4-[2-methyl-4-(5-methyl-1,2,4-oxadiazol-3-yl)phenyl]phenyl]-(1'-methylspiro [6,7-dihydro-2H-furo [2,3-f]indole-3,4'-piperidine]-5-yl)methanone) from APEX BIO (catalog #B3459 and B6641, respectively). OxB (RSGPPGLQGRAQRLQASGNHAAGILTM-NH₂), A β 40 (rat: DAEPFGHDSGFVVRHQKLVFFAEDVGSNKGAIIGLMVGGVV; human: DAEFRHDSGYEVVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) and A β 42 (rat: DAEPFGHDSGFVVRHQKLVFFAEDVGSNKGAIIGLMVGGVVA; human: DAEFRHDSGYEVVHHQKLVFFAEDVGSNKGAIIGLMVGGVVA) were synthesized by Shangting (Shanghai, China). The Flp-InTM T-RExTM 293 cells, Lipofectamine[®]2000 transfection reagent, and cell culture materials were from Invitrogen (Thermo Fisher Scientific Inc., United States). The anti-VSV-G antibody (catalog #V5507) was obtained from Sigma-Aldrich, and the anti-ERK1/2-MAP kinase (catalog # 9102S) and anti-phospho-ERK1/2-MAP kinase (catalog # 9101S) antibodies were from Cell Signaling Technology (Nottingham, United Kingdom).

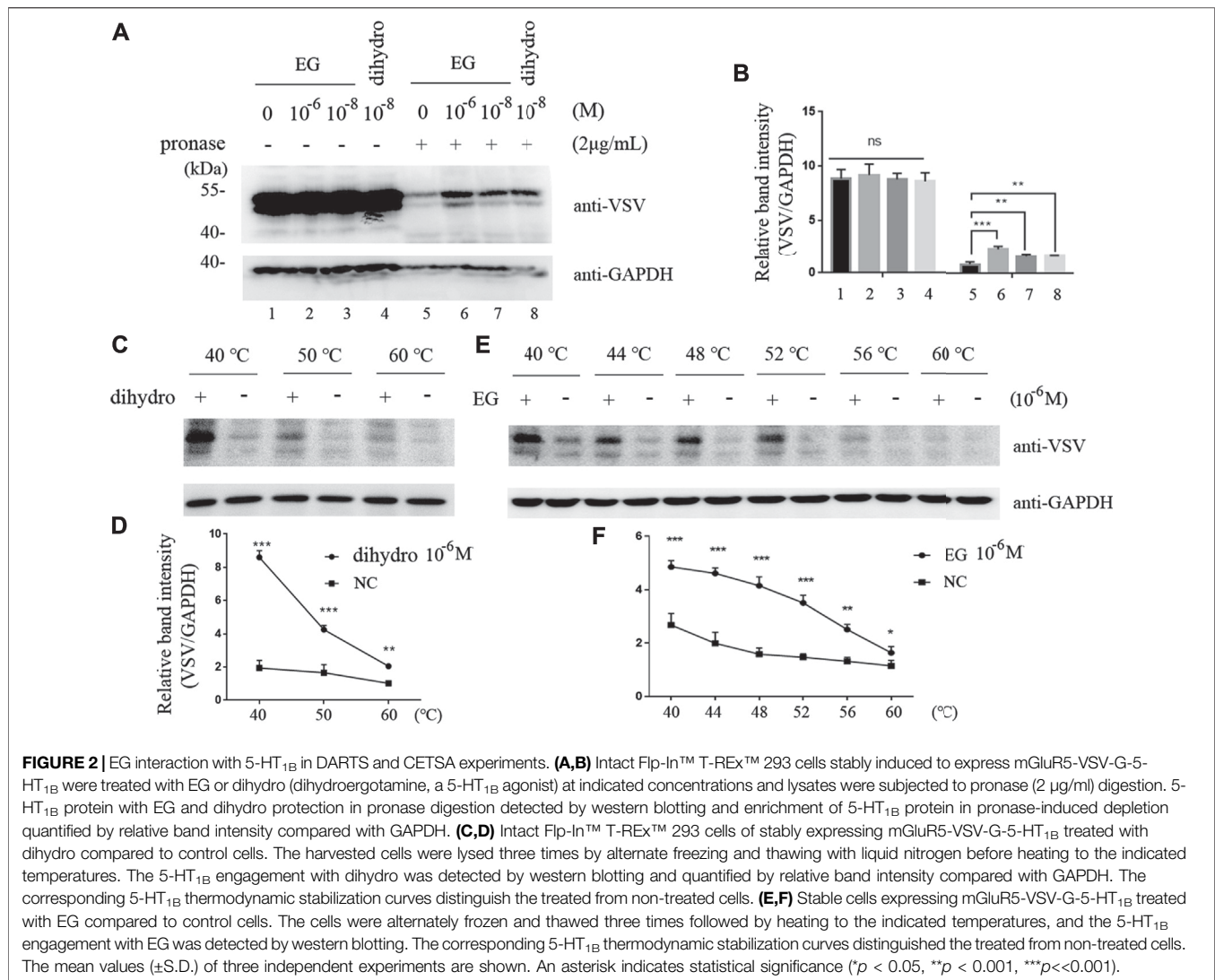
DNA Constructs

The VSV-G-mGluR5-5-HT_{1B}, VSV-G-mGluR5-5HT_{2A}, D₂, MT₂, and OX₂ constructs were established by inserting human 5-HT_{1B} cDNA into a pcDNA5/FRT/TO vector (Invitrogen) using the In-Fusion[®] PCR Cloning System (Clontech, United States) as previously described (Xu et al., 2012). Constructs were introduced into HEK293T cell lines for preliminary transient transfection studies and then into Flp-InTM T-RExTM 293 cells to generate inducible stable cell lines (Ward et al., 2011).

Generation and Maintenance of Stable Flp-InTM T-RExTM 293 Cells

The cells were co-transfected with the pOG44 plasmid and the desired protein cDNA in pcDNA5/FRT/TO in a 9:1 ratio using Lipofectamine[®]2000 to generate Flp-InTM T-RExTM 293 cells that can inducibly express the indicated constructs (Ward et al., 2011). After 48 h, the medium was supplemented with 200 ng ml⁻¹ hygromycin to select stable transfected cells. The cell pools were tested for inducible expression by adding 100 ng ml⁻¹ doxycycline for 12 h, followed by western blot analysis for phosphorylation of extracellular regulated ERKs





corresponding to GPCR activation and VSV-G protein expression.

Cell Culture and Transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g · L⁻¹ L-glutamine, whereas PC12 and SH-SY5Y cells were maintained in PRIM 1640 medium and 10% (v/v) fetal bovine serum (FBS) at 37°C in a 5% CO₂ humidified atmosphere. The cells were transfected with the indicated constructs using Lipofectamine®2000.

Molecular Docking Process

The structure of 5-HT_{1B}, access number 5V54 (Name: Crystal structure of 5-HT_{1B} receptor in complex with methiothepin), was obtained from the PDB database (<http://www.rcsb.org/pdb/home/home.do>). The 3D structures and properties of CP-94253 and EG were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). The free molecular docking software AutoDock Tools-1.5.6 was employed, and the Autodock 4 (Department of Molecular

Biology of Scripps Research Institute, La Jolla, CA and Department of Cognitive Science of University of California, San Diego, La Jolla, CA) was used for flexible docking, as previously reviewed and discussed (Beuming et al., 2015; Kontoyianni, 2017).

Cell Lysates and Western Blotting

Cells were washed once in cold phosphate-buffered saline (PBS) (120 mM NaCl, 25 mM KCl, 10 mM Na₂HPO₄, and 3 mM KH₂PO₄, pH 7.4) and harvested with ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate, 10 mM NaF, 5 mM EDTA, 10 mM NaH₂PO₄, and 5% ethylene glycol pH 7.4) supplemented with a protease inhibitor cocktail, as previously described. After heating the samples at 37°C for 5 min, the cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred onto a nitrocellulose membrane, which was then blocked (5% fat-free milk powder in PBS containing 0.1% Tween-20) at room temperature on a rotating

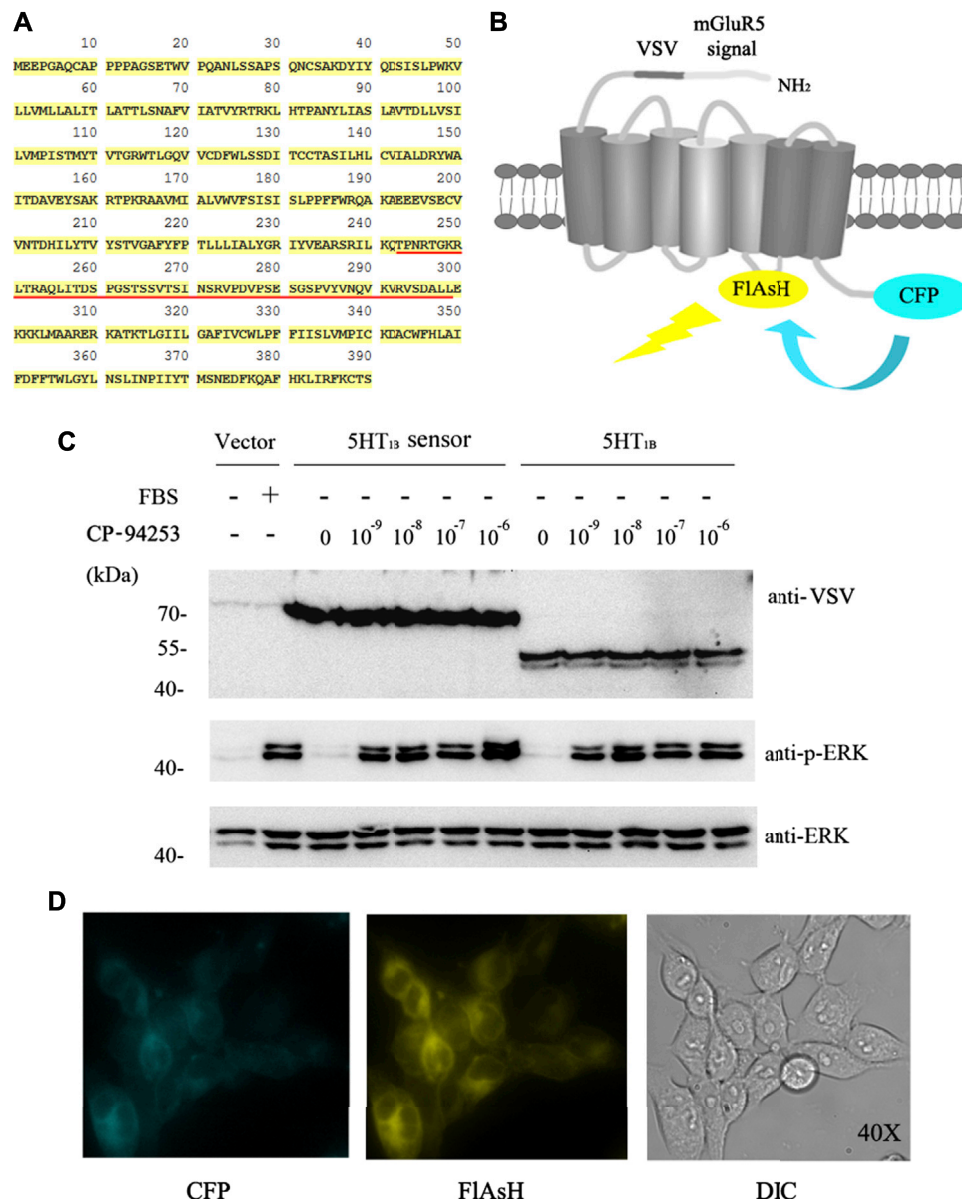
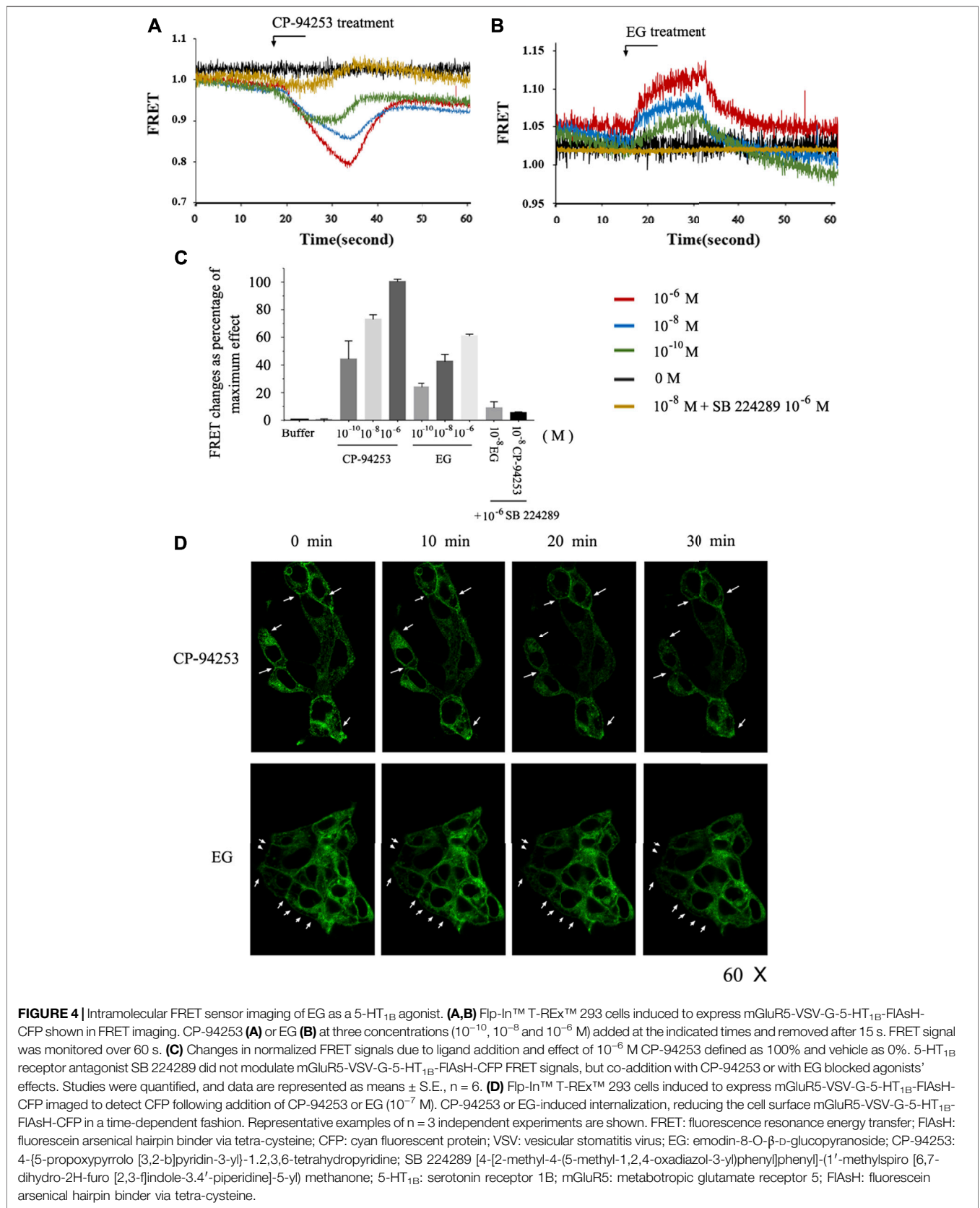
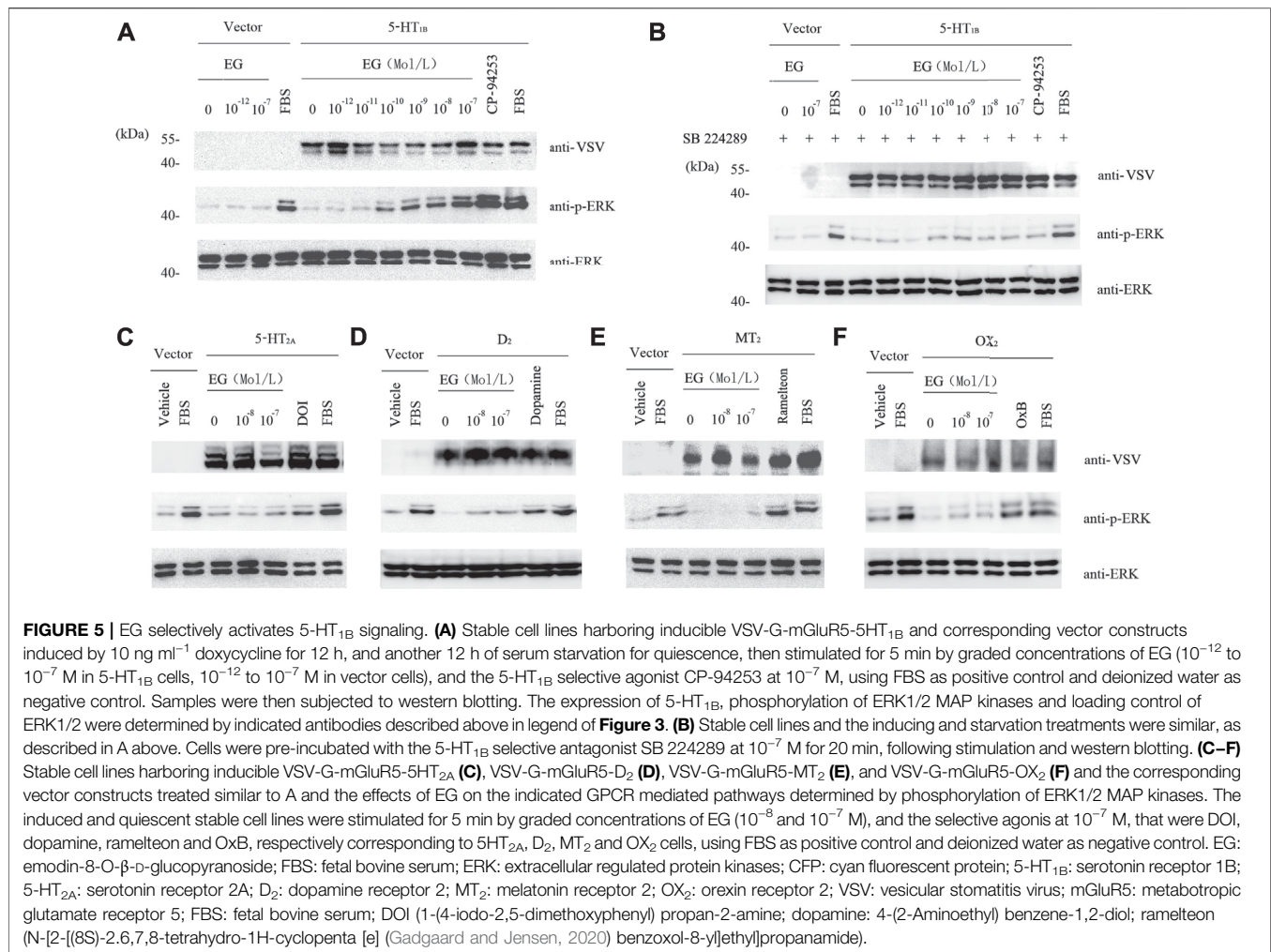


FIGURE 3 | 5-HT_{1B} FRET sensor construction. **(A)** Primary human 5-HT_{1B}-receptor amino acid sequence. FIAsh motif replaces FRET sensor sequence (underlined in red). **(B)** N terminus modification of human 5-HT_{1B} receptor by adding a leader sequence from mGluR5 followed by the VSV-G peptide sequence, while CFP was added at C terminus. FIAsh motif sequence FLNCCPGCCMEP introduced into third intracellular loop-linking transmembrane domains V and VI. Energy transfer from CFP to FIAsh and subsequent output is illustrated. **(C)** Stable cell lines harboring inducible 5-HT_{1B} FRET sensor, VSV-G-mGluR5-5-HT_{1B}, and corresponding vector constructs induced by 10 ng ml⁻¹ doxycycline for 12 h, followed by 12 h of serum starvation quiescence, and 5 min stimulation by graded concentrations (10⁻⁹ to 10⁻⁶ M) of the 5-HT_{1B} selective agonist CP-94253, using FBS as the positive control and deionized water as negative control in vector construct cells. Samples were then subjected to western blotting. The expression of 5-HT_{1B} was determined by its fusion protein tag using VSV antibody. Phosphorylation of ERK 1/2 MAP kinases was detected by a phospho-ERK1/2-specific antibody (anti-p-ERK), and the anti-ERK1/2 antibody was used to assess the protein loading equivalence. **(D)** 5-HT_{1B} FRET sensor was cloned into the inducible Flp-InTM T-RExTM locus of Flp-InTM T-RExTM 293 cells, and expression was induced by doxycycline. Imaging of CFP (left), labeled FIAsh (middle), and light field (right) demonstrated effective delivery of this sensor to the cell surface. FRET: fluorescence resonance energy transfer; VSV: vesicular stomatitis virus; FIAsh: fluorescein arsenical hairpin binder via tetra-cysteine; ERK: extracellular regulated protein kinases; CFP: cyan fluorescent protein; VSV: vesicular stomatitis virus; EG: emodin-8-O-β-D-glucopyranoside; CP-94253: 4-[5-propoxypropyl]-[3,2-b]pyridin-3-yl]-1,2,3,6-tetrahydropyridine; 5-HT_{1B}: serotonin receptor 1B; FBS: fetal bovine serum; DIC: differential interference contrast; mGluR5: metabotropic glutamate receptor 5.

shaker for 1 h, washed twice in tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20, and incubated with the appropriate primary antibody for 2 h, followed by incubation with a secondary antibody for 1 h at room temperature. Subsequently, the proteins

of interest were visualized using the ECL Chemiluminescence System (Santa Cruz Biotechnology) according to the manufacturer's instructions. Protein levels were quantified by band intensity using the Quantity One 1D Analysis Software.





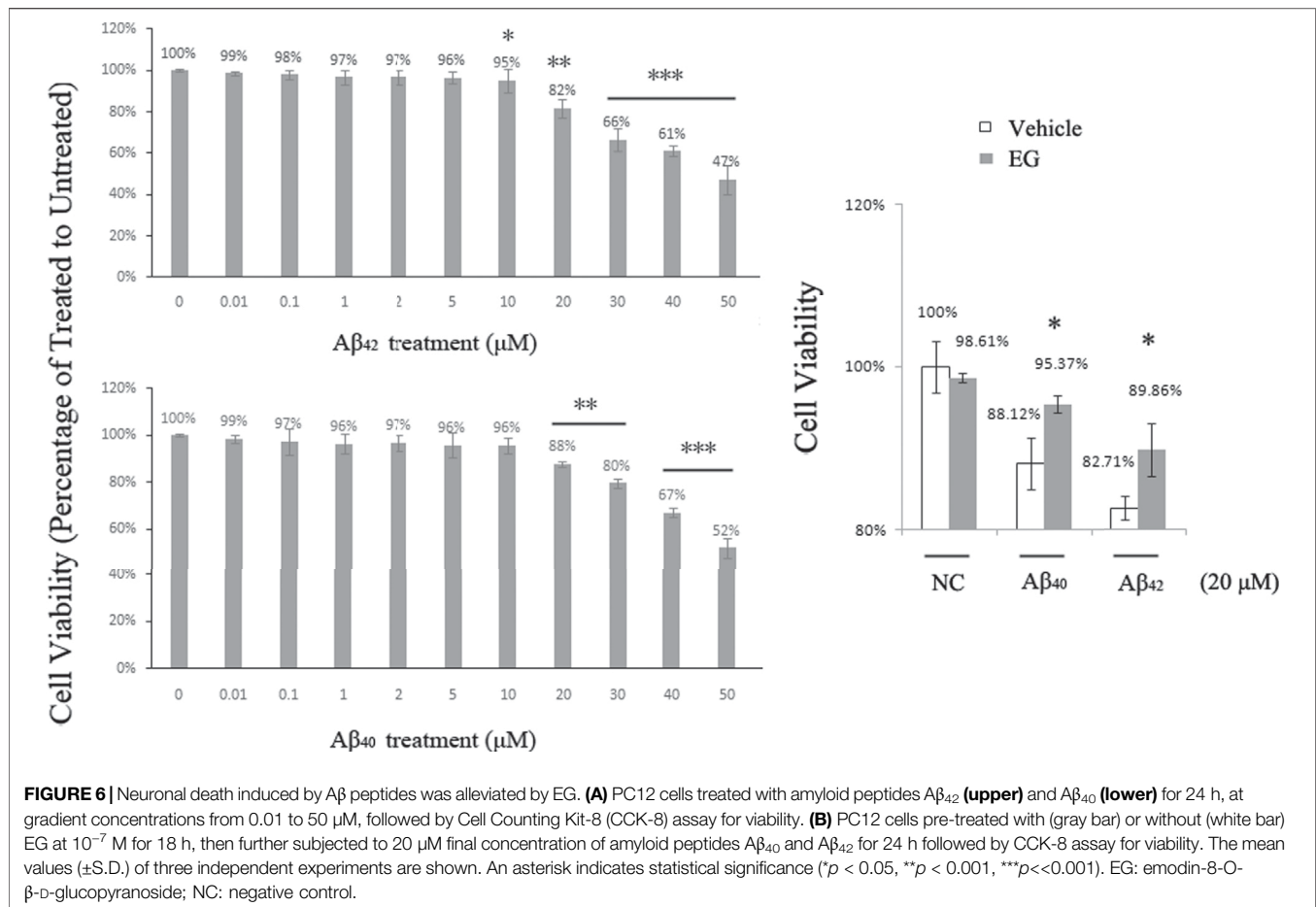
DARTS and CETSA

Intact Flp-InTM T-RExTM 293 cells induced to express mGluR5-VSV-G-5-HT_{1B} were treated with EG, dihydroergotamine, CP-94253, or the vehicle at the indicated concentrations and used for subsequent studies. In the DARTS experiment, cell lysates were subjected to pronase digestion at room temperature for 3 min, then loaded for western blotting to detect the 5-HT_{1B} protein. In the CETSA experiment, the cells were scraped off and lysed via alternate freezing and thawing thrice with liquid nitrogen, then heated to varying temperatures for 5 min. The 5-HT_{1B} protein was detected with western blotting. In both experiments, GAPDH was used as the loading control for quantification.

FRET Experiments

The 5-HT_{1B} FRET sensor-expressing cells were placed in a microscope chamber containing physiological HEPES-buffered saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 10 mM D-glucose, pH 7.4). The cells were then imaged using an inverted Nikon TE2000-E microscope (Nikon Instruments) equipped with a 40× (numerical aperture = 1.3) oil immersion Fluor lens, as previously described (Xu et al., 2012). The monochromator was set at 427 nm/bandwidth (BW) 5 and 504 nm/

BW 5 nm to visualize the surface located cyan fluorescent protein (CFP) and FIAsh separately. FRET and donor emission images were recorded simultaneously using a Quadview 2 (QV2) image splitting device (Photometrics, United Kingdom), coupled to a CoolSnap-HQ2 camera connected to the microscope. The FRET and donor signals were detected simultaneously using the following Chroma (Burlington, VT) ET dichroic and emitter series mounted in the QV2 cube: ET t505LPXR dichroic, ET535/30 nm, and ET 632/60 nm. Using the streaming capability of the multiple dimensional wavelength acquisition modules of MetaMorph, the ligand-induced changes in intramolecular FRET were recorded directly in the computer's hard drive at 40 ms intervals during excitation with 427 nm light. The Cool Snap-HQ2 camera was operated in the 14-bit mode, and exposure time, 8 × 8 binning, and camera gain, were kept constant for all streaming experiments. Computerized control of all electronic hardware and camera streaming acquisition was achieved using the MetaMorph software (version 7.7.5 Molecular Devices, Sunnydale, CA). A peristaltic pump, operated at a 5 ml/min flow rate, was used to rapidly add or remove test ligands to or from the imaging chamber. The ratiometric quantification of intramolecular charge changes was then calculated as the average 535 nm emission intensity divided by the average 470 nm emission



intensity. The FRET ratiometric was set to 1.0 at the onset of each experiment and plotted over time.

ERK1/2 MAP Kinase Phosphorylation

Cells stably expressing inducible GPCRs were placed in 6-well plates and allowed to grow overnight. Expression of the constructs was induced by adding 10 ng ml⁻¹ doxycycline for 12 h and rendered quiescent via serum starvation for 12 h, then stimulated for 5 min by the indicated agonist using FBS as the positive control and deionized water as the negative control. Cells were then placed on ice and harvested with ice-cold RIPA buffer. Phosphorylation of ERK1/2 MAP kinases was detected via western blotting using a phospho-ERK1/2-specific antibody. The nitrocellulose membranes were subsequently stripped of immunoglobulins and re-probed using an anti-ERK1/2 antibody to assess the protein loading equivalence.

Quantitative RT-PCR

Total RNA was isolated from PC12 or worm model cells treated as previously indicated, using TRIzol (Ambion Life Technologies), and the mRNA was reversed-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, catalog #K1621) according to the manufacturer's instructions. The primer sequences for quantitative PCR were as follows: TNF-α F (5'-CCCTCACACTCAGATCATCTTCT-3'), TNF-α R

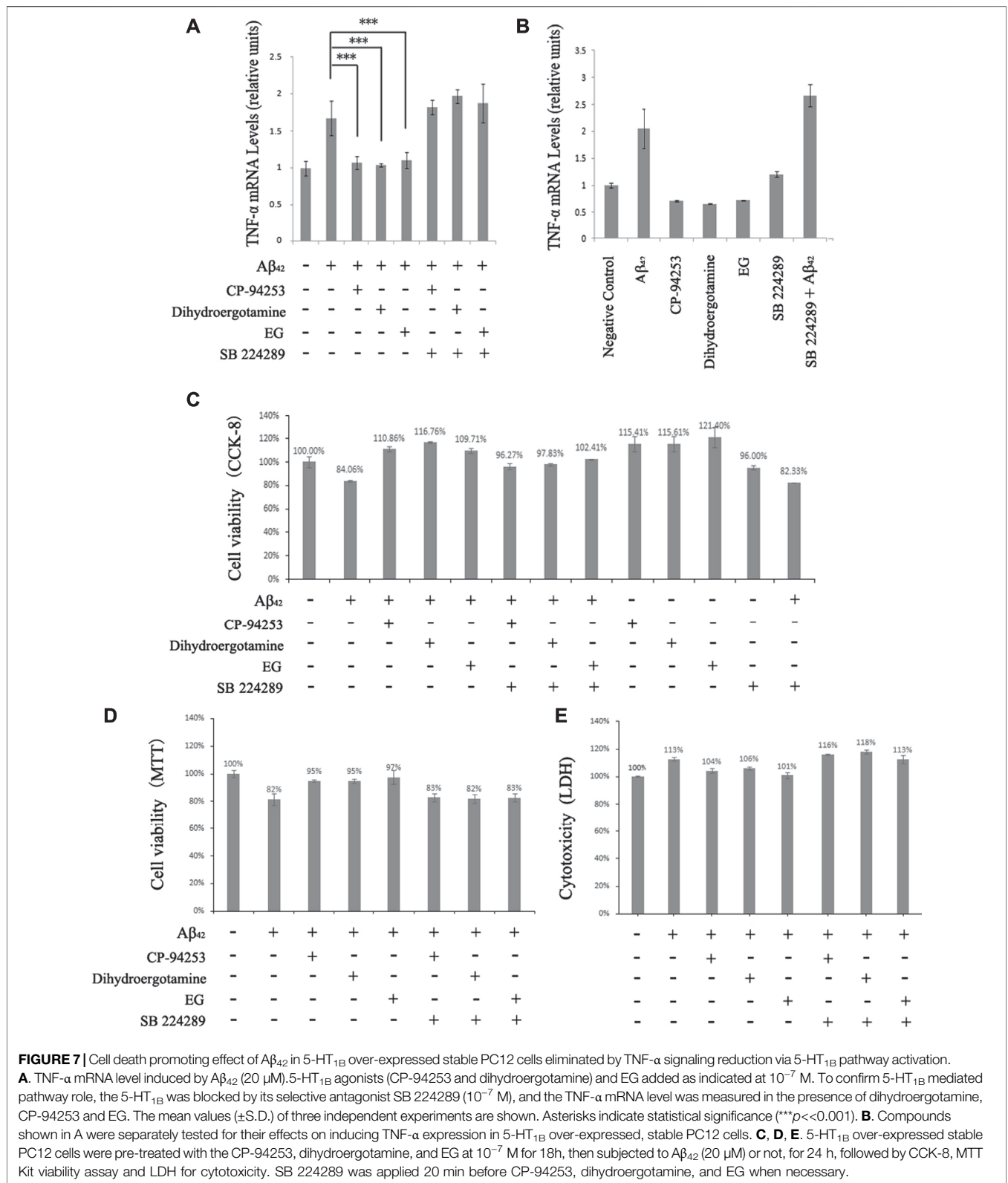
(3'-GCTACGACGTGGGCTACAG-5'), β-Actin F (5'-GAT TACTGCTCTGGCTCCTAGC-3'), and β-Actin R (3'-GAC TCATCGTACTCCTGCTTGC-5'). Previously described primers were used for the serotonin receptor subtypes (Koizumi and Nakajima, 2014) and the worm model (Bellier et al., 2009; Sahu et al., 2012). Three independent experiments were performed on triplicate samples. PCR amplification was performed using the SYBR Green PCR Master Mix Kit (Thermo Fisher). All quantifications were normalized to *GAPDH* levels.

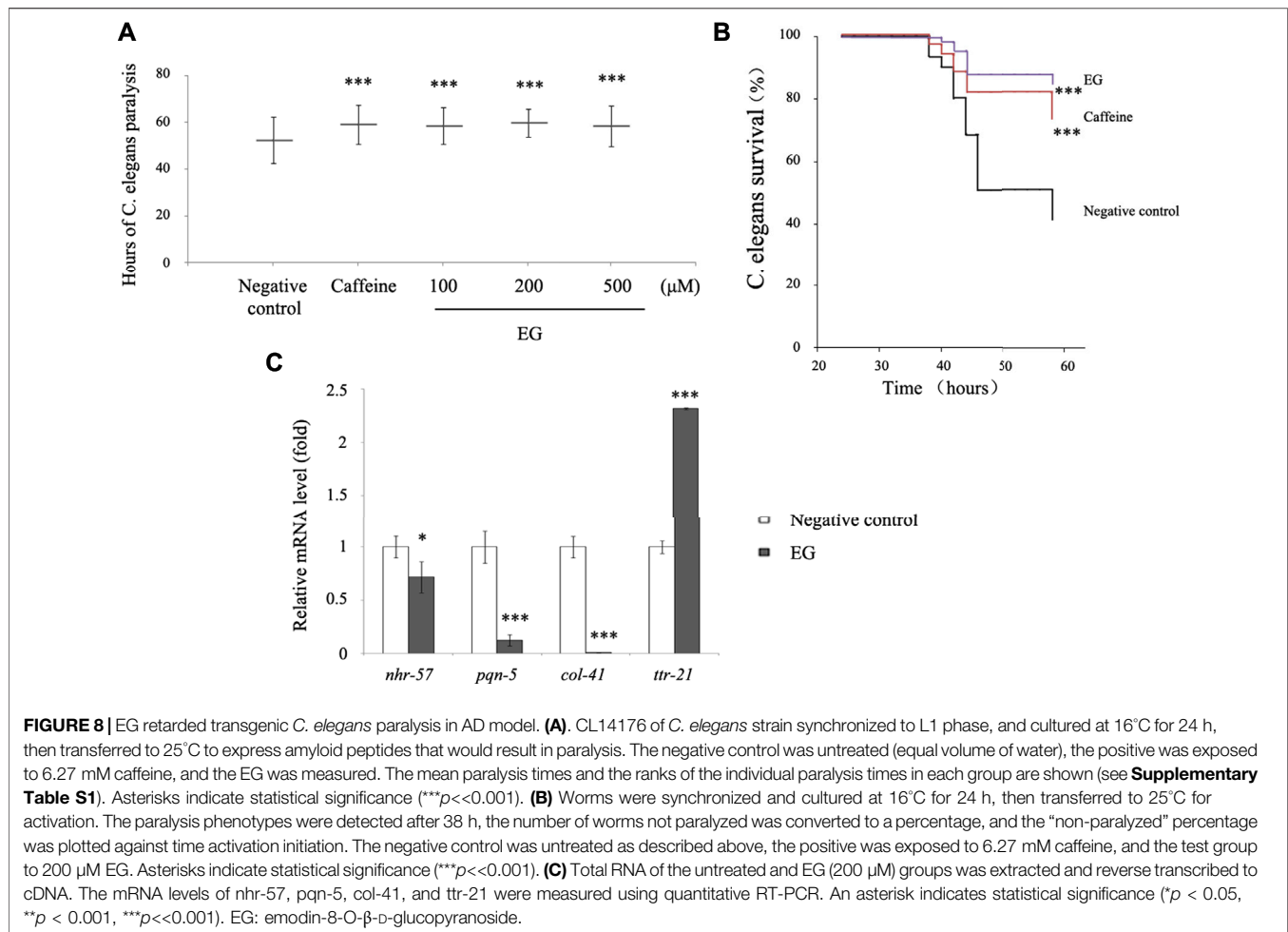
CCK-8, MTT, and LDH Assays

The PC12 cells were seeded in a 96-well plate at a 5 × 10³ density; 12 h after seeding, CP-94253 or EG was added with or without SB 224289. After 24 h of seeding, the amyloid peptides were added, and after 18 h, the cells were subjected to CCK-8 (catalog #C0037, Beyotime, China), MTT (catalog #M1025, Solarbio, Beijing, China), and LDH (catalog # BC0680, Solarbio, Beijing, China) assays following the manufacturers' instructions.

C. Elegans Assay for AD Protective Compounds

The *C. elegans* CL14176 strain was synchronized to the L1 phase and allocated to 160 μL S media with the appropriate treatment





drug concentrations. The worms were cultured at 16°C for 24 h and transferred to 25°C, at which point they were activated to express amyloid peptides that would result in paralysis (Dostal and Link, 2010). At 38 h, the worms were counted for the paralysis phenotype. Each group contained approximately 60 worms. The positive control group was set by adding 6.27 mM caffeine.

Statistical Analysis

Variables were compared between non-treated and treated groups using Student's *t*-tests. Statistical differences in protein expression, cell viability, and relative mRNA levels between two groups were analyzed using a One-way Analysis of variance (ANOVA). Data were analyzed using GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA, United States). Data are expressed as mean ± S.D. of values from at least three independent experiments. A value of $p < 0.05$ was considered statistically significant.

RESULTS

EG Targets 5-HT_{1B} Receptor

Molecular docking is widely used to identify specific molecular targets, such as GPCRs (Beuming et al., 2015; Kontoyianni, 2017).

We used this technique to determine whether EG binds to the human GPCR 5-HT_{1B}. The binding affinity of EG to 5-HT_{1B} was similar to that of the agonist CP-94253, as the binding energies were -8.09 and -8.48 kcal mol⁻¹, respectively. EG (Figures 1D,E) docked into the same 5-HT_{1B} pocket as CP-94253 (Figures 1A,B). In addition, EG (Figure 1F) interacted with 5-HT_{1B} similarly to the agonist CP-94253 (Figure 1C). The specific interaction parameters are shown in Supplementary Figure S1. The results suggested that EG might target 5-HT_{1B} and act similarly to CP-94253 as a ligand of this receptor.

To verify the interaction of EG with the 5-HT_{1B} receptor, we applied DARTS and CETSA, previously reported methods (Chang et al., 2016), for identifying target proteins of natural products without chemical modifications. Unlike that with the single target protein, the conformation and stabilization of the compound-target complex are altered in either DARTS or CETSA, and the interaction between the compound and target protein can be visualized via western blotting (Lomenick et al., 2009; Molina et al., 2013; Chang et al., 2016). In this study, DARTS was performed on the whole-cell lysate of cells stably induced to express 5-HT_{1B}. The proteins were digested with pronase, whereas EG, CP-94253, and dihydroergotamine (a 5-HT_{1B} agonist, FDA-approved for migraine) were used to prevent

target depletion. The 5-HT_{1B} protein in the band protected by EG (10⁻⁶ M) was enriched approximately 2-fold compared with that in the vehicle, and EG showed a similar protective effect with dihydroergotamine at a low concentration (10⁻⁸ M). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was resistant to pronase under this condition and served as a loading indicator (Figures 2A,B). The 5-HT_{1B} protein protected by EG, compared with CP-94253, from digestion is shown in Supplementary Figure S2A. CETSA was performed using intact cells induced to express 5-HT_{1B}. The dihydroergotamine- or vehicle-treated cells were heated to varying temperatures as indicated. The 5-HT_{1B} protein in the soluble fractions was separated from the precipitated destabilized protein and detected with western blotting (Figure 2C). Figure 2D shows ligand-target interaction plotted against temperature to display the shifts. The results revealed a physical interaction between the agonist dihydroergotamine and its target protein 5-HT_{1B} in intact cells. Additionally, EG-induced thermodynamic stabilization of 5-HT_{1B} was observed (Figures 2E,F). The 5-HT_{1B} protein thermodynamically stabilized by EG, compared with CP-94253, is shown in Supplementary Figure S2B.

Intramolecular FRET sensors have been used as real-time optical tools for GPCR ligand binding (Xu et al., 2012; Lohse et al., 2008). We successfully produced effective 5-HT_{1B} sensor constructs using previously described methods (Xu et al., 2012). The full-length human 5-HT_{1B} receptor was added to CFP in-frame with the C-terminal tail (Figures 3A,B), and the 12-amino acid sequence (FLNCCPGCCMEP) containing the fluorescein arsenical hairpin binder via tetra-cysteine (FlAsH) labeling sequence (CCPGCC) was inserted into the third intracellular loop of the receptor. In addition, it contained the VSV-G peptide epitope within the extracellular N-terminal domain (Figure 3B). Functionality of the 5-HT_{1B} sensor has been previously assessed in a study on ERK1/2 MAP kinase phosphorylation (p-ERK) (Xu et al., 2012). The full-length coding sequence of the human 5-HT_{1B} receptor without alterations to the receptor's intracellular segments was also cloned into the pcDNA5/FRT/TO vector described previously (Xu et al., 2012) and in the Methods section. Then, these constructs were transfected into Flp-InTM T-RExTM 293 cells to generate stable cell lines. In these cells, the doxycycline-induced expression of either the 5-HT_{1B} sensor or the full-length human 5-HT_{1B} was examined using an anti-VSV antibody. The 5-HT_{1B} sensor performed effectively in response to the selective 5-HT_{1B} agonist CP-94253, compared with the full-length human 5-HT_{1B}, at the p-ERK level (Figure 3C). The 5-HT_{1B} sensor was effectively delivered to the cell surface, on which its normal functions are based (Figure 3D). These results showed that the sensor was functionally similar and virtually identical to the wild-type receptor.

To study the effects of EG on the 5-HT_{1B} receptor, we imaged the intramolecular rearrangement of the 5-HT_{1B} sensor in response to EG, compared with CP-94253, by measuring emission from CFP and FlAsH. The normalized basal FRET signal in cells induced to express the 5-HT_{1B} sensor decreased immediately upon the addition of CP-94253, then rapidly restored agonist withdrawal in a concentration-dependent

manner. In contrast, addition of the 5-HT_{1B} antagonist SB 224289 (10⁻⁶ M) did not produce a significant alteration in sensor response beyond the vehicle effect, and, as anticipated, blocked the effect of CP-94253 (Figure 4A). This was similar to the results obtained using EG, although the FRET signal changed in the opposite direction to that of CP-94253 (Figure 4B). Moreover, the variability of FRET changes from EG was approximately 60% of CP-94253 (Figure 4C), illustrating that structure changes in the receptor triggered by binding of CP-94253 and EG were distinct in amplitude and distance, at least between the third intracellular loop and C-terminal, where CFP and FlAsH are located, respectively. 5-HT_{1B} has been previously shown to be internalized from the cell surface via agonist binding in a time-dependent manner (Janoshazi et al., 2007). Our findings revealed that EG induced 5-HT_{1B} internalization, similarly to CP-94253 (Figure 4D). These results demonstrated that EG could be a 5-HT_{1B} agonist.

EG Showed Selective Agonist Activity Toward 5-HT_{1B} Receptor Rather Than Other GPCRs

To further study the EG target, we used stable cell lines that were induced to overexpress serotonergic-related GPCRs, including the serotonin receptors 1B and 2A (5-HT_{1B} and 5-HT_{2A}), dopamine receptor 2 (D₂), melatonin receptor 2 (MT₂), and orexin receptor 2 (OX₂). In the stable cell lines that overexpressed 5-HT_{1B}, EG promoted the phosphorylation of ERK1/2 MAP kinases in a concentration-dependent manner. This activation through the 5-HT_{1B} receptor-mediated pathway was similar to the 5-HT_{1B} selective agonist, i.e., the positive control CP-94253 (Figure 5A). In addition, the 5-HT_{1B} selective antagonist SB224289 at 10⁻⁷ M inhibited the effect of the concentration-dependent promotion of the ERK1/2 MAP kinase phosphorylation by EG and CP-94253 activity (Figure 5B). However, no response to the EG of other 5-HT-related GPCRs activities, such as 5-HT_{2A}, D₂, MT₂, or OX₂, was observed (Figures 5C–F). These results showed that EG exhibited agonistic activity toward 5-HT_{1B} and could selectively activate the 5-HT_{1B}-mediated signaling pathway.

EG Alleviates A β -Induced Cell Mortality

A β -induced neuronal toxicity is an important pathology in AD, and there are two principal variants of amyloid peptides in humans, of which A β ₄₂ is more toxic than A β ₄₀ (Hamley, 2012). EG and its derivatives exhibit neural protection activity and can be used in AD treatment (Lin et al., 2015). We applied A β ₄₀ and A β ₄₂ to PC12 (with rat A β) and SH-SY5Y (with human A β) cell lines, respectively. The cells were subsequently assessed using the Cell Counting Kit-8 (CCK-8) for viability assay. Cell survival decreased with increased A β concentration in both PC12 (Figure 6A) and SH-SY5Y cells (Supplementary Figure S3). No significant differences in viability were observed in the treatments with <5 μ M A β ₄₂ or <10 μ M A β ₄₀. PC12 cell death was observed at 10 μ M A β ₄₂ and reached approximately 18% at 20 μ M, whereas cell death in the A β ₄₀ treatment group began at 20 μ M. At 50 μ M, 47% of the A β ₄₂-treated and 52% of the A β ₄₀-treated cells

survived. These results suggest that A β peptides dose-dependently induce neuronal toxicity, and support the hypothesis that A β ₄₂ is more toxic than A β ₄₀. To determine the effects of EG on neural cells, we pre-treated PC12 cells with or without EG, followed by exposure to the amyloid peptides A β ₄₀ and A β ₄₂, respectively. The results showed that EG could alleviate the reduction in A β ₄₀- and A β ₄₂-induced cell viability by approximately 8.22 and 8.64%, respectively (Figure 6B).

Reduction in A β -Induced Pro-Inflammatory TNF- α Signaling Through 5-HT_{1B} Pathway by EG

As EG could selectively activate 5-HT_{1B} signaling and alleviate A β -induced mortality, we further investigated the underlying mechanisms. Activation of 5-HT_{1B} by its agonist reduces the levels of pro-inflammatory TNF- α in rat nerve cells (Khalilzadeh et al., 2018). The inflammatory cascade is one of the most important processes in A β -induced toxicity, and the A β oligomer-elevated TNF- α signaling is a cause of memory loss in mice (Lourenco et al., 2013); thus, stimulation of 5-HT_{1B} may relieve A β -induced TNF- α signaling. The two amyloid peptides A β ₄₂ and A β ₄₀ induced the death of PC12 cells to a similar extent (Figure 6A). Therefore, we conducted the following experiments with A β ₄₂ because it exhibited neuronal toxicity at a lower concentration than A β ₄₀.

To evaluate TNF- α levels under 5-HT_{1B}-related conditions, we used quantitative RT-PCR to detect TNF- α mRNA levels in 5-HT_{1B} over-expressing stable PC12 cells (Supplementary Figure S4B) with A β ₄₂ and with or without 5-HT_{1B} activators and blockers (Figure 7A). PC12 cells have endogenous 5-HT_{1B} as well as 5-HT_{2A/3/6} receptor subtypes (Supplementary Figure S4A). We used 5-HT_{1B}-over-expressing stable PC12 cells to accentuate the effect of 5-HT_{1B}. Consistent with previous studies (Shrewsbury et al., 2008), our results showed that A β ₄₂ elevated TNF- α expression, which could be reversed by treatment with the 5-HT_{1B} agonist CP-94253 and dihydroergotamine and the novel 5-HT_{1B} activator EG. Moreover, the selective serotonin 5-HT_{1B} antagonist SB 224289 blocked EG, CP-94253, and dihydroergotamine-mediated TNF- α restraint under A β ₄₂ conditions. The 5-HT_{1B} activators alone could decrease TNF- α levels in the 5-HT_{1B} over-expressed PC12 cells, whereas the antagonist SB 224289 contributed to pro-inflammatory TNF- α signaling and was synergistic with A β ₄₂ (Figure 7B). These results suggested that EG resorted to the 5-HT_{1B} pathway to reduce A β -induced pro-inflammatory TNF- α signaling.

EG Eliminates the A β ₄₂-Induced Cytotoxic Effect via 5-HT_{1B} Pathway

The A β -induced inflammatory response and subsequent neuronal death are considered important events in AD pathology (Hamley, 2012; Unzeta et al., 2016). We found that the death of A β ₄₂-induced 5-HT_{1B} over-expressing stable PC12 cells was significantly reversed by the 5-HT_{1B} agonists and EG, as assessed via the CCK-8 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Figures 7C,D,

Supplementary Figure S5A). However, SB 224289 alone did not lead to significant cell death under the indicated conditions (10⁻⁷ M, 42 h, Supplementary Figure S6). EG and the 5-HT_{1B} agonists reduced the A β ₄₂-induced cytotoxicity that could be blocked by SB 224289 (Figure 7E, Supplementary Figure S5B).

EG Protection Against Morbidity in *C. Elegans* AD Model

To further explore the ability of EG to afford protection against amyloid peptides, we tested transgenic *C. elegans* for resistance to the induced A β expression. The *C. elegans* CL14176 strain (Link et al., 2003), which could be induced to express A β by modulating the temperature and leads to paralysis in 2–3 d, was used. This paralysis phenotype could serve as a measurement of A β toxicity to assay the effects of compounds protective against A β , using the paralysis delay to indicate the suppression of A β toxicity in the worm model (Dostal and Link, 2010). The results showed that EG at different concentrations retarded the paralysis time from 6 to 7 h compared with that for the untreated group (~52 h). The performance of the 200 μ M EG group was superior to that of the positive control caffeine group (Supplementary Table S1, Figure 8A). In this model, the survival rate of the EG group at 200 μ M was significantly prolonged (Figure 8B). The expression of immune response-related genes that are analogs of the inflammatory effectors in humans, including the nuclear receptor nhr-57, effectors of hypoxia pathway, pqn-5 (prion-like glutamine (Q)/asparagine (N) -5) gene, part of the unfolded protein response pathway that responded to endoplasmic reticulum stress, the collagen gene col-41, and transthyretin-like protein gene ttr-21, between the untreated and EG groups was observed via quantitative RT-PCR (Bellier et al., 2009; Sahu et al., 2012). The EG could significantly reduce the A β -induced expression of the immune response-related genes nhr-57, pqn-5, and col-41. However, it failed to suppress ttr-21 expression (Figure 8C), which suggested that EG protected the worm model from AD in a complex manner rather than arbitrarily suppressing all the immune response pathways.

DISCUSSION

The serotonin receptor 5-HT_{1B} is an important part of the serotonergic system, which regulates crucial processes of the CNS such as cognition, satiety, anxiety, depression, and sleep. 5-HT_{1B} has been considered a drug target for serotonergic system-related disorders (Gadgaard and Jensen, 2020; Monti and Jantos, 2008; Tiger et al., 2018). We identified 5-HT_{1B} as a target of EG, an active ingredient in several Chinese medicinal plants with antidepressant and neuroprotective effects, by using molecular docking, DARTS, and CETSA. Molecular docking provided a structural basis for EG recognition in 5-HT_{1B}. Additionally, EG docked into the same 5-HT_{1B} pocket as CP-94253, a 5-HT_{1B} agonist (Figure 1). DARTS allows for the determination of the direct binding of natural products and their extracts to their protein targets. As the conformation and stabilization of the compound-target complex, compared with those of the control protein target, are altered by protease-induced digestion, the compound and target

protein interactions can be visualized using western blotting (Chang et al., 2016). The CETSA method can detect the physical interaction between a ligand and target protein in intact cells. The thermodynamic stability of target proteins in drug- or vehicle-treated intact cells varies over changing temperatures, and the target proteins can be monitored using western blotting (Chang et al., 2016). The DARTS results showed that EG could protect the 5-HT_{1B} protein from pronase digestion, compared with 5-HT_{1B} agonists dihydroergotamine (Figures 2A,B) and CP-94253 (Supplementary Figure S2A). In addition, EG promoted thermodynamic stabilization of the 5-HT_{1B} protein compared with dihydroergotamine (Figures 2C–F) and CP-94253 (Supplementary Figure S2B). These methods can also be used to identify other 5-HT_{1B} agonists.

We further studied the structural changes in the 5-HT_{1B} receptor after EG binding using FRET. Intramolecular FRET sensors provide real-time optical evidence for GPCR ligand binding and activation kinetics and can be used as screening tools for molecular ligands toward a particular receptor (Lohse et al., 2008; Xu et al., 2012). We produced an effective 5-HT_{1B} sensor (Figure 4) and detected EG as a ligand with 5-HT_{1B} (Figure 5). However, the changes in FRET signals during treatment with CP-94253 (Figure 4A) and EG (Figure 4B) were in opposite directions, demonstrating that binding of CP-94253 closed the distance between the third intracellular loop and C-terminal of the 5-HT_{1B} receptor, whereas EG extended the distance. Additionally, we found that 5-HT_{1B} could be induced to internalization by agonist CP-94253 and EG (Figure 5), suggesting that EG might be an agonist of 5-HT_{1B}. Selective activation of the 5-HT_{1B}-mediated ERK1/2 signaling pathway (Figure 6) by EG established its agonist activity toward 5-HT_{1B}. Together, the intramolecular FRET 5-HT_{1B} sensor and the subsequent tests of 5-HT_{1B}-induced ERK1/2 signaling activation provide a strong evidence that 5-HT_{1B} is a target of and is activated by EG. Further biological functions of EG targeting and 5-HT_{1B} stimulation need to be elucidated.

Overall, activation of central 5-HT_{1B} may have antidepressant (Sanchez et al., 2015), anti-aggression (de Almeida et al., 2001), and antinociceptive effects (Labastida-Ramírez et al., 2020). The therapeutic use of those herbal antidepressants that contain EG has been suggested for the treatment of AD (Jeon et al., 2019; Chen et al., 2020; Li et al., 2020). In this study, the 5-HT_{1B}-mediated ERK1/2 phosphorylation by EG and the antifungal (Qi et al., 2005) as well as acetylcholinesterase I (AChE I)-inhibiting (Lin et al., 2015) activities of EG led us to investigate the EG and 5-HT_{1B} interaction in the context of AD. Activation of ERK1/2 signaling is related to the inhibition of A β -induced apoptosis (Galvão et al., 2019). Additionally, a series of interesting studies showed that A β might act as an antimicrobial peptide, thereby protecting the CNS from infections in mouse models and innate immunity in worms, whereas dysregulated A β leads to AD pathology (Kumar et al., 2016). AChE I has been used as a biomarker in AD diagnosis, and AChE I inhibitors have been clinically used for AD treatment (Lin et al., 2015). Both the classic 5-HT_{1B} agonists (CP-94253 and dihydroergotamine) and EG could singly suppress the expression of pro-inflammatory TNF- α and correspondingly improve the survival rates of 5-HT_{1B} over-expressing stable PC12 cells compared with untreated negative control (Figures 7B,C,

Supplementary Figure S5A, B). This result might agree with the finding that activated serotonergic pathways promote proliferation (Chilmonczyk et al., 2017). The data in wild-type PC12 cells pointed toward the same trend, although not as significant as those in the 5-HT_{1B} over-expressing stable PC12 cells. EG alleviated A β ₄₂-induced cell death and increased cell viability from approximately 82.71–89.86% (Figure 6B), whereas in 5-HT_{1B} over-expressing stable PC12 cells, the increase was from 84.06 to 109.71% (Figure 7C). In addition, PC12 cells possessed some endogenous serotonin receptor subtypes, including 5-HT_{1B}, 5-HT_{2A}, 5-HT₃, and 5-HT₆ (Supplementary Figure S4A), that could explain why the wild-type PC12 cells also responded to the classic 5-HT_{1B} agonists (data not shown) and EG in reducing TNF- α levels and were pro-survival. Cell viability in the EG treatment (in A β ₄₂ conditions) was greater than that in the untreated control, indicating the protective effects of EG via the 5-HT_{1B} pathway.

This study provides evidence that 5-HT_{1B} receptor is one of the targets for compounds in Chinese medicinal plant ingredients with cognitive deficit attenuating and antidepressant effects in the treatment of AD. Molecular docking, DARTS, CETSA, FRET, and ERK1/2 phosphorylation tests are all relatively economic, fast, and accurate methods. Further biological functions of the compound targeting 5-HT_{1B} was tested with AD models in cells and worms. However, a variety of herbal compounds are reported to possess cognitive deficit attenuating and antidepressant effects, and the ability of these compounds to target and activate the 5-HT_{1B} still needs to be investigated. Interestingly, caffeine, also a herbal ingredient used as positive control in the worm experiments, was also docked into the 5-HT_{1B} receptor, similar with that of EG, CP94253, and dihydroergotamine (data not shown). These results suggest that caffeine might also be an agonist toward 5-HT_{1B}, and support the 5-HT_{1B} agonists' potential for treatment of AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was funded by The Fund of Yunnan Basic Research Program (202001AS070024 and 202001AT0700) and conducted at the University Based Provincial Key Laboratory of Screening and Utilization of Targeted Drugs of Yunnan.

ACKNOWLEDGMENTS

The transgenic *C. elegans* CL14176 strain was a gift from Ping Yang at the Shanghai Research Center For Model Organisms, Pudong New Area, Shanghai, China.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2021.735876/full#supplementary-material>

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