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Physical Interaction of Jab1 with Human Serotonin 6 G-protein-coupled Receptor and Their Possible Roles in Cell Survival*[S]

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The 5-HT$_6$ receptor (5-HT$_6$R) is one of the most recently cloned serotonin receptors, and it plays important roles in Alzheimer disease, depression, and learning and memory disorders. However, unlike the other serotonin receptors, the cellular mechanisms of 5-HT$_6$R are poorly elucidated relative to its significance in human brain diseases. Here, using a yeast two-hybrid assay, we found that the human 5-HT$_6$R interacts with Jun activation domain-binding protein-1 (Jab1). We also confirmed a physical interaction between 5-HT$_6$R and Jab1 using glutathione *S*-transferase pulldown, fluorescence resonance energy transfer, co-immunoprecipitation, and immunocyto(histo)-chemistry assays. The manipulation of Jab1 expression using Jab1 small interference RNA decreased 5-HT$_6$R-mediated activity and cell membrane expression of 5-HT$_6$R, whereas overexpression of Jab1 produced no significant effect. In addition, we demonstrated that the activation of 5-HT$_6$R induced the translocation of Jab1 into the nucleus and increased c-Jun phosphorylation and the interaction between Jab1 and c-Jun. Furthermore, we found that 5-HT$_6$R and Jab1 were up-regulated in middle cerebral artery occlusion-induced focal cerebral ischemic rats and in cultured cells exposed to hypoxic insults, suggesting possible protective roles for 5-HT$_6$R and Jab1. These findings suggest that Jab1 provides a novel signal transduction pathway for 5-HT$_6$R and may play an important role in 5-HT$_6$R-mediated behavior changes in the brain.

Neurotransmitters and neurohormones regulate diverse and myriad brain functions ranging from rapid modulation of ligand-gated ion channels to long term modulation such as gene expression, behavior, and mood changes. Serotonin (5-HT)[2] is known to be one of the key neurotransmitters related to mood changes. The serotonergic system has also been implicated in the neurobiological control of learning and memory. Therefore, it emerges as a key player in affective, cognitive, complex sensory, and motor functions (1). 5-HT mediates its diverse physiological responses through seven distinct receptor families: the 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, and 5-HT$_7$ receptors. With the exception of the 5-HT$_3$ receptor, all of these receptors are members of the G-protein-coupled receptor (GPCR) superfamily (2). Among the 5-HT receptors, the 5-HT$_6$ receptor (5-HT$_6$R) is coupled to a stimulatory Gα (Gα_S) protein. This receptor increases cAMP formation and then activates cAMP-dependent protein kinase (3). 5-HT$_6$R is abundantly distributed in the brain, especially in the limbic region (4), and it has a high affinity for antipsychotic compounds and tricyclic antidepressants (5). These preliminary reports imply that 5-HT$_6$R has a significant role in the control of mood and emotion in the central nervous system. To date, most findings suggest that 5-HT$_6$R plays crucial roles in neurological disorders, including Alzheimer disease, depression, and learning and memory disorders (6–9). Selective antagonists of 5-HT$_6$R improved memory and cognition in aged rats (10) and in Alzheimer disease patients (11). In an effort to elucidate possible underlying mechanisms of 5-HT$_6$R antagonist-mediated cognition enhancement, microdialysis studies have shown that a 5-HT$_6$R blockade elevates cholinergic or glutaminergic neurotransmission in the brain (12–14). However, the role of 5-HT$_6$R is not clearly defined in the context of depression due to contradictory results. Although it has been reported that selective 5-HT$_6$R antagonists produced antidepressant-like effects (15, 16), Svenningsson *et al.* reported that the 5-HT$_6$R agonist 2-ethyl-5-methoxy-*N*,*N*-dimethyltryptamine induced antidepressant-like biochemical and behavioral effects (9). Alzheimer disease, depression, and learning and memory disorders are important severe neurological diseases that cause extensive human suffering. Therefore, it is very important to elucidate

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[2] The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); CHO/5-HT$_6$R, Chinese hamster ovary cells stably expressing human 5-HT$_6$R; CT, C terminus of 5-HT$_6$R; CT-Jab1, C-terminal part of Jab1; iL2, intracellular loop 2 of 5-HT$_6$R; iL3, intracellular loop 3 of 5-HT$_6$R; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; GST, glutathione *S*-transferase; HEK/HA-5-HT$_6$R, HEK293 cells stably expressing human HA-tagged 5-HT$_6$R; HIF-1α, hypoxia-inducible factor-1α; Jab1, Jun activation domain-binding protein-1; MCAO, middle cerebral artery occlusion; NT-Jab1, N-terminal part of Jab1; OGD, oxygen-glucose deprivation; CSN, COP9 signalosome; siRNA, small interference RNA; HA, hemagglutinin; CMV, cytomegalovirus; eYFP, enhanced yellow fluorescent protein; eGFP, enhanced green fluorescent protein; eCYP, enhanced cyan fluorescent protein; PBS, phosphate-buffered saline; rLHR, rat lutropin/choriogonadotropin receptor; PAR-2, protease-activated receptor-2.

the mechanisms responsible for 5-HT$_6$R-mediated cognition and mood changes in the brain. However, the cellular mechanisms of 5-HT$_6$R-mediated signal pathways are not well explored except the common Gα_S-protein-mediated PKA pathway.

Jun activation domain-binding protein-1 (Jab1) was initially identified as a protein that interacts with c-Jun and stimulates the binding of c-Jun and JunD to AP-1 sites, potentiating them as transcription factors (17). Jab1 is also known as CSN5, which is the fifth member of the COP9 signalosome (CSN) complex consisting of eight subunits, CSN1–CSN8 (18). Jabl exists and functions both as a monomer and as a subunit of the CSN complex. Although Jab1 has been shown to be a key player in plant light signaling, development, cell cycle control, and stability of various proteins in a number of systems (see reviews in Refs. 19, 20), the specific role of Jab1 and its cellular mechanism in the central nervous system still remain obscure.

In this study, we discovered a novel interaction between Gα_S-family GPCR 5-HT$_6$R and Jab1, and we observed Jab1-mediated modulation of the membrane expression and activity of 5-HT$_6$R. In addition, we found that 5-HT$_6$R affects the cytosol/nuclear distribution of Jab1 as well as the interaction between Jab1 and c-Jun, a target protein downstream of Jab1. Furthermore, we demonstrated that 5-HT$_6$R and Jab1 play important roles under conditions of *in vitro* hypoxia and *in vivo* cerebral ischemia.

MATERIALS AND METHODS

Antibodies and Reagents—Anti-HA, -Myc, -β-tubulin, -histone H3, -c-Jun, and -*p*-c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-5-HT$_6$R was generated by Lab Frontier Co. (Seoul, Korea) or purchased from GeneTex Inc. (San Antonio, TX). Sources for other antibodies were as follows: FLAG (Sigma), GST (Novagen, Madison, WI), His$_6$ (Roche Diagnostics GmbH, Mannheim, Germany), and Jab1 (Novus Biologicals, Littleton, CO). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA) and Abcam (Cambridge, UK). Protease inhibitor mixture was from BioVision (Mountain View, CA). 5-HT, $CoCl_2$, cycloheximide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma. Diaminobenzidine was from Roche Applied Science. The ECL kit was from iNtron Biotechnology (Seoul, Korea) and Millipore (Bedford, MA). Fluo-4-AM, Pluronic F-127, and WGA, Alexa Fluor 350 conjugate were from Molecular Probes (Eugene, OR).

Plasmid Constructs and siRNAs—Human 5-HT$_6$R and HA-tagged human 5-HT$_6$R cDNAs were purchased from UMR cDNA Resource Center (Miner Circle Rolla, MO). The Myc-tagged 5-HT$_6$R plasmid was constructed by recloning the human 5-HT$_6$R cDNA into the EcoRI/XhoI sites of the pCMV-Tag 3B vector. 5-HT$_6$R-eGFP was subcloned (Fw, 5′-CCG-CTCGAGATGGTCCCAGAGCC-3′; Rv, 5′-CCGGAATTCG-GTTCGTGGGGATGCC-3′) into the XhoI/EcoRI sites of pEGFP-N1 (BD Biosciences, Clontech, Palo Alto, CA). 5-HT$_6$R-eYFP was recloned from 5-HT$_6$R-eGFP into the XhoI/EcoRI sites of pEYFP-N1 (Clontech). cDNA fragments encoding the iL2, iL3, and CT domains of 5-HT$_6$R were subcloned into the EcoRI/BamHI sites of the Gal4 activation domain vector pGBKT7 (Clontech) with the following sets of primers: iL2 (Fw, 5′-CCGGAATTCATGGACCGCTACCTG-3′; Rv, 5′-CGGGATCCGCAGGGCACGCAG-3′), iL3 (Fw, 5′-CCGGA-ATTCATGTGCAGGATCCTG-3′; Rv, 5′-CGGGATCCGCT-TCAGGGCCTTC-3′), and CT (Fw, 5′-CCGGAATTCATGC-CACTCTTCATG-3′; Rv, 5′-CGCGGATCCTCAGTTC-GTGGG-3′).

GST-iL2, -iL3, and -CT were kindly provided by Dr. Yu (Kookmin University, Korea). FLAG-Jab1, GST-Jab1, and eYFP-Jab1 were kindly provided by Drs. J. Song (Sungkyunkwan University, Korea) and K. S. Kwon (Korea Research Institute of Bioscience and Biotechnology). eCFP-Jab1 was recloned from eYFP-Jab1 into the XhoI/BamHI sites of pECFP-C1 (Clontech). His-Jab1 was recloned from GST-Jab1 into the BamHI/EcoRI sites of pET-28a(+) (Novagen, Madison, WI). His-NT-Jab1 (Fw, 5′-CGCGGATCCATGGCAGCTTCCG-3′; Rv, 5′-CCGGAATTCTCAATCAATCCCGGAGAG-3′) and His-CT-Jab1 (Fw, 5′-CGCGGATCCGTTAGTACACAGA-TGC-3′; Rv, 5′-CCGGAATTCCTAAGCAACGTTAATCTG-3′) were constructed by cloning from His-Jab1 into the BamHI/EcoRI sites of the pET-28a(+) vector (Novagen).

siRNA sequences were designed using siRNA target finder software (Turbo si-Designer, Bioneer, Korea), and two single-strand RNA oligonucleotides were chemically synthesized and annealed to form the siRNA duplex. The sequences of siRNA for *5-HT$_6$R* and *Jab1* genes were as follows: 5-HT$_6$R (sense, 5′-CUGUAACAGCACCAUGAACdTdT-3′; antisense, 5′-GUUC-AUGGUGCUGUUACAGdTdT-3′), Jab1 (sense, 5′-CUACAAA-CCUCCUGAUGAAdTdT-3′; antisense, 5′-UUCAUCAGGAG-GUUUGUAGdTdT-3′; or sense, 5′-GCUCAGAGUAUCGAUG-AAAdTdT-3′; antisense, 5′-UUUCAUCGAUACUCUGAGCd-TdT-3′), and negative control siRNA (sense, 5′-CCUACGCCAC-CAAUUUCGUdTdT-3′; antisense; 5′-ACGAAAUUGG-UGGCGUAGGdTdT-3′).

Yeast Two-hybrid Screening—The bait plasmids, pGBKT7/iL2, iL3, or CT of 5-HT$_6$R, were stably expressed in yeast strain AH109 and did not display a self-transcriptional activity. The prey plasmid, human brain cDNA library/pACT2, was transformed into yeast strain Y187. All yeast two-hybrid screening was performed as described previously (21).

GST Pulldown Assays—GST, GST-tagged proteins, and His-tagged proteins were transformed into BL21 (DE3) and induced by adding 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C during the midlog phase. The cells were sonicated in lysis buffer (1× PBS, pH 7.4, 1 mM dithiothreitol, 0.01% Triton X-100, and protease inhibitor mixture). All His-tagged proteins formed inclusion bodies except GST and GST-tagged protein. The inclusion bodies were isolated and dissolved as previously described (22). After GST, the GST-tagged proteins and the His-tagged proteins were purified and GST pulldown assays were performed using the Profound Pulldown GST Protein:Protein Interaction kit (Pierce). In detail, after GST, GST-iL2, GST-iL3, and GST-CT were expressed in *Escherichia coli*, the proteins were immobilized on glutathione gel, and purified His-tagged Jab1 was incubated with the glutathione gel bound to GST or GST-tagged proteins. GST pulldown assays using CHO/K1 cells and rat brain lysates were performed as described previously (21).

Cell Culture and Transfection—HEK393 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10%

fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. HEK293 cell lines stably expressing HA-5-HT$_6$R (HEK/HA-5-HT$_6$R) were selected with 800 μg/ml G-418 and maintained with 400 μg/ml G-418. CHO cells stably expressing 5-HT$_6$R (CHO/5-HT$_6$R) cells were kindly provided by Dr. M. Teitler (Albany Medical College, Albany, NY), and the cells were maintained with 400 μg/ml G-418. Cultured hippocampal neurons were dissected from 16- to 18-day fetal Sprague-Dawley rats and dissociated using trypsin and trituration through a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in Neurobasal/B27 medium (Invitrogen) containing 0.5 mM L-glutamine, 25 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. Experiments were carried out on neurons after 14 days *in vitro*. For transient transfection, cells were transfected using Lipofectamine 2000 (Invitrogen).

FRET Microscopy—HEK293 cells were transfected with eCFP-Jab1 (donor) and 5-HT$_6$R-eYFP (acceptor), fixed 24 h after transfection in 4% paraformaldehyde and PBS for 20 min at room temperature, and then washed three times in PBS. The coverslips were mounted on slides using CRYSTAL/MOUNT™ (Biomeda Corp., Foster City, CA) and examined under Olympus FluoView FV1000 confocal microscope (Tokyo). A fluorescence resonance energy transfer (FRET) signal is defined as the light emitted by 5-HT$_6$R-eYFP at 535–565 nm in response to the light emitted at 480–495 nm by eCFP-Jab1. The background FRET signal was detected when eYFP and eCFP vectors were expressed. FRET signal was also detected by the acceptor photobleaching method as previously reported (23, 24). This method measures the release of donor quenching after acceptor photobleaching, thus providing a measurement of close co-localization of the two fusion proteins.

Co-immunoprecipitation—HEK293 cells were gently lysed with lysis buffer (1× PBS, pH 7.4, 10 mM NaF, 5 mM dithiothreitol, 0.5% Triton X-100, 0.5% Nonidet P-40, and protease inhibitor mixture) for 30 min on ice and then centrifuged at 20,000 × *g* and 4 °C for 15 min. The supernatant was then collected. Rat brain was homogenized in lysis buffer added to 0.32 mM sucrose. Cell and brain lysates were precleared with 50 μl of ImmunoPure immobilized Protein G Plus (Pierce) for 2 h, and the precleared lysates were incubated with 2 μg of each specific antibody overnight at 4 °C. The lysates were then were incubated with 50 μl of ImmunoPure immobilized Protein G Plus for 4 h at 4 °C and were washed six times. Immune complexes were eluted by boiling for 10 min at 95 °C in SDS sample buffer, followed by immunoblotting.

Immunocyto(histo)chemistry—The immunocytochemistry procedure was performed as described previously (21). In detail, HEK293 or CHO/K1 cells were cultured and fixed with 4% paraformaldehyde in PBS 24 h after transfection. Cultured hippocampal neurons were grown until 14 days *in vitro* and then fixed with 4% paraformaldehyde in PBS. For immunohistochemistry, adult male Sprague-Dawley rats were perfused with saline and then fixed with 4% paraformaldehyde in PBS. Brains were sectioned at 10 μm in a cryostat at −20 °C. Sections were collected on a coated glass slide and dried at room temperature before being returned to −70 °C for storage. Slides were post-fixed with acetone for 30 min on ice.

Assay of 5-HT$_6$R Activity Using an FDSS6000 System—5-HT$_6$R activity was measured using an FDSS6000 96-well fluorescence plate reader (Hamamatsu Photonics, Japan) as previously described (21, 25). Briefly, HEK293 cells were transiently transfected with human 5-HT$_6$R and Gα_{15} protein using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were seeded into 96-well black wall clear-bottom plates, and 5-HT$_6$R activity was measured the next day. After the cells were loaded with 5 μM Fluo-4/AM and 0.001% Pluronic F-127 for 60 min at 37 °C in an HEPES-buffered solution (115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 20 mM HEPES, and 13.8 mM glucose, pH 7.4), cells were assayed with the FDSS6000 system. After determination of a short baseline, 10 μM or one of various indicated doses of 5-HT was added to HEK293 cells, and the Ca^{2+} response was measured at 480 nm. All data were collected and analyzed using the FDSS6000 system and related software (Hamamatsu Photonics).

Nuclear/Cytoplasmic Fractionation—After CHO/5-HT$_6$R cells were treated with 5-HT in serum-free Dulbecco's modified Eagle's medium, the cells were harvested by centrifugation at 600 × *g* for 5 min at 4 °C. Nuclear and cytoplasmic fractions were separated using the Nuclear/Cytosol Fractionation Kit (BioVision), following the manufacturer's protocol.

Transient Focal Cerebral Ischemia—Sprague-Dawley rats weighing 250–300 g were used for a rat model of focal cerebral ischemia. The animals were anesthetized by inhalation of 1.5% isoflurane and were submitted to 2 h of ischemia by occlusion of the middle cerebral artery with intraluminal Mononylon 4.0 sutures introduced through the internal cervical carotid artery form. After 2 h of MCAO, the intraluminal filament was withdrawn from the internal cervical carotid artery to allow reperfusion for 1 day.

Hypoxia Conditions—HEK293 cells and HEK/HA-5-HT$_6$R cells were exposed to various doses of $CoCl_2$ or oxygen-glucose deprivation (OGD) as described previously (26) with a minor modification. Briefly, the cells were washed two times with a glucose-free HEPES-buffered solution and then immersed in deoxygenated glucose-free HEPES-buffered solution bubbled with nitrogen gas for 30 min to remove residual oxygen. The cells were transferred to an anaerobic chamber (Billups-Rothenberg Inc., Del Mar, CA), exposed to the anaerobic gas mixture (5% CO_2, 10% H_2, and 85% N_2), and maintained at 37 °C for 6 h. After incubation, the cells were removed from the anaerobic chamber, and the media was replaced with normal Dulbecco's modified Eagle's medium-based culture media. Cells were allowed to recover for 12 h before being analyzed.

Cell viability was measured by the detection of dehydrogenase activity retained in living cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (50 μl, 5 mg/ml) was added to the cells, and the formazan was dissolved by adding DMSO after the media was removed. The optical densities were measured at 570 nm.

Statistical Analysis—The optical intensity was measured using the AlphaEase program (Version 5.1, Alpha Innotech, San Leandro, CA) and was analyzed using the Prism Version 4

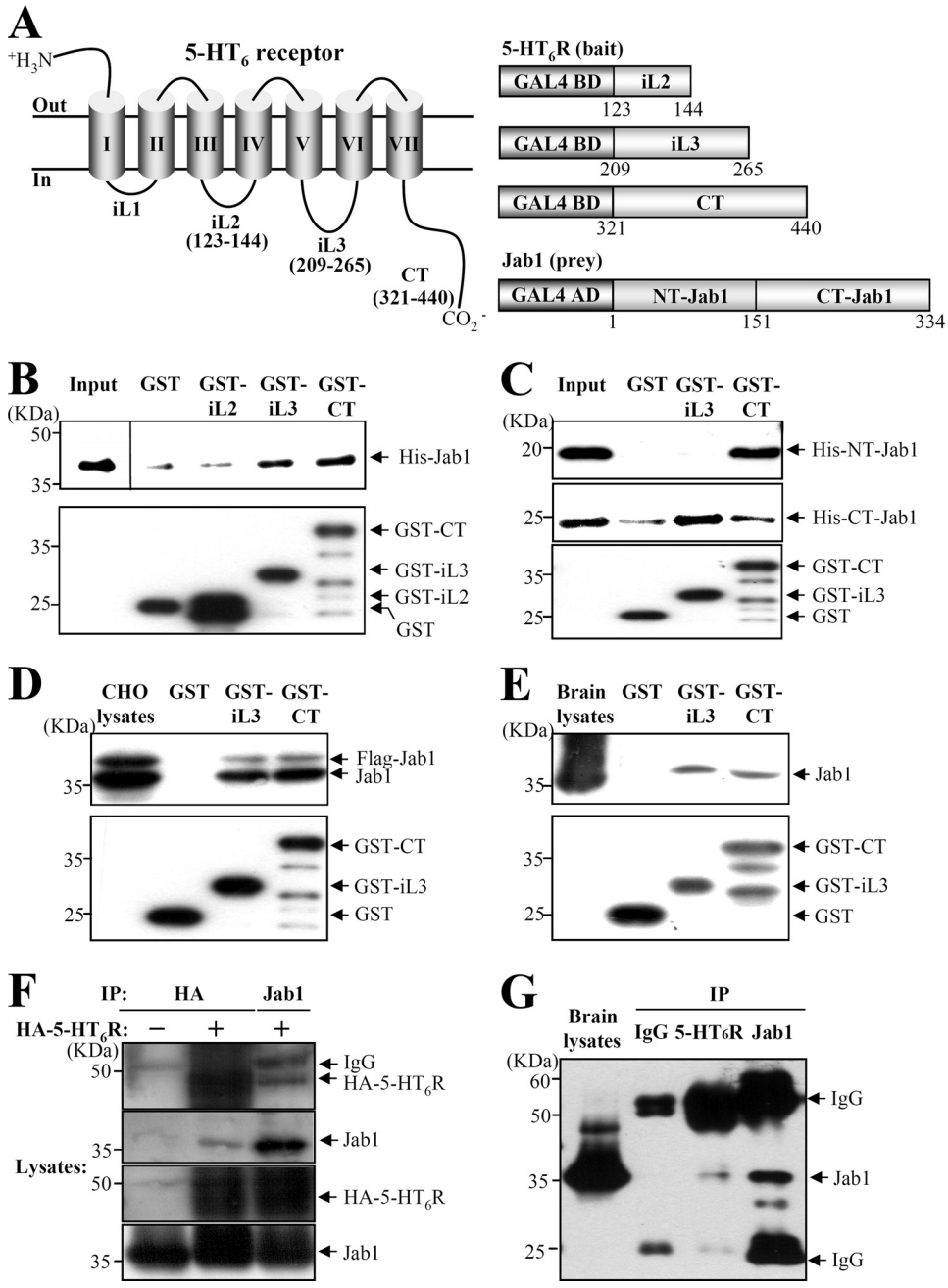


FIGURE 1. **Specific interaction between Jab1 and human 5-HT$_6$R identified using GST pulldown and co-immunoprecipitation assays.** *A*, *left*, schematic diagrams showing Gα_s-family GPCR 5-HT$_6$R characterized by seven helical transmembrane domains; *right*, schematic diagrams showing iL2 (intracellular loop 2), iL3 (intracellular loop 3), and CT (C terminus) of 5-HT$_6$R as bait and Jab1 protein as prey. *NT-Jab1* and *CT-Jab1* represent two fragments of Jab1, amino acids 1–151 and 152–334, respectively. *B*, immobilized GST, GST-iL2, GST-iL3, or GST-CT was incubated with purified full-length His-tagged Jab1, and then the retained proteins were analyzed with anti-His$_6$ or anti-GST antibodies. *C*, immobilized GST, GST-iL3, or GST-CT was incubated with NT-Jab1 or CT-Jab1, and then the retained proteins were analyzed with anti-His$_6$ or anti-GST antibodies. *D*, CHO/K-1 cells were transfected with FLAG-Jab1. After 24 h, the cells were lysed, and the lysates were incubated with immobilized GST, GST-iL3, or GST-CT. The retained proteins were analyzed by immunoblotting with anti-Jab1 or anti-GST antibodies. *E*, purified GST, GST-iL3, or GST-CT was immobilized and incubated with whole rat brain lysates. The retained proteins were detected with anti-Jab1 or anti-GST antibodies. *F*, HEK293 cells were transfected with HA-5-HT$_6$R. After 24 h, immunoprecipitation was carried out using anti-HA or anti-Jab1 antibodies. The immune complexes were then analyzed by immunoblotting with anti-HA or anti-Jab1 antibodies. Proper expression of transiently transfected HA-5-HT$_6$R and endogenous Jab1 in cell lysates was identified with specific antibodies. *G*, the rat brain lysates were immunoprecipitated with anti-rabbit IgG, anti-5-HT$_6$R, and anti-Jab1 antibodies. The immune complexes and the brain lysates were analyzed by immunoblotting with anti-Jab1 antibodies.

program (GraphPad Software Inc., San Diego, CA). All numeric values are represented as the mean ± S.E. The statistical significance of the data was determined using a Student's unpaired *t* test.

RESULTS

Jab1 Specifically Interacts with the Human 5-HT$_6$ Receptor—We previously demonstrated that the C-terminal region of human 5-HT$_6$R interacts with the Fyn tyrosine kinase. We also characterized the signaling pathways downstream of 5-HT$_6$R activation via a direct interaction with Fyn (21). In the present study, we found a new binding protein, Jab1, that bound to 5-HT$_6$R in a yeast two-hybrid screening assay using a human brain cDNA library. We found that Jab1 binds to both the intracellular loop 3 (iL3) and the C-terminal (CT) region of human 5-HT$_6$R based on the yeast two-hybrid screening assay (Fig. 1*A*). To verify a specific interaction between Jab1 and 5-HT$_6$R, we attempted to determine whether Jab1 selectively binds to 5-HT$_6$R using a GST pulldown assay. As shown in Fig. 1*B*, His-tagged Jab1 specifically interacted with GST-iL3 or GST-CT, which contain the iL3 and CT regions of 5-HT$_6$R, respectively, but Jab1 did not interact with GST alone or with GST-iL2 (intracellular loop 2 of 5-HT$_6$R). We then sought to identify the specific residues within Jab1 that are responsible for binding to 5-HT$_6$R. After Jab1 was arbitrarily divided into two constructs based on amino acid size (the N-terminal portion of Jab1, NT-Jab1: amino acids 1–151 and the C-terminal portion of Jab1, CT-Jab1: amino acids 152–334, Fig. 1*A*), and GST pulldown assays were again performed using His-tagged NT-Jab1 or His-tagged CT-Jab1. The Jab1 fragments were detected with anti-His antibodies. As shown in Fig. 1*C*, we found that CT-Jab1 and NT-Jab1 selectively interacted with GST-iL3 and GST-CT of 5-HT$_6$R, respectively. These GST pulldown results were also confirmed in a

mammalian system using CHO/K-1 cells transiently transfected with FLAG-tagged Jab1 and in rat brain lysates. Endogenous and full-length exogenous Jab1 from CHO/K-1 cells (Fig. 1*D*) and endogenous Jab1 from rat brain (Fig. 1*E*) bound to both GST-iL3 and GST-CT of 5-HT$_6$R, whereas no signal was detected using GST alone.

To show the relevance of the specific binding of Jab1 to 5-HT$_6$R, it is necessary to demonstrate the association of the two full-length proteins in mammalian cells using an *in vivo* co-immunoprecipitation assay. After HA-tagged full-length 5-HT$_6$R was transiently transfected into HEK293 cells, cell lysates were prepared, immunoprecipitated with anti-HA antibodies, and subsequently immunoblotted with anti-Jab1 antibodies. As shown in Fig. 1*F*, the transfected HA-5-HT$_6$R was able to bind to endogenous Jab1 (the *middle lane* of the *second row*) in HEK293 cells, whereas no signal was detected from nontransfected cells. When co-immunoprecipitation was performed in reverse with anti-Jab1 antibodies, followed by immunoblotting with anti-HA antibodies, the same result was obtained (the *last lane* of the *first row* in Fig. 1*F*). We also detected the association of 5-HT$_6$R and Jab1 in native tissues using rat brain lysates. As shown in Fig. 1*G*, endogenous 5-HT$_6$R selectively binds to endogenous Jab1 (*third lane*) in the rat brain, whereas no signal was detected in immunoprecipitates using control IgG antibody (*second lane*). These results strongly demonstrate that Jab1 selectively interacts with human 5-HT$_6$R in mammalian cell lines and in native tissues.

Jab1 Co-localizes with 5-HT$_6$ Receptors in Mammalian Cell Lines and in Rat Brains—FRET has been used as a spectroscopic ruler to measure molecular proximity (27). Monitoring energy transfer between versatile green fluorescent proteins has frequently been used to detect molecular interactions between proteins. We therefore utilized FRET techniques using enhanced cyan fluorescence protein (eCFP)-tagged Jab1 (eCFP-Jab1) and enhanced yellow fluorescence protein (eYFP)-tagged 5-HT$_6$R (5-HT$_6$R-eYFP). When eCFP and eYFP control constructs were co-expressed in HEK293 cells and imaged, no FRET signal was observed (Fig. 2*A*). However, when eCFP-Jab1 and 5-HT$_6$R-eYFP were co-expressed and imaged under the same experimental conditions, excitation of the eCFP component of Jab1 allowed detection of eYFP with FRET, which is represented by pseudocolor images (the *last column* in Fig. 2*A*). This FRET-based molecular proximity was also confirmed using an acceptor photobleaching method (23, 24). If eCFP and eYFP are in close proximity, the donor (eCFP) fluorescence should increase in the region where the acceptor (eYFP) has been bleached. After bleaching eYFP-5-HT$_6$R, we observed increased fluorescence of eCFP-Jab1 in the region of acceptor bleaching when compared with the pre-bleaching images (Fig. 2*B* and supplemental Fig. S1*A*). These results indicate that 5-HT$_6$R-eYFP and eCFP-Jab1 were in close proximity in the cells.

We next examined the co-localization of 5-HT$_6$R and Jab1 using double immunofluorescence staining in various mammalian cell lines. In HEK293 and CHO/K1 cells, we examined the co-localization of endogenous Jab1 and exogenously transfected 5-HT$_6$R. As shown in Fig. 2*C* and supplemental Fig. S1*B*, HA-5-HT$_6$R was predominantly localized at the plasma membrane, and Jab1 was shown to be diffusely distributed in the cytosol and nucleus, as well as at the plasma membrane. The *merged images* showed a prominent co-localization between 5-HT$_6$R and Jab1 at or near the plasma membrane in HEK293 and CHO-K1 cells. In cultured rat hippocampal neurons, we also examined the co-localization of the two proteins their endogenous native forms. Although the staining of 5-HT$_6$R at the plasma membrane in the neurons was not as clear as that observed in the two stable cell lines, double immunofluorescence labeling also revealed clear co-localization of 5-HT$_6$R and Jab1 in hippocampal neurons. Immunohistochemistry was also performed to examine any relationship in the regional and cellular distributions of 5-HT$_6$R and Jab1 in the rat brain. As shown in Fig. 2*D* and supplemental Fig. S1*C*, we used anti-5-HT$_6$R and anti-Jab1 antibodies to map the regional expression of 5-HT$_6$R and Jab1, respectively, in coronal sections of adult rat brains. The use of anti-5-HT$_6$R and anti-Jab1 antibodies led to intensive staining in the cortex, hippocampus, and hypothalamus and provided similar expression profiles for the two proteins. When immunohistochemical images from the hippocampus were magnified, both 5-HT$_6$R and Jab1 were prominently expressed in the dentate gyrus and in all three CA regions of the hippocampus. Taken together, these results suggest the clear co-localization of 5-HT$_6$R and Jab1 *in vivo*.

The Expression Level of Jab1 Modulates G-protein-mediated 5-HT$_6$ Receptor Activities—Based on the evidence of a physical interaction between 5-HT$_6$R and Jab1, we next examined whether overexpression of Jab1 affects 5-HT$_6$R activity. In our previous studies, we demonstrated that the activity of 5-HT$_6$R can be measured as 5-HT-induced Ca^{2+} increases using a promiscuous $G\alpha_{15}$ protein (21) or a chimeric $G\alpha_{qG66Ds5}$ protein (25), which allows coupling of $G\alpha_S$-coupled receptors to phospholipase C and consequent intracellular Ca^{2+} release. HEK293 cells were transfected with 5-HT$_6$R and a promiscuous $G\alpha_{15}$ protein for 24 h. 5-HT-induced Ca^{2+} increases via activation of 5-HT$_6$R were measured using the Fluo-4-AM fluorescence dye with the FDSS6000 system. Fluorescence levels peaked within 20–30 s after the treatment with 5-HT. Under these conditions, the overexpression of Jab1 did not change 5-HT-induced Ca^{2+} responses following a single dose (10 μM, Fig. 3*A*) or a full dose (0.1 nM-10 μM, Fig. 3*B*) of 5-HT. However, the overexpression of Fyn significantly increased 5-HT-induced Ca^{2+} signals (Fig. 3*C*) as previously reported (21). We next attempted to examine whether the selective knockdown of endogenous Jab1 using siRNA-mediated slicing of Jab1 messenger modulates 5-HT$_6$R activity. As shown in Fig. 3*D* and supplemental Fig. S2*A*, the knockdown of endogenous Jab1 significantly reduced 10 μM 5-HT-induced Ca^{2+} increases as compared with the negative control. When examined at a full dose of 5-HT, the transfection of Jab1 siRNA produced a decreased maximum in concentration-response curves without any significant change in apparent 5-HT$_6$R affinity. The calculated EC_{50} values were 8.6 ± 0.8 nM in the presence of negative control siRNA and 7.7 ± 0.4 nM in the presence of Jab1 siRNA ($n = 3$, $p = 0.36$, Fig. 3*E*). The expression level of Jab1 was confirmed by immunoblotting analysis; transfection of Jab1 siRNA significantly reduced endogenous Jab1 expression in

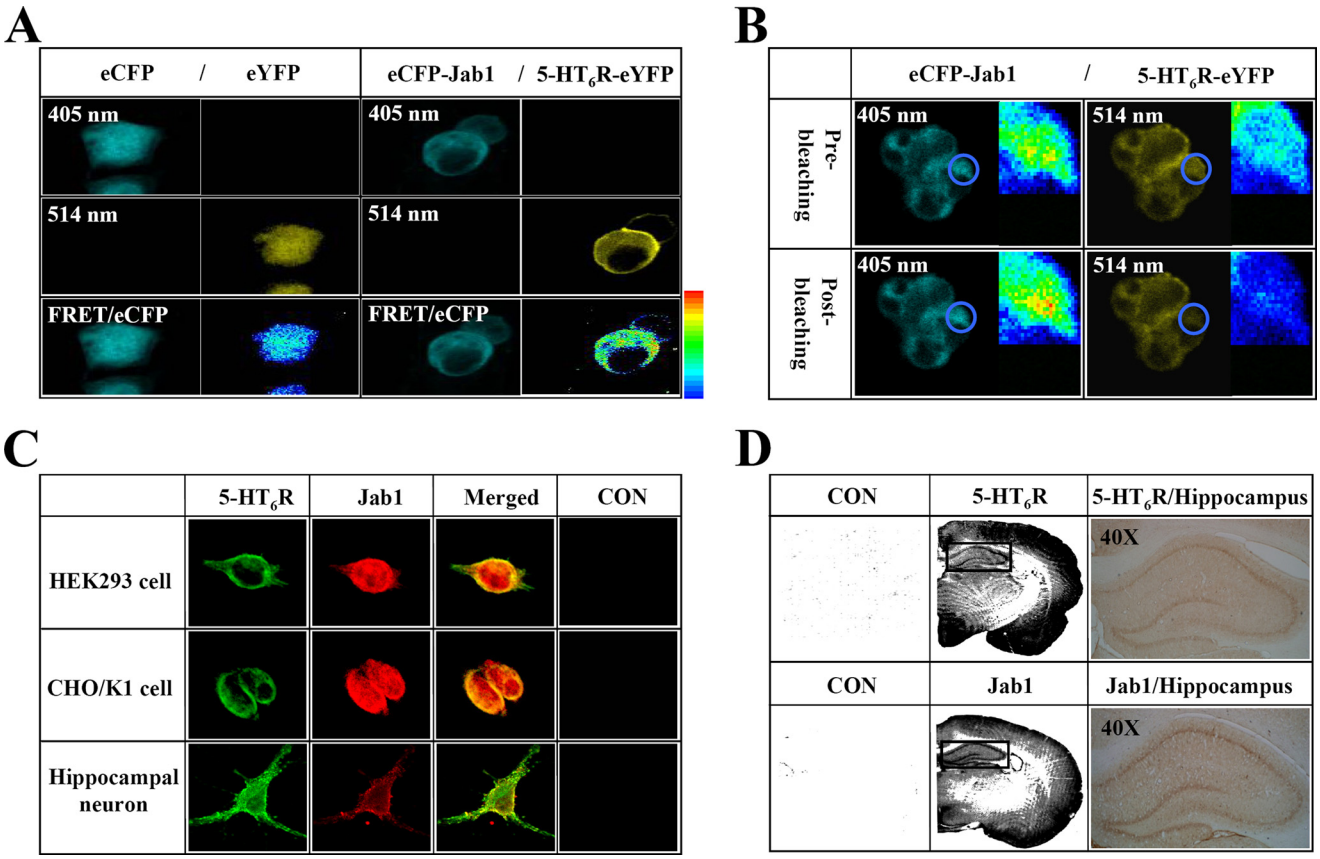


FIGURE 2. **Jab1 shows co-localization with human 5-HT$_6$R in diverse cells and similar distribution with 5-HT$_6$R in the rat brain.** *A*, HEK293 cells were transfected with eCFP/eYFP or eCFP-Jab1/5-HT$_6$R-eYFP. After 24 h, the cells were fixed and then eCFP, eYFP, eCFP-Jab1, or 5-HT$_6$R-eYFP fluorescence was monitored. The pseudocolor for visualization of fluorescence intensity represents FRET efficiency calculated as FRET/eCFP ratio. *B*, HEK293 cells were transfected with eCFP-Jab1 and 5-HT$_6$R-eYFP. After 24 h, the cells were fixed, and eCFP-Jab1 or 5-HT$_6$R-eYFP fluorescence was monitored. Fluorescence intensity was visualized using pseudocolor, and the bleached region is indicated by a *circle*. *C*, immunofluorescence analysis in HEK293 cells, CHO/K-1 cells, and cultured rat hippocampal neurons. HEK293 and CHO/K-1 cells were transfected with HA-5-HT$_6$R. After 24 h of transfection, the cells were fixed and permeabilized. HA-5-HT$_6$R (*green*) was immunostained with rabbit anti-HA followed by fluorescein isothiocyanate-conjugated secondary antibodies, and Jab1 (*red*) was immunostained with mouse anti-Jab1, followed by rhodamine-conjugated secondary antibodies. In cultured rat hippocampal neurons, endogenous 5-HT$_6$R (*green*) was immunostained with rabbit anti-5-HT$_6$R, followed by fluorescein isothiocyanate-conjugated secondary antibodies. The *third panels* show the merged images of the *first* and *second panels*. Negative control experiments (*CON*) were processed with only fluorescein isothiocyanate- and rhodamine-conjugated IgG antibodies. *D*, immunohistochemical assay showing regional distribution of 5-HT$_6$R and Jab1. Coronal sections of adult rat brains were immunostained with rabbit anti-5-HT$_6$R and mouse anti-Jab1 antibodies. The *third panels* show magnified images of the hippocampus. Negative control experiments (*CON*) were processed with only horseradish peroxidase-conjugated rabbit or mouse IgG antibodies.

HEK293 cells (42.5 ± 6.4% of the control, $n = 3$, $p < 0.001$, Fig. 3*F* and supplemental Fig. S2*B*).

We next examined whether the knockdown of endogenous Jab1 affects the expression level of 5-HT$_6$R in HEK/HA-5-HT$_6$R cells (HA-5-HT$_6$R was stably expressed in HEK293 cells). We found that the transfection of Jab1 siRNA induced a significant decrease in the expression level of 5-HT$_6$R (74.3 ± 5.5% of the control, $n = 3$, $p < 0.01$, Fig. 4*A*), whereas the overexpression of Jab1 did not affect the expression level of 5-HT$_6$R (100.2 ± 5.1% of the control, $n = 3$, Fig. 4*B*). To examine whether the Jab1 modulates 5-HT$_6$R stability, the turnover of 5-HT$_6$R was measured after the pretreatment with cycloheximide for 0–6 h to prevent *de novo* receptor synthesis. As shown in Fig. 4*C*, 5-HT$_6$R expression levels were significantly decreased over 3 h in Jab1 knockdown cells as compared with the negative control. However, 5-HT$_6$R expression levels were not significantly changed in Jab1-overexpressed cells as compared with FLAG-vector control (Fig. 4*D*). These data suggest that the regulation of 5-HT$_6$R expression levels by Jab1 is due to effects on 5-HT$_6$R stability. The immunofluorescent images using 5-HT$_6$R-eGFP, and the associated pixel line scans, also clearly demonstrate that less 5-HT$_6$R is seen at the HEK293 cell membrane when cells have been transfected with Jab1 siRNA (Fig. 4, *E* and *F*, and supplemental Fig. S3). Taken together, these results suggest that the expression level of 5-HT$_6$R and its functional activity can be regulated by its binding protein Jab1.

Activation of 5-HT$_6$ Receptor Affects the Distribution of Jab1 and the Interaction between Jab1 and c-Jun—We next examined whether the activation of 5-HT$_6$R influences Jab1 activity. We first addressed the question of whether the activation of 5-HT$_6$R influences the distribution of endogenous Jab1, because Jab1 is known to be a nuclear/cytoplasmic shuttle protein (28, 29). We found that treatment with 5-HT (20 μM, 60 min) in CHO/5-HT$_6$R cells (CHO cells stably expressing 5-HT$_6$R) induces more condensed staining of Jab1 in the nucleus as compared with control cells, which display broad staining for Jab1 in both the nucleus and cytoplasm as shown in Fig. 5*A*. This altered distribution of Jab1 was also examined under time- and fraction-dependent experiments using immunoblotting assays. As shown Fig. 5*B*, the treatment of CHO/5-

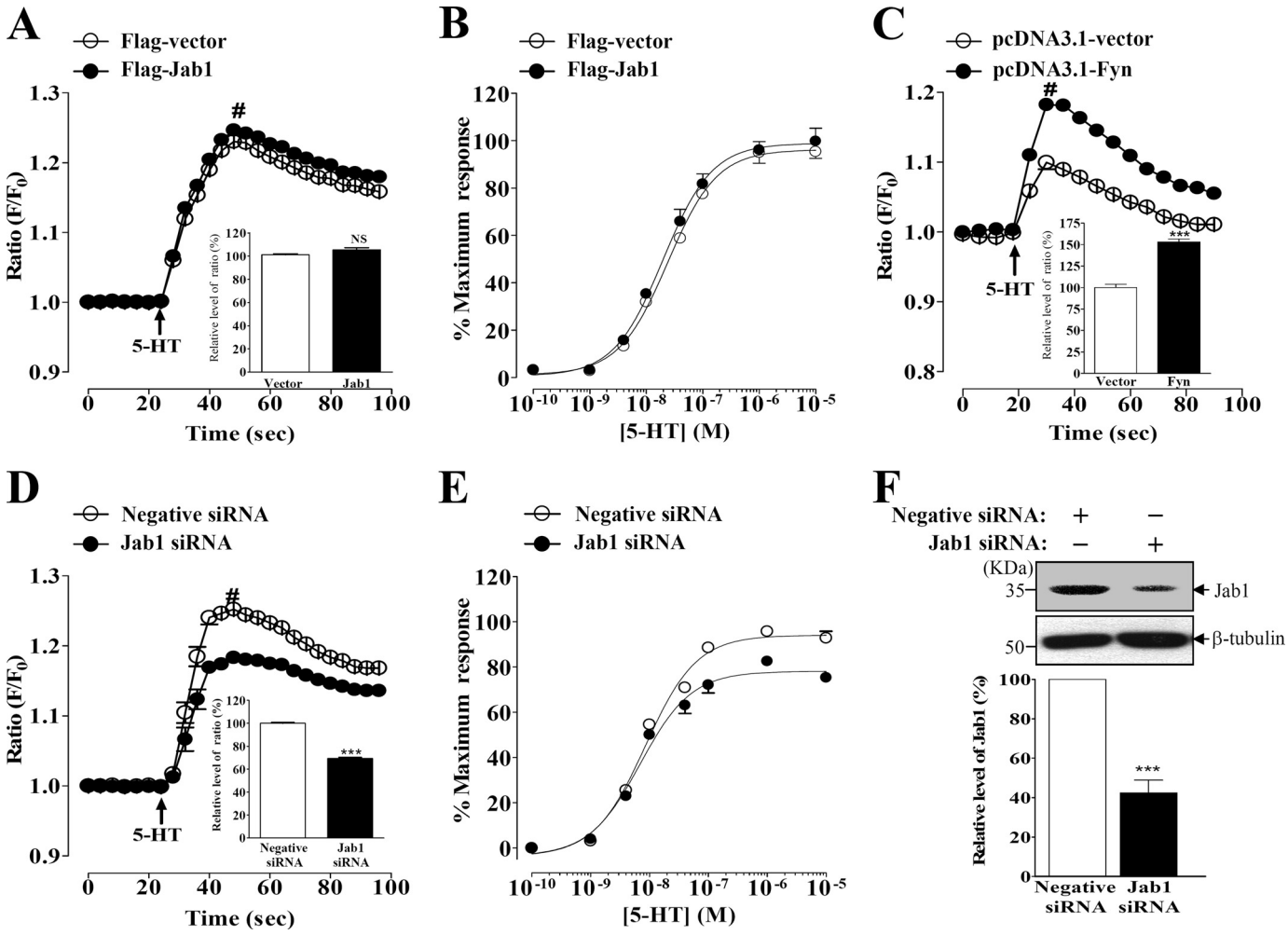


FIGURE 3. **The expression level of Jab1 tightly regulates the activity of 5-HT$_6$R.** *A*, HEK293 cells were transiently transfected with 5-HT$_6$R and Gα_{15} in the presence of FLAG-vector (*open circles*) or FLAG-Jab1 (*closed circles*) for 24 h. The Ca^{2+} responses by 10 μM 5-HT were measured using an FDSS6000 system. *F* is the fluorescence intensity, and F_0 is the initial fluorescence intensity at 480 nm. *Inset*, pooled results showing the mean relative change of the ratio (F/F_0) measured at the indicated time (#). The mean relative change of ratio by Jab1 was 105.4 ± 1.8% of the control ($n = 4$, $p = 0.07$). *B*, dose-response relationship of 5-HT$_6$R-mediated Ca^{2+} responses in the absence (*open circles*) and in the presence (*closed circles*) of Jab1 overexpression. *Best-fit lines* were computed for all concentration-response curves using the equation, $y/y_{max} = 1/(1 + (k_{1/2}/[5\text{-HT}])^{n_H})$, where y_{max} is the maximum response, $_{1/2}$ is the concentration for half-maximum response (EC_{50}), and n_H is the Hill coefficient. Intracellular Ca^{2+} changes are expressed as a percentage of the maximum response obtained at a 10 μM concentration of 5-HT. *C*, the changes in intracellular Ca^{2+} mediated by 10 μM 5-HT in 5-HT$_6$R- and Gα_{15}-transfected HEK293 cells in the absence (*open circles*) and in the presence (*closed circles*) of Fyn overexpression were recorded using an FDSS6000 system. The mean relative change in the ratio mediated by Fyn was 156.5 ± 3.7% of the control ($n = 4$; ***, $p < 0.001$). *D*, the changes in intracellular Ca^{2+} mediated by 10 μM 5-HT in 5-HT$_6$R- and Gα_{15}-transfected HEK293 cells with negative control siRNA (*open circles*) or with Jab1 siRNA (*closed circles*) were recorded using an FDSS6000 system. The mean relative change of ratio following treatment with Jab1 siRNA was 69.3 ± 1.0% of the control ($n = 4$; ***, $p < 0.001$). *E*, dose-response relationship of 5-HT$_6$R-mediated Ca^{2+} responses with negative control siRNA (*open circles*) or with Jab1 siRNA. The changes in Ca^{2+} responses are expressed as a percentage of the maximum response observed with a 10 μM concentration of 5-HT. *F*, the expression level of Jab1 after transfecting with Jab1 siRNA was confirmed by immunoblotting using an anti-Jab1 antibody. β-Tubulin was detected by immunoblotting on the same sample to normalize the amount of lysates. ***, $p < 0.001$ compared with control.

HT$_6$R cells with 20 μM 5-HT induced a time-dependent changes in both the cytoplasmic and nuclear fractions of Jab1. Upon treatment with 5-HT, endogenous amounts of Jab1 protein were significantly decreased in the cytoplasm (85.3 ± 3.4% of the control at 60 min, $n = 3$, $p < 0.05$) and were significantly increased in the nucleus (114.4 ± 3.8% of the control at 30 min, $n = 3$, $p < 0.05$; 122.7 ± 6.1% of the control at 60 min, $n = 3$, $p < 0.05$), suggesting that Jab1 is translocated into the nucleus upon activation of 5-HT$_6$R.

Jab1 was initially identified as a protein that interacts with c-Jun and enhances endogenous phospho-c-Jun levels (17, 30). After observing increases in the nuclear distribution of Jab1, we first examined whether the activation of 5-HT$_6$R can increase c-Jun phosphorylation in CHO/5-HT$_6$R cells. As shown in Fig. 5*C*, the treatment of CHO/5-HT$_6$R cells with 20 μM 5-HT induced a time-dependent increase in c-Jun phosphorylation (164.6 ± 5.2% of the control at 15 min, $n = 3$, $p < 0.001$). We next examined whether 5-HT also modulates an interaction between Jab1 and c-Jun via an activation of 5-HT$_6$R by using co-immunoprecipitation assays. Fig. 5*D* shows that 20 μM 5-HT increased the interaction between Jab1 and c-Jun in a time-dependent manner in CHO/5-HT$_6$R cells. Immunocomplexes levels were significantly higher following treatment with 5-HT (125.6 ± 7.6% of the control at 30 min, $n = 3$, $p < 0.05$; 133.9 ± 8.5% at 60 min, $n = 3$, $p < 0.05$). In contrast, treatment with 5-HT decreased the interaction between 5-HT$_6$R and Jab1, as shown in Fig. 5*E*. Taken together, these data obviously demonstrate that the activation of 5-HT$_6$R can affect Jab1 activities

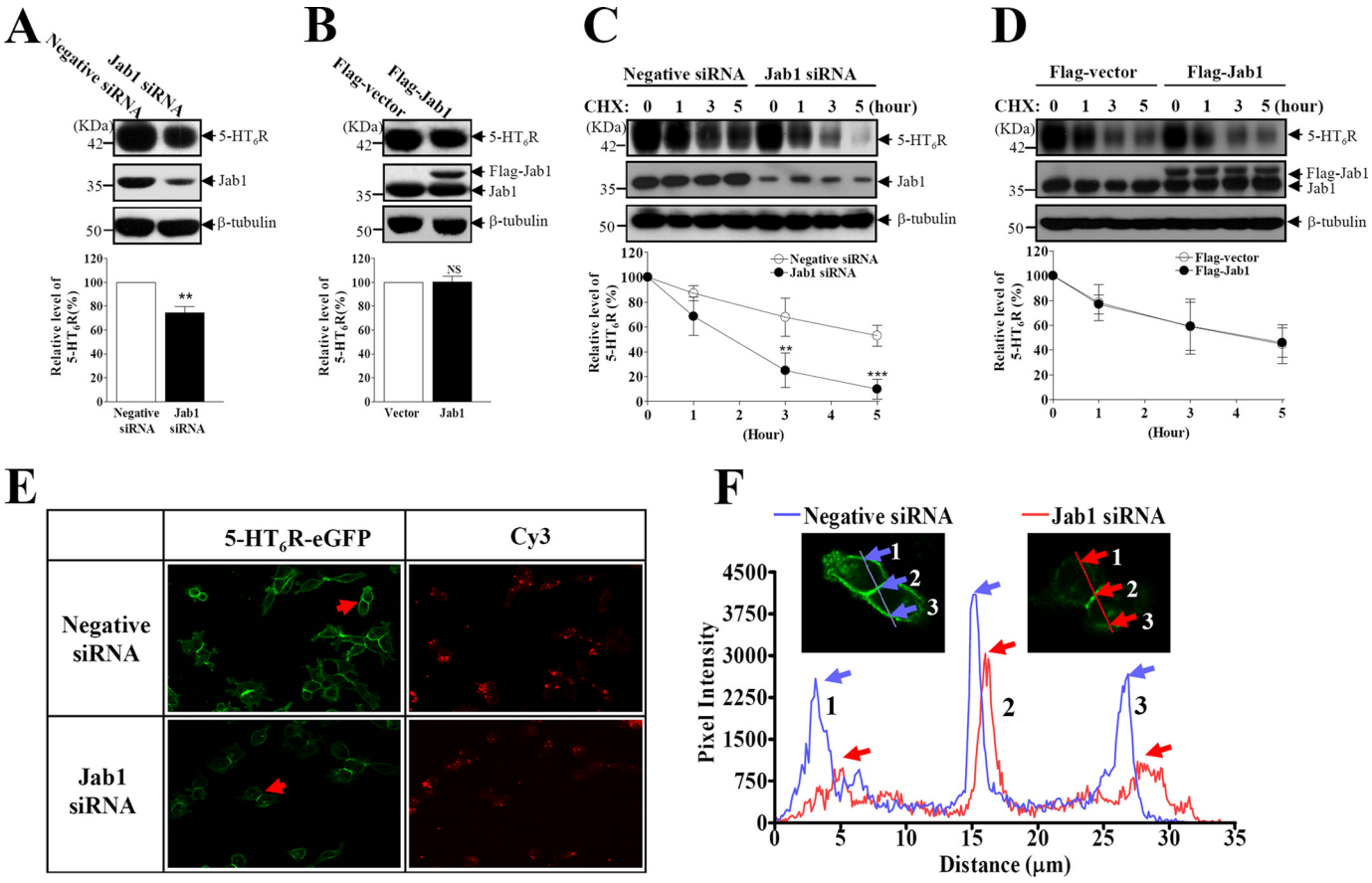


FIGURE 4. **Jab1 modulates the expression levels of 5-HT$_6$R by regulating stability of 5-HT$_6$R.** After HEK/HA-5-HT$_6$R cells were transiently transfected with negative control siRNA or Jab1 siRNA in *A* and with FLAG-vector or FLAG-Jab1 in *B* for 24 h, the total expression level of 5-HT$_6$R was analyzed by immunoblotting cell lysates. Representative immunoblotting results were detected using anti-HA, anti-Jab1, and anti-β-tubulin antibodies. **, $p < 0.01$ compared with control. After HEK/HA-5-HT$_6$R cells were transiently transfected with negative control siRNA or Jab1 siRNA in *C* and with FLAG-vector or FLAG-Jab1 in *D* for 24 h, cycloheximide (*CHX*) was added at 100 μg/ml. Then total proteins were isolated at 0, 1, 3, and 6 h after CHX treatment. The expression level of 5-HT$_6$R was analyzed by immunoblotting. Representative immunoblotting results were detected using anti-HA, anti-Jab1, and anti-β-tubulin antibodies. The data are represented as relative percentages of the control. **, $p < 0.01$; ***, $p < 0.001$ compared with control. *E*, HEK293 cells were transiently transfected with 5-HT$_6$R-eGFP and negative control siRNA-cy3 or with 5-HT$_6$R-eGFP and Jab1 siRNA-cy3 for 24 h. After the cells were fixed, eGFP fluorescence was excited (Ex: 488 nm) and detected (Em: 507 nm). Cy3 fluorescence showed that negative control siRNA and Jab1 siRNA were transfected in HEK293 cells. *F*, magnified images of HEK293 cells transfected with 5-HT$_6$R-eGFP and negative control siRNA-cy3 (*left*, *blue color*) or with 5-HT$_6$R-eGFP and Jab1 siRNA-cy3 (*right*, *red color*) are marked with *arrows* in *E*. The *graph* shows pixel-by-pixel fluorescence intensity measured along the *line* drawn across the cells.

such as the translocation of Jab1 and the interaction between Jab1 and c-Jun, suggesting that Jab1 plays an important role as a novel 5-HT$_6$R-specific signal mediator.

Correlation of 5-HT$_6$ Receptor and Jab1 Expression Patterns under Focal Cerebral Ischemia—Jab1 was previously reported to interact with hypoxia-inducible factor-1α (HIF-1α) and to increase HIF-1α stability under hypoxic conditions (31). To elucidate the function of 5-HT$_6$R and Jab1 in the brain, we examined expression levels of 5-HT$_6$R and Jab1 in brain sections obtained from middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemic rats, in which the HIF-1α protein is up-regulated (32). After confirming the damaged brain areas using cresyl violet staining, we immunostained coronal brain slices with anti-5-HT$_6$R and anti-Jab1 antibodies. As shown in Fig. 6*A*, we found that the damaged ischemic areas produced increasing levels of 5-HT$_6$R and Jab1, as compared with the ipsilateral undamaged control area. The damaged cortex and striatum areas were measured quantitatively. Fig. 6*B* represents the pooled data from three different experiments. Based on cresyl violet staining, the regions of striatum and cortex subjected to MCAO exhibited severe brain damage compared with controls (cortex: 58.3 ± 4.2% of the control, striatum: 37.1 ± 6.1% (Fig. 6B_1)). As shown in Fig. 6, B_2 and B_3, cerebral ischemia significantly increased the expression levels of both 5-HT$_6$R (cortex: 176.9 ± 6.6%, $p < 0.01$; striatum: 155.3 ± 5.6%, $p < 0.05$) and the interacting protein Jab1 (cortex: 171.0 ± 9.9%, $p < 0.05$; striatum: 127.8 ± 7.3%, $p < 0.05$), as compared with the control. These results indicate that the expression levels of 5-HT$_6$R and Jab1 are correlatively altered in cerebral ischemia and that these two proteins might play important roles in pathophysiological conditions such as ischemic disorders.

5-HT$_6$R and Jab1 Enhance Cell Survival under Hypoxic Conditions—To further explore the potential roles of 5-HT$_6$R and Jab1 as related to the *in vivo* ischemic results, we examined the expression level of 5-HT$_6$R and Jab1 under hypoxic conditions. We used a hypoxia-mimicking agent or an *in vitro* OGD model in cultured cells to induce ischemic effects. Treatment with $CoCl_2$, a known hypoxia-mimicking agent and a chemical inducer of HIF-1α (33, 34), for 18 h significantly increased the

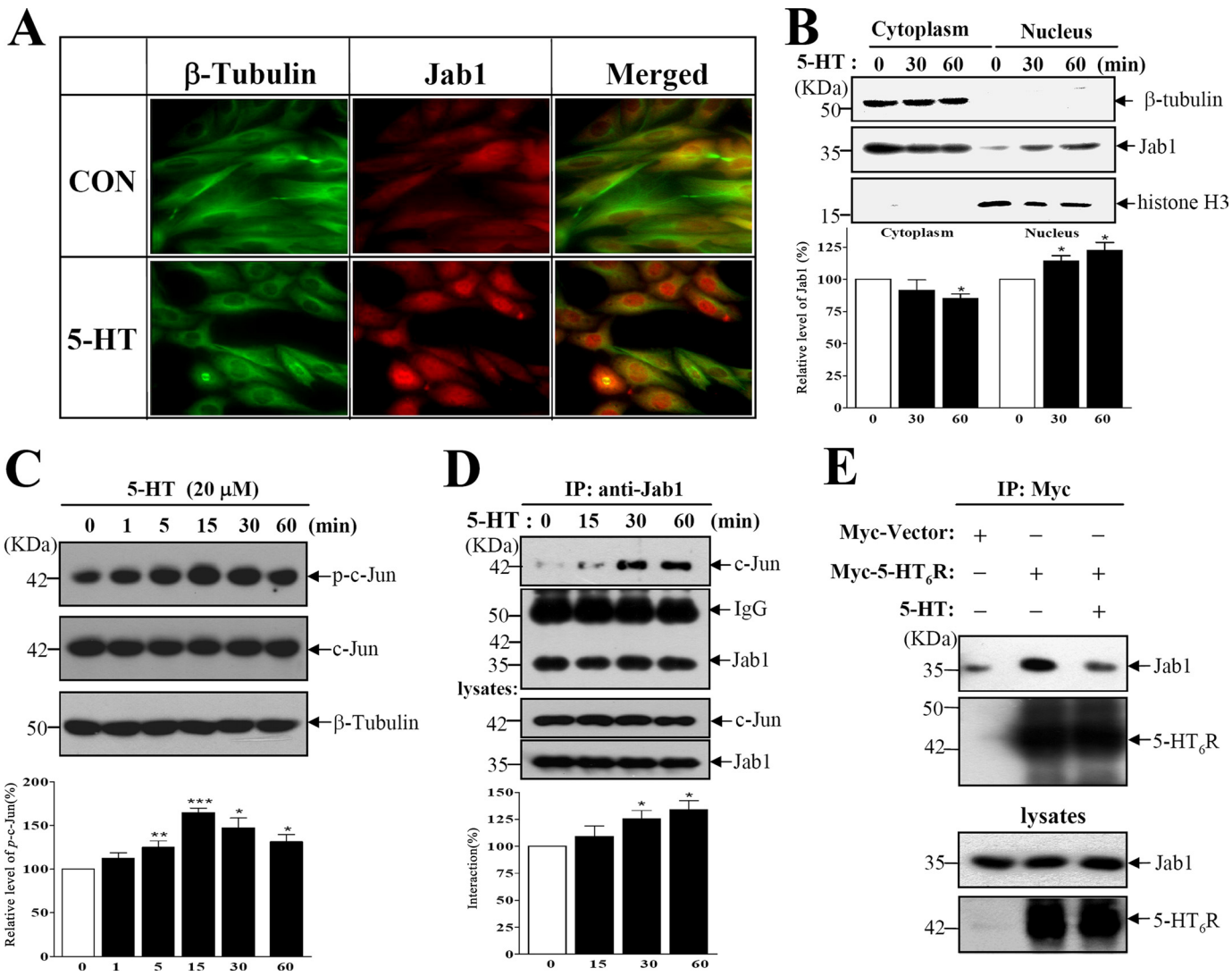


FIGURE 5. **The activation of 5-HT$_6$R affects the Jab1 distribution and the interaction with c-Jun or 5-HT$_6$R.** *A*, after CHO/5-HT$_6$R cells were treated with 20 μM 5-HT for 60 min, the cells were fixed and permeabilized. Jab1 (*red*) and β-tubulin (*green*) were stained using mouse anti-Jab1 and rabbit anti-β-tubulin antibodies. Fluorescence of these proteins was visualized with cy5.5-conjugated and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. *B*, after CHO/5-HT$_6$R cells were treated with 20 μM 5-HT for the indicated times, the Jab1 contents were analyzed by immunoblotting with anti-Jab1 in separated cytoplasmic or nuclear fractions. β-Tubulin and histone H3 were used as cytoplasmic and nuclear markers, respectively. *C*, the cells were treated with 20 μM 5-HT for the indicated times and were assayed to detect phospho-c-Jun (*p-c-Jun*) and c-Jun. Representative immunoblotting results were analyzed by using anti-phospho-c-Jun (*p-c-Jun*), anti-c-Jun, and anti-β-tubulin antibodies. *D*, under the same experimental conditions, the cell lysates were immunoprecipitated with anti-Jab1 antibodies, and the immunocomplexes were assayed using anti-Jab1 and anti-c-Jun antibodies. Proper expression of endogenous Jab1 and c-Jun in cell lysates was identified with specific antibodies. The data shown in the *bar graph* were normalized as relative percentages of the control (no treatment with 5-HT). *, $p < 0.05$ compared with control. *E*, HEK293 cells were transiently transfected with Myc-vector or Myc-5-HT$_6$R, treated with 20 μM 5-HT for 30 min, and then immunoprecipitated with anti-Myc or anti-Jab1 antibodies. The immunocomplexes were then analyzed by immunoblotting with anti-Myc or anti-Jab1 antibodies. The proper expression of transiently transfected or endogenous protein in cell lysates was identified with the specific antibodies.

expression level of 5-HT$_6$R in HEK/HA-5-HT$_6$R cells in a dose-dependent manner (Fig. 7, A_1 and A_2). In addition, the expression level of Jab1 was also significantly increased by 300 and 600 μM $CoCl_2$ (133.1 $\pm$ 9.5% of the control at 300 μM; 146.4 $\pm$ 11.6% of the control at 600 μM, Fig. 7, A_1 and A_3). These results are consistent with those obtained from ischemic models; the expression levels of 5-HT$_6$R and Jab1 are also increased by a chemically induced hypoxic insult *in vitro*.

5-HT$_6$R and Jab1 can increase as a consequence of hypoxic insults or can increase as the result of an adaptation mechanism that protects cells from hypoxic-mediated cell death. If 5-HT$_6$R plays a critical role in protection of cells from hypoxic-mediated cell death, we hypothesize that HEK/HA-5-HT$_6$R cells exhibit less cell death during hypoxia than do native HEK293 cells that do not express 5-HT$_6$R proteins. As shown in Fig. 7*B* and supplemental Fig. S4*A*, HEK/HA-5-HT$_6$R cells showed significantly less cell death than did native HEK293 cells at all tested doses of $CoCl_2$. We confirmed these results using an *in vitro* OGD model with cultured cells instead of using the hypoxia-mimicking agent $CoCl_2$. When exposed to an anaerobic gas mixture (5% CO_2, 10% H_2, and 85% N_2) in a glucose-free solution, HEK/HA-5-HT$_6$R cells exhibited less cell death than did native HEK293 cells (Fig. 7*C*). To explore the involvement of Jab1 in hypoxia-mediated cell death, Jab1 was selectively knocked down using siRNA methods. When HEK/HA-5-HT$_6$R cells were transiently transfected with negative control or Jab1

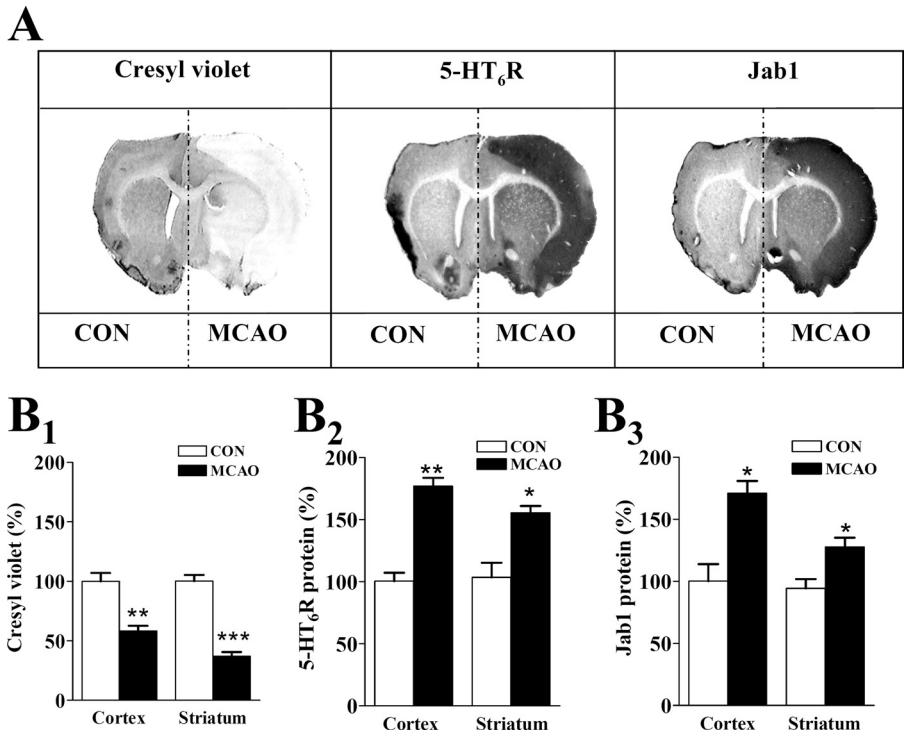


FIGURE 6. **The increased expression levels of 5-HT$_6$R and Jab1 in a rat model of focal cerebral ischemia.** Rats subjected to MCAO for 2 h were reperfused for 1 day. *A*, coronal sections of the rat brain were stained with cresyl violet (*left*) or immunostained with anti-5-HT$_6$R (*middle*) or anti-Jab1 antibodies (*right*). *B*, the *bar graph* represents the density of cresyl violet staining (B_1) and the quantification of the expression of 5-HT$_6$R (B_2) and Jab1 (B_3) in the cortex and striatum. The data shown in the *bar graph* were normalized as relative percentages of the ipsilateral undamaged control area (*CON*). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with control.

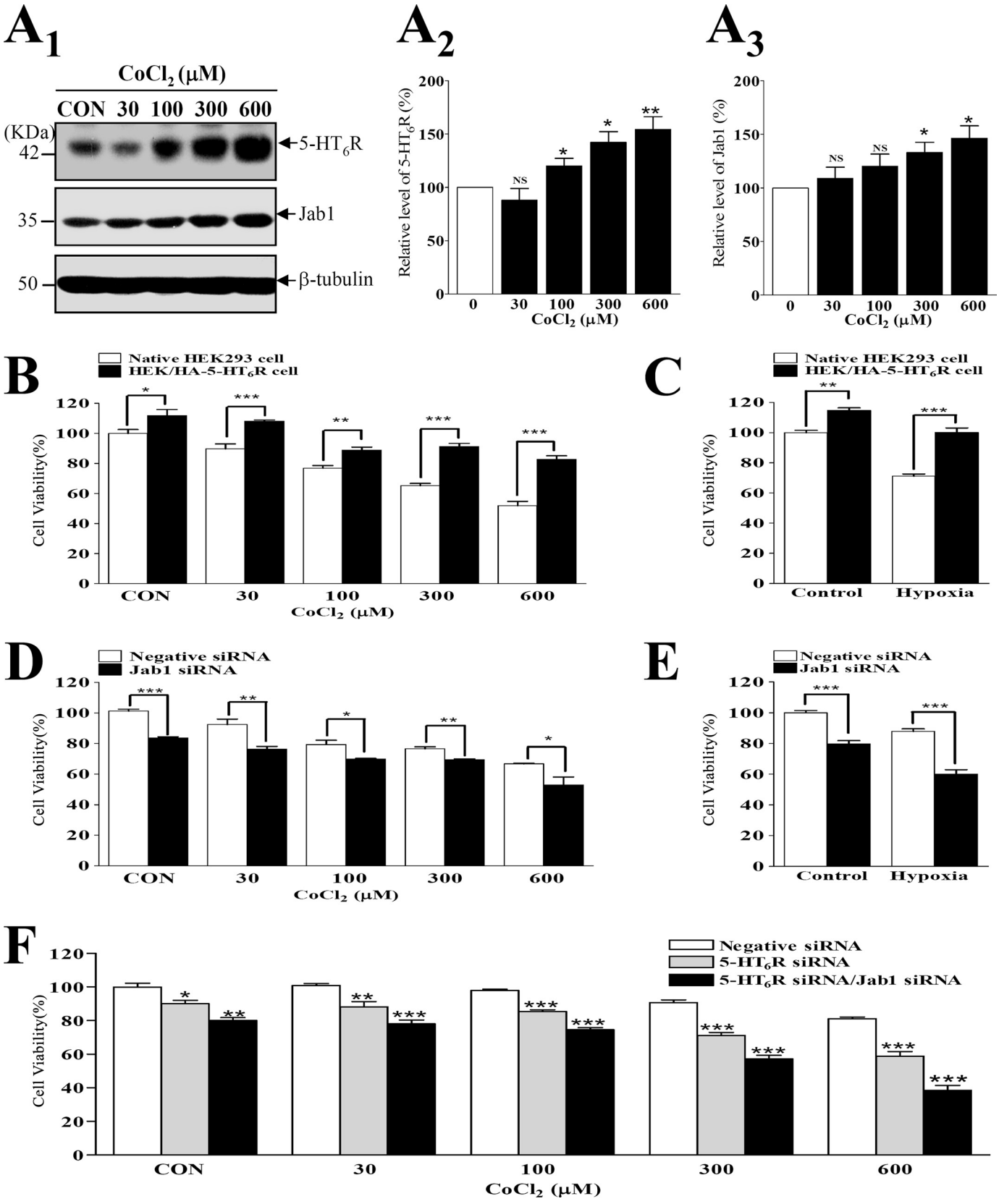
siRNA, negative control siRNA-transfected cells showed less cell death than did the Jab1 knockdown cells at all tested doses of $CoCl_2$ (Fig. 7*D*) and *in vitro* OGD model cultured cells (Fig. 7*E*). Because it is not clear whether Jab1 is involved in the hypoxia-induced cell death as shown in Fig. 7*D* and supplemental Fig. S4*B*, we performed double knockdown experiments using 5-HT$_6$R and Jab1 siRNAs and examined cell viability in HEK/HA-5-HT$_6$R cells. As shown in Fig. 7*F* and supplemental Fig. S4*C*, the involvement of 5-HT$_6$R in the hypoxia-induced cell death was also confirmed in a direct 5-HT$_6$R knockdown experiment using 5-HT$_6$R siRNA. However, the involvement of Jab1 was also observed when both *5-HT$_6$R* and *Jab1* genes were down-regulated in double knockdown experiments. It is interesting that the presence of *5-HT$_6$R* or *Jab1* genes themselves caused greater cell survival in control cells that were not exposed to hypoxic insults, as compared with cells lacking *5-HT$_6$R* genes or both *5-HT$_6$R* and *Jab1* genes (Fig. 7, *B–F*, and supplemental Fig. S4, *A–C*). Taken together, these results demonstrate that chemically induced *in vitro* hypoxia increased expression of both 5-HT$_6$R and Jab1. The presence of these two proteins protected against hypoxia-induced cell death, suggesting important roles for 5-HT$_6$R and Jab1 as cell protectors in the brain.

DISCUSSION

In the present study, we provided clear evidence of a physical and functional interaction between 5-HT$_6$R and Jab1. This is an original report demonstrating the direct interaction of a $G\alpha_S$-coupled membrane receptor, 5-HT$_6$R, and a co-activator of c-Jun-mediated transcription, Jab1. The 5-HT$_6$R·Jab1 complex was confirmed using a yeast two-hybrid assay and with GST pulldown, FRET, and co-immunoprecipitation assays in two different mammalian clonal cell lines as well as in the adult rat brain. Using a yeast two-hybrid screen, two bait fragments of 5-HT$_6$R (iL3 and CT) were independently found to interact with Jab1, suggesting the possibility of multiple 5-HT$_6$R domains that contribute to the 5-HT$_6$R-Jab1 interaction. Another GST pulldown assay using two fragments of Jab1 (CT-Jab1 and NT-Jab1), which were arbitrarily divided, demonstrated that both the iL3 and CT portions of 5-HT$_6$R are required and selectively interact with other parts of Jab1: iL3 of 5-HT$_6$R binds to CT-Jab1 and CT of 5-HT$_6$R binds to NT-Jab1. These data imply that 5-HT$_6$R and Jab1 may modulate each other's activity. In the case of 5-HT$_6$R, the iL3 and CT domains of 5-HT$_6$R are known to be necessary for interaction with a $G\alpha_S$-protein (22) and a Fyn tyrosine kinase (21), respectively. With regard to Jab1, NT-Jab1 (amino acids 1–151) contains a Jab1/MPN domain metalloenzyme motif (amino acids 138–151), which is functional only in the context of the CSN complexes (35). Jab1 has been also known to interact with the rat lutropin/choriogonadotropin receptor (rLHR) and with human protease-activated receptor-2 (PAR-2), which are GPCRs (36, 37). Interestingly, these authors independently reported that, for both LHR and PAR-2, the iL3 and the CT domains are primarily responsible for the interaction with Jab1. Such binding sites are consistent with our data; we further provided specific binding sites on Jab1 for the interaction: the iL3 and CT domains of 5-HT$_6$R selectively bind to CT-Jab1 and NT-Jab1, respectively. Based on these observations, we aligned the amino acid sequences of the iL3 and the CT regions of three different GPCRs (Fig. 8). We found some interesting homologies both in the iL3 (Fig. 8*A*) and CT (Fig. 8*B*) regions of the receptors. These motifs aligned well and showed many identical and homologous residues in both the iL3 and CT regions. Therefore, it will be interesting to determine whether a new GPCR-binding protein aligns well with these sequences and to propose a consensus binding domain through which GPCRs bind with Jab1.

Because Jab1 was first reported as a nuclear exporter and an activator of the degradation of binding protein p27^{Kip1}, the cyclin-dependent-kinase inhibitory protein (29), there have been many reports showing the function of Jab1 as a nuclear exporter and inducer of the cytoplasmic degradation of several proteins,

including p53, p27, Smad4/7, and West Nile Virus Capsid (38–40). Because Jab1 does not have a transmembrane region due to its hydrophilic amino acid sequence, it is mainly localized to the nucleus and cytosol. Therefore, the distribution of Jab1 at or near the plasma membrane suggests that Jab1 localizes near the membrane by binding with plasma membrane proteins and



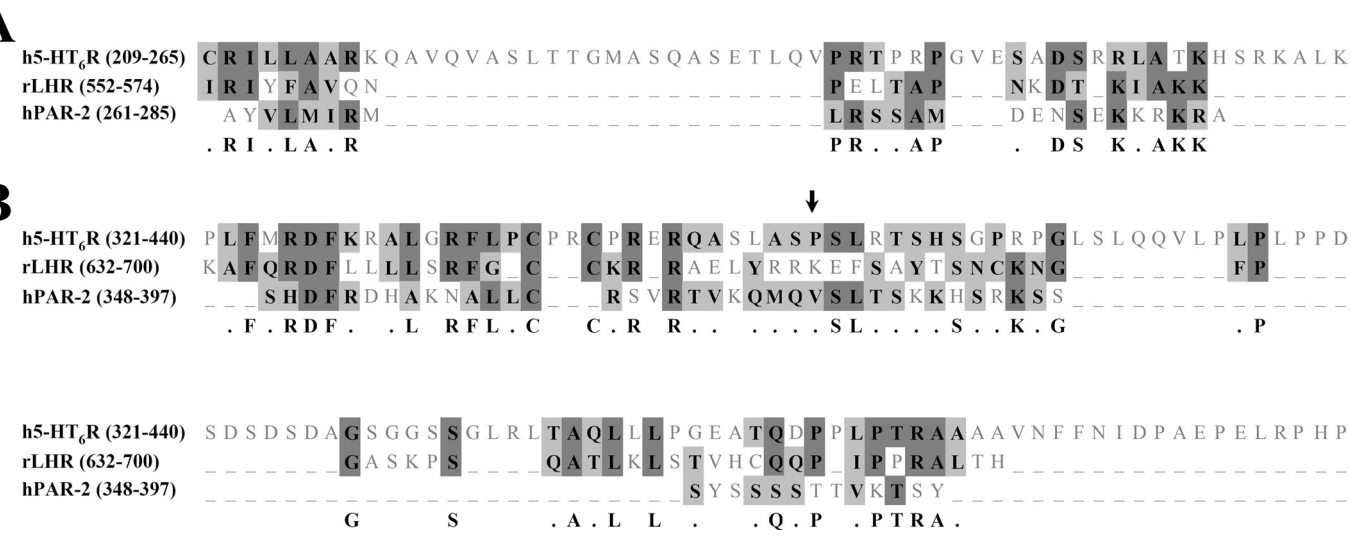


FIGURE 8. **Primary amino acid alignment of h5-HT$_6$R, rLHR, and hPAR-2, which interact with Jab1.** *A*, the intracellular loop 3 (iL3) of human 5-HT$_6$R (h5-HT$_6$R; 209–265 region) was aligned to the iL3 region of rat LHR (rLHR; 552–574 region) and to human PAR-2 (hPAR-2; 261–285 region). *B*, the C-terminal (CT) region of h5-HT$_6$R (321–440 region) was aligned to the CT of rLHR (632–700 region) and hPAR-2 (348–397 region). An *arrow* indicates the starting point of rLHR previously aligned with human p27 and human c-Jun, which interact with Jab1 (Li, *et al.* (36)). The primary amino acid alignment as shown in *A* and *B* was obtained using the ClustalW2 program. Identical or homologous residues are *shaded* in *dark* or *light gray*, respectively. Gaps introduced for optimal alignment are represented by *dashes*. Identical sequences in at least two of the three sequences are represented by *bold characters*, and homologous residues are indicated by *dots* at the *bottom*.

modulating their activities. Our binding data strongly suggest that 5-HT$_6$R is the target protein that allows localization of Jab1 at or near the plasma membrane. Then what is the role of Jab1 as a partner of membrane protein 5-HT$_6$R? Based on our results, we proposed three new functions for Jab1 in the mammalian system.

First, the Jab1 protein is necessary to maintain the activity and expression of endogenous 5-HT$_6$R. Overexpression of Jab1 in HEK293 cells produced no effect on the ability of cells to respond to a 5-HT$_6$R agonist. However, inhibition of Jab1 expression by Jab1 siRNA decreased the activity of 5-HT$_6$R without changing the affinity of 5-HT for the receptor. Thus, it is possible that Jab1 may inhibit the activity of 5-HT$_6$R through an inhibition of 5-HT$_6$R expression. Western blot and the immunofluorescent image experiments suggest this possibility. Jab1 knockdown using siRNA decreased cell surface expression of 5-HT$_6$R as well as total expression of 5-HT$_6$R. Most GPCRs, including 5-HT$_6$R, are rapidly desensitized (14), and the iL3 and CT of GPCRs are important regions for the interaction with G-proteins and other regulators as well as for GPCR desensitization, internalization, and degradation (41, 42). We previously found that Fyn interacts with the CT of 5-HT$_6$R and that the presence of Fyn increased the surface expression of 5-HT$_6$R (21). These results suggest that the CT region of 5-HT$_6$R is essential to maintain 5-HT$_6$R at the plasma membrane, specifically, to facilitate binding with interacting proteins such as Fyn or Jab1. Interestingly, expression of Jab1 leads to a reduction in the steady-state levels of the rLHR precursor by direct interaction but has no effect on the level of the mature cell surface rLHR or on the ability of cells to respond to an LHR agonist with increased cAMP accumulation (36). Therefore, further investigation is necessary to elucidate whether Jab1 prevents 5-HT$_6$R desensitization, internalization, and degradation by direct binding to the iL3 and CT regions of 5-HT$_6$R, or whether Jab1 modulates the stability of 5-HT$_6$R precursor molecules, as is the case for rLHR.

Second, Jab1 provides a novel signal transduction pathway for 5-HT$_6$R-mediated pathways. The serotonergic system is implicated in the neurobiological control of learning and memory. In contrast to the implications of 5-HT$_6$R in Alzheimer disease and depression (6–9), the cellular mechanism of 5-HT$_6$R activity has not yet been elucidated due to a lack of selective agonists for 5-HT$_6$R. In addition to the 5-HT$_6$R-mediated increase of cAMP, which implies function as a $G\alpha_S$-protein-coupled receptor, our previous study first reported that 5-HT$_6$R increases Fyn phosphorylation and ERK1/2 phosphorylation via a Fyn-dependent pathway (21). In the present study, we further elucidated that Jab1 mediates 5-HT$_6$R signals from the cytoplasm to the nucleus. We found that activation of 5-HT$_6$R induced the translocation of Jab1 from the cytoplasm to the nucleus and facilitated the interaction between Jab1 and c-Jun. It has been reported that Jab1 activates c-Jun N-terminal kinase activity and enhances c-Jun phosphorylation (30). Our data demonstrated that treatment with 5-HT increased c-Jun

FIGURE 7. **The presence of 5-HT$_6$R with Jab1 increases cell survival under hypoxia.** *A*, the HEK/HA-5-HT$_6$R cells were treated with the indicated $CoCl_2$ dose for 6 h and then were allowed to recover for 12 h before being analyzed. A_1, representative immunoblotting was detected using anti-HA, anti-Jab1, and anti-β-tubulin antibodies. A_2 and A_3, the data shown in the *bar graphs* were normalized as relative percentages of the control. The viability of cells exposed to various doses of $CoCl_2$ or OGD conditions (5% CO_2, 10% H_2, and 85% N_2 in a glucose-free solution) for 6 h was measured using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after recovery in normal Dulbecco's modified Eagle's medium culture media for 12 h. Native HEK293 and HEK/HA-5-HT$_6$R cells were used in *B* and *C*, HEK/HA-5-HT$_6$R cells transfected with Jab1 siRNA or negative control siRNA were used in *D* and *E*, and HEK/HA-5-HT$_6$R cells transfected with 5-HT$_6$R siRNA, 5-HT$_6$R siRNA·Jab1 siRNA, or negative control siRNA were used in *F*. The data are represented as relative percentages of the control. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with control.

phosphorylation via the activation of 5-HT$_6$R, which is the first report of 5-HT$_6$R-mediated c-Jun phosphorylation. To support these results, the interaction between 5-HT$_6$R and Jab1 and the interaction between Jab1 and c-Jun should change upon activation of 5-HT$_6$R. We observed a reduced level of interaction between 5-HT$_6$R and Jab1 and an increased level of interaction between Jab1 and c-Jun following a 30-min treatment with 5-HT. What are the consequences of Jab1 translocation into the nucleus and increased interaction between Jab1 and c-Jun upon activation of 5-HT$_6$R? These results suggest that 5-HT$_6$R can play a role in the regulation of gene expression via Jab1, which may provide a crucial mechanism in 5-HT$_6$R-related conditions such as Alzheimer disease, depression, and learning and memory disorders. Therefore, we are currently examining whether 5-HT$_6$R can modulate AP-1 activity via an interaction between 5-HT$_6$R and Jab1.

Finally, Jab1, along with its binding partner 5-HT$_6$R, is up-regulated and seems to protect against cell death in response to hypoxic insult. Jab1 overexpression is reported in many human cancers (43–45), but its physiological significance remains to be investigated. We demonstrated the up-regulation of 5-HT$_6$R and Jab1 in MCAO-induced focal cerebral ischemia as well as in *in vitro* hypoxic cells. This is the first report to demonstrate that a pathophysiological condition such as hypoxia can increase the expression of Jab1 and 5-HT$_6$R in the brain. Why do hypoxic insults increase 5-HT$_6$R and Jab1 protein levels both *in vitro* and *in vivo*? We propose that Jab1 plays an important role as a cell protector in hypoxic-mediated cell death. We observed more cell death after hypoxic insult in cell lines that did not express *5-HT$_6$R* and *Jab1* genes. Even with no hypoxic insult, the presence of *5-HT$_6$R* and *Jab1* genes increased cell survival in comparison to cells lacking *5-HT$_6$R* and *Jab1* genes. Similar results observed in two different studies support our data. In pancreatic cancer cell lines, it was reported that Jab1 knockdown resulted in impaired cell proliferation and increased apoptosis (46). On the other hand, cell death induced by H_2O_2 was reduced when Jab1 was overexpressed and was dramatically increased when Jab1 was suppressed in U2OS cells (38). However, there is no previous study that directly reports the role of 5-HT$_6$R as a cell protector. One relevant report was from the recent study on 5-HT$_4$ receptors, which belong to the same GPCR family as 5-HT$_6$ receptors. Liu *et al.* (47) recently showed 5-HT$_4$ receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice.

Therefore, one possible candidate that can explain the role of the 5-HT$_6$R·Jab1 complex in the protection of cells in the central nervous system is HIF-1α, a transcription factor that controls the transcriptional activity of a number of genes that respond to low cellular oxygen tension. Because the expression level of HIF-1α is increased in MCAO-induced focal cerebral ischemia, and because Jab1 is able to interact with HIF-1α and control HIF-1α stability and activity (31, 32), there is a possibility that the 5-HT$_6$R·Jab1 complex plays an important role in hypoxic-mediated cell death. Under hypoxic condition, cells either die by apoptosis or adapt to the hypoxic conditions through a series of compensatory mechanisms. HIF-1α is a transcription factor involved in both processes, but the exact mechanisms regulating whether the cells adapt or die by apoptosis are largely unknown. Interestingly, Larsen *et al.* (48) have established a working hypothesis with regard to the mechanism that may control whether HIF-lα/hypoxia induces hypoxic adaptation or hypoxic apoptosis. The hypothesis is that the interaction of Jab1 with HIF-lα promotes adaptation to hypoxia, whereas the interaction of p53 and HIF-lα at the same domain promotes apoptosis. At this point, we do not have any direct evidence to support their hypothesis. However, our data may help elucidate the adaptive role of the 5-HT$_6$R·Jab1 complex in the context of hypoxic insults. Furthermore, our data suggest that 5-HT$_6$R plays a more dominant role in cell protection than Jab1 does, because the involvement of Jab1 was clearly seen in both 5-HT$_6$R and Jab1 double knockdown experiments (Fig. 7 and supplemental Fig. S4).

In summary, the significance of the present study not only lies in providing direct evidence for 5-HT$_6$R-mediated cellular events via an interaction with Jab1 but also in providing evidence for the correlation between 5-HT$_6$R and Jab1 under ischemic conditions. A recent study has suggested that Jab1 is involved in the onset of neuronal diseases such as Alzheimer disease and Parkinson disease through interaction with the endoplasmic reticulum stress transducer IRE1 (49). Therefore, these data provide new insights into biology at the cellular level and the physiological roles of 5-HT$_6$R and Jab1 in the central nervous system.

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