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

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Hyung-Mun Yun†§, Ja-Hyun Baik†, In Sug Kang†, Changbae Jin‡ and Hyewhon Rhim††

From the †Life Sciences Division, Korea Institute of Science and Technology, Seoul 136-791, the ‡School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, the §College of Medicine, KyungHee University, Seoul 130-702, and the ¶Doping Control Center, Korea Institute of Science and Technology, Seoul 136-791, Korea

The 5-HT₆ receptor (5-HT₆R) 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-HT₆R are poorly elucidated relative to its significance in human brain diseases. Here, using a yeast two-hybrid assay, we found that the human 5-HT₆R interacts with Jun activation domain-binding protein-1 (Jab1). We also confirmed a physical interaction between 5-HT₆R 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-HT₆R-mediated activity and cell membrane expression of 5-HT₆R, whereas overexpression of Jab1 produced no significant effect. In addition, we demonstrated that the activation of 5-HT₆R 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-HT₆R 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-HT₆R and Jab1. These findings suggest that Jab1 provides a novel signal transduction pathway for 5-HT₆R and may play an important role in 5-HT₆R-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-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇ receptors. With the exception of the 5-HT₃ receptor, all of these receptors are members of the G-protein-coupled receptor (GPRC) superfamily (2). Among the 5-HT receptors, the 5-HT₆ receptor (5-HT₆R) is coupled to a stimulatory Go(αs) protein. This receptor increases cAMP formation and then activates cAMP-dependent protein kinase (3). 5-HT₆R 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-HT₆R has a significant role in the control of mood and emotion in the central nervous system. To date, most findings suggest that 5-HT₆R plays crucial roles in neurological disorders, including Alzheimer disease, depression, and learning and memory disorders (6–9). Selective antagonists of 5-HT₆R improved memory and cognition in aged rats (10) and in Alzheimer disease patients (11). In an effort to elucidate possible underlying mechanisms of 5-HT₆R antagonist-mediated cognition enhancement, microdialysis studies have shown that a 5-HT₆R blockade elevates cholinergic or glutaminergic neurotransmission in the brain (12–14). However, the role of 5-HT₆R is not clearly defined in the context of depression due to contradictory results. Although it has been reported that selective 5-HT₆R antagonists produced antidepressant-like effects (15, 16), Svenssonsson et al. reported that the 5-HT₆R 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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‡ To whom correspondence should be addressed: Life Sciences Division, Korea Institute of Science and Technology, 39-1 Hawholgok-dong, Sungbuk-gu, Seoul 136-791, Korea. Tel.: 82-2-958-5923; Fax: 82-2-958-5909; E-mail: hrhim@kist.re.kr.

§ The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); CHO/5-HT₆R, Chinese hamster ovary cells stably expressing human 5-HT₆R; CT, C terminus of 5-HT₆R; CT-Jab1, C-terminal part of Jab1; IL2, intracellular loop 2 of 5-HT₆R; IL3, intracellular loop 3 of 5-HT₆R; FRET, fluorescence resonance energy transfer; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; HEK/5-HT₆R, HEK293 cells stably expressing human HA-tagged 5-HT₆R; 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 interfering 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-HT₆R-mediated cognition and mood changes in the brain. However, the cellular mechanisms of 5-HT₆R-mediated signal pathways are not well explored except the common Go₅-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). Jab1 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 Go₅-family GPCR 5-HT₆R and Jab1, and we observed Jab1-mediated modulation of the membrane expression and activity of 5-HT₆R. In addition, we found that 5-HT₆R 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-HT₆R 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-HT₆R 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), His₆ (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, CoCl₂, 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-HT₆R and HA-tagged human 5-HT₆R cDNAs were purchased from UMR cDNA Resource Center (Miner Circle Rolla, MO). The Myc-tagged 5-HT₆R plasmid was constructed by recoding the human 5-HT₆R cDNA into the EcoRI/Xhol sites of the pCMV-Tag 3B vector. 5-HT₆R-eGFP was subcloned (Fw, 5’-CCGGATCCAGATGTTCCAGGCC-3’; Rv, 5’-CCGGATCCAGATGTTCCAGGCC-3’). 5-HT₆R-eYFP was recloned from 5-HT₆R-eGFP into the Xhol/EcoRI sites of pEGFP-N1 (BD Biosciences, Clontech). cDNA fragments encoding the iL2, iL3, and CT domains of 5-HT₆R were subcloned into the EcoRI/BamHI sites of the Gal4 activation domain vector pGBK7T (Clontech) with the following sets of primers:

- iL2 (Fw, 5’-CCGGATTTGACGAGCGCTACCTG-3’; Rv, 5’-CCGGATCCAGGCGACGCAG-3’), iL3 (Fw, 5’-CCGGATTTGACGAGCGCTACCTG-3’; Rv, 5’-CCGGATCCAGGCGACGCAG-3’), and CT (Fw, 5’-CCGGATTTGAGCGTTCCTTGC-3’; Rv, 5’-CCGGATTTGAGCGTTCCTTGC-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 Xhol/BamHI sites of pECPF-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’-CCGGATTTGACGAGCGCTACCTG-3’; Rv, 5’-CCGGATTTGAGCGTTCCTTGC-3’; Rv, 5’-CCGGATTTGAGCGTTCCTTGC-3’), and His-CT-Jab1 (Fw, 5’-CCGGATTTGAGCGTTCCTTGC-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 oligonucleotides were chemically synthesized and annealed to form the siRNA duplex. The sequences of siRNA for 5-HT₆R and jab1 genes were as follows: 5-HT₆R (sense, 5’-CUGUAAACGCAACAGACGTTD-3’; antisense, 5’-GUCAUGGUGUCUGUAACGTTD-3’), jab1 (sense, 5’-CAUACAAACCCUCUGAAGTADT-3’; antisense, 5’-UCUACGAAGCUGUUGATADT-3’; or sense, 5’-GCUCAUGUACGUGAAUADT-3’; antisense, 5’-UUUCAUGUAACUGAGCTG-3’), and negative control siRNA (sense, 5’-CCUACGGCCCAUUUUGUADT-3’; antisense, 5’-ACGAAAUUGGUUCGGUGGADT-3’).

**Yeast Two-hybrid Screening**—The bait plasmids, pGBK7T/iL2, iL3, or CT of 5-HT₆R, 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-galactopyranoside at 18°C during the midlog phase. The cells were sonicated in lysis buffer (1× PBS, pH 7.4, 1 mM dithiotreitol, 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%
Novel Interaction between 5-HT$_6$ Receptor and Jab1

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$^{TM}$ (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 for 15 min. The supernatant was then collected. Rat 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).

**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 Go$_{a_1}$ 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 cerebral carotid artery form. After 2 h of MCAO, the intraluminal filament was withdrawn from the internal cerebral 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-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (50 µg, 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.
RESULTS

Jab1 Specifically Interacts with the Human 5-HT₆ Receptor—We previously demonstrated that the C-terminal region of human 5-HT₆R interacts with the Fyn tyrosine kinase. We also characterized the signaling pathways downstream of 5-HT₆R activation via a direct interaction with Fyn (21). In the present study, we found a new binding protein, Jab1, that bound to 5-HT₆R 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-HT₆R based on the yeast two-hybrid screening assay (Fig. 1A).

To verify a specific interaction between Jab1 and 5-HT₆R, we attempted to determine whether Jab1 selectively binds to 5-HT₆R using a GST pulldown assay. As shown in Fig. 1B, His-tagged Jab1 specifically interacted with GST-iL3 or GST-CT, which contain the iL3 and CT regions of 5-HT₆R, respectively, but Jab1 did not interact with GST alone or with GST-iL2 (intracellular loop 2 of 5-HT₆R). We then sought to identify the specific residues within Jab1 that are responsible for binding to 5-HT₆R. 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. 1A), 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. 1C, we found that CT-Jab1 and NT-Jab1 selectively interacted with GST-iL3 and GST-CT of 5-HT₆R, respectively.

These GST pulldown results were also confirmed in a
mammalian system using CHO/K-1 cells transiently transfected with FL-AG-tagged Jab1 and in rat brain lysates. Endogenous and full-length exogenous Jab1 from CHO/K-1 cells (Fig. 1D) and endogenous Jab1 from rat brain (Fig. 1E) bound to both GST-iL3 and GST-CT of 5-HT₆R, whereas no signal was detected using GST alone.

To show the relevance of the specific binding of Jab1 to 5-HT₆R, 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-HT₆R 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. 1F, the transfected HA-5-HT₆R 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. 1F). We also detected the association of 5-HT₆R and Jab1 in native tissues using rat brain lysates. As shown in Fig. 1G, endogenous 5-HT₆R 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-HT₆R in mammalian cell lines and in native tissues.

Jab1 Co-localizes with 5-HT₆ 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-HT₆R (5-HT₆R-eYFP). When eCFP and eYFP control constructs were co-expressed in HEK293 cells and imaged, no FRET signal was observed (Fig. 2A). However, when eCFP-Jab1 and 5-HT₆R-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. 2A). 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-HT₆R, we observed increased fluorescence of eCFP-Jab1 in the region of acceptor bleaching when compared with the pre-bleaching images (Fig. 2B and supplemental Fig. S1A). These results indicate that 5-HT₆R-eYFP and eCFP-Jab1 were in close proximity in the cells.

We next examined the co-localization of 5-HT₆R 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-HT₆R. As shown in Fig. 2C and supplemental Fig. S1B, HA-5-HT₆R 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-HT₆R 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-HT₆R 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-HT₆R and Jab1 in hippocampal neurons. Immunohistochemistry was also performed to examine any relationship in the regional and cellular distributions of 5-HT₆R and Jab1 in the rat brain. As shown in Fig. 2D and supplemental Fig. S1C, we used anti-5-HT₆R and anti-Jab1 antibodies to map the regional expression of 5-HT₆R and Jab1, respectively, in coronal sections of adult rat brains. The use of anti-5-HT₆R 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-HT₆R 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-HT₆R and Jab1 in vivo.

The Expression Level of Jab1 Modulates G-protein-mediated 5-HT₆ Receptor Activities—Based on the evidence of a physical interaction between 5-HT₆R and Jab1, we next examined whether overexpression of Jab1 affects 5-HT₆R activity. In our previous studies, we demonstrated that the activity of 5-HT₆R can be measured as 5-HT-induced Ca²⁺ increases using a promiscuous Go₁₅ protein (21) or a chimeric Go₁₅/S₁₅D₅ protein (25), which allows coupling of Gαₛ-coupled receptors to phospholipase C and consequent intracellular Ca²⁺ release. HEK293 cells were transfected with 5-HT₆R and a promiscous Go₁₅ protein for 24 h. 5-HT-induced Ca²⁺ increases via activation of 5-HT₆R were measured using the fluoro-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 Ca²⁺ responses following a single dose (10 μM, Fig. 3A) or a full dose (0.1 nM–10 μM, Fig. 3B) of 5-HT. However, the overexpression of Fyn significantly increased 5-HT-induced Ca²⁺ signals (Fig. 3C) 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-HT₆R activity. As shown in Fig. 3D and supplemental Fig. S2A, the knockdown of endogenous Jab1 significantly reduced 10 μM 5-HT-induced Ca²⁺ 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-HT₆R affinity. The calculated EC₅₀ 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. 3E). The expression level of Jab1 was confirmed by immunoblotting analysis; transfection of Jab1 siRNA significantly reduced endogenous Jab1 expression in
HEK293 cells (42.5 ± 6.4% of the control, n = 3, p < 0.001, Fig. 3F and supplemental Fig. S2B).

We next examined whether the knockdown of endogenous Jab1 affects the expression level of 5-HT6R in HEK/HA-5-HT6R cells (HA-5-HT6R was stably expressed in HEK293 cells). We found that the transfection of Jab1 siRNA induced a significant decrease in the expression level of 5-HT6R (74.3 ± 5.5% of the control, n = 3, p < 0.01, Fig. 4A), whereas the overexpression of Jab1 did not affect the expression level of 5-HT6R (100.2 ± 5.1% of the control, n = 3, Fig. 4B). To examine whether the Jab1 modulates 5-HT6R stability, the turnover of 5-HT6R was measured after the pretreatment with cycloheximide for 0–6 h to prevent de novo receptor synthesis. As shown in Fig. 4C, 5-HT6R expression levels were significantly decreased over 3 h in Jab1 knockdown cells as compared with the negative control. However, 5-HT6R expression levels were not significantly changed in Jab1-overexpressed cells as compared with FLAG-vector control (Fig. 4D). These data suggest that the regulation of 5-HT6R expression levels by Jab1 is due to effects on 5-HT6R stability. The immunofluorescent images using 5-HT6R-eGFP, and the associated pixel line scans, also clearly demonstrate that less 5-HT6R 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-HT6R and its functional activity can be regulated by its binding protein Jab1.

Activation of 5-HT6 Receptor Affects the Distribution of Jab1 and the Interaction between Jab1 and c-Jun—We next examined whether the activation of 5-HT6R influences Jab1 activity. We first addressed the question of whether the activation of 5-HT6R 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-HT6R cells (CHO cells stably expressing 5-HT6R) induces more condensed staining of Jab1 in the nucleus as compared with control cells, which display broad staining for Jab1 in the nucleus as shown in Fig. 5A. This altered distribution of Jab1 was also examined under time- and fraction-dependent experiments using immunoblotting assays. As shown Fig. 5B, the treatment of CHO/5-
HT6R cells with 20 μM 5-HT induced a time-dependent change 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-HT6R.

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-HT6R can increase c-Jun phosphorylation in CHO/5-HT6R cells. As shown in Fig. 5C, the treatment of CHO/5-HT6R 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-HT6R by using co-immunoprecipitation assays. Fig. 5D shows that 20 μM 5-HT increased the interaction between Jab1 and c-Jun in a time-dependent manner in CHO/5-HT6R cells. Immunocomplexes 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-HT6R and Jab1, as shown in Fig. 5E. Taken together, these data obviously demonstrate that the activation of 5-HT6R can affect Jab1 activities.
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-HT₆R-specific signal mediator.

**Correlation of 5-HT₆ 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-HT₆R and Jab1 in the brain, we examined expression levels of 5-HT₆R and Jab1 in brain sections obtained from middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemic rats, in which the HIF-1α protein is upregulated (32). After confirming the damaged brain areas using cresyl violet staining, we immunostained coronal brain slices with anti-5-HT₆R and anti-Jab1 antibodies. As shown in Fig. 6A, we found that the damaged ischemic areas produced increasing levels of 5-HT₆R and Jab1, as compared with the ipsilateral undamaged control area. The damaged cortex and striatum areas were measured quantitatively. Fig. 6B 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)). As shown in Fig. 6, B and C, cerebral ischemia significantly increased the expression levels of both 5-HT₆R (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-HT₆R and Jab1 are coordinately altered in cerebral ischemia and that these two proteins might play important roles in pathophysiological conditions such as ischemic disorders.

**5-HT₆R and Jab1 Enhance Cell Survival under Hypoxic Conditions**—To further explore the potential roles of 5-HT₆R and Jab1 as related to the in vivo ischemic results, we examined the expression level of 5-HT₆R 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 CoCl₂, a known hypoxia-mimicking agent and a chemical inducer of HIF-1α (33, 34), for 18 h significantly increased the

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**FIGURE 4.** Jab1 modulates the expression levels of 5-HT₆R by regulating stability of 5-HT₆R. After HEK/HA-5-HT₆R 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-HT₆R 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-HT₆R 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-HT₆R 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-HT₆R-eGFP and negative control siRNA-cy3 or with 5-HT₆R-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-HT₆R-eGFP and negative control siRNA-cy3 (left, blue color) or with 5-HT₆R-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.
The expression level of 5-HT₆R in HEK/HA-5-HT₆R cells in a dose-dependent manner (Fig. 7, A₁ and A₂). In addition, the expression level of Jab1 was also significantly increased by 300 and 600 μM CoCl₂ (133.1 ± 9.5% of the control at 300 μM; 146.4 ± 11.6% of the control at 600 μM, Fig. 7, A₁ and A₃). These results are consistent with those obtained from ischemic models; the expression levels of 5-HT₆R and Jab1 are also increased by a chemically induced hypoxic insult in vitro.

5-HT₆R 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-HT₆R plays a critical role in protection of cells from hypoxic-mediated cell death, we hypothesize that HEK/HA-5-HT₆R cells exhibit less cell death during hypoxia than do native HEK293 cells that do not express 5-HT₆R proteins. As shown in Fig. 7B and supplemental Fig. S4A, HEK/HA-5-HT₆R cells showed significantly less cell death than did native HEK293 cells at all tested doses of CoCl₂. We confirmed these results using an in vitro OGD model with cultured cells instead of using the hypoxia-mimicking agent CoCl₂. When exposed to an anaerobic gas mixture (5% CO₂, 10% H₂, and 85% N₂) in a glucose-free solution, HEK/HA-5-HT₆R cells exhibited less cell death than did native HEK293 cells (Fig. 7C). To explore the involvement of Jab1 in hypoxia-mediated cell death, Jab1 was selectively knocked down using siRNA methods. When HEK/HA-5-HT₆R cells were transiently transfected with negative control or Jab1

![Image of Figure 5: The activation of 5-HT₆R affects the Jab1 distribution and the interaction with c-Jun or 5-HT₆R. A, after CHO/5-HT₆R 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-HT₆R 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-HT₆R, 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.
siRNA, negative control siRNA-transfected cells showed less cell death than did the Jab1 knockdown cells at all tested doses of CoCl₂ (Fig. 7D) and in vitro OGD model cultured cells (Fig. 7E). Because it is not clear whether Jab1 is involved in the hypoxia-induced cell death as shown in Fig. 7D and supplemental Fig. S4B, we performed double knockdown experiments using 5-HT₆R and Jab1 siRNAs and examined cell viability in HEK/HA-5-HT₆R cells. As shown in Fig. 7F and supplemental Fig. S4C, the involvement of 5-HT₆R in the hypoxia-induced cell death was also confirmed in a direct 5-HT₆R knockdown experiment using 5-HT₆R siRNA. However, the involvement of Jab1 was also observed when both 5-HT₆R and Jab1 genes were down-regulated in double knockdown experiments. It is interesting that the presence of 5-HT₆R or Jab1 genes themselves caused greater cell survival in control cells that were not exposed to hypoxic insults, as compared with cells lacking 5-HT₆R genes or both 5-HT₆R 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-HT₆R and Jab1. The presence of these two proteins protected against hypoxia-induced cell death, suggesting important roles for 5-HT₆R 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-HT₆R and Jab1. This is an original report demonstrating the direct interaction of a Gα₅-coupled membrane receptor, 5-HT₆R, and a co-activator of c-Jun-mediated transcription, Jab1. The 5-HT₆R-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-HT₆R (iL3 and CT) were independently found to interact with Jab1, suggesting the possibility of multiple 5-HT₆R domains that contribute to the 5-HT₆R-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-HT₆R are required and selectively interact with other parts of Jab1: iL3 of 5-HT₆R binds to CT-Jab1 and CT of 5-HT₆R binds to NT-Jab1. These data imply that 5-HT₆R and Jab1 may modulate each other’s activity. In the case of 5-HT₆R, the iL3 and CT domains of 5-HT₆R are known to be necessary for interaction with a Gα₅-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-HT₆R 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. 8A) and CT (Fig. 8B) 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 p27Kip1, 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,
Novel Interaction between 5-HT_6 Receptor and Jab1

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

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**A**

A1: CoCl₂ (μM) CON 30 100 300 600 (KDa) 42 35 50 → 5-HT₆R → Jab1 → β-tubulin

A2: Relative level of 5-HT₆R(%) 0 30 100 300 600

A3: Relative level of Jab1 (%) 0 30 100 300 600

**B**

B: Cell Viability(%) CON 30 100 300 600

**C**

C: Cell Viability(%) Control Hypoxia

**D**

D: Cell Viability(%) CON 30 100 300 600

**E**

E: Cell Viability(%) Control Hypoxia

**F**

F: Cell Viability(%) CON 30 100 300 600
modulating their activities. Our binding data strongly suggest that 5-HT6R 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-HT6R? 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-HT6R. Overexpression of Jab1 in HEK293 cells produced no effect on the ability of cells to respond to a 5-HT6R agonist. However, inhibition of Jab1 expression by Jab1 siRNA decreased the activity of 5-HT6R without changing the affinity of 5-HT for the receptor. Thus, it is possible that Jab1 may inhibit the activity of 5-HT6R through an inhibition of 5-HT6R expression. Western blot and the immunofluorescent image experiments suggest this possibility. Jab1 knockdown using siRNA decreased cell surface expression of 5-HT6R as well as total expression of 5-HT6R. Most GPCRs, including 5-HT6R, 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-HT6R and that the presence of Fyn increased the surface expression of 5-HT6R (21). These results suggest that the CT region of 5-HT6R is essential to maintain 5-HT6R 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-HT6R desensitization, internalization, and degradation by direct binding to the iL3 and CT regions of 5-HT6R, or whether Jab1 modulates the stability of 5-HT6R precursor molecules, as is the case for rLHR.

Second, Jab1 provides a novel signal transduction pathway for 5-HT6R-mediated pathways. The serotonergic system is implicated in the neurobiological control of learning and memory. In contrast to the implications of 5-HT6R in Alzheimer disease and depression (6–9), the cellular mechanism of 5-HT6R activity has not yet been elucidated due to a lack of selective agonists for 5-HT6R. In addition to the 5-HT6R-mediated increase of cAMP, which implies function as a Gαq-protein-coupled receptor, our previous study first reported that 5-HT6R increases Fyn phosphorylation and ERK1/2 phosphorylation via a Fyn-dependent pathway (21). In the present study, we further elucidated that Jab1 mediates 5-HT6R signals from the cytoplasm to the nucleus. We found that activation of 5-HT6R 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...
phosphorylation via the activation of 5-HT₆R, which is the first report of 5-HT₆R-mediated c-Jun phosphorylation. To support these results, the interaction between 5-HT₆R and Jab1 and the interaction between Jab1 and c-Jun should change upon activation of 5-HT₆R. We observed a reduced level of interaction between 5-HT₆R 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-HT₆R? These results suggest that 5-HT₆R can play a role in the regulation of gene expression via Jab1, which may provide a crucial mechanism in 5-HT₆R-related conditions such as Alzheimer disease, depression, and learning and memory disorders. Therefore, we are currently examining whether 5-HT₆R can modulate AP-1 activity via an interaction between 5-HT₆R and Jab1.

Finally, Jab1, along with its binding partner 5-HT₆R, 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-HT₆R 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-HT₆R in the brain. Why do hypoxic insults increase 5-HT₆R 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-HT₆R and Jab1 genes. Even with no hypoxic insult, the presence of 5-HT₆R and Jab1 genes increased cell survival in comparison to cells lacking 5-HT₆R 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 H₂O₂ 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-HT₆R as a cell protector. One relevant report was from the recent study on 5-HT₄ receptors, which belong to the same GPCR family as 5-HT₆ receptors. Liu et al. (47) recently showed 5-HT₄ 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-HT₆R-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-HT₆R-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-1α/hypoxia induces hypoxic adaptation or hypoxic apoptosis. The hypothesis is that the interaction of Jab1 with HIF-1α promotes adaptation to hypoxia, whereas the interaction of p53 and HIF-1α 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-HT₆R-Jab1 complex in the context of hypoxic insults. Furthermore, our data suggest that 5-HT₆R plays a more dominant role in cell protection than Jab1 does, because the involvement of Jab1 was clearly seen in both 5-HT₆R 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-HT₆R-mediated cellular events via an interaction with Jab1 but also in providing evidence for the correlation between 5-HT₆R 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-HT₆R and Jab1 in the central nervous system.

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