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Physical and Functional Interaction between 5-HT$_6$ Receptor and Nova-1

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5-HT$_6$ receptor (5-HT$_6$R) is implicated in cognitive dysfunction, mood disorder, psychosis, and eating disorders. However, despite its significant role in regulating the brain functions, regulation of 5-HT$_6$R at the molecular level is poorly understood. Here, using yeast two-hybrid assay, we found that human 5-HT$_6$R directly binds to neuro-oncological ventral antigen 1 (Nova-1), a brain-enriched splicing regulator. The interaction between 5-HT$_6$R and Nova-1 was confirmed using GST pull-down and co-immunoprecipitation assays in cell lines and rat brain. The splicing activity of Nova-1 was decreased upon overexpression of 5-HT$_6$R, which was examined by detecting the spliced intermediates of gonadotropin-releasing hormone (GnRH), a known pre-mRNA target of Nova-1, using RT-PCR. In addition, overexpression of 5-HT$_6$R induced the translocation of Nova-1 from the nucleus to cytoplasm, resulting in the reduced splicing activity of Nova-1. In contrast, overexpression of Nova-1 reduced the activity and the total protein levels of 5-HT$_6$R. Taken together, these results indicate that when the expression levels of 5-HT$_6$R or Nova-1 protein are not properly regulated, it may also deteriorate the function of the other.

Key words: Serotonin, 5-HT$_6$ receptor, Neuro-oncological ventral antigen 1, RNA binding proteins, Neurological diseases

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is best known as a neurotransmitter that modulates neural activity and a wide range of neuropsychological process [1]. Until now, seven distinct subfamilies of 5-HT receptor, 5-HT$_{1-7}$ receptors, were found in mammals and these are a group of G-protein-coupled receptors (GPCRs), except 5-HT$_3$ receptor that is a ligand-gated ion channel [2]. Among them, 5-HT$_6$ receptor (5-HT$_6$R) is coupled to a stimulatory Gα protein, which increases cAMP formation and then activates cAMP-dependent protein kinase A (PKA) [3, 4]. 5-HT$_6$R is exclusively expressed within the central nervous system (CNS) [4], while most other 5-HT receptors are widely expressed throughout organs [5, 6]. Recent studies have revealed that 5-HT$_6$R is implicated in the brain functions such as eating behavior, movement, cognition and mood [7-9], and selective antagonists of 5-HT$_6$R improve cognitive function in aged animals [10, 11] and in patients with Alzheimer disease [12, 13]. Also, 5-HT$_6$R has high af-

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finity for several antipsychotic and antidepressant drugs [7, 8] and modulation of 5-HT_6R showed antidepressant and anxiolytic-like effects, [14-16] suggesting that it is a potential therapeutic target for psychological disorders. However, little is known about the role of 5-HT_6R in these diseases or normal physiology, except the fact that it functions through the binding to Gα protein [4]. Therefore, we aimed to identify the binding partners of 5-HT_6R to understand the 5-HT_6R-mediated physiological responses. In an effort for this, our group previously reported that Fyn, a member of the Src family of non-receptor protein-tyrosine kinase, Jun activation domain-binding protein-1 (Jab1) and microtubule-associated protein 1B (MAP1B) directly interact with 5-HT_6R and play roles in 5-HT_6R-mediated signaling pathways in CNS [17-19]. In present study, we identified neuro-oncological ventral antigen 1 (Nova-1) as a novel interacting partner of 5-HT_6R.

Nova was first identified as an onconeural antigen in paraneoplastic opsoclonus-myoclonus ataxia (POMA) patients who harbored a high-titer termed Ri antibody [20]. This Ri antigen that reacts with Ri antibody was named afterward as Nova, and two isoforms, Nova-1 and Nova-2 were identified [21]. Nova-1 and Nova-2 are expressed only in CNS, but interestingly, their expression patterns within CNS are mutually exclusive; Nova-1 is expressed in hindbrain and spinal cord, whereas Nova-2 is expressed in the brain regions where Nova-1 is not present, such as neocortex and thalamus [22, 23]. Nova-1 is a member of RNA binding proteins (RBPs) and has three typical RNA binding domains, so called K homology (KH) motif [24-26]. The consensus binding sequences of Nova-1 are well-defined as YCAY element [27-30], and among RNA metabolism, Nova-1 regulates pre-mRNA splicing and produces mature mRNA. Especially, through alternative splicing of pre-mRNA, Nova-1 contributes to proteome complexity and functional diversity [31]. Pre-mRNA targets of Nova-1 are mainly proteins involved in synapse formation or synaptic transmission, including inhibitory $GABA_A$ receptor γ2 ($GABA_A$Rγ2), glycine receptor α2 (GlyRα2) [27-31]. Nova also actively shuttles from nucleus to cytoplasm, as far as synaptic contact in dendrites, along its target RNAs, where Nova may contribute to mRNA localization [29]. This result implies that the role of Nova-1 can extend beyond the established boundary of RNA splicing. Although Nova-1 and 5-HT_6R belong to a different functional category with distinct subcellular localization, we demonstrated that Nova-1 directly binds to 5-HT_6R both *in vitro* and *in vivo*. In particular, overexpression of 5-HT_6R reduced the splicing activity of Nova-1 and triggered the translation of Nova-1 from nucleus to cytoplasm. In contrast, overexpression of Nova-1 weakened the activity and stability of 5-HT_6R via promoting the proteasomal degradation of 5-HT_6R.

MATERIALS AND METHODS

Plasmid constructs

A human brain cDNA library in the GAL4 activation domain vector pACT2 was purchased from BD biosciences (Palo Alto, CA). Full-length non-tagged and HA-tagged human 5-HT_6R cDNA in pcDNA3.1 were purchased from UMR cDNA Resource Center (Miner Circle Rolla, MO). The Myc-tagged 5-HT_6R plasmid was constructed by recloning the human 5-HT_6R cDNA into pCMV-Tag3B vector (Stratagene, La Jolla, CA). cDNA fragment encoding the carboxyl terminus of 5-HT_6R (6RCT) was subcloned into pGBKT7 (Clontech, Palo Alto, CA). GST-6RCT was kindly provided by Dr. Y. G. Yu (Kookmin Univ., Korea). His-tagged 6RCT was constructed by subcloning the 6RCT into pET28a (+) vector. The CTs of 5-HT_4R and 5-HT_{7B}R (4RCT and 7BRCT) were cloned into pGEX4T-1 (Amersham, Piscataway, NJ) to express them in a GST-fusion form. The primer sequences used were as follows: 4RCT (Fw, 5'-GAATTCCCGAGACGTGCCTTCCTC TC-3'; Rv, 5'-CTCGAGAGTGTCACTGGGCTGAGCAG-3') and 7BRCT (Fw, 5'-GAATTCCCGGACCTGAGGACCACCTATC-3'; Rv, 5'-CTCGAGCAGCACAAACTCAGGTC-3'). Full length human Nova-1 in pCMV6-XL5 was purchased from Origene (Rockville, MD). Flag-tagged Nova-1 was constructed by recloning into pCMV-Tag2B (Stratagene). GST-Nova239-419 (239-419 amino acids of mouse Nova-1) and GST-KH3 (KH3 domain, 420-507 amino acid of mouse Nova-1) were constructed by subcloning from full length Nova-1 into pGEX4T-1 vector. Nova-1 full length, Nova-1-ΔKH1 and Nova-1-ΔKH1/2 constructs were generated by fusing a HA tag to either full length or partially truncated human Nova-1 constructs.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed using the Matchmaker GAL4 two-hybrid system 3 (Clontech). The bait plasmid, pGBKT7/CT of 5-HT_6R, was stably expressed in yeast strain AH109 and did not have 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 [17].

Cell culture and transfection

GT1-1, NIH3T3, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GenDEPOT) and 100 units/ml penicillin/streptomycin (Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C. HEK293 cell lines stably expressing the human 5-HT_6R (HEK293/6R cells) were selected and maintained with 800 μg/ml

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and 400 μg/ml of G418, respectively. For the transient transfection, cells were plated in 6-well plates and grown to 70-90% confluence in 1 day. The cells were transfected with plasmid DNA using Lipofectamine PLUS reagent (Invitrogen). Six hours after transfection, the medium was changed with complete medium. For the transfection into GT1-1 cells, Lipofectamine reagent was treated for 4 h. The cells were analyzed after 24 h of recovery in normal medium.

GST pull-down assays

GST-mediated pull-down assay was performed using the Profound Pull-down GST Protein:Protein Interaction kit (Pierce, Rockford, IL). GST and GST-6RCTs plasmids were transformed into bacterial strain BL21 (DE3) and their protein expressions were induced by adding 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG, Sigma) at 25°C during mid-log phase. GST and GST-6RCTs proteins were immobilized by glutathione gel. To prepare prey protein, Flag-Nova-1 plasmid was transfected into HEK293 cells and cell lysates were harvested after 24 h of transfection. For GST pull-down assay using rat brain lysates, the lysates were prepared from age- and weight controlled adult male SD rats (60-70 days old, 230-260 g) as previously described [17]. Prepared cell or brain lysates were incubated with immobilized GST proteins. Bound proteins were eluted by boiling for 10 min at 95°C in SDS sample buffer followed by immunoblotting with anti-Nova-1 (Upstate Biotechnology Inc., Lake Placid, NY) and anti-GST antibodies (Novagen, Madison, WI).

Co-immunoprecipitation

Co-immunoprecipitation was performed as described by Yun et al. [18]. Soluble HEK293 cell lysates were precleared with 50 μl of ImmunoPure immobilized protein G Plus (Pierce) and 2 μg of rabbit normal IgG for 1 h. Precleared lysates were incubated with 4 μg of anti-Myc, anti-HA (Cell Signaling Technology, Beverly, MA), or anti-Flag (Sigma-Aldrich, St. Louis, MO) antibodies overnight at 4°C. Then the lysates were added with 50 μl of ImmunoPure immobilized Protein G Plus and incubated for 4 h at 4°C and washed three times with lysis buffer. Immune complexes were eluted by boiling for 5 min at 95°C in SDS sample buffer, followed by Western blot analysis. For co-immunoprecipitation using rat brain lysates, the lysates were immunoprecipitated with 10 μg of anti-5-HT$_6$R antibody (GeneTex Inc., San Antonio, TX).

RT-PCR analysis

Total RNA was extracted using Easy-spin total RNA extraction kit (Intron biotechnology, Korea). To eliminate possible DNA contamination, 1 μg of RNA samples were treated with 0.2 units of DNase I (NEB, Beverly, MA) for 10 min at 37°C. Then the RNA was heated for 10 min at 75°C to inactivate DNase I and reverse-transcribed using random hexamers. The primers used for RT-PCR were as follows: 6R E1-F, 5'-TGTCTGCTTTGGACCGCCTT-3'; 6R IA-F, 5'-GCTTCAGCTGGGGATCAGTCAA-3'; 6R E2-R, 5'-ATGCTGGCCACAAAGAAGGG-3'. The primers for GnRH E1-up, IA-up, and E3-dn were synthesized referring to the sequences previously described [32]. The primer sequences used are as follows: E1-up, 5'-GGAAGACATCAGTGTCCCAGA-3'; IA-up, 5'-TACCTCTGCAGTTTCTGTGA-3'; E3-dn, 5'-GAAGTGCTGGGGTTCTGC-3'. GAPDH was used as an internal control for semi-quantitative RT-PCR. The GAPDH primers were forward primer 5'-CTCTCCAGAACATCATCCCTG-3' and reverse primer 5'-CACCCTGTT GCTGTAGCCAAA-3'.

Western blot analysis

For immunoblotting, proteins were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with Tris-buffered saline containing 5% skim milk (BD Difco) and 0.1% Tween 20 (Sigma) for 1 h at room temperature (RT). Then, the membranes were incubated with the respective primary antibodies overnight. After three washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT. The immune complexes were visualized with an ECL detection kit (Millipore). The following antibodies were used for western blot: anti-5-HT$_6$R (GeneTex Inc., San Antonio, TX); Anti-Nova-1 (Novus Biologicals, USA); anti-HA (Cell Signaling Technology, Beverly, MA); anti-Actin (sigma); anti-β-Tublin (Cell Signaling Technology); anti-histone H3 (Cell Signaling Technology); anti-phospho-ERK (Cell Signaling Technology); and anti-ERK (Cell Signaling Technology).

Immunofluorescence

HEK293, GT1-1 cells or cortical neurons were grown on coverslips (Corning, Corning, NY). Twenty four hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT. The cells were washed with PBS and permeabilized with 0.2% Triton X-100 (Sigma) for 15 min. Then the cells were blocked with 3% bovine serum albumin (GenDEPOT) for 1 h at RT and incubated overnight with rabbit anti-HA (Sigma) and mouse anti-Flag antibodies (1:500) at 4°C. After three washes, the cells were incubated with anti-rabbit IgG (FITC) and anti-mouse IgG (rhodamine) secondary antibodies (1:400; Jackson ImmunoResearch) for 2 h at RT. The cells were washed three times and counter-stained with DAPI (Sigma) for 10 min. After mounting on glass slide using ProLong Antifade reagent (Invitrogen), the cells were examined

with a FluoView® Confocal Laser Scanning Microscope (Olympus, Japan).

Nuclear/cytoplasmic fractionation

Twenty four hour after transfection, HEK293 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, Mountain View, CA), following the manufacturer's protocol.

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 [18, 33]. Briefly, HEK293/6R stable cells were transiently transfected with Gα_{15} protein using Lipofectamine Plus (Invitrogen). After 6 h, the cells were transferred into 96-well black wall/clear bottom plate and cultured overnight. The cells were loaded with Ca^{2+} indicator dye Fluo-4-AM (5 μM) and 0.001% Pluronic F-127 (Molecular Probes, Eugene, OR) and incubated in a HEPES-buffered solution (150 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM HEPES, 13.8 mM glucose and 2 mM $CaCl_2$, pH 7.4) for 1 h at 37°C. After three washes, 10 μM of 5-HT (Sigma) was added to the cells and Ca^{2+} response was measured at 480 nm. All data were collected and analyzed using the FDSS system and related software (Hamamatsu Photonics).

Statistical analysis

The intensity of bands was measured with Image J software (National Institute of Health, Bethesda, MD) and analyzed using the GraphPad Prism Version 4 (GraphPad Software Inc., San Diego, CA). All numeric values are represented as the mean±S.E.M. Data were analyzed by Student's t-test, one-way analysis of variance (ANOVA) followed by Duncan's post-hoc tests or two-way ANOVA followed by Newman-Keuls post hoc tests.

RESULTS

Nova-1 directly binds to the 5-HT$_6$R in vitro and in vivo

Our group reported previously that Fyn, Jab1 and MAP1B directly interact with 5-HT$_6$R *in vitro* and *in vivo* and play roles in 5-HT$_6$R-mediated signal transduction [17-19]. In this study, we also identified Nova-1 as a new binding protein of 5-HT$_6$R, through a yeast two-hybrid screening assay using the C-terminal (CT) region of human 5-HT$_6$R (bait) and the human brain cDNA library. We confirmed this specific interaction by a separate yeast two-hybrid assay in which the CT of 5-HT$_6$R (bait) and full-length Nova-1 cDNA (prey) were transformed in the AH109 and Y187 yeast strains, respectively (Fig. 1A), and a blue color colony was detected after mating the two strains (Fig. 1B). GST pull-down assay also verified the direct binding between Nova-1 and 5-HT$_6$R. GST-fused to the CT of 5-HT$_6$R (GST-6RCT) bound to the Flag-tagged-Nova-1 (Flag-Nova-1), but GST protein did not (Fig. 1C), indicating that 5-HT$_6$R directly binds to Nova-1 via the CT region. However, given that this interaction occurred with partial fragment of 5-HT$_6$R outside cells, we attempted to determine whether full-length 5-HT$_6$R binds to Nova-1 in a specific manner in mammalian cells using co-immunoprecipitation. After full-length Myc-tagged 5-HT$_6$R (Myc-5-HT$_6$R) and Flag-Nova-1 or empty vector (Flag-V) were transiently transfected into HEK293 cells, cell lysates were prepared, immunoprecipitated with anti-Flag antibodies, and subsequently immunoblotted with anti-Myc antibodies. Myc-5-HT$_6$R band was visible only in the HEK293 lysates containing Flag-Nova-1 protein (Fig. 1D). When co-immunoprecipitation was performed in reverse with anti-Myc antibodies followed by immunoblotting with anti-Flag antibodies, Myc-5-HT$_6$R immunoprecipitated with Flag-Nova-1 protein (Fig. 1E). These results confirm the direct interaction between Nova-1 and 5-HT$_6$R in mammalian cells.

Next, we examined whether the interaction between 5-HT$_6$R and Nova-1 occurs *in vivo* using the rat brain lysates. As previously reported, 5-HT$_6$R was widely expressed in the brain while Nova-1 was preferentially expressed in the brainstem and hypothalamus (Fig. 1F). Similarly to the GST pull-down assay in HEK293 cells, GST-6RCT pulled down Nova-1 in the rat brain lysate, but GST protein did not (Fig. 1G). We further confirmed this interaction *in vivo* using co-immunoprecipitation assay. Rat whole brain lysates were immunoprecipitated with anti-5-HT$_6$R antibody and subsequently immunoblotted with anti-Nova-1 antibody. As shown in Fig. 1H, endogenous Nova-1 signal was selectively detected in the sample immunoprecipitated with anti-5-HT$_6$R. These results consistently showed that 5-HT$_6$R interacts with Nova-1 under physiological conditions. To prove the specific binding between Nova-1 and 5-HT$_6$R, we also examined whether Nova-1 interacts with other types of serotonin receptors. Among several 5-HT receptors, we chose 5-HT$_4$R and 5-HT$_{7B}$R given that these both receptors belong to the Gα_s-family similar to 5-HT$_6$R, and used their intracellular domain CT regions as bait proteins for GST pulldown assay. As in Fig. 1I, CT of 5-HT$_4$R (4R) and CT of 5-HT$_{7B}$R (7BR) did not bind to Nova-1 protein, while CT of 5-HT$_6$R (6RCT) showed the distinct binding signal to Nova-1. Taken together, these results suggest that the 5-HT$_6$R selectively and directly binds to Nova-1 *in vitro* and *in vivo*.

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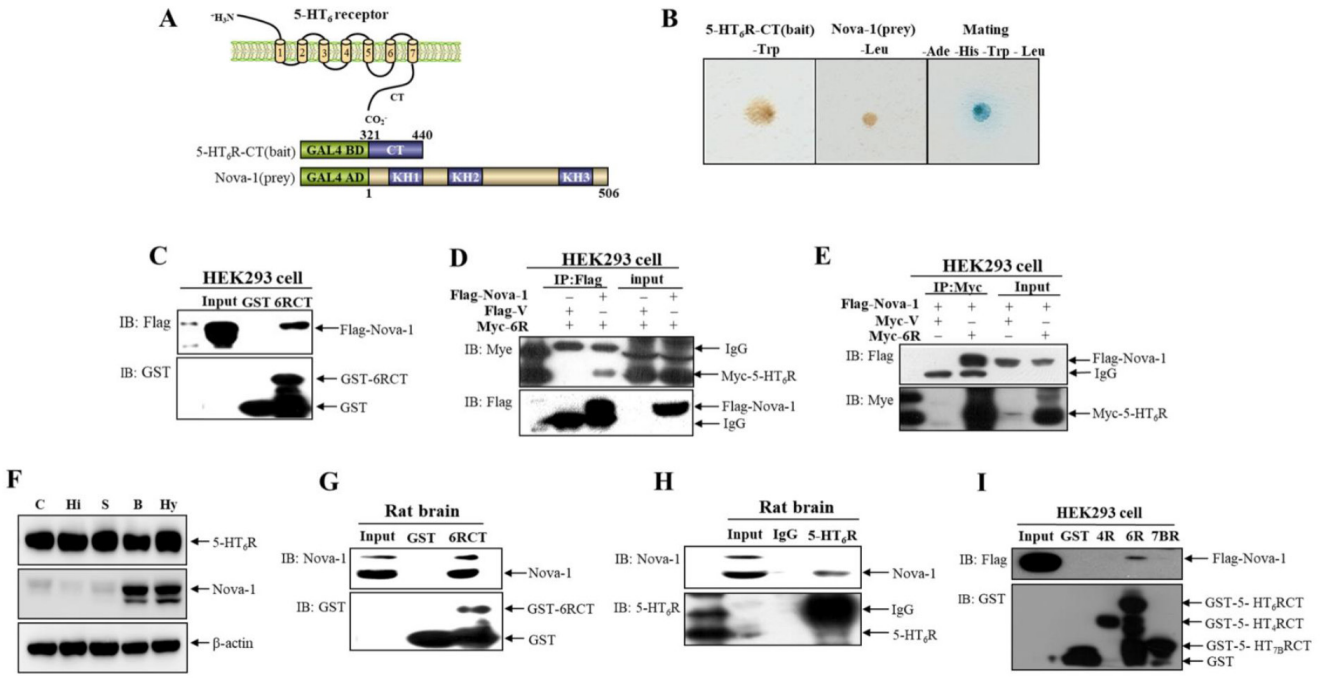

Fig. 1. Nova-1 interacts with 5-HT$_6$R *in vitro* and *in vivo*. (A) Schematic diagrams showing the structure of 5-HT$_6$R, the CT (carboxyl-terminus) of 5-HT$_6$R as bait and complete Nova-1 protein as prey. (B) When the AH109 strain with 5-HT$_6$R CT and the Y187 yeast strain with Nova-1 were mated, a blue color was detected. (C) GST pull-down assay showed that Flag-Nova-1 specifically interacted with GST-fused CT of 5-HT$_6$R (GST-6RCT). (D) Full-length Myc-tagged 5-HT$_6$R (Myc-5-HT$_6$R) signal was detected in the immunoprecipitation with anti-Flag. (E) Flag-Nova-1 signal was detected in the immunoprecipitation with anti-Myc. (F) Western blot with anti-5-HT$_6$R or anti-Nova-1 antibody showed the expression patterns of 5-HT$_6$R and Nova-1 in the rat brain. (G) GST-6RCT interacted with endogenous Nova-1 in the rat brain. (H) Co-immunoprecipitation assay confirmed the interaction between endogenous 5-HT$_6$R and Nova-1 in the rat brain. (I) When GST pull-down assays were performed between Nova-1 and CTs of several serotonin receptors (GST-4RCT, GST-6RCT and GST-7BRCT), Nova-1 only interacted with 6RCT but not 4RCT and 7BRCT. GAL4, galactose-responsive transcription factor GAL4; BD, binding domain; AD, activation domain; C, Cortex; Hi, Hippocampus; S, Striatum; B, Brainstem; Hy, Hypothalamus.

Identification of binding domain of Nova-1 that interacts with 5-HT$_6$R

Then, we tried to identify the binding sites of Nova-1 protein that interact with 5-HT$_6$R. Nova-1 has three KH domains that recognize and bind to target RNA [24-26]. On the basis of KH domains, three different Nova-1 constructs were generated in HA- and venus C-terminal (VC)-tagged form; full length Nova-1 (amino acid 1-507), Nova-1-ΔKH1 (amino acid 118~507, including KH2 and KH3 domains), and Nova-1-ΔKH1/2 (amino acid 239~507, including KH3 domain) (Fig. 2A). These constructs were transiently transfected into HEK293 cells and then their expressions were analyzed by immunoblotting with anti-HA antibody (Fig. 2B). To identify which domain binds to 5-HT$_6$R, HEK293 cell lysates containing HA-VC-tagged full length or truncated Nova-1 proteins were incubated with GST or GST-6RCT, and then GST pull-down assay was performed. As shown in Fig. 2C, CT of 5-HT$_6$R interacted with all three different Nova-1 proteins (full length and two kinds of truncated Nova-1 proteins). Also, when immunoprecipitation was carried out using anti-HA antibody, full length and two truncated Nova-1 proteins pulled down GST-6RCT protein (Fig. 2D). To verify the interaction between three different Nova-1 constructs and 5-HT$_6$R *in vivo*, immunoprecipitation with anti-5-HT$_6$R antibody was performed between rat brain and HEK293 lysates containing full length or truncated Nova-1 proteins. As seen in Fig. 2E, endogenous 5-HT$_6$R in the brain bound to all HA-VC-tagged full length and truncated Nova-1 proteins, whereas control IgG showed no binding signal. Taken together, these results suggest that 5-HT$_6$R is likely to interact with KH3 domain or upstream flanking region of KH3 because these domains are present in all of three Nova-1 constructs.

To further narrow down the specific binding site of Nova-1, 239-507 aa of Nova-1 was divided into two fragments, 239-419 aa and KH3 domain (420-507 aa) in a GST-fusion form (Fig. 2F, *left*). Then, we examined which domain binds to the 5-HT$_6$R using GST pull-down assay. GST-fused Nova-1 protein fragments and His-tagged 6RCT were expressed in *E. coli* and protein-protein interaction was examined. As a result of GST pull-down, CT of 5-HT$_6$R bound to only KH3 domain of Nova-1 while no signal was detect-

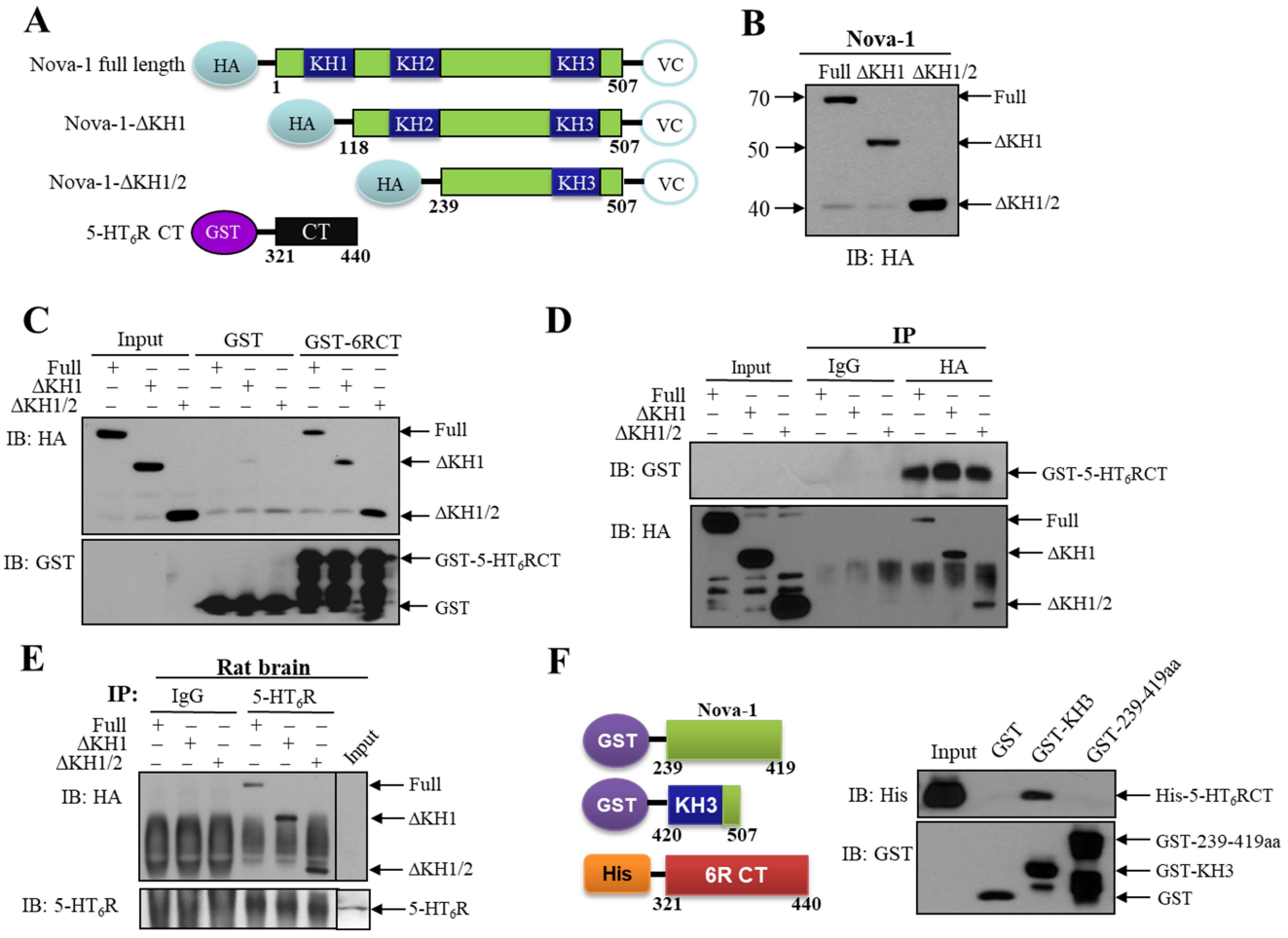

Fig. 2. The KH3 domain of Nova-1 is responsible for 5-HT$_6$R binding. (A) Schematic diagram of HA- and venus C-terminal (VC)-tagged full length and truncated Nova-1 constructs (top). GST-tagged CT of 5-HT$_6$R (GST-6RCT) (bottom). (B) The expression of HA-VC-tagged full length or truncated Nova-1 constructs was identified by immunoblotting with anti-HA antibody. (C) GST pull-down assay showed that GST-6RCT interacted with all three different Nova-1 constructs. (D) Specific interaction between GST-6RCT and three different Nova-1 constructs was validated by co-immunoprecipitation. (E) Co-immunoprecipitation assay showed that endogenous 5-HT$_6$R interacted with all three different Nova-1 constructs. (F) 5-HT$_6$R CT interacted with GST-KH3 but not with GST-Nova-1 (239-419 aa).

ed in control GST and GST-239-419 (Fig. 2F, *right*). These results indicate that 5-HT$_6$R binds to Nova-1 via KH3 domain.

Translocation of Nova-1 from nucleus to cytoplasm by 5-HT$_6$R overexpression

Based on evidence for a physical interaction between 5-HT$_6$R and Nova-1 and, we next examined whether the function of Nova-1 is affected by 5-HT$_6$R. Nova-1 is a neuron-specific RNA binding protein and regulates RNA splicing [27]. Therefore, we investigated whether there is any change in splicing activity of Nova-1 when 5-HT$_6$R is overexpressed, using gonadotropin-releasing hormone (GnRH) transcript that was reported as a splicing target of Nova-1 [32]. GnRH minigene and Nova-1 DNA were transiently transfected into NIH3T3 cells, and after 24 h, competitive RT-PCR was performed using three primers to evaluate the relative amount of spliced intermediates of target pre-mRNA (Fig. 3A). As described previously [32], Nova-1 facilitated the rate of intron A excision of GnRH pre-mRNA (Fig. 3B). However, when 5-HT$_6$R DNA was co-transfected with Nova-1, splicing activity of Nova-1 was significantly suppressed (8.0±1.1% of control at 1 μg of 5-HT$_6$R DNA, Fig. 3C). Meanwhile, truncated 5-HT$_6$R (5-HT$_6$RΔCT) lacking the CT region, a binding domain of 5-HT$_6$R with Nova-1, did not affect Nova-1-mediated intron A excision of GnRH, implying that binding of two proteins via CT region regulates the splicing activity of Nova-1 (Fig. 3D). In GT1-1 cells in which GnRH and Nova-1 are endogenously expressed, a consistent pattern of splicing intermediates was observed. As seen in Fig. 3E and F, 5-HT$_6$R overexpression suppressed the splicing activity of Nova-

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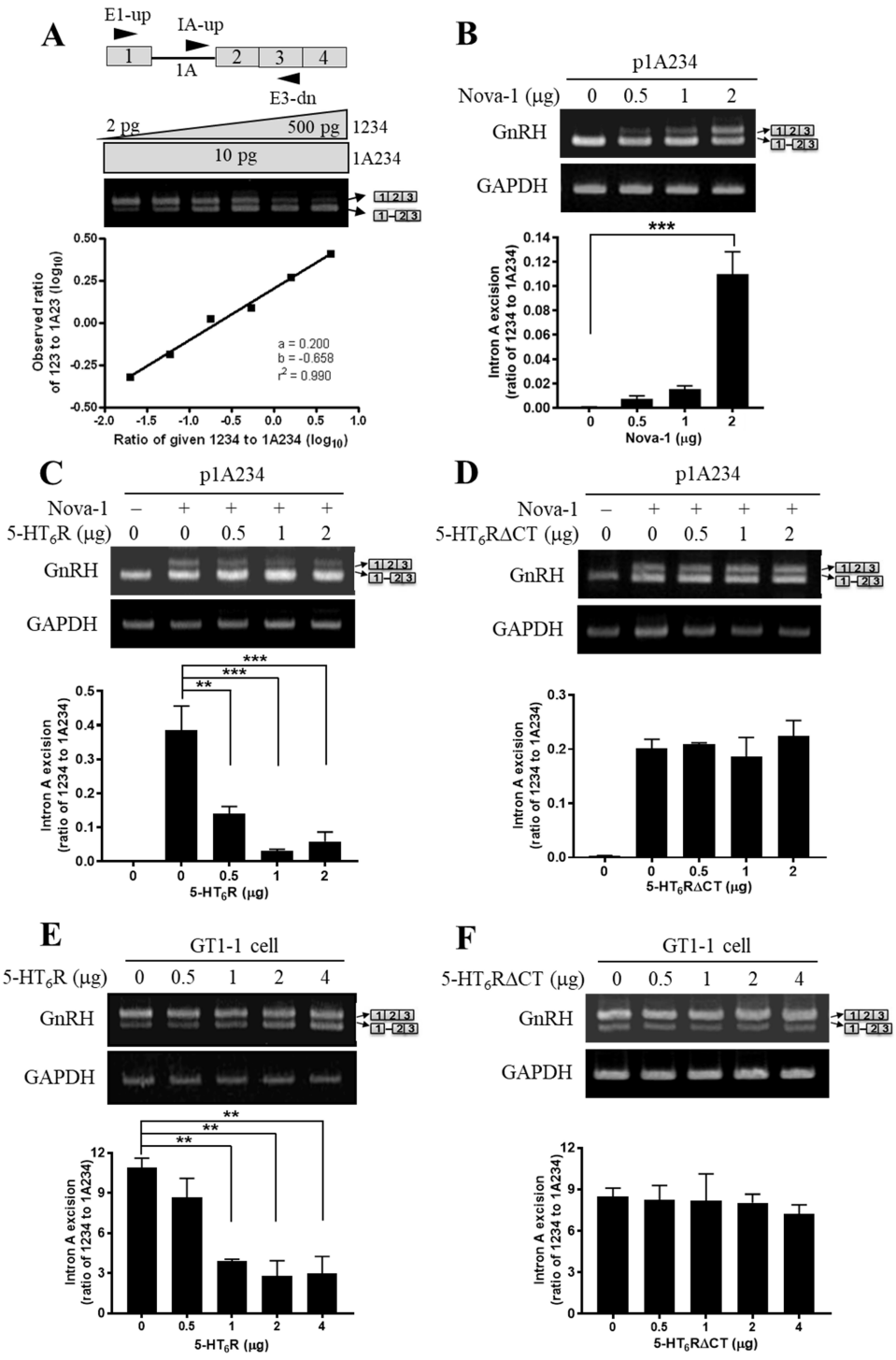

Fig. 3. The 5-HT$_6$R overexpression attenuates the splicing activity of Nova-1 centered on the excision of GnRH intron A. (A) The structure of the GnRH gene and positions of the primers (top). A standard curve was constructed to analyze the quantitative ratio of 1234 and 1A234 cDNAs by competitive PCR using 10 pg of the 1A234 and serial dilutions of the 1234 gene (bottom). (B) Nova-1 increased the rate of intron A excision of GnRH gene in dose-dependent manner when GnRH minigene and Nova-1 were transiently transfected into NIH3T3 cells ($F_{(3, 8)}$=29.79, p=0.0001; Dunnett's post hoc test: ***p<0.001). (C) 5-HT$_6$R decreased the splicing activity of Nova-1 in dose-dependent manner ($F_{(4,10)}$=18.92, p=0.0001; Dunnett's post hoc test: **p<0.01, ***p<0.001). (D) C-terminal truncated 5-HT$_6$R (5-HT$_6$RΔCT) did not affect the splicing activity of Nova-1 ($F_{(3, 8)}$=0.4302, p=0.7371). (E) 5-HT$_6$R dose-dependently decreased the splicing activity of Nova-1 in GT1-1 neuronal cell line in which GnRH and Nova-1 are endogenously expressed ($F_{(4, 10)}$=12.69, p=0.0006; Dunnett's post hoc test: **p<0.01). (F) The splicing activity of Nova-1was not affected by 5-HT$_6$RΔCT in GT1-1 neuronal cell line ($F_{(4. 5)}$=0.194, p=0.9314). Data are expressed as means±S.E.M. (n=3 per group).

1 (25.7±10.4% of control at 2 μg of 5-HT$_6$R DNA), but truncated 5-HT$_6$R (5-HT$_6$RΔCT) did not affect the intron A excision rate of GnRH. Taken together, these results suggest that the increased interaction between Nova-1 and 5-HT$_6$R induced by overexpression of 5-HT$_6$R hampers the splicing activity of Nova-1, and the CT region of 5-HT$_6$R plays a critical role in mediating this interaction as well as the regulation of Nova-1 function.

Translocation of Nova-1 from nucleus to cytoplasm by 5-HT$_6$R overexpression

We next examined how interaction with 5-HT$_6$R could affect the splicing function of Nova-1. At first, we assessed whether total protein levels of Nova-1 were changed by 5-HT$_6$R expression in GT1-1 cells, and found that the expression levels of Nova-1 remained unchanged, regardless of 5-HT$_6$R expression levels (Fig. 4A). Interestingly, however, Nova-1 was translocated from nucleus to cytoplasm when co-expressed with 5-HT$_6$R. In contrast, Nova-1 remained in nucleus when the control empty vector was co-transfected (Fig. 4B). This translocation of Nova-1 by 5-HT$_6$R expression was observed in HEK293 cells as well (Fig. 4C). Moreover, nuclear/cytoplasmic fractionation result also demonstrated that Nova-1 was mainly located in nucleus (183.4±2.3% compared by cytosol), but when co-expressed with 5-HT$_6$R, the cellular distribution of Nova-1 was significantly changed from nucleus to cytoplasm (nucleus, 38.9±10.2% compared by cytosol, Fig. 4D). Taken together, these results suggest that overexpression of 5-HT$_6$R interferes with the proper subcellular localization of Nova-1 to execute its function.

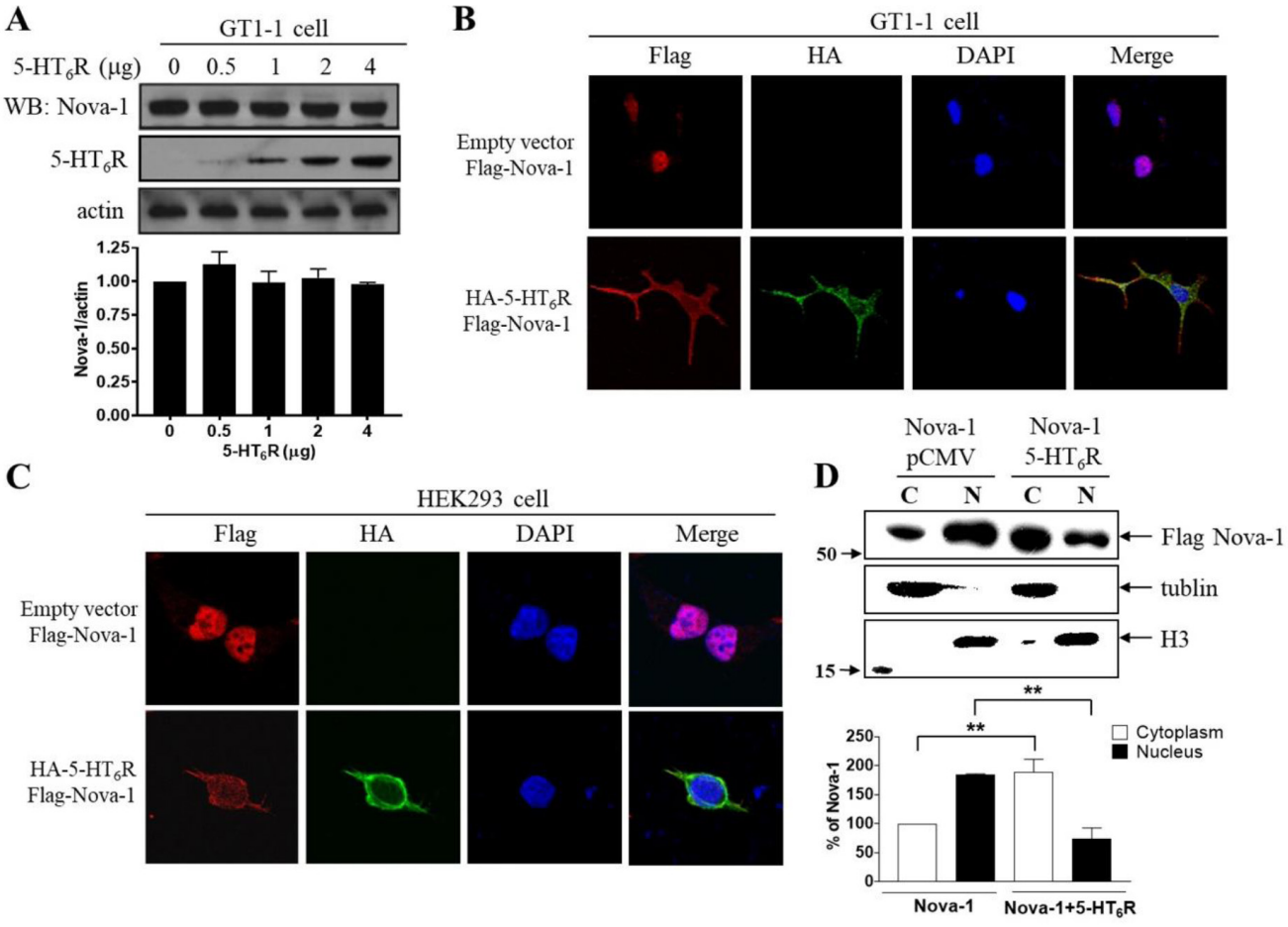

Fig. 4. The subcellular localization of Nova-1 is shifted from nucleus to cytoplasm upon 5-HT$_6$R overexpression. (A) Overexpression of 5-HT$_6$R in GT1-1 cells did not affect the expression levels of Nova-1 ($F_{(4,5)}$=0.8457, p=0.5520). (B) Translocation of Nova-1 (red) to cytoplasm was detected in GT1-1 when co-expressed with 5-HT$_6$R (green). (C) Translocation of Nova-1 to cytoplasm was confirmed in HEK293 cells. (D) Nova-1 was transported from nucleus to the cytoplasm fraction when co-expressed with 5-HT$_6$R ($F_{(3,9)}$=18.31, p=0.0004; Dunnett's post hoc test: **p<0.01). β-Tubulin and histone H3 were used as cytoplasmic and nuclear markers, respectively. Data are expressed as means±S.E.M. (n=3 per group).

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The effect of Nova-1 overexpression on 5-HT$_6$R activity and its total protein levels

We then examined whether overexpression of Nova-1 has an effect on 5-HT$_6$R activity. We previously developed the assay system to assess the activity of 5-HT$_6$R [34]. In this assay system, co-transfected promiscuous Gα_{15} along with 5-HT$_6$R facilitates coupling of Gα_s–coupled receptors to phospholipase C and subsequent intracellular Ca^{2+} release, which is detected using an FDSS6000 96-well fluorescence plate reader [34]. Using this system, the effect of interaction with Nova-1 on the activity of 5-HT$_6$R was examined. Twenty-four hours after transfection of 5-HT$_6$R and promiscuous Gα_{15} into HEK293 cells, 5-HT-induced Ca^{2+} increases via 5-HT$_6$R were measured with FDSS6000 system. Fluorescence peaked within 20-30 s after 5-HT treatment and gradually decreased with time. Compared to control, when Nova-1 was co-expressed, 5-HT-induced Ca^{2+} signal was significantly reduced (48.5±7.2% of control, n=10, Fig. 5A, B). This reduction of Ca^{2+} release by Nova-1 co-expression was not observed when 5-HT$_4$R or 5-HT$_{7B}$R was co-expressed with Nova-1 (Fig. 5C). As an alternative method to detect 5-HT$_6$R activity, we measured 5-HT-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) via 5-HT$_6$R in HEK293/6R stable cells, given that activated 5-HT$_6$R phosphorylates ERK1/2 to mediate signal transduction [17, 33]. 5-HT-induced ERK1/2 phosphorylation levels were decreased as the levels of Nova-1 were increased (38.7±3.4% of control at 4 μg on Nova-1 DNA, Fig. 5D). In addition, the expression levels of

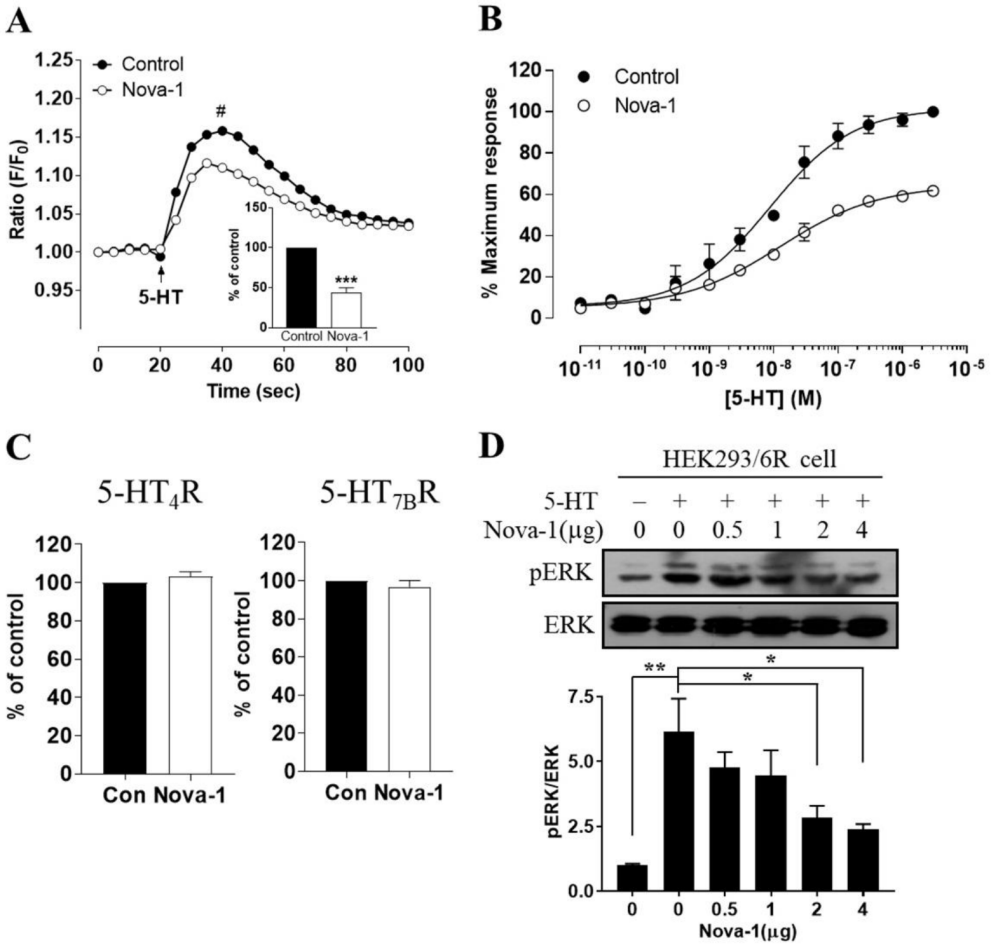

Fig. 5. Nova-1 overexpression decreases functional activity of 5-HT$_6$R. (A) Overexpression of Flag-Nova-1 (open circle) suppressed the Ca^{2+} responses induced by 5-HT (10 μM) compared to control (closed circle). *F* is the fluorescent intensity, and *F0* is the initial fluorescent intensity at 480 nm. *Inset*, The average of ratio (F/F0) measured at the indicated time (#) was significantly reduced by Nova-1 (t=9.834, ***p<0.001; n=10 per group). (B) Expression of Nova-1 reduced 5-HT-induced Ca^{2+} responses (effect of treatment, $F_{(1,96)}$=131, p<0.0001; effect of 5-HT concentration, $F_{(11,96)}$=106.8, p<0.0001; interaction between treatment and 5-HT concentration, $F_{(11,96)}$=7.91, p<0.0001; n=12 per group). (C) Nova-1 did not affect 5-HT$_4$R (t=1.237, p=0.2224; n=24 per group) and 5-HT$_{7B}$R-mediated Ca^{2+} responses (t=0.9698, p=0.3386; n=19 per group). (D) Expression of Nova-1 decreased 5-HT-induced ERK phosphorylation levels in a dose-dependent manner ($F_{(5,12)}$=6.523, p=0.0037; Dunnett's post hoc tests, *p<0.05, **p<0.001; n=3 per group). Data are expressed as means±S.E.M..

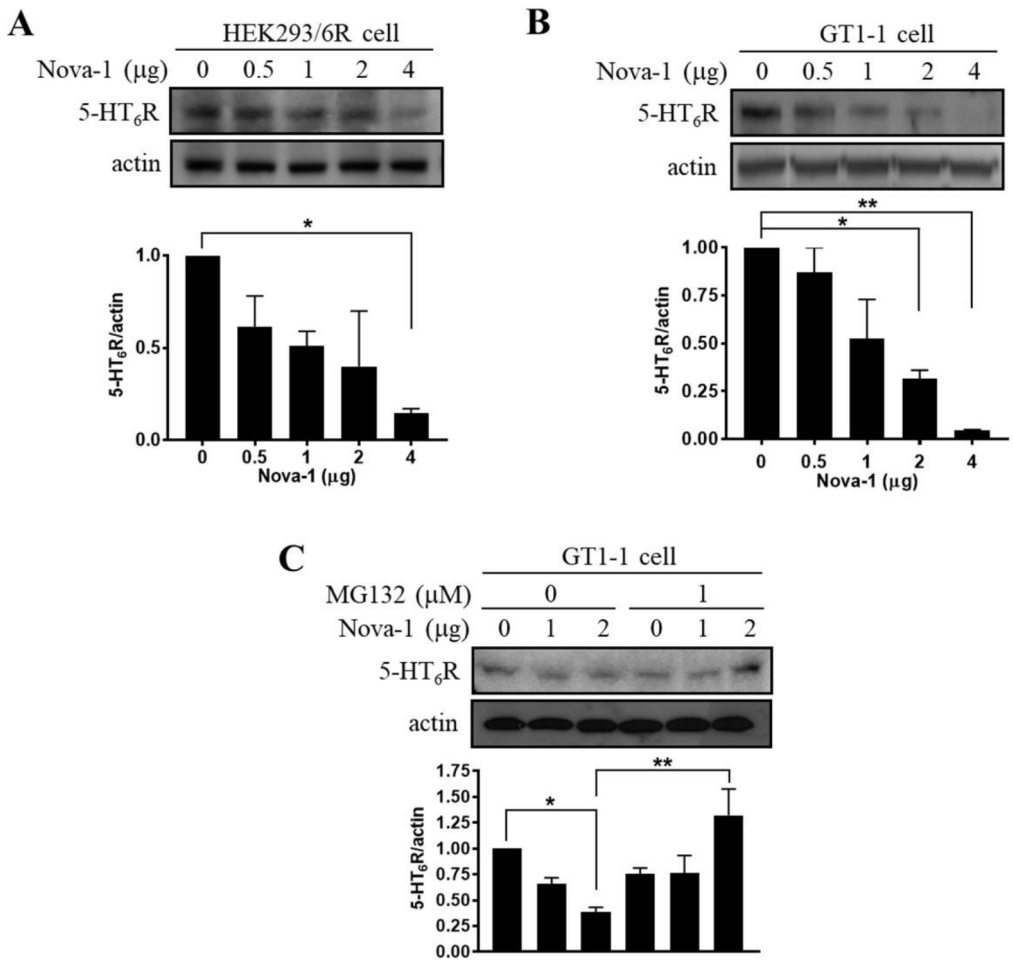

Fig. 6. Nova-1 overexpression decreases the expression levels of 5-HT_6R. (A, B) 5-HT_6R levels were reduced upon overexpression of Nova-1 in HEK293/6R stable cells ($F_{(4, 5)}$=3.959, p=0.0818; Dunnett's post hoc test: *p<0.05; A) as well as in GT1-1 cells ($F_{(4, 5)}$=12.62, p=0.008; Dunnett's post hoc test: *p<0.05, **p<0.01; B). (C) Reduced 5-HT_6R expression caused by Nova-1 was rescued by MG132, a proteasome inhibitor (effect of MG132, $F_{(1, 12)}$=0.9567, p=0.0281; effect of Nova-1, $F_{(2, 12)}$=0.9567, p=0.4116; interaction between MG132 and Nova-1, $F_{(2, 12)}$=10.85, p=0.0020; Student-Newman-Keuls post hoc test: *p<0.01, **p<0.01). Data are expressed as means±S.E.M. (n=3 per group).

5-HT_6R were inversely correlated with Nova-1 expression levels both in HEK293/6R cells and in GT1-1 cells (HEK293/6R cells, 14.5±2.5% of control at 4 μg of Nova-1 DNA; GT1-1 cells, 4.5±0.5% of control at 4 μg of Nova-1 DNA, Fig. 6A, B). Thus, these results suggest that the activity and expression levels of 5-HT_6R are inversely related to the expression of Nova-1. To examine whether the reduction in 5-HT_6R levels upon overexpression of Nova-1 is mediated by proteasome-mediated protein degradation, its total protein levels were measured in the presence of proteasome inhibitor, MG132. Interestingly, the reduction of 5-HT_6R in the presence of Nova-1 overexpression was rescued by MG132, indicating that overexpression of Nova-1 seems to make 5-HT_6R unstable and vulnerable to degradation via proteasome (Fig. 6C). Interestingly, Nova-1 is also subject to proteasomal degradation. However, upon the degradation of 5-HT_6R, Nova-1 is likely to shuttle back to the nucleus from cytoplasm (Fig. 4D), thus potentially making Nova-1 less sensitive to proteasome activity. Taken together, these results suggest that the Nova-1 causes the 5-HT_6R degradation and overall decrease of 5-HT_6R function.

DISCUSSION

Although previous studies reported the implication of 5-HT_6R in eating behavior, movement, cognition and mood [7-9], the mechanisms underlying how 5-HT_6R-mediated signal pathway is involved in these functions remain elusive. Previously, our group demonstrated that Fyn, Jab 1, and MAP1B directly interact with CT of 5-HT_6R [17-19]. In this study, we also revealed Nova-1 as a novel binding protein of 5-HT_6R. Physical interaction between Nova-1 and 5-HT_6R was confirmed both *in vitro* and *in vivo*. Par-

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ticularly, Nova-1 bound to the CT of 5-HT$_6$R, but not to those of 5-HT$_4$R and 5-HT$_{7B}$R that are known to belong to the Gα_s-family like to 5-HT$_6$R [8]. Nova-1 has three KH domains, and among them, the KH3 domain is necessary for binding pre-mRNA targets and for mediating alternative RNA splicing [26, 35]. We found that KH3 domain of Nova-1 is required for binding to the CT of 5-HT$_6$R. These findings indicate the direct and specific interaction between Nova-1 and 5-HT$_6$R.

Previous studies have demonstrated that Nova-1 regulates RNA splicing such as GABA$_A$Rγ2, GlyRα2, and GnRH pre-mRNAs [27, 32, 35]. Consistent with previous findings, we showed that Nova-1 increases the rate of intron A excision of GnRH pre-mRNA. Surprisingly, this splicing activity of Nova-1 was suppressed by overexpression of 5-HT$_6$R. Thus, to the best of our knowledge, this work is the first report to show that overexpression of 5-HT$_6$R can lead to the loss of Nova-1 activity.

Nova-1 is primarily localized to the nucleus, but is also found within cytoplasm and dendrites [29]. In addition, Nova-1 contains nuclear localization sequences (NLS; 25-40 aa) and nuclear export sequences (NES; 318-335 aa) which regulate its cellular localization [29]. In present study, we observed that Nova-1 is mainly expressed in the nucleus. However, overexpression of 5-HT$_6$R changed the subcellular localization of Nova-1 from the nucleus to cytoplasm without affecting the expression levels of Nova-1. Although yet to be proved, translocation of Nova-1 into the cytoplasm could attenuate the binding to its RNA splicing targets in the nucleus, implying that suppression of Nova-1 function is related to its translocation from the nucleus to cytoplasm induced by 5-HT$_6$R overexpression.

The expression levels of Nova-1 also influence the 5-HT$_6$R function. Firstly, overexpression of Nova-1 decreased 5-HT-induced Ca^{2+} via 5-HT$_6$R and ERK 1/2 phosphorylation that is known to be activated by 5-HT$_6$R, indicating that Nova-1 regulates the activity of 5-HT$_6$R. These results suggest that Nova-1 makes 5-HT$_6$R vulnerable to degradation, and consequently total protein levels and activity of 5-HT$_6$R are decreased. Although Nova-1 is known to regulate RNA splicing, it is not clear whether Nova-1 is implicated in protein degradation pathways. Further study is required to determine the mechanisms underlying how Nova-1 induces the degradation of 5-HT$_6$R.

We observed that physical and function interaction between Nova-1 and 5-HT$_6$R. However, the functional significance of this interaction in the CNS remains to be established. Nova-1 is a member of RNA binding proteins that regulate RNA splicing. Interrupting the function of a RNA binding protein can significantly influence the post-transcriptional processing of its target RNAs which is crucial to modulate the expression and function of proteins [36, 37]. Recent genetic and proteomic studies have found that aberrant functions of RNA binding proteins are associated with a wide range of human disorders including neurologic disorders, muscular atrophies and cancer [36-38]. In POMA, autoantibodies generated by cancer cells recognize Nova-1 as an antigen which prompts an autoimmune response and consequently induces attacks of neurons expressing Nova-1. POMA patients suffer from movement dysfunctions and show neural cell death in the brainstem and spinal cord where Nova-1 is mainly expressed [36, 37]. Nova-1 null mice die postnatally and display apoptotic neuronal death and splicing defects of GABA$_A$Rγ2 [27] and GlyRα2 [28]. In addition, we previously reported 5-HT$_6$Rs are involved in cell survival [18]. Based on these findings and our data, dysfunction of Nova-1 caused by 5-HT$_6$R overexpression might influence neural cell survival and inhibitory synaptic transmission. Our previous studies identified 5-HT$_6$R-associated partners such as Fyn, Jab1, and MAP1B which activate 5-HT$_6$R function [17-19], which are implicated in cognition, neurodevelopment, and neurodegeneration [8, 39-42]. Thus, it is possible that impaired function of 5-HT$_6$R by Nova-1 can affect 5-HT$_6$R-mediated pathways and might be associated with neurological diseases. Although we induced overexpression of 5-HT$_6$R or Nova-1 to examine the effects of their interaction on the function of the other, it remains to be determined which conditions induce increased expression levels of 5-HT$_6$R or Nova-1. Previous studies have shown that disturbances of hypothalamic-pituitary-adrenal (HPA) axis could influence the expression levels of 5-HT$_6$R and Nova-1[32, 43, 44]. In a condition of decreased glucocorticoid levels, such as adrenalectomy, up-regulation of the 5-HT$_6$R mRNA expression was observed in the hippocampus [43, 44]. In addition, the treatment of dexamethasone, a synthetic glucocorticoid, reduced mRNA and protein levels of Nova-1 in GT1-1 cells [32]. Based on these reports, we can speculate that expression levels of 5-HT$_6$R and Nova-1 could be improperly regulated under dysfunction of HPA.

In conclusion, we demonstrate that Nova-1 directly interacts with 5-HT$_6$R, and that dysregulated expression of Nova-1 or 5-HT$_6$R interferes with the function of the other. Although the precise pathophysiological significance of the interaction between Nova-1 and 5-HT$_6$R remains to be determined, this study provides new evidence on the important role of Nova-1 in regulating 5-HT$_6$R-mediated signaling events.

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