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Differential Agonist-Mediated Internalization of the Human 5-HT$_7$ Receptor Isoforms

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Trafficking of 5-HT$_7$ Receptor Isoforms

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List of non-standard abbreviations: 5-HT, 5-hydroxytryptamine; SCN, suprachiasmatic nucleus; DMEM, Dulbecco's Modification of Eagle's Medium; FBS, fetal bovine serum; DAPI, 4',6-Diamidino-2-phenylindole; 5-CT, 5-carboxamidotryptamine; SB 269970, (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl) phenol; AEBSF 4-(2-aminoethyl)-benzenesulfonyl fluoride;

ABSTRACT

The human 5-HT_7 serotonin receptor is a class A G-protein coupled receptor that has three isoforms, 5-$HT_{7(a)}$, 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$, which are produced by alternative splicing. The 5-HT_7 receptors are expressed in discrete areas of the brain and in both vascular and gastrointestinal smooth muscle. Central nervous system 5-HT_7 receptors may play a role in mood and sleep disorders. 5-HT_7 receptors show high affinity for a number of antidepressants and typical and atypical antipsychotics. We report here that the human 5-$HT_{7(d)}$ isoform expressed in HEK 293 cells exhibits a pattern of receptor trafficking in response to agonist that differs from 5-$HT_{7(a)}$ or 5-$HT_{7(b)}$ isoforms. We employed a modification of a live cell labeling technique to demonstrate that surface 5-$HT_{7(d)}$ receptors are constitutively internalized in the absence of agonist. This is in contrast to 5-$HT_{7(a)}$ and 5-$HT_{7(b)}$ isoforms, which do not show this profound agonist-independent internalization. Indeed, the 5-$HT_{7(d)}$ isoform displays this internalization in the presence of a 5-HT_7 – specific antagonist. In addition, the human 5-$HT_{7(d)}$ isoform shows a diminished efficacy in stimulation of cAMP-responsive reporter gene activity in transfected cells compared to 5-$HT_{7(a)}$ or 5-$HT_{7(b)}$ receptors expressed at comparable levels. Thus, the carboxy-terminal tail of 5-$HT_{7(d)}$, which is the longest among known human 5-HT_7 isoforms, may contain a motif that interacts with cellular transport mechanisms that is distinct from 5-$HT_{7(a)}$ and 5-$HT_{7(b)}$.

Serotonin (5-hydroxytryptamine, 5-HT) is a small molecule involved in a number of physiological and pathological processes. Fourteen subtypes of receptors and the serotonin transporter mediate these processes.

The human 5-HT_7 receptor was cloned in 1993 (Bard et al., 1993). 5-HT_7 receptors are expressed in the central nervous system (thalamus, hypothalamus, limbic and cortical regions), but also in peripheral tissues (vascular and gastrointestinal smooth muscle and certain leukocytes) (Eglen et al., 1997). A variety of approaches has provided evidence that 5-HT_7 receptors may be involved in regulation of affect, body temperature, circadian rhythms and REM sleep, primary sensory neuronal sensitivity, and relaxation of smooth muscle in a variety of tissues (Terron, 1996; Prins et al., 1999; Vanhoenacker et al., 2000; De Ponti and Tonini, 2001; Terron, 2002; Inoue et al., 2003; Centurion et al., 2004; Janssen et al., 2004; Thomas and Hagan, 2004; Varnas et al., 2004) Three splice variants of the human 5-HT_7 receptor have been described (Heidmann et al., 1997; Jasper et al., 1997; Stam et al., 1997) and designated 5-$HT_{7(a)}$, 5-$HT_{7(b)}$ and 5- $HT_{7(d)}$, which differ only in the length and amino acid composition of their carboxy-terminal tail. Although levels of expression differed, all three isoforms were detected in most tissues examined(Heidmann et al., 1998).

In previous studies, the three isoforms showed pharmacology and functional coupling to adenylyl cyclase to be essentially identical. However, the human 5-$HT_{7(d)}$ isoform did display a diminished 5-HT-mediated stimulation of adenylyl cyclase activity (Krobert et al., 2001). Levy's group also extensively characterized the ability of the three isoforms to constitutively activate adenylyl cyclase and the inverse agonist properties of a series of antagonists. Again, 5-$HT_{7(a)}$, 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ displayed similar profiles for both constitutive activity and inverse agonist responses (Krobert and Levy, 2002).

Previous studies have all failed to show any differences in pharmacology or function among the three isoforms of the human 5-HT_7 receptors. One investigation, in the laboratory of Levy and colleagues (Krobert et al., 2001), showed that the 5-$HT7_{(d)}$ isoform had reduced efficacy in 5-HT-mediated stimulation of adenylyl cyclase. We chose to examine the trafficking of the human 5-HT_7 receptors in response to treatment with agonist to see if there were differences among the three isoforms. Receptor trafficking has been investigated and shown to occur among other members of the serotonin receptor family, particularly 5-$HT_{1(a)}$, 5-$HT_{2(a)}$ and 5-$HT_{2(c)}$ receptors (Xia et al., 2003; Schlag et al., 2004; Zimmer et al., 2004). In this study we demonstrate that the 5-$HT7_{(d)}$ isoform exhibits receptor trafficking that is distinct from that of 5-$HT7_{(a)}$ or 5-$HT7_{(b)}$. Human 5-$HT7_{(d)}$ receptors display agonist-independent internalization and indeed internalize even in the presence of antagonist. We employed a novel double labeling procedure to label two populations (surface and internal) of receptor carrying the same epitope tag. These studies reveal near total removal of 5-$HT7_{(d)}$ receptors initially on the surface of the cell to the interior and replacement with a population of receptors previously found inside the cell. Under identical conditions, the majority of 5-$HT7_{(a)}$ and 5-$HT7_{(b)}$ remain on the surface. The consequence of this removal of 5-$H7_{(d)}$ receptors from the surface is a reduced efficacy of a cAMP-responsive reporter gene activity. Agonist-independent internalization provides a possible mechanism for the previous observation that the human 5-$HT7_{(d)}$ isoform has a reduced ability to stimulate the second messenger pathway. These data further show that the 5-$HT7_{(d)}$ carboxy tail may mediate an interaction with a trafficking pathway for internalization other than that used by. 5-$HT7_{(a)}$ and 5-$HT7_{(b)}$ receptors.

METHODS

Construction of plasmids. Human 5-$HT_{7(a)}$ (h5-$HT_{7(a)}$) receptors with a c-terminal epitope tag derived from influenza hemagglutinin (HA) were constructed by PCR from a construct containing the previously cloned h5-$HT_{7(a)}$ cDNA (Heidmann et al., 1997) in the pcD- SRα expression vector (Takebe et al., 1988). The sequence MGYPYDVPDYA with a consensus Kozak sequence and a 5' *Eco* RI site was added at the amino terminus of the receptor coding sequence. This primer has the sequence, 5'GAATTCGAATTCATCATGGGGTACCCATATGACGTCCCAGACTACG CCATGGACGTTAACAGCAGCGGC3'. The primer for the 3' end of the PCR product was designed to hybridize to a sequence surrounding the pcD- SRαmultiple cloning site with the sequence 5'ATGTCTGGATCCTCGACGAGCTCG3'. The product was cut with *Eco* RI and *Bam* HI and ligated back into pcD- SRα vector with the resulting expression vector being designated SRα-HA 5-HT7A. Epitope –tagged expression vectors for the 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ were generated by domain swapping coding sequence corresponding to the c-terminus of the 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ isoforms with SRα-HA 5-HT7A to generate SRα-HA 5-HT7B and SRα-HA 5-HT7D. Constructs were validated by restriction analysis and sequencing.

Determination of relative abundance of mRNA isoforms with RT-PCR. Total RNA was extracted from homogenized tissues or cells using a guanidine isothiocyanate:phenol extraction protocol (Chomczynski and Sacchi, 1987),digested for 30 min with ribonuclease-free DNaseI (Roche, Indianapolis, IN) and heat denatured for 15 min at 65°C. Poly$(A)^+$ RNA was prepared using Dynabead Oligo (dT)25 selection (Dynal Biotech, Brown Deer, WI). Poly$(A)^+$ RNA (1 mg) was reverse transcribed to cDNA as described previously (Heidmann et al., 1997). cDNA was synthesized using SuperScript™ II reverse transriptase (Invitrogen, Carlsbad. CA).

Radiolabeled PCR was performed using the primers: 184F 3'AGTACCGGAATATCAACCGGAAG 5' and 184 R 3'GTTTATTTCATCTCCATTGTTCTGC 5'. This produces three products, amplified from a single template corresponding to the three human isoforms of the 5-HT_7 receptor. Products were separated on 12% polyacrylamide gels. Relative abundance of the isoforms was determined by phosphorimaging and densitometry using the GS363 Molecular Imaging System (Bio-Rad Hercules, CA).

Generation of stable cell lines. The expression vectors for selection of HA-5-$HT_{7(a)}$, HA-5-$HT_{7(b)}$ and HA-5-$HT_{7(d)}$ stable cell lines were constructed by subcloning of the epitope tagged receptors (SRα-HA-5-HT7A,-7B,-7D) into the expression vector pIRESpuro (Clontech, Palo Alto, CA). 293T cells, an SV40 T antigen-expressing variant of the 293 adenovirus-transformed human embryonic kidney cell line HEK 293 (ATCC CRL-1573) were transfected using the calcium phosphate method. Positive clones were selected and maintained in Dulbecco's Modification of Eagle's Medium (DMEM) + 10% fetal bovine serum + penicillin(100 I.U./mL)/streptomycin (100μg/mL) + 10μg/mL puromycin. Serum products contain large amounts of serotonin, making it necessary to remove serum for experiments. Therefore, 24 hours prior to experiments, cell cultures were washed extensively and fed with COMPLETE™ serum-free medium (Mediatech, Herndon, VA).

Radioligand binding assay. Saturation binding assays were performed as described previously (Heidmann et al., 1998). Briefly, stable cell lines were grown in COMPLETE™ serum-free medium for 24 hrs, harvested, and crude membranes prepared by Polytron homogenization. Aliquots of membrane suspension were incubated with increasing concentrations of [^{3}H]5-HT [26-30 Ci/mmole] (PerkinElmer, Boston, MA) in a total volume of

0.5 mL, filtered on GF/C filters, washed and counted by liquid scintillation. Non-specific binding was determined in the presence of 2μM methiothepin. BioRad Protein Assay was used to determine protein content. Saturation binding data was analyzed using non-linear regression analysis in GraphPad Prism.

Reporter gene assays. 293T cells were plated in 24 well dishes at 1 x 10^5 cells/well in DMEM + 10% FBS and grown at 37°C/5%CO_2 for 18 hrs. Medium was reduced to 250μL and cells were transfected with expression vectors for the three 5-HT_7 isoforms (SRα5-HT7a, 7b, 7d) by calcium phosphate precipitation. A total of 250ng of DNA was added to each well, which, in addition to the 5-HT_7 receptor expression vectors, contained 2.5 ng of an $\tilde{\alpha}$168 luciferase reporter construct and 40 ng of RSV-β-galactosidase as a control for transfection efficiency. After overnight transfection, culture medium was changed to COMPLETE™ serum-free medium for 24 hrs prior to drug treatment. Treatments were performed in triplicate wells and luciferase and β-galactosidase activities in cell extracts were measured on a luminometer (Berthold AutoLumat 953). Dose response curves were generated and statistical analyses performed using GraphPad Prism.

Immunocytochemistry. These experiments were performed using two different techniques. The first was a standard technique where cells were grown on coverslips and subjected to various treatments. The cells were then fixed, permeabilized and labeled with primary antibodies directed at epitope tags or organelles, followed by detection with appropriate fluorochrome-conjugated secondary antibodies. The second approach was to label receptors on the surface of the cell with primary and secondary antibodies prior to any treatments. Cells were removed from incubator, placed on ice to inhibit internalization of receptors. Primary and secondary antibodies were applied, cells were returned to the incubator and drug treatments were initiated. Upon

completion of treatment, cells were fixed, permeabilized and receptors that were not on the surface initially were labeled as above.

Total receptor stain. Stable cell lines expressing the three HA-5HT$_7$ isoforms were grown on 12mm glass coverslips. The slides were coated with 100µg/mL poly-D-lysine for 60 min prior to plating cells. After 24 hrs in DMEM + 10% FBS, cells were washed three times with DMEM and grown for 24 hrs in COMPLETE™ serum-free medium (Mediatech). Cells were drug treated at 37° C for 120 min, then washed 2X with PBS/Ca^{2+}/Mg^{2+} (PBS+0.1g/L $CaCl_2$+1.0g/L $MgCl_2$) on ice. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Coverslips were washed 4 X 5 min with PBS/Ca^{2+}/Mg^{2+}. Cells were permeabilized in PBS/Ca^{2+}/Mg^{2+} + 0.1% Triton X-100 for 15 min. Coverslips were blocked with PBS/Ca^{2+}/Mg^{2+} + 10% normal goat serum for 60 min. Coverslips were incubated in primary antibody, 3F10 anti-HA rat monoclonal (Roche, Indianapolis, IN), 1:1000 in PBS/Ca^{2+}/Mg^{2+} + 10% normal goat serum for 60 min. Slides were then washed 3X 5 min with PBS/Ca^{2+}/Mg^{2+}, and re-blocked for 15 min. Secondary antibody, goat-anti rat–Alexa Fluor® 488 (Molecular Probes, Eugene OR), 1:2000 in PBS/Ca^{2+}/Mg^{2+} + 10% normal goat serum was added and slides were incubated for 20 min. Coverslips were again washed 3X 5 min with PBS/Ca^{2+}/Mg^{2+}, counterstained with 4',6-Diamidino-2-phenylindole (DAPI) and mounted with ProLong Antifade (Molecular Probes, Eugene OR).

Immunocytochemistry. Double stain of HA-tagged 5-HT$_7$ surface receptors vs. HA-tagged 5-HT$_7$ cytoplasmic receptors.(Fig. 5B & Fig. 6). This modification of a live cell surface labeling protocol allowed us to examine two populations of human 5-HT$_7$ receptors carrying the same epitope tag. Therefore, the images and data in Fig.6 differ from Fig. 3 in that the receptors labeled green are those that were on the cell surface prior to any treatments.

Stable cell lines were grown on coverslips and serum starved as above. Cells were removed from the incubator and placed on ice for 15min, then washed 1X with cold COMPLETE™. Cells were incubated on ice with primary antibody, 3F10 anti-HA rat monoclonal, (Roche) 1:1000 in COMPLETE™ for 60 min. Cells were washed 3X with COMPLETE™. Secondary antibody, goat-anti rat–Alexa Fluor® 488 , 1:1000 in COMPLETE™ was applied for 60 min on ice. Cells were then removed to incubator, where pre-warmed medium with or without appropriate drugs was added. After 120 min, cells were immediately fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized in PBS/Ca^{2+}/Mg^{2+} + 0.1% Triton X-100 for 15 min. The second primary antibody, HA.11 anti-HA mouse monoclonal, (CRP, Cumberland, VA) 1:1000 in COMPLETE™ was applied for 60 min at room temperature. Cells were washed 3X 5 min with PBS/Ca^{2+}/Mg^{2+}, and re-blocked for 15 min in COMPLETE™. Secondary antibody, goat-anti mouse-Alexa Fluor® 568, 1:2000 in COMPLETE™ was applied for 30 min at room temperature. . Cells were again washed 3X 5 min with PBS/Ca^{2+}/Mg^{2+}, counterstained with DAPI and mounted with ProLong Antifade.

Immunofluorescence microscopy. Observation and imaging of fixed or live cells were done on a Nikon Eclipse TE300 epi-fluorescent microscope. Image acquisition, processing and analysis were done with IPLab (Scanalytics, Fairfax, VA) and Adobe Photoshop. Images (512X512 pixels) were acquired using a Photometrics SenSys™ cooled CCD camera and IPLab acquisition software. Images were deconvolved using MicroTome™ deconvolution software (Scanalytics, Fairfax, VA). Fluorescence intensity measurements were made by hand-segmenting images into plasma membrane (300nm wide) and perinuclear regions. Values are expressed as a percentage of total fluorescence intensity for each region. Each cell was segmented separately,

15 – 30 fields (2-10 cells/field) were measured for each data set. Data sets were analyzed using the Mann-Whitney Test, data expressed as a mean ± standard error of the mean.

Surface Biotinylation Assay. For surface biotinylation experiments, cells were grown to 80% confluence on 100 mm dishes, and serum starved overnight by washing 3X and feeding with COMPLETE™ serum-free medium. Plates were chilled to 4°C and washed twice with PBS/Ca^{2+}/Mg^{2+}. Plates were then incubated with 300 μg/mL sulfo-NHS-SS-biotin (Sigma) in PBS/Ca^{2+}/Mg^{2+} for 30 minutes. Unreacted biotin was quenched with by washing 3X with TBS (10mm Tris pH 7.4, 154mM NaCl). Plates were returned to the incubator in COMPLETE™ serum-free medium containing agonist (5-CT), antagonist (SB269970), or no drugs (UNTREATED) for 120 minutes. Plated were then removed and chilled to 4°C. Remaining surface biotin was cleaved by incubating in glutathione reducing buffer (50 mM glutathione, 75mM NaCl, 75 mM NaOH, 10% FBS) for 30 minutes followed by quenching of unreacted glutathione with iodoacetamide buffer (0.9% iodoacetamide, and 1% BSA in PBS/Ca^{2+}/Mg^{2+}). The cells were then immediately lysed in cold lysis buffer (TBS, 0.1% Triton X-100, 3 μg/mL aprotonin, 2 μg/mL pepstatin, 1 μg/mL leupeptin, and 1mM AEBSF) in a volume of 0.5 mL. Proteins were measured using the BioRad protein assay. Equivalent amounts of protein were loaded onto NeutrAvidin agarose (Pierce), incubated overnight at 4°C. The beads were washed three times with lysis buffer and biotinylated proteins were eluted with SDS sample buffer, resolved with SDS-PAGE and detected using anti-HA (HA.11 Covance) antibody.

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RESULTS

Relative expression of the three human 5-HT$_7$ isoforms in various cell lines and tissues. Radiolabeled amplification of cDNAs reverse transcribed from poly (A)+ RNA isolated from a range of tissue types known to express the 5-HT$_7$ receptor (Bard et al., 1993). The primer set used (Heidmann et al., 1997) produced three different bands corresponding to the differing c-termini of the 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ serotonin receptors. This permitted an examination of the relative abundance of the three human 5-HT$_7$ isoforms within each tissue or cell (Fig 1.). Each tissue examined expressed all three isoforms. Even when we examined transformed cell lines (various blood cell types) and a primary culture of aortic smooth muscle cells, all three isoforms were found. In all cases the 5-HT$_{7(d)}$ isoform was the least abundant. It was observed that while the 5-HT$_{7(b)}$ isoform was the most prevalent, the relative amounts of 5-Ht$_{7(a)}$ and 5-HT$_{7(d)}$ differed by tissue type, with the 5-HT$_{7(d)}$ isoform being most abundant in smooth muscle and least common in brain tissues.

HA-tagged 5-HT$_7$ receptors are functionally equivalent to the native 5-HT$_7$ receptors. A cAMP-responsive luciferase reporter gene assay was used to evaluate the functionality of the 5-HT$_{7(a)}$ serotonin receptor with an n-terminal influenza hemagglutinin epitope tag, HA-5-HT$_{7(a)}$. 293T human embryonic kidney cells were transiently transfected with vectors that expressed either native 5-HT$_{7(a)}$ or HA-tagged 5-HT$_{7(a)}$ receptors. Treatment of cells with the selective agonist 5-carboxamidotryptamine (5-CT) (Fig. 2A) showed a dose-dependent activation of the reporter gene to similar magnitude and with comparable agonist potency for the native (EC_{50}=.31nM) and the HA-tagged (EC_{50}=.46nM) 5-HT$_7$ receptors. Likewise, cells treated with 5-CT and the 5-HT$_7$-specific antagonist SB 269970 (R)-3-(2-(2-(4-methylpiperidin-1-yl)ethyl)pyrrolidine-1-sulfonyl) phenol displayed dose dependent inhibition with similar

inhibition potency. While the HA-tagged 5-$HT_{7(a)}$ receptor was slightly less sensitive to SB 269970 inhibition,EC_{50}=7nM vs. EC_{50}=.93nM for the native receptor, both displayed complete inhibition of 5-CT stimulated activity.

5-HT_7 receptors internalize in response to agonist. Stable cell lines over-expressing HA-5-$HT_{7(a)}$, HA-5-$HT_{7(b)}$ and HA-5-$HT_{7(d)}$ receptors (304-482 fmol/mg) were examined using epi-fluorescence microscopy. After 24 hours in serum-free medium, cells were subjected to one of three treatments for two hours: UNTREATED (change to fresh medium), 1 μM 5-CT (agonist), or 1 μM SB 269970 (antagonist). Cells on coverslips were fixed, stained with anti-HA antibody and examined using epi-fluorescent microscopy. Images were acquired with a cooled CCD camera and deconvolved with MicroTome™ deconvolution software. In the untreated condition (Fig. 3A) both HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ have the majority of their receptors in the plasma membrane and fewer in the cytoplasm. In contrast, HA-5-$HT_{7(d)}$ receptors have a greater proportion in an internal compartment. When treated with agonist (5-CT), all three isoforms present a picture with many receptors localized to a perinuclear compartment. Antagonist (SB 269970) treatment results in a distribution of HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ signal that closely resembles the untreated state. Again, HA-5-$HT_{7(d)}$ differs, now exhibiting more receptor staining on the plasma membrane than in the untreated condition. Fig. 3(B) is a quantitative representation of fifteen fields of cells (avg. 5 cells/field) for each condition. A biochemical assay of 5-HT_7 receptor internalization using surface biotinylation (Fig. 5), showed that while HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ show some constitutive internalization, the HA-5-$HT_{7(d)}$ isoform has a much larger proportion of receptors in the cytoplasm in the UNTREATED condition. We observe a translocation of receptors into the cytoplasm with agonist (5-CT) exposure for HA-5-

$HT_{7(a)}$ and HA-5-$HT_{7(b)}$. The HA-5-$HT_{7(d)}$ isoform shows an apparent retention of receptors in the cytoplasm in the presence of antagonist (SB 269970).

HA-5-$HT_{7(d)}$ receptors have diminished activity to activate a cAMP-responsive reporter gene. The observation that HA-5-$HT_{7(d)}$ had a greater proportion of receptors localized in the cytoplasm than did either HA-5-$HT_{7(a)}$ or HA-5-$HT_{7(b)}$ led to further investigation into the functional activity of these stable cell lines. We found that the three isoforms had similar agonist potency: HA-5-HT(7a) (304 ±46fmol/mg), HA-5-HT(7b) (310 ±10fmol/mg) and HA-5-HT(7d) (482 ±39fmol/mg). However, the maximum response of the HA-5-$HT_{7(d)}$ isoform was only about half that of the HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ isoforms (Fig. 4). This result was consistent over repeated experiments with different stable clones of each isoform. HA-5-$HT_{7(d)}$ receptors have reduced activity, possibly due to fewer receptors at the cell surface at any given time.

Double labeling of external vs. internal populations of HA-5-HT_7 receptors. In order to determine whether HA-5-$HT_{7(d)}$ receptors were never reaching the plasma membrane or if they were being internalized in an agonist-independent fashion, we devised a method to double label plasma membrane vs. cytoplasmic receptors. We used a rat anti-HA monoclonal antibody, 3F10 (Roche), to label surface receptors on live cells, then rapidly fixed and permeabilized the cells and labeled internal receptors with a mouse anti-HA monoclonal antibody. HA.11 (Covance). This method provided good resolution of the two populations of HA-tagged 5-HT_7 receptors, and revealed that at the time of labeling, before any treatment, all three isoforms have a population of receptors on the surface and a population in the perinuclear region(Fig. 6, top row).

HA-5-$HT_{7(d)}$ receptors are constitutively internalized in the absence of agonist. The above double labeling protocol enabled us examine more closely the data presented in Fig. 3. This experiment (Fig. 6) is different in that we could examine receptors that were on the cell surface

prior to treatment (those with the green label) independently from those that were initially inside the cell (those with the red label). After labeling the surface receptors and simply returning the cells to 37°C for two hours (UNTREATED), two different patterns of receptor distribution were evident. HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ receptors that began on the cell surface (green) remained there, but clustered into some sort of membrane micro-domain. The surface population (green) of HA-5-$HT_{7(d)}$ receptors was nearly all inside the cell and had been replaced with others (red) that were inside at the beginning of the experiment. Still, the majority of these (red) HA-5-$HT_{7(d)}$ receptors either remains in or has returned to the cytoplasm. HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ expressing cells assume this state when they are exposed to agonist (5-CT) and surface receptors internalize in response. Agonist-treated HA-5-$HT_{7(d)}$ cells (5-CT) look very much like the untreated condition. When HA-5-$HT_{7(d)}$ cells are exposed to antagonist (SB 269970), a significant fraction of surface-labeled receptors (green) remain at the plasma membrane. Conversely, HA-5-$HT_{7(a)}$ and HA-5-$HT_{7(b)}$ expressing cells appear much as they do in the untreated condition, with receptor clustering (green) but not much internalization.

DISCUSSION

We have found that the three splice variants of the human 5-HT_7 receptor display different trafficking patterns in response to agonist treatment. The 5-$HT_{7(d)}$ isoform is continuously internalized in an agonist-independent fashion. In fact, 5-$HT_{7(d)}$ receptors internalize in the presence of a potent 5-HT_7 receptor antagonist. 5-$HT_{7(d)}$ receptors are properly displayed on the cell surface and respond to agonist treatment with activation of a cAMP-responsive reporter gene. The constitutive internalization of the 5-$HT_{7(d)}$ isoform is distinct from the signaling pathway as untreated cells show no cAMP mediated response and a 5-HT7 receptor antagonist

inhibits agonist-mediated reporter gene activity in a dose-dependent manner. While no such trafficking differences have been reported among the serotonin receptors, similar trafficking patterns have been reported for the α-$_1$ adrenergic receptors (Hirasawa et al., 1997; Chalothorn et al., 2002; Hague et al., 2003; Morris et al., 2004). However, these α-$_1$ adrenergic receptors are transcribed from three separate genes.

We established stable cell lines expressing 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ receptors. A functional assay that measured the response of a cAMP-responsive luciferase gene, revealed a reduced efficacy of the 5-HT$_{7(d)}$ isoform. The response was only 50% that of the 5-HT$_{7(a)}$ or 5-HT$_{7(b)}$ isoforms, even though the 5-HT$_{7(d)}$ receptors were expressed at equivalent or slightly greater levels. Imaging studies revealed a greater proportion of 5-HT$_{7(d)}$ receptors in the perinuclear region relative to the plasma membrane in cells that have not been exposed to agonist than what is seen in cell lines expressing 5-HT$_{7(a)}$ or 5-HT$_{7(b)}$ receptors. Two possible explanations for this distribution pattern were endocytosis of surface receptors without agonist exposure, or failure of receptors to exit the Golgi and reach the surface. Double labeling studies of populations of receptors residing at the either at the surface or inside the cell prior to treatment eliminated the second possibility, as receptors that had been inside the cell moved to the surface. This was true for all three isoforms (Fig. 6A, red label). The difference in trafficking between 5-HT$_{7(d)}$ receptors and 5-HT$_{7(a)}$ or 5-HT$_{7(b)}$ was that the 5-HT$_{7(d)}$ receptors cycled to the perinuclear region without agonist exposure (Fig. 6A, green label). Surface biotinylation experiments showed some constitutive internalization of all three isoforms but the proportion of 5-HT$_{7(d)}$ receptors inside the cell compared to total receptor is much greater. In addition, this internalization of 5-HT$_{7(d)}$ receptors takes place even in the presence of antagonist. It has been demonstrated by Morello and co-workers that cell permeant antagonists can act as 'chemical

chaperones' to bring receptors trapped intracellularly to the surface (Morello et al., 2000). Both our imaging and surface biotinylation studies show that while there may be some of this going occurring with SB 269970 exposure, certainly many of the 5-$HT_{7(d)}$ receptors remain inside the cell. Thus, there may be fewer 5-$HT_{7(d)}$ receptors at the surface of the cell that are available for signaling at any given time, resulting in diminished efficacy in signal transduction via the 5-$HT_{7(d)}$ receptor.

Amplification of the different carboxy-termini of the three isoforms revealed the presence of 5-$HT_{7(a)}$, 5-$HT_{7(b)}$ and 5-$HT_{7(d)}$ receptors in each tissue or cell type evaluated. The relative abundance of the three isoforms was variable in the three tissue types investigated, with the greatest relative abundance of the 5-$HT_{7(d)}$ isoform found in smooth muscle tissues. The significance of these distribution differences is not clear, for example, they could indicate specialized functions in tissue such as smooth muscle where expression is greater. Our previous work looking for cellular differences in splicing in rat brain was not able to show any differences (Heidmann et al., 1998).Further examination of individual cell types rather than tissues may reveal single isoform expression.

In this investigation, we have observed that the human 5-$HT_{7(d)}$ isoform of the 5-HT_7 serotonin receptor has reduced activity when compared to either the 5-$HT_{7(a)}$ or the 5-$HT_{7(b)}$ isoform. Our results suggest that this is due to a reduced population of receptors on the surface of the plasma membrane at any given time because of a constitutive agonist-independent endocytosis of the 5-$HT_{7(d)}$ receptor.

The functional significance of the reduced signaling efficacy of the 5-$HT_{7(d)}$ receptor on the regulation of serotonin signaling though the 5-HT_7 receptor is unclear. Elucidation of the roles of

the human 5-HT_7 receptor variants and their interaction with other cellular proteins remains an objective of further investigation.

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LEGENDS FOR FIGURES

Fig. 1. *Relative expression levels of 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$.* cDNAs prepared from Poly(A)$^+$ RNA isolated from various tissues and cell types were amplified with primers that produced different bands for each of the three isoforms of the 5-HT$_7$ receptor. Autoradiograms were analyzed using densitometry to determine the relative abundance of the three 5-HT$_7$ receptor isoforms in each tissue or cell type examined. Expression levels are reported as a percentage within each tissue or cell type. Combined values for three tissue types, brain, smooth muscle, or blood are shown below each group of bars. Values reported are from a single experiment representative of three separate experiments.

Fig.2. *Functional characterization of HA-tagged 5-HT$_7$receptors.* Functional reporter gene assays of a cAMP- responsive luciferase gene show influenza virus hemagglutinin (HA) epitope tag does not interfere with normal function of 5-HT$_7$ receptor protein. (A) Transiently transfected 293T cells were grown, serum starved for 24 hours, then stimulated with increasing concentrations of the 5-HT$_7$ agonist 5-carboxyamidotryptamine (5-CT). Cells were harvested and cell extracts assayed for luciferase activity. Agonist potencies were similar for the native (EC_{50}=.31nM) and the HA-tagged (EC_{50}=.46nM) 5-HT$_7$ receptors. (B) Cells as described above were exposed to increasing concentrations of the 5-HT$_7$-selective antagonist SB 269970 and to 100nM 5-CT. Both the native (EC_{50}=.93nM) and HA-tagged (EC_{50}=7nM) 5-HT$_7$ receptors activity was completely inhibited by the 5-HT$_7$-specific antagonist SB 269970.

Fig. 3. *Total 5-HT$_7$ receptor localization and trafficking* **.** 293T stable cell lines expressing 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$ receptors. (A) Epi-fluorescent photomicrographs of HA-5-HT$_7$ receptors. In the UNTREATED condition, 5-HT$_{7(d)}$ displays more perinuclear cytoplasmic localization than do 5-HT$_{7(a)}$ or 5-HT$_{7(b)}$. Upon agonist treatment (5-CT), 5-HT$_{7(a)}$ and 5-HT$_{7(b)}$ show a redistribution of receptor from the plasma membrane to the peri-nuclear region. 5-HT$_{7(d)}$ distribution is unchanged from the untreated condition. Treatment of 5-HT$_{7(d)}$ with antagonist (SB269970) results in some redistribution of receptor from the cytoplasm to the plasma membrane. Antagonist-treated 5-HT$_{7(a)}$ and 5-Ht$_{7(b)}$ cells look much like their untreated condition. Scale bar = 10μm. (B) Quantitation of photomicrographs using IPLab (Scanalytics) software analysis. Fifteen fields for each condition were segmented and fluorescence intensity of plasma membrane and cytoplasmic receptors was measured. ** P = .0017, ***P = <.0001

Fig.4. *Functional characterization of HA-5-HT$_{7(a)}$, 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ stable cell lines.* Stable cell lines HA-5-HT$_{7(a)}$ (304 ±46fmol/mg), HA-5-HT$_{7(b)}$ (310 ±10fmol/mg) and HA-5-HT$_{7(d)}$ (482 ±39fmol/mg) were grown, serum starved for 24 hours, then stimulated with increasing concentrations of the 5-HT$_7$ agonist 5-carboxyamidotryptamine (5-CT). After 6 hours cells were harvested and cell extracts assayed for luciferase activity. Agonist potency was similar for the three isoforms: HA-5-HT$_{7(a)}$ (EC$_{50}$=.27nM), 5-HT$_{7(b)}$ (EC$_{50}$=.20nM) and 5-HT$_{7(d)}$ (EC$_{50}$=.56nM). However, the maximal response of the 5-HT$_{7(d)}$ response was only half that of 5-HT$_{7(a)}$ and 5-HT$_{7(b)}$. Data is from combined experiments of two different cell lines of each isoform and representative of multiple experiments.

Fig.5. *Internalization of biotinylated 5-HT$_7$ receptors.* Endocytosis of 293T stable cell lines expressing 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$ receptors were analyzed by surface biotinylation assay. UNTREATED TOTAL represents total biotinylated receptor (surface biotin was not stripped after treatment). UNTREATED represents constitutively internalized receptors. 5-CT and SB 269970 represent internalized receptors in response to agonist or antagonist treatment respectively

Fig. 6. *Surface 5-HT$_7$ receptor localization and trafficking.* 293T stable cell lines expressing 5-HT$_{7(a)}$, 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$ receptors. (A) Epi-fluorescent photomicrographs of HA-5-HT$_7$ receptors. The top row shows the Pretreat Fix control cells where the cells were immediately fixed on ice with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. This control shows a good separation of the two populations of receptors, plasma membrane (green)and cytoplasm (red). In the UNTREATED (2 hours at 37° after surface receptor labeling) condition, 5-HT$_{7(a)}$ and 5-HT$_{(7b)}$ receptors (green) display movement into localized domains, but remain on the surface of the cell. In contrast 5-HT$_{(7d)}$ receptors (green) translocate to the perinuclear region. Upon agonist treatment (5-CT) many of the 5-HT$_{7(a)}$ and 5-HT$_{(7b)}$ receptors translocate into the cytoplasm while 5-HT$_{(7d)}$ receptors (green) look much as they did in the untreated condition. Antagonist-treated (SB 269970) 5-HT$_{7(a)}$ and 5-HT$_{(7b)}$ cells look much like their untreated condition. Antagonist treatment appears to cause some retention of 5- HT$_{(7d)}$ receptors (green) on the cell surface. Scale bar =10μm. (B) Quantitation of photomicrographs using IPLab (Scanalytics) software analysis. Only the green channel (HA-5-HT$_{(7)}$ receptors on the cell surface at the beginning of the experiment) was measured. Fifteen to thirty-five fields for each condition

were segmented and fluorescence intensity of plasma membrane and cytoplasmic receptors was measured. * P = .0391, ***P = <.0001

Fig.1

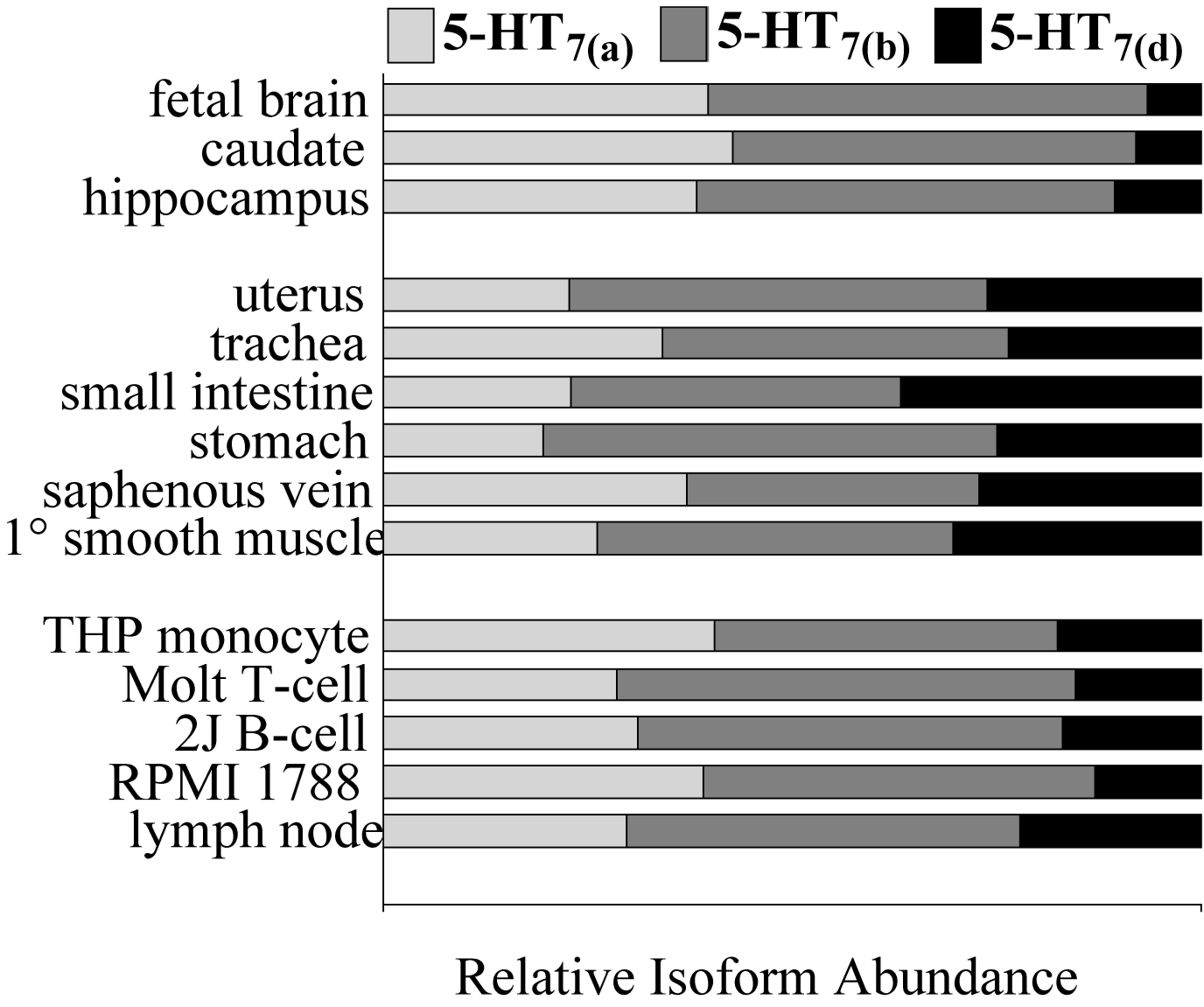

5-HT7(a)
5-HT7(b)
5-HT7(d)
fetal brain
caudate
hippocampus
uterus
trachea
small intestine
stomach
saphenous vein
1° smooth muscle
THP monocyte
Molt T-cell
2J B-cell
RPMI 1788
lymph node
Relative Isoform Abundance

Fig. 2

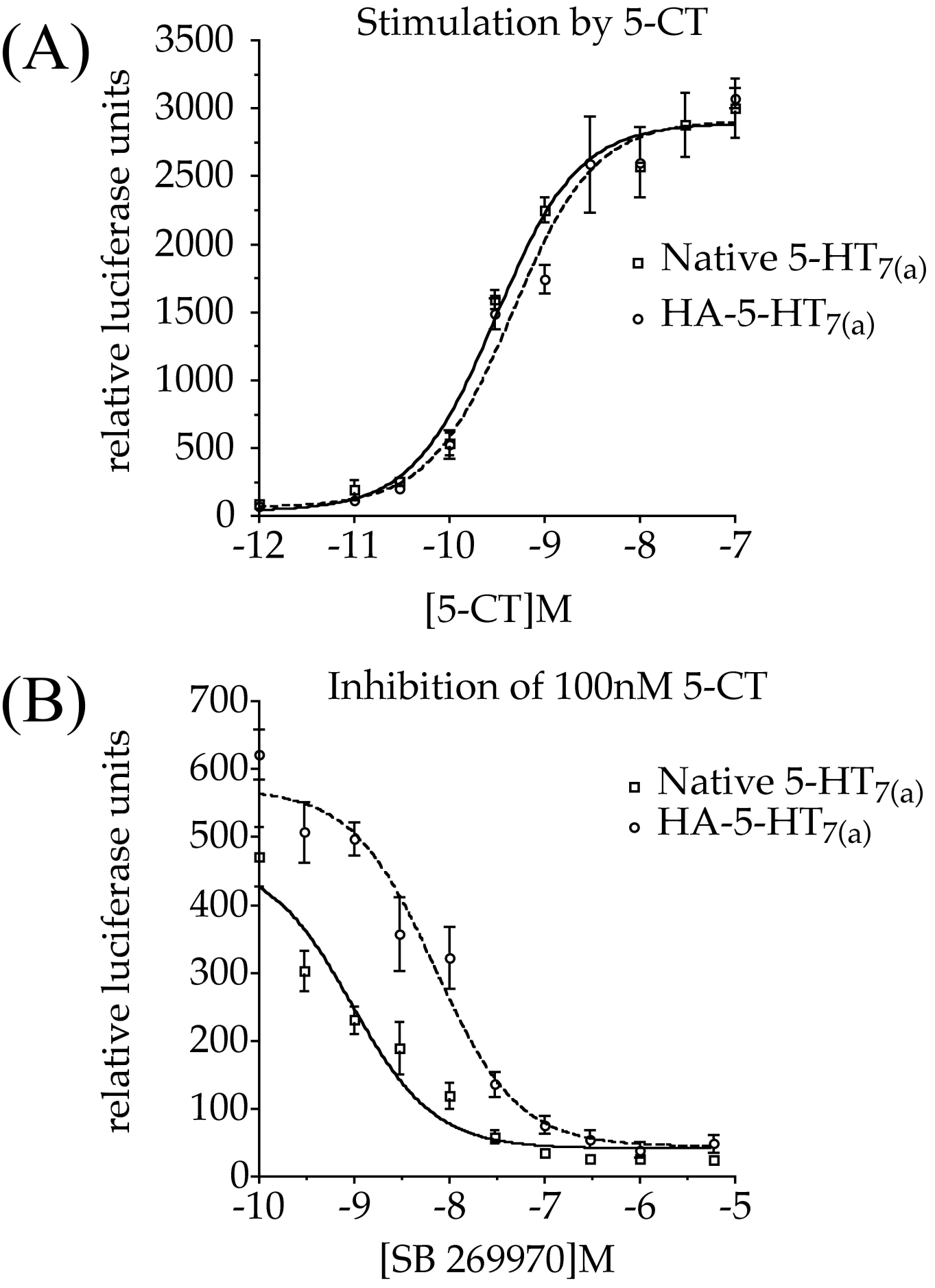
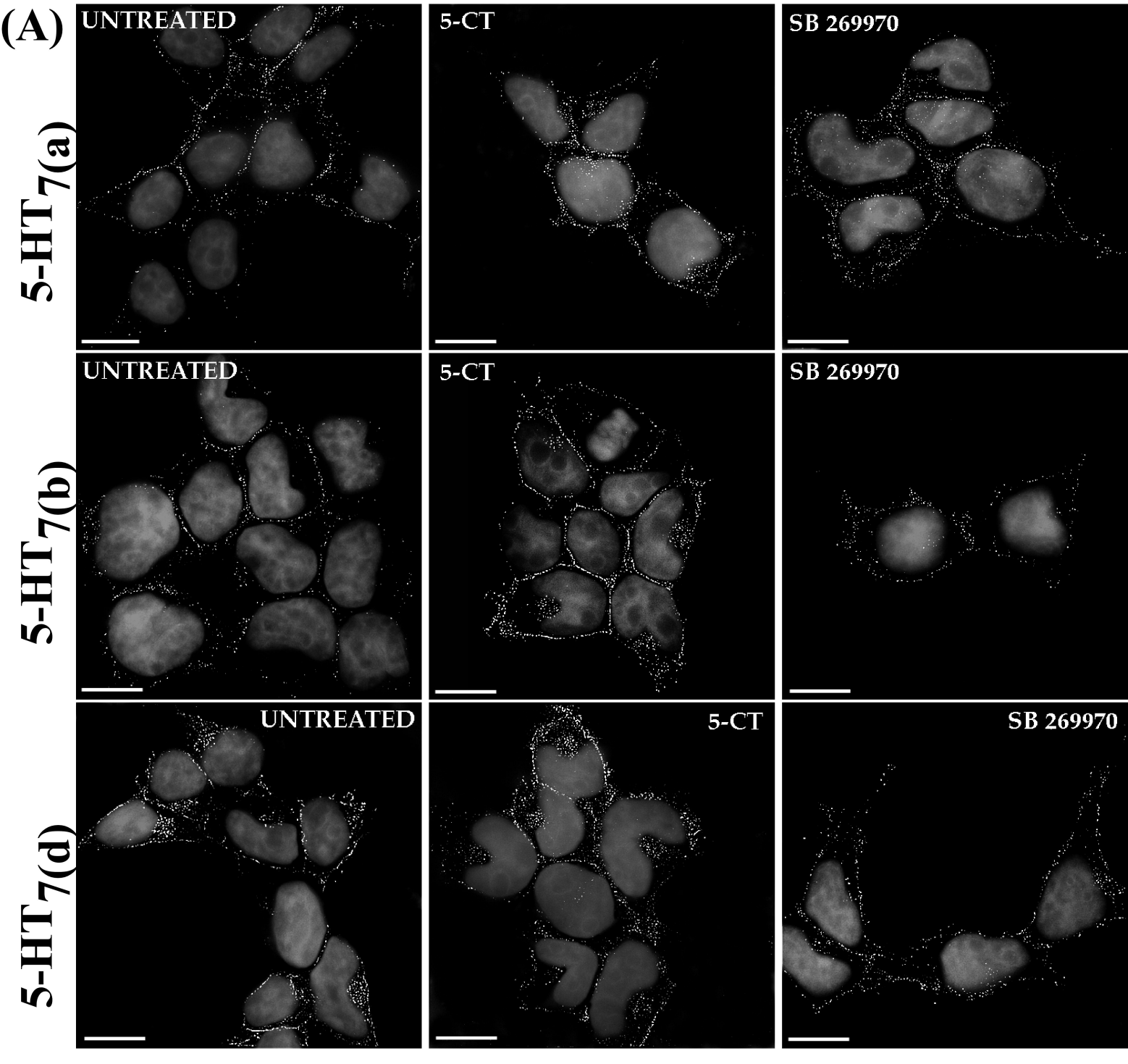
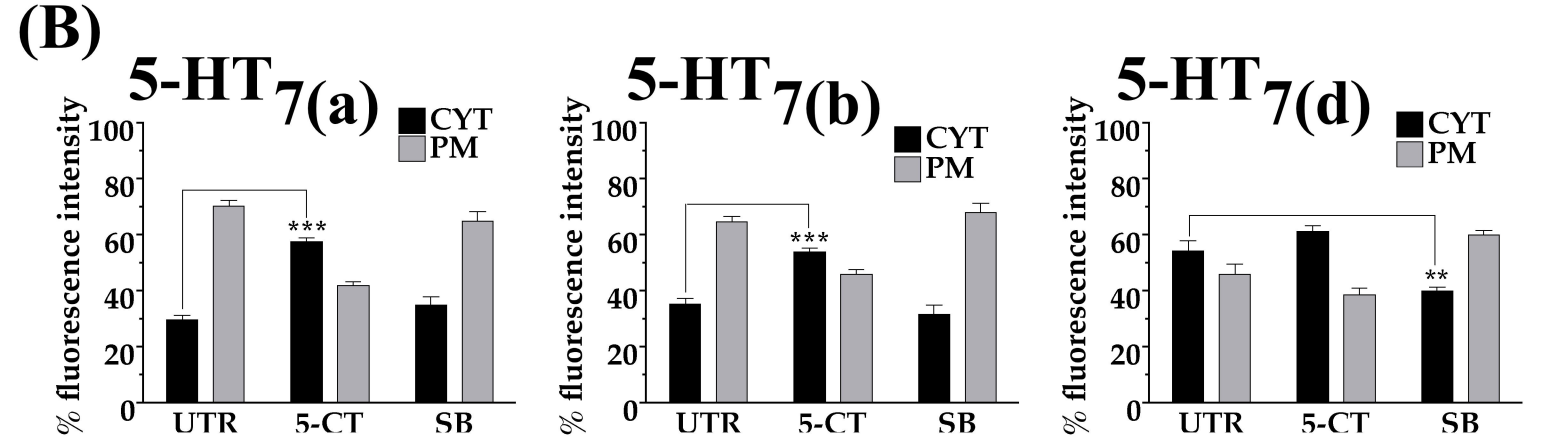

(A)
Stimulation by 5-CT
relative luciferase units
3500
3000
2500
2000
1500
1000
500
0
-12
-11
-10
-9
-8
-7
[5-CT]M
Native 5-HT7(a)
HA-5-HT7(a)
(B)
Inhibition of 100nM 5-CT
relative luciferase units
700
600
500
400
300
200
100
0
-10
-9
-8
-7
-6
-5
[SB 269970]M
Native 5-HT7(a)
HA-5-HT7(a)

Fig. 3


(A)
UNTREATED
5-CT
SB 269970
5-HT7(a)
UNTREATED
5-CT
SB 269970
5-HT7(b)
UNTREATED
5-CT
SB 269970
5-HT7(d)


(B)
5-HT7(a)
CYT
PM
% fluorescence intensity
100
80
60
40
20
0
***
UTR
5-CT
SB
5-HT7(b)
CYT
PM
% fluorescence intensity
100
80
60
40
20
0
***
UTR
5-CT
SB
5-HT7(d)
CYT
PM
% fluorescence intensity
100
80
60
40
20
0
**
UTR
5-CT
SB

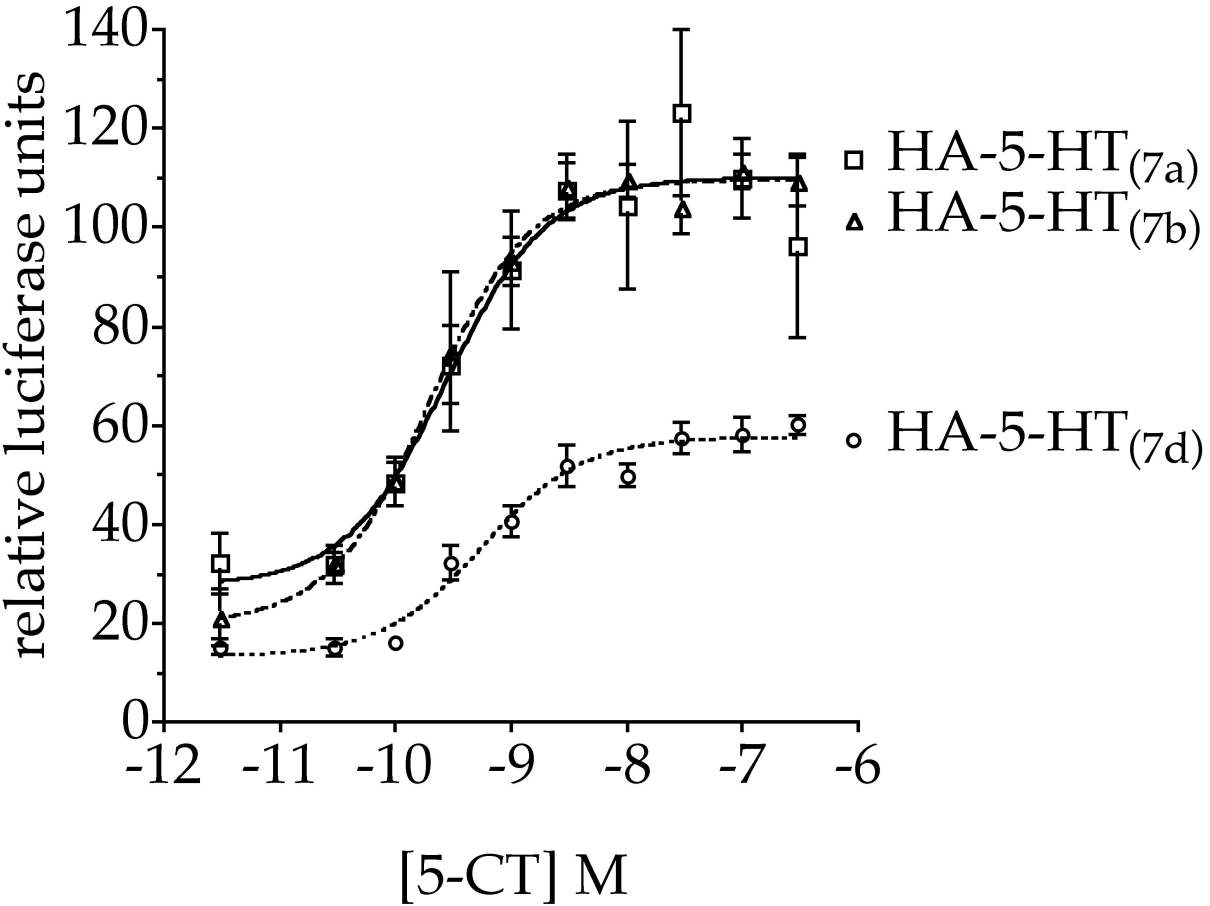

relative luciferase units
140
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
[5-CT] M
HA-5-HT(7a)
HA-5-HT(7b)
HA-5-HT(7d)

Fig. 4

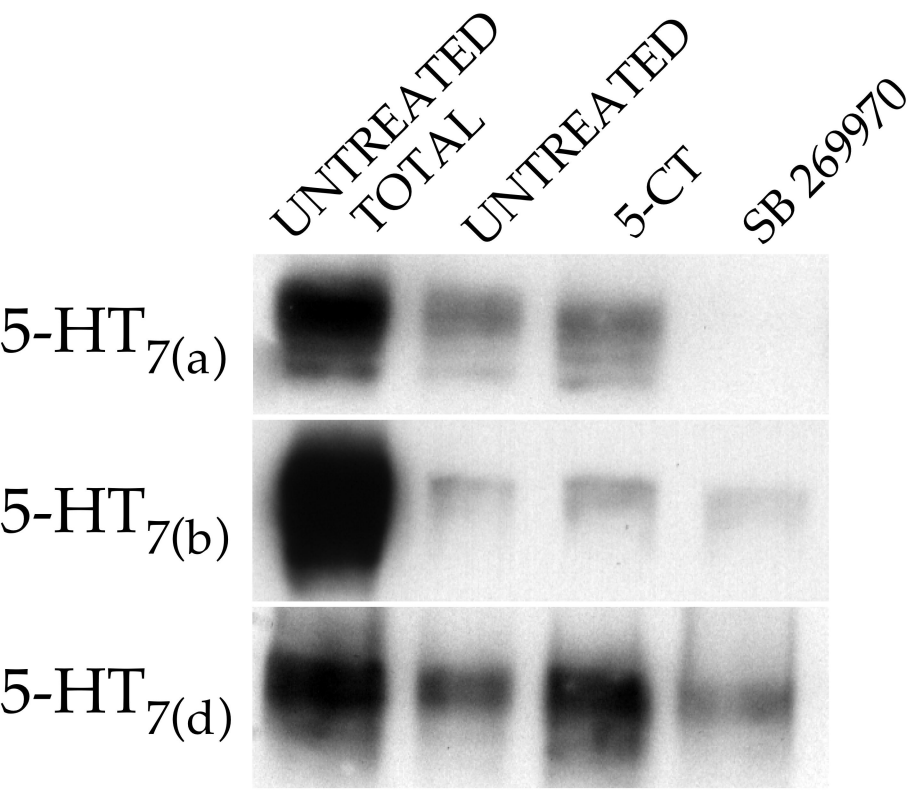


Fig. 5

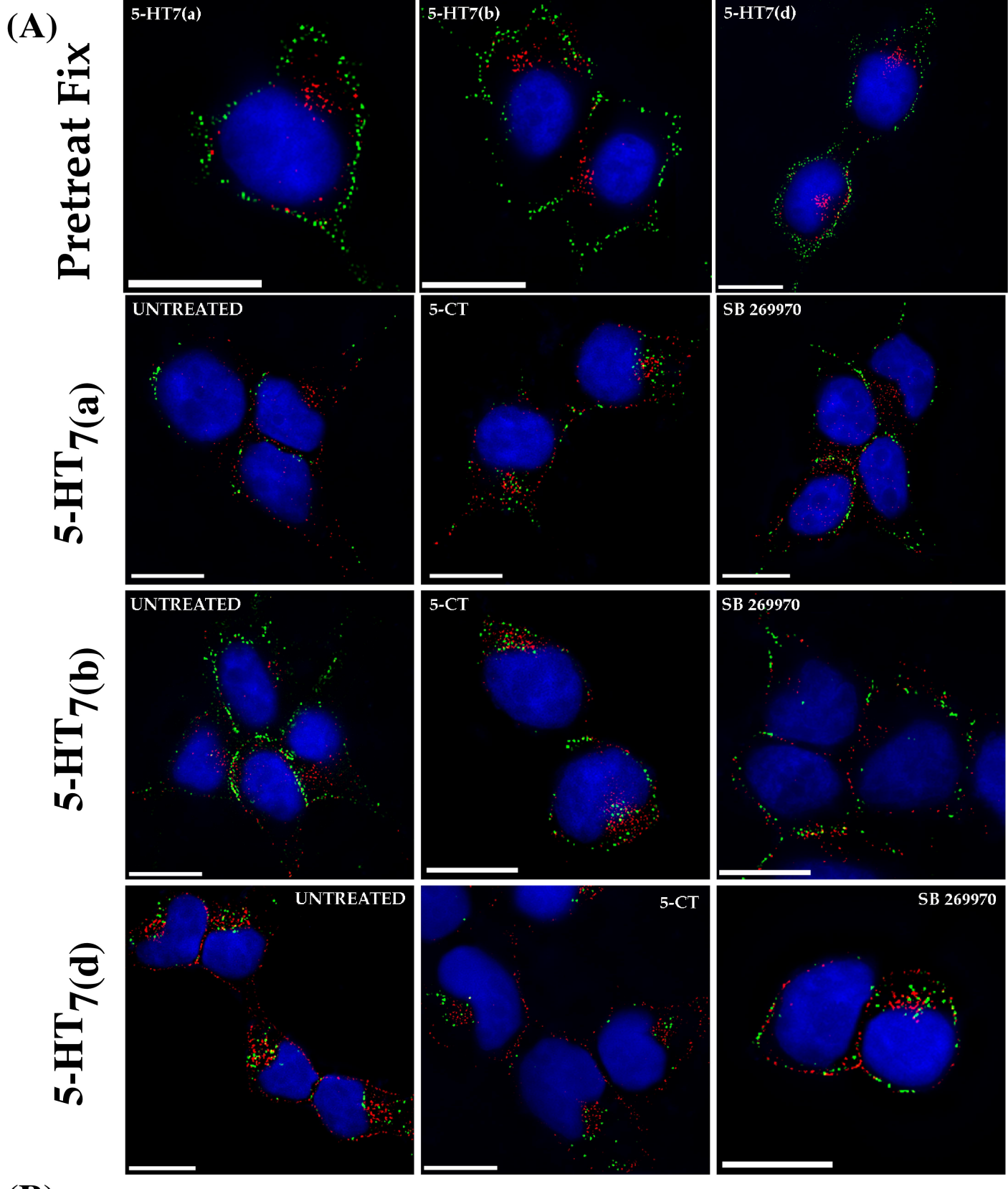

(A)
Pretreat Fix
5-HT7(a)
5-HT7(b)
5-HT7(d)
UNTREATED
5-CT
SB 269970
5-HT7(a)
5-HT7(b)
5-HT7(d)
(B)
5-HT7(a)
5-HT7(b)
5-HT7(d)
CYT
PM
% fluorescence intensity
UTR
5-CT
SB
***
*

Fig. 6