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RESEARCH PAPER

Agonist- and antagonist-induced up-regulation of surface 5-HT_3A receptors

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BACKGROUND AND PURPOSE
The 5-HT_3 receptor is a member of the pentameric ligand-gated ion channel family and is pharmacologically targeted to treat irritable bowel syndrome and nausea/emesis. Furthermore, many antidepressants elevate extracellular concentrations of 5-HT. This study investigates the functional consequences of exposure of recombinant 5-HT_3A receptors to agonists and antagonists.

EXPERIMENTAL APPROACH
We used HEK cells stably expressing recombinant 5-HT_3A receptors and the ND7/23 (mouse neuroblastoma/dorsal root ganglion hybrid) cell line, which expresses endogenous 5-HT_3 receptors. Surface expression of recombinant 5-HT_3A receptors, modified to contain the bungarotoxin (BTX) binding sequence, was quantified using fluorescence microscopy to image BTX-conjugated fluorophores. Whole cell voltage-clamp electrophysiology was used to measure the density of current mediated by 5-HT_3A receptors.

KEY RESULTS
5-HT_3A receptors were up-regulated by the prolonged presence of agonists (5-HT and m-chlorophenylbiguanide) and antagonists (MDL-72222 and morphine). The up-regulation of 5-HT_3A receptors by 5-HT and MDL-72222 was time- and concentration-dependent but was independent of newly translated receptors. The phenomenon was observed for recombinant rodent and human 5-HT_3A receptors and for endogenous 5-HT_3 receptors in neuronal ND7/23 cells.

CONCLUSIONS AND IMPLICATIONS
Up-regulation of 5-HT_3A receptors, following exposure to either agonists or antagonists suggests that this phenomenon may occur in response to different therapeutic agents. Medications that elevate 5-HT levels, such as the antidepressant inhibitors of 5-HT reuptake and antiemetic inhibitors of 5-HT_3 receptor function, may both raise receptor expression. However, this will require further investigation *in vivo*.

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Abbreviations

BTX, α-bungarotoxin, WGA, wheat germ agglutinin; CHX, cycloheximide; HEK-3A/BBS, HEK-293 cells stably expressing the mouse 5-HT3A subunit tagged with the α-bungarotoxin binding sequence (BBS) on the extracellular C-terminus; HEK-h3A, HEK-293 cells stably expressing human 5-HT$_3$A receptors; mCPBG, m-Chlorophenylbiguanide; ND7/23, mouse neuroblastoma/rat dorsal root ganglion neuron hybrid; pLGICs, pentameric ligand-gated ion channels; SFGM, serum-free growth media

Tables of Links

TARGETS
Ligand gated ion channels
5-HT$_3$ receptors
GABA$_A$ receptors
nACh receptors

LIGANDS
5-HT
Citalopram
mCPBG, m-chlorophenylbiguanide

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The 5-HT$_3$ receptor is a member of the cysteine (Cys)-loop pentameric ligand-gated ion channel (pLGIC) family, which includes the nicotinic acetylcholine (nACh) receptors, GABA$_A$ receptors, glycine receptors and Zn^{2+}-activated ion channels (Barnes *et al.*, 2009). Five 5-HT3 subunit genes have been cloned, which encode subunits termed 5-HT3A, B, C, D and E (Maricq *et al.*, 1991; Davies *et al.*, 1999; Karnovsky *et al.*, 2003; Niesler *et al.*, 2003). The 5-HT3A subunit can form functional homomeric receptors, whereas the 5-HT3B subunit requires the presence of 5-HT3A subunits to form heteromeric receptors with altered channel properties compared with homomeric 5-HT$_3$A receptors (Davies *et al.*, 1999; Karnovsky *et al.*, 2003; Niesler *et al.*, 2003). The functional consequences of the other receptor combinations are less well established (Barnes *et al.*, 2009).

5-HT$_3$ receptors are highly expressed in the peripheral nervous system and in the gut where they regulate the vomiting reflex and gut motility (Glatzle *et al.*, 2002; Raybould *et al.*, 2003). Antagonists to 5-HT$_3$A receptors are used clinically to treat irritable bowel syndrome as well as nausea and emesis following either chemotherapy or surgical anaesthesia (Machu, 2011). 5-HT$_3$A receptors have also been implicated in visceral pain and inflammation processes (Costall and Naylor, 2004) and may participate in some of the maladaptive effects of chronic morphine analgesia, such as hyperalgesia, tolerance (Liang *et al.*, 2011) and dependence (Chu *et al.*, 2009). Morphine has both competitive and non-competitive antagonist actions on 5-HT$_3$ receptors (Fan, 1995; Baptista-Hon *et al.*, 2012).

5-HT$_3$A receptors are expressed throughout the CNS and are localized both pre- and post-synaptically (Kilpatrick *et al.*, 1987; van Hooft and Vijverberg, 2000; Morales and Wang, 2002). Mice lacking the 5-HT$_3$A receptor show sex-dependent alterations in depression and anxiety-like behaviours (Bhatnagar *et al.*, 2004), and 5-HT$_3$ receptors have been implicated in alcohol intake in humans and rats (Li *et al.*, 2001). 5-HT plays a major role in mood and most clinically used anti-depressants elevate extracellular levels of this amine. Although the anti-depressant effects are not mediated by 5-HT$_3$ receptors (Millan *et al.*, 2003), it remains unclear whether 5-HT$_3$A receptors are modulated by prolonged exposure to agonist.

Receptor surface expression is a highly regulated process, and changes in surface expression can produce significant alterations in downstream signalling. Sustained activation of GPCRs often leads to the internalization of surface receptors, leading, in turn, to a down-regulation of surface receptors (Ferguson, 2001). However, prolonged exposure to agonist can increase the surface expression of nACh (see Govind *et al.*, 2009) and GABA$_A$ receptors (Eshaq *et al.*, 2010). It has been proposed that intracellular nicotine or GABA can act as a molecular chaperone within the endoplasmic reticulum aiding the assembly of subunits, leading to an increase in functional surface receptors (Lester *et al.*, 2009; Eshaq *et al.*, 2010). We have previously reported that 24 h of exposure to 100 μM 5-HT produced an increase in maximal binding of radioligands to 5-HT$_3$A receptors expressed in HEK-293 cells (Sanghvi *et al.*, 2009). However, it is unclear whether this elevated binding reflects an increase in functional cell surface receptors. In this study, we used fluorescence microscopy and whole-cell electrophysiology to investigate the functional cell surface expression of recombinant 5-HT$_3$A receptors stably expressed in HEK-293 and receptors native to ND7/23 (mouse neuroblastoma/rat dorsal root ganglion hybrid) cells.

Methods

Cell cultures

HEK-293 cells and ND7/23 cells were purchased from American Type Culture Collection (Manassas, VA, USA). HEK-293 cells and ND7/23 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum and 1% penicillin/streptomycin/glutamate incubated at 37°C and in a humid environment at 5% CO_2.

The mouse 5-HT3A subunit was tagged with a linker sequence and two bungarotoxin pharmatope-tags on the receptor C-terminus (5-HT3A/BBS) in pCDNA3.1. The human 5-HT3A subunit was tagged with the hemagglutinin (HA) epitope in pCDNA3.1 vector on the N-terminus. Mouse 5-HT3A/BBS and human 5-HT3A subunits were stably expressed in HEK-293 cells by transfection with polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA) (150 μM per 1 μg DNA) or lipofectamine (Invitrogen) respectively. Cells were incubated for 24–48 h at 37°C in 5% CO_2 after transfection to allow for expression, yielding HEK-3A/BBS or HEK-h3A cells respectively. Approximately 1000 cells were seeded in a 10 cm dish and cultured in the presence of 400 or 1000 $\mu g \cdot mL^{-1}$ G418 (Gibco Invitrogen, Carlsbad, CA, USA) for HEK-3A/BBS and HEK-h3A respectively. Individual colonies from HEK-293 cells transfected with 5-HT3A/BBS were tested for expression using BTX/555 labelling (Sanghvi *et al*., 2009; Morton *et al*., 2011). For HEK-h3A cells, expression levels were determined under voltage clamp using the patch-clamp technique. In experiments investigating the effects of 5-HT pre-incubation, a saturating concentration of 5-HT (30 or 100 μM) was added from frozen stocks of 5-HT to the culture medium.

Microscopy

Surface $5\text{-}HT_3A$/BBS receptors were labelled with α-bungarotoxin (BTX) conjugated to Alexa fluorophores at 1 $\mu g \cdot mL^{-1}$ for 30 min at 4°C. Cell membranes were labelled with wheat germ agglutinin (WGA) conjugated to Alexa-555 at 10 $\mu g \cdot mL^{-1}$ for 30 min at 4°C (Invitrogen). Cells were fixed with 4% paraformaldehyde for 30 min at 25°C. Confocal images were collected using an inverted Zeiss LSM 510 (Zeiss USA, Thornwood, NY, USA). For green fluorescence, an argon laser was used with an excitation filter of 480/35 and an emission filter of 535/30. For red fluorescence, a HeNe laser was used with an excitation filter of 540/25 and an emission filter of 605/55. Images were acquired with an oil immersion Plan-Apochromat 63× 1.4 NA objective using LSM imager software. Laser intensity, gain and offset were set using a time zero or no agonist control and maintained throughout each experiment. The fluorescence intensities for the control conditions were comparable between replicate experiments.

Surface receptors were quantified, as previously described (Morton *et al*., 2011), by drawing a segmented line along the cell membrane using the WGA fluorescence as a marker in ImageJ software. The pixel intensities were averaged for the BTX/488 fluorescence along that line in three separate optical sections within each cell. The average fluorescence along that line was averaged from all three optical slices to estimate the surface expression of $5\text{-}HT_{3A}$/BBS receptors for an entire cell. All surface expression calculations were normalized to the time zero no agonist control for each individual experiment.

Electrophysiology

The whole-cell configuration of the patch-clamp technique was used to record currents mediated by $5\text{-}HT_3A$/BBS and $5\text{-}HT_3A$-HA receptors in HEK-293 cells, or $5\text{-}HT_3$ receptors native to ND7/23 cells. Cells were seeded at low densities in 35-mm-diameter culture dishes and visualized with a Nikon Eclipse TE300 microscope (Chiyoda, Tokyo, Japan) equipped with a Plan Fluor 20× objective with NA of 0.45. Cells were held at −60 mV and continuously superfused with extracellular solution (in mM): NaCl, 140 or 150; KCl, 2.5 or 2.8; $MgCl_2$, 2 or 2.5; $CaCl_2$, 1 or 2.5; glucose, 10; and HEPES, 10 (pH 7.4 with NaOH). Patch pipettes were pulled from thin wall filament-containing borosilicate capillary glass with a Sutter Flaming-Brown P-97 multi-stage puller. Patch pipettes were filled with intracellular solution containing either (in mM) CsCl, 150; BAPTA, 0.2; $MgCl_2$, 1; Mg ATP, 3; GTP, 0.3; HEPES, 10 (pH 7.2 with CsOH) for HEK-3A/BBS; or CsCl, 140; $MgCl_2$, 2; $CaCl_2$, 0.1; EGTA, 1.1; and HEPES, 10 (pH 7.4 with CsOH) for HEK-h3A and ND7/23 cells. Cells were lifted clear of the dish bottom to facilitate rapid drug application. Unless otherwise stated, a maximally efficacious concentration of 5-HT was used to evoke currents. Agonist was applied locally by rapid perfusion exchange using a SF-77B fast step perfusion system (Warner Instruments, Hamden, CT, USA) that is able to achieve complete whole-cell solution exchange in ~20 ms (Moykkynen *et al*., 2003). Cells were held for 2–5 min after establishing the whole-cell configuration and prior to recording to allow complete exchange of ions between the electrode and the cell interior.

Currents were amplified with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), low-pass filtered at 1 kHz and digitized at 12.5 kHz using a Digidata 1322A interface (Molecular Devices). Data were collected, displayed and analysed using pCLAMP8.2 software. Experiments were performed at room temperature (20–24°C). No correction was made for the compensation for liquid junction potential, which had a calculated value of −5.4 mV. Pre-agonist baseline currents were leak-subtracted and peak amplitudes were measured between cursors placed at the baseline and at the end of the sweep. In all cases, mean current amplitudes are presented as current densities, by normalizing the peak amplitude to the cell capacitance.

Data analysis

All data points represent individual cells from at least three separate experiments and averaged data are presented as mean ± SEM. All statistical analyses were performed in GraphPad Prism 5.0 (San Diego, CA, USA) or SigmaPlot software (Systat Software Inc, London, UK). Statistical significance was determined by either a one-way ANOVA with a *post hoc* Tukey's test or a Student *t*-test. $P < 0.05$ was considered statistically significant.

Materials

All compounds used in these experiments were supplied by Sigma-Aldrich.

Results

Prolonged agonist exposure enhances $5\text{-}HT_3$ receptor-mediated current density

Agonist-induced up-regulation of $5\text{-}HT_3A$ receptors was investigated using multiple cell lines expressing both human (HEK-h3A) and mouse (HEK3A/BBS) isoforms of $5\text{-}HT_3A$ receptors with pre-incubation with 5-HT at 100 or 30 μM respectively. The functional consequence of agonist pre-incubation was investigated by whole-cell voltage-clamp elec-

trophysiology in HEK-293 cells stably expressing human 5-HT$_3$A receptors (HEK-h3A) that were pre-incubated with a saturating concentration of 5-HT (100 μM) for 24 h (Figure 1A). There was a significant increase in maximal current density (pA/pF) evoked by rapid application of 5-HT (100 μM) to cells incubated in 5-HT compared with control cells. This phenomenon was also tested in rodent ND7/23 cells that endogenously express 5-HT$_3$A receptors. Once again, 5-HT induced a significant up-regulation of the density of current mediated by 5-HT$_3$ receptors (Figure 1B).

Prolonged agonist exposure increases surface 5-HT$_3$A receptors in HEK cells

The surface expression of 5-HT$_3$A receptors was investigated using a heterologous HEK-293 system that stably expresses the mouse 5-HT3A subunit tagged with the α-bungarotoxin binding sequence (BBS) on the extracellular C-terminus (HEK-3A/BBS). Our previous studies demonstrated that the addition of the BBS to 5-HT$_3$A receptors does not alter receptor function even in the presence of bound BTX (Sanghvi *et al.*, 2009; Morton *et al.*, 2011). This experimental design allows us to label only the surface receptors with fluorescently conjugated BTX and measure surface expression using fluorescent microscopy. Following a 24 h pre-incubation with 5-HT (30 μM), the surface expression of 5-HT$_3$A/BBS receptors was measured in HEK-3A/BBS cells. Surface receptors were visualized following pre-incubation with BTX/488 and the cell membrane with BTX/488 and WGA conjugated to Alexa 555 (WGA/555) respectively. Following fixation, cells were imaged using confocal microscopy. Representative images of cells with or without 24 h 5-HT pre-incubation are shown in Figure 2A. Surface receptor expression was determined by BTX/488 fluorescence, averaged along the cell surface, as previously described (Morton *et al.*, 2011). Exposure to 5-HT for 24 h increased surface expression of 5-HT$_3$A/BBS receptors by approximately 30% (Figure 2B). These data suggest that prolonged exposure to 5-HT can induce the up-regulation of surface 5-HT$_3$A/BBS receptors.

Agonist-induced up-regulation in serum-free growth medium

Agonist-induced up-regulation was assessed in the absence of serum to eliminate any confounding effects of 5-HT in serum (Mothersill *et al.*, 2010). We used a chemically defined medium CD-293 (Invitrogen), which lacks 5-HT, but maintains growth and survival of cells without serum [serum-free growth medium (SFGM)]. HEK-3A/BBS cells were incubated in SFGM for 24 h in the absence or presence of 5-HT (30 μM), and 5-HT$_3$A surface expression was measured via fluorescence microscopy (Figure 3A and B). Incubation in SFGM alone significantly reduced the surface fluorescence of 5-HT$_3$A/BBS receptors. However, similar to our previous data, pre-incubation with 5-HT in SFGM increased surface 5-HT$_3$A receptors to a similar magnitude (Figure 3C). The functional consequences of SFGM with or without 30 μM 5-HT incubation were assessed with whole-cell voltage-clamp electrophysiology. Representative current traces elicited by a saturating concentration of 5-HT (30 μM) are shown in Figure 3D. Pre-incubation with 5-HT (30 μM) in SFGM significantly increased the current densities from HEK-3A/BBS cells (Figure 3D).

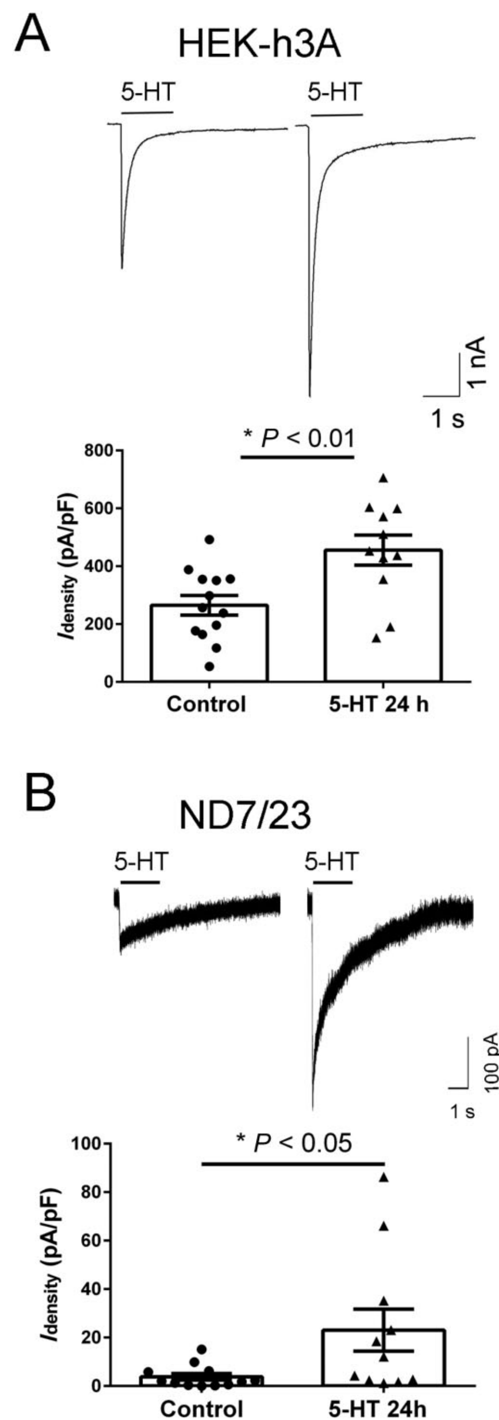

Figure 1

Pre-incubation of HEK-h3A cells and ND7/23 cells with 5-HT for 24 h increases functional 5-HT$_3$A receptors. (A) Representative traces from HEK-h3A cells (top panel) and current density measurements of control and 5-HT (100 μM) pre-incubation (bottom panel) (each symbol represents an individual cell $n = 11$). (B) Representative traces from ND7/23 cells (top panel) and current density measurements (bottom panel) following pre-incubation (100 μM) for 24 h. Individual data points represent individual cells from several experiments. Means with SEM are shown; significantly different as indicated; unpaired Student's *t*-test.

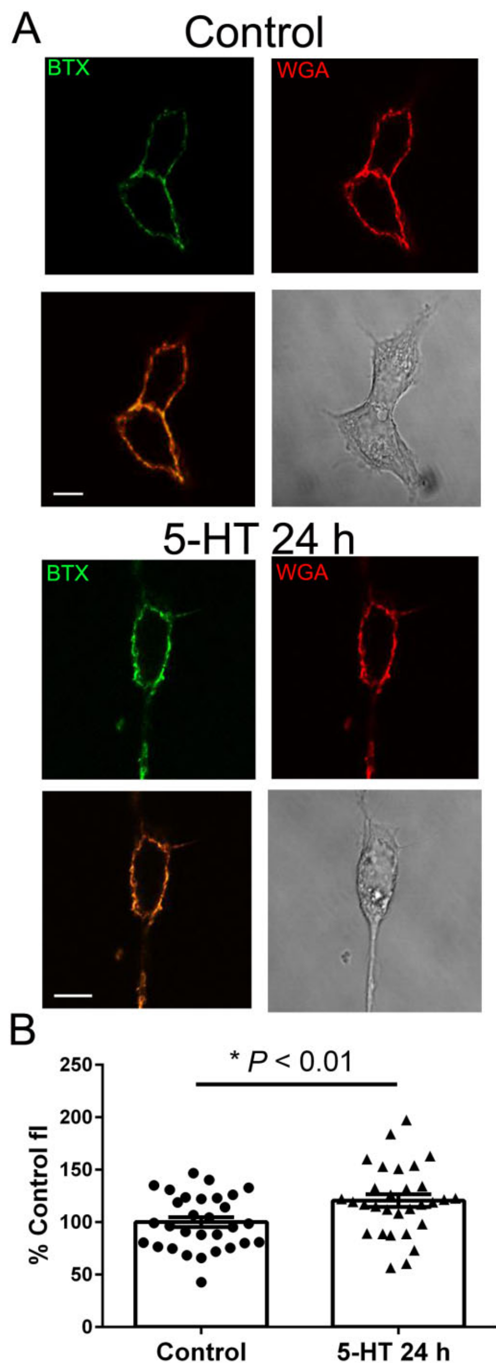

Figure 2

Pre-incubation of HEK-3A/BBS cells with 5-HT for 24 h increases surface 5-HT_3A/BBS receptors. (A) Representative confocal images of HEK-3A/BBS cells with or without pre-incubation of 5-HT (30 μM) for 24 h. Green = BTX/Alexa488-labelled surface 5-HT_3A receptors; red = WGA/Alexa555 labelling of plasma membrane; and grey = DIC image (scale bar = 10 μm). (B) Averaged BTX/488 pixel intensities along WGA/555-labelled cell membrane normalized to control cells. Data are presented as percentage of control ($n = 30$). Individual data points represent individual cells from several experiments. Means with SEM are shown; significantly different as indicated; unpaired Student's *t*-test.

Agonist-induced up-regulation is dependent on both time and concentration

HEK-3A/BBS cells were exposed to 0, 1, 3, 30 and 100 μM 5-HT for 24 h in SFGM to determine whether agonist-induced up-regulation was concentration-dependent. The concentrations used span the 5-HT_3A concentration response relationship previously described (Schreiter *et al.*, 2003). Indeed, higher concentrations of 5-HT induced a larger up-regulation (Figure 4A). There was no significant up-regulation when the cells were pre-incubated with 1 or 3 μM 5-HT. However, after pre-incubation with 30 and 100 μM 5-HT, significant up-regulation was observed. The magnitude of up-regulation was similar when treated with 30 and 100 μM 5-HT, concentrations that produce maximal receptor activation, suggesting that maximal up-regulation occurs at concentrations that produce maximal receptor occupancy.

Our findings suggest that the length of agonist exposure and concentration are important and led us to examine whether a lower concentration of agonist could induce up-regulation of 5-HT_3A/BBS receptors after longer periods of time. HEK-3A/BBS cells were exposed to 1 μM 5-HT for 48 h in SFGM and surface receptors were quantified by fluorescence. The lower concentration of 5-HT induced a significant up-regulation of 5-HT_3A/BBS receptors after 48 h albeit to a smaller magnitude compared with higher concentrations for shorter periods (Figure 4B).

The time scale of 5-HT-induced up-regulation was examined by pre-incubating HEK-3A/BBS cells in SFGM supplemented with 30 μM 5-HT for 0, 4, 8 and 24 h. Surface receptor expression was again quantified by fluorescence analysis and normalized to time zero. Similar to our previous findings, 4 h of pre-incubation with 5-HT did not result in an increase in surface 5-HT_3A/BBS receptors (Figure 4C). However, 8 and 24 h of pre-incubation with 5-HT significantly up-regulated surface 5-HT_3A/BBS receptors (Figure 4C). It is possible that the signalling process required to induce receptor up-regulation could occur in the first few hours, but the remaining 4–20 h may be required for synthesis of new receptors or trafficking of more receptors to the plasma membrane. To examine this possibility, we pre-incubated cells with 5-HT (30 μM) in SFGM for 4 h and then incubated in SFGM only for an additional 20 h before measuring surface 5-HT_3A/BBS receptors. We were unable to detect any difference in surface receptor expression after 20 h following the 4 h exposure compared with the time zero time point (Figure 4C). The time course of up-regulation was also assessed by incubating HEK-h3A cells in 5-HT (100 μM) for varying durations and functional receptors were assessed with whole-cell electrophysiology. While there was a trend to enhanced current density at shorter exposure durations, the difference became significant ($P < 0.05$) only after 24 h (Figure 4D). These data suggest that optimal up-regulation requires the presence of a saturating concentration of agonist for at least 8 h.

We investigated whether surface receptor expression returned to baseline levels following removal of 5-HT from the culture media. In both HEK-3A/BBS and ND7/23 cells, pre-incubation with 5-HT, 30 and 100 μM, respectively, resulted in significant up-regulation (Figure 4E and F). Within 2 h of 5-HT washout, the current densities in both cell lines

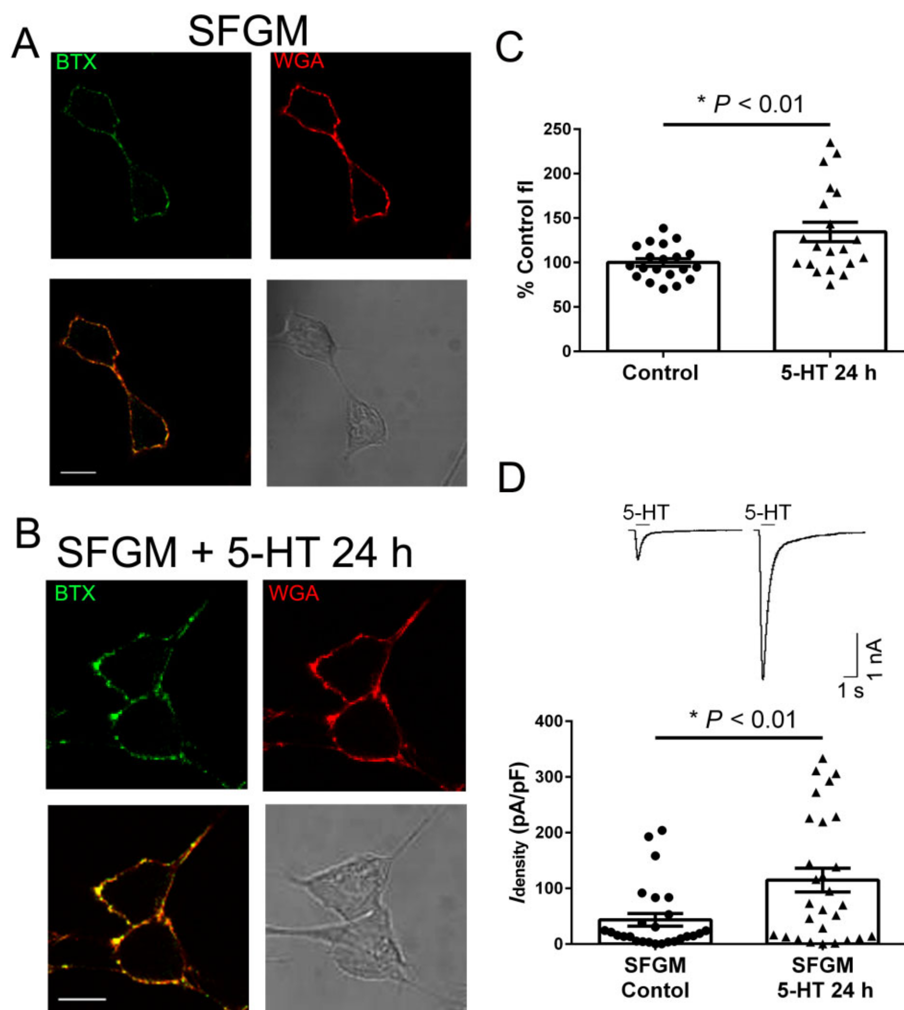

Figure 3

Agonist-induced up-regulation of mouse 5-HT$_3$A receptors in the absence of serum in HEK-3A/BBS cells. Representative images of HEK-3A/BBS cells in the absence (A) or presence (B) of 5-HT (30 μM) for 24 h. Green = BTX/Alexa488-labelled surface 5-HT$_3$A receptors; red = WGA/Alexa555 labelling of plasma membrane; and grey = DIC image (scale bar = 10 μm). (C) Averaged surface fluorescence from HEK-3A/BBS cells with or without pre-incubation with 5-HT (30 μM) for 24 h. Data are presented as percentage of control (n = 30). (D) Representative traces (top panel) and current densities (bottom panel) elicited by 30 μM 5-HT following pre-incubation in SFGM with or without 5-HT (30 μM). Data are expressed as current densities (pA/pF) (n = 26). Means with SEM are shown; significantly different as indicated; unpaired Student's t-test.

were reduced, and within 4 h, the current densities had returned to baseline levels (Figure 4E and F). These data suggest that the agonist-induced up-regulation is reversible.

Antagonist-induced up-regulation of 5-HT$_3$/BBS receptors

A possible mechanism for the up-regulation of 5-HT$_3$A receptors is that the persistent opening of the receptor channel leads to an influx of Ca^{2+} that could activate downstream signalling and transcriptional mechanisms, resulting in an increase in 5-HT$_3$A receptor expression. If the up-regulation were activity-dependent, then a competitive antagonist would be expected to inhibit up-regulation. HEK-3A/BBS cells were pre-incubated in SFGM with 5-HT (30 μM) and MDL-72222 (1 μM), a 5-HT$_3$-specific competitive antagonist, for 24 h before surface expression was assessed by fluorescence. Interestingly, MDL-72222 did not block the 5-HT-induced up-regulation (Figure 5A). In fact, 24 h of exposure to 1 μM MDL-72222 alone caused a smaller but significant increase in surface expression of 5-HT$_3$A/BBS receptors (Figure 5A). By contrast, picrotoxin, a non-competitive antagonist of 5-HT$_3$A receptors (Das *et al.*, 2003), did not increase surface expression of 5-HT$_3$A/BBS receptors after 24 h of exposure (Figure 5B), consistent with the hypothesis that up-regulation requires occupancy of the orthosteric binding site.

The antagonist modulation of functional receptors was also assessed with whole–cell voltage-clamp electrophysiology. HEK-3A/BBS cells were exposed for 24 h to MDL-72222 in SFGM. Using a Mann–Whitney t-test, we determined that MDL-72222 significantly increased the current density in HEK-3A/BBS cells, suggesting that the presence of MDL-72222 alone induces up-regulation of functional surface

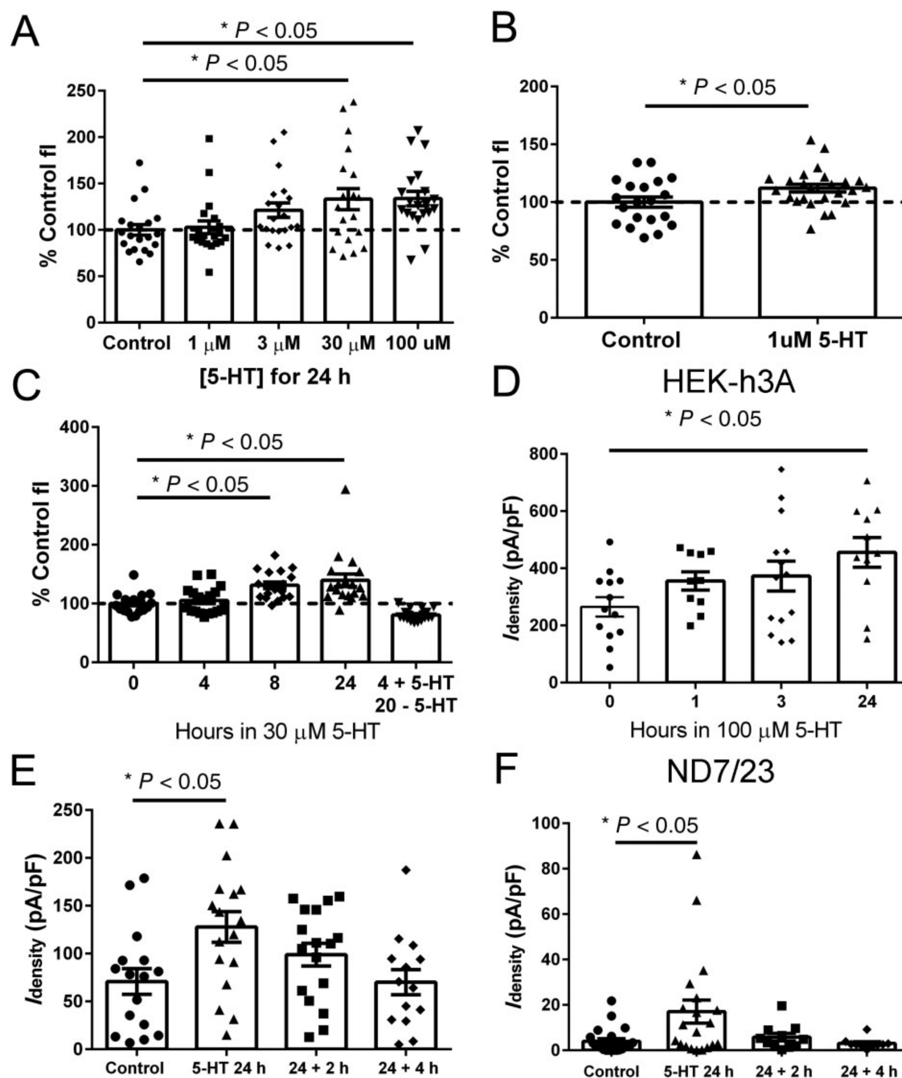

Figure 4

Agonist-induced up-regulation of 5-HT$_3$A receptors is both concentration- and time-dependent. (A) HEK-3A/BBS cells were pre-incubated in SFGM containing varying concentrations of 5-HT. Surface mouse 5-HT$_3$A receptors were quantified by BTX/488 fluorescence, normalized to the no 5-HT exposure condition (n = 20). (B) HEK-3A/BBS cells were pre-incubated in SFGM with or without 1 μM 5-HT for 48 h, and surface mouse 5-HT$_3$A receptors were quantified by BTX/488 fluorescence. Data are normalized to control cells and presented as percentage of control (n = 20). (C) HEK-3A/BBS cells were pre-incubated with 5-HT (30 μM) for varying amounts of time and surface receptors were quantified by fluorescence. The last bar represents surface expression of 5-HT$_3$A receptors in HEK-3A/BBS cells that were exposed to 5-HT for 4 h followed by 20 h in the absence of 5-HT (n = 18). Data are presented as percentage of control. (D) HEK-h3A cells were exposed to 5-HT (100 μM) for varying lengths of time and current densities were recorded (n = 10). HEK-3A/BBS (E) (n = 14) and ND7/23 (F) (n = 10) cells were exposed to 5-HT (30 or 100 μM, respectively) for 24 h and current densities were measured every 2 h following the removal of 5-HT. Statistical significance was determined using an ANOVA with a Tukey's *post hoc* comparison or an unpaired Student's *t*-test.

receptors (Figure 5C). We also tested whether morphine, another antagonist of 5-HT$_3$A receptors, which has competitive and non-competitive actions (Fan, 1995; Baptista-Hon *et al*., 2012), could induce up-regulation of 5-HT$_3$A receptors using the HEK-h3A cell line. Exposure of HEK-h3A cells to 10 μM morphine for 24 h also induced up-regulation of 5-HT$_3$A receptors (Figure 5D).

Receptor occupancy *per se* may also play a role in the antagonist-induced up-regulation. To test this possibility, HEK-3A/BBS cells were exposed to 0.1, 0.3 or 1 μM MDL-72222 for 24 h and surface expression was assessed by fluorescence. As observed with receptor agonists, the antagonist-induced up-regulation was concentration-dependent (Figure 5E and F). Lower concentrations of MDL-72222 induced a smaller but significant up-regulation of 5-HT$_3$A receptors.

Agonist- and antagonist-induced up-regulation is independent of protein translation

We tested if the agonist-induced up-regulation was due to translation of new receptors by blocking protein translation

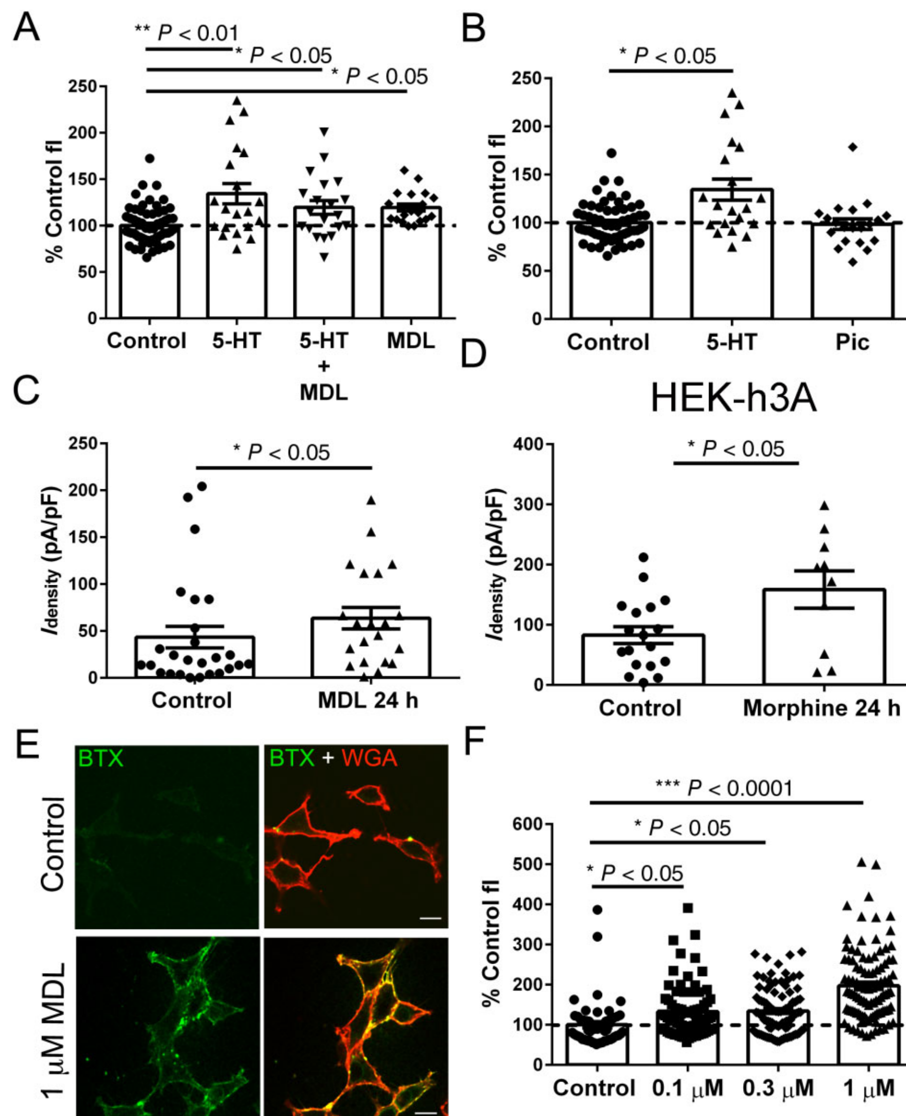

Figure 5

Up-regulation of 5-HT$_3$A receptors was not blocked by MDL-72222 and was induced by either MDL-72222 or morphine. Surface receptors were quantified by BTX/488 fluorescence and normalized to control cells in HEK-3A/BBS cells that were pre-incubated in SFGM for 24 h with 30 μM 5-HT (n = 20), 30 μM 5-HT + 1 μM MDL-72222 (n = 20), 1 μM MDL-72222 (n = 20) (A) or with 30 μM 5-HT, and 100 μM picrotoxin (Pic) (B) (n = 20). (C) Mouse 5-HT$_3$A/BBS currents were elicited by 5-HT (30 μM) following pre-incubation with or without MDL-72222 (n = 21) and expressed as current densities. (D) Current densities from HEK-h3A cells pre-incubated with or without 10 μM morphine for 24 h (n = 9). HEK-3A/BBS cells were exposed to varying concentrations of MDL-72222 for 24 h and surface receptors were quantified by fluorescence. Representative images of HEK-3A/BBS cells in the presence or absence of MDL-72222 (1 μM) are shown in (E) and averaged data in (F). Means with SEM are shown; significantly different as indicated; ANOVA with Tukey's *post hoc* comparison or unpaired Student's *t*-test.

with cycloheximide (CHX). HEK-3A/BBS cells were pre-incubated in SFGM without or with agonist in the absence or presence of 7 μM CHX for 24 h. This concentration of CHX has been shown to block translation-dependent up-regulation of dopamine receptors in HEK cells (Filtz *et al.*, 1994). Surface 5-HT$_3$A/BBS receptors were analysed via fluorescence, and we found that CHX alone did not alter the surface expression of 5-HT$_3$A/BBS receptors compared with control. Furthermore, the presence of CHX did not block the agonist-induced up-regulation (Figure 6A). These data suggest that the up-regulation of surface receptors induced by 5-HT is not due to newly translated receptors, but rather receptors previously translated and stored intracellularly.

The presence of intracellular 5-HT$_3$A/BBS receptors was examined in HEK-3A/BBS cells by labelling surface 5-HT$_3$A/BBS receptors with BTX/488, fixing and permeabilizing the membranes, then labelling all intracellular receptors with BTX/555. A representative image is shown in Figure 6B, indicating that there is a significant intracellular pool of 5-HT$_3$A/

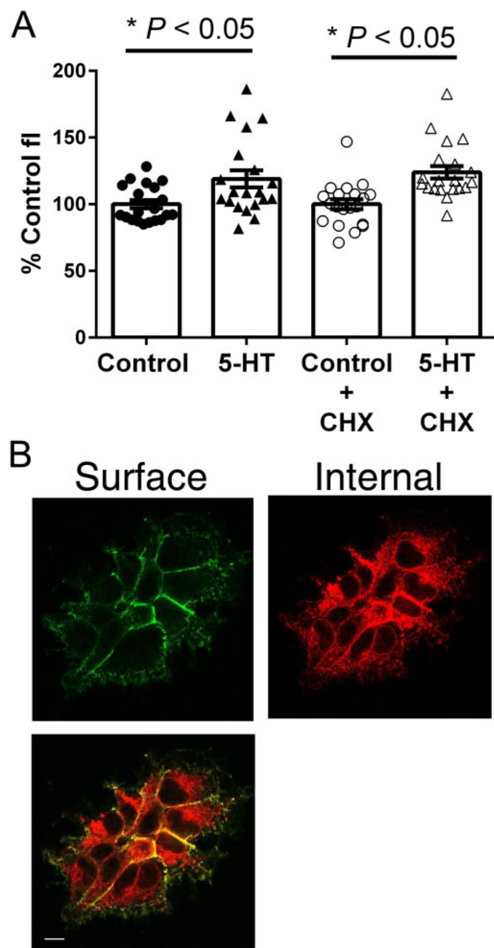

Figure 6

Up-regulation is independent of receptor translation. (A) HEK-3A/BBS cells were pre-incubated in SFGM alone or SFGM containing 5-HT (30 μM). The same conditions were also performed in the presence of 7 μM CHX. Surface 5-HT_3A receptors were quantified by BTX/488 fluorescence normalized to control cells (n = 20). Means with SEM are shown; significantly different as indicated; unpaired Student's t-test. (B) Surface 5-HT_3A/BBS receptors in HEK-3A/BBS cells were labelled with BTX/488, cells were fixed, permeabilized and intracellular receptors were labelled with BTX/555. Representative images of HEK-3A/BBS cells are shown (green = BTX/488-labelled surface receptors; red = BTX/555-labelled intracellular receptors). Scale bar = 10 μm.

BBS receptors. Together, these data suggest that agonist induces the assembly and/or trafficking of intracellular receptors to the cell surface.

Active transport of 5-HT is not necessary for up-regulation

It has been suggested that nicotine and GABA cause the up-regulation of nACh and $GABA_A$ receptors, respectively, by entering the cell and acting as molecular chaperones within the ER enhancing subunit assembly. Unlike nicotine, 5-HT and GABA cannot cross the cell membrane without transporters. $GABA_A$ receptor up-regulation may be induced by GABA

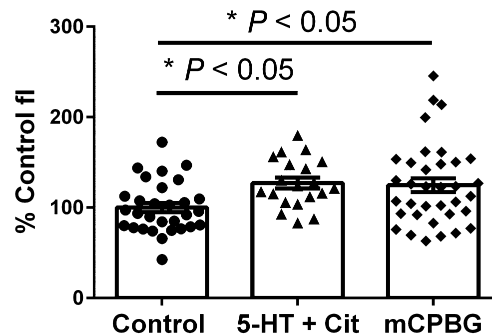

Figure 7

Active transport of 5-HT is not required for the up-regulation of 5-HT_3A/BBS receptors. HEK-3A/BBS cells were pre-incubated for 24 h in SFGM alone (CTR) (n = 30), SFGM + 30 μM 5-HT + 1 μM citalopram (Cit) (n = 20) or 1 μM mCPBG (n = 35). Surface 5-HT_3A/BBS receptors were quantified by BTX/488 fluorescence and normalized to control cells. Means with SEM are shown; significantly different as indicated; ANOVA with Tukey's *post hoc* comparison.

being synthesized by the cell, or by being actively transported across its membrane (Eshaq *et al*., 2010). We examined 5-HT-induced up-regulation in the presence of a 5-HT transporter inhibitor. Citalopram (1 μM) had no effect on the 5-HT-induced up-regulation of 5-HT_3A/BBS receptors (Figure 7). We also pre-incubated HEK-3A/BBS cells with m-chlorophenylbiguanide (mCPBG), a selective 5-HT_3 receptor agonist. Due to the difference in chemical structure, it is unlikely to be transported by any of the monoamine transporters. Like 5-HT, mCPBG in SFGM resulted in the up-regulation of surface 5-HT_3A/BBS receptors after 24 h (Figure 7), demonstrating that transport is not required for the increase in receptor expression.

Discussion

In this study, we have demonstrated that recombinant and endogenous 5-HT_3A receptors were up-regulated in the prolonged presence of the endogenous agonist 5-HT, or the 5-HT_3A receptor specific agonist mCPBG. The up-regulation of 5-HT_3A receptors was dependent upon the concentration and duration of agonist exposure. Furthermore, antagonists (MDL-72222 and morphine) also induced a significant up-regulation of functional surface receptors. Up-regulation was independent of receptor translation but was most likely generated from intracellular pools of receptors. Furthermore, our findings suggest that the up-regulation of 5-HT_3A receptors is not mediated by the molecular chaperone mechanism.

Prolonged exposure to agonists has been shown to increase the surface expression of other members of the Cys-loop pLGIC family. Nicotine, an agonist for nACh receptors, is well known to increase the surface expression of nACh receptors in cultured cells (Bencherif *et al*., 1995), rodents (Schwartz and Kellar, 1985; Wonnacott, 1990) and human smokers (Benwell *et al*., 1988; Perry *et al*., 1999; Govind *et al*., 2009). Recently, Eshaq *et al*. (2010) showed that the $GABA_A$ receptors expressed in HEK cells are also up-regulated in the

presence of GABA. Our results demonstrate that recombinant 5-HT$_3$A receptors are also up-regulated in the prolonged presence of agonists or antagonists. These findings are consistent with our previous observation that exposure to 5-HT (100 μM) for 24 h leads to approximately a threefold increase in total binding to cells stably expressing 5-HT$_3$ receptors (Sanghvi *et al*., 2009). Furthermore, Eshaq *et al*. (2010) demonstrated that 5-HT increases surface levels of 5-HT$_3$A receptors, GABA was unable to up-regulate 5-HT$_3$ receptors and 5-HT was unable to up-regulate GABA$_A$ receptors, suggesting that this phenomenon requires a ligand that binds to the appropriate orthosteric site.

Maximal up-regulation of 5-HT$_3$A/BBS receptors by 5-HT (30 or 100 μM) occurred within 24 h. A smaller up-regulation could be achieved in response to lower 5-HT concentrations over a longer time course. Similarly, the up-regulation of nACh and GABA$_A$ receptors requires saturating concentrations of agonist (Peng *et al*., 1994; Wang *et al*., 1998; Kuryatov *et al*., 2005; Vallejo *et al*., 2005; Eshaq *et al*., 2010). Up-regulation of nACh receptors by nicotine requires the presence of nicotine for at least 6–8 h (Wang *et al*., 1998; Kuryatov *et al*., 2005; Vallejo *et al*., 2005), similar to the time course presented here for 5-HT$_3$A receptors. It remains unclear if the up-regulation of GABA$_A$ receptors is dependent upon agonist concentration. However, GABA$_A$ receptors were up-regulated with 1 h of GABA exposure followed by 5 h in the absence of agonist (Eshaq *et al*., 2010). This differs from the results with nACh and 5-HT$_3$A receptors, which require the presence of agonist for several hours of exposure. This difference could be due to the proposed requirement for transport or synthesis of GABA intracellularly.

Up-regulation of 5-HT$_3$A/BBS receptors by 5-HT was not blocked by MDL-72222, and MDL-72222 alone resulted in an increase in functional receptors. Furthermore, morphine was capable of inducing the up-regulation of functional 5-HT$_3$A receptors. In contrast, the non-competitive antagonist picrotoxin did not induce up-regulation of 5-HT$_3$A receptors. Morphine has competitive and non-competitive modes of inhibition at 5-HT$_3$A receptors, (Baptista-Hon *et al*., 2012) and it is likely that both sites will be occupied in the absence of added 5-HT. As 5-HT and the competitive antagonist MDL-72222 both up-regulate functional receptors, it is likely that the ability of morphine to increase 5-HT$_3$A receptor expression is also mediated through the orthosteric site. Recent data suggest that 5-HT$_3$A receptors are involved in opioid-induced hyperalgesia, tolerance (Liang *et al*., 2011) and dependence (Chu *et al*., 2009). Morphine-induced up-regulation of functional 5-HT$_3$A receptors may contribute to this effect. Antagonist-induced up-regulation has also been shown for GABA$_A$ receptors with a competitive antagonist (Eshaq *et al*., 2010). Mutations of the agonist-binding site of GABA$_A$ receptors completely blocked agonist-induced up-regulation (Eshaq *et al*., 2010). Competitive antagonists for nACh receptors are unable to induce up-regulation; however, non-competitive antagonists significantly up-regulate nACh receptors (Peng *et al*., 1994; Kuryatov *et al*., 2005). It could be that the non-competitive antagonists for nACh receptors are more effective than competitive antagonists at promoting subunit assembly in the endoplasmic reticulum.

It has been well established that agonist-induced up-regulation of both nACh receptors and GABA$_A$ receptors is independent of receptor translation (Peng *et al*., 1994; Wang *et al*., 1998; Kuryatov *et al*., 2005; Lester *et al*., 2009; Eshaq *et al*., 2010). Many hypotheses for the mechanism of the translation-independent up-regulation of nACh receptors have been put forward, including alterations in surface turnover (Peng *et al*., 1994) and the enhancement of subunit assembly by nicotine (chaperone effect) in the endoplasmic reticulum (Kuryatov *et al*., 2005; Lester *et al*., 2009). Furthermore, GABA$_A$ receptors are also thought to be up-regulated by a chaperone mechanism because up-regulation could be induced when cells synthesized GABA or expressed the GABA transporter (Eshaq *et al*., 2010). Our results are in agreement that the up-regulation of 5-HT$_3$A receptors is independent of translation. However, our data also suggest that 5-HT$_3$A receptor up-regulation is not due the transport of 5-HT across the cell membrane to act as an intracellular chaperone. Structural studies with the ACh-binding protein have shown that there are slight structural differences around the binding site between agonist bound and competitive antagonist-bound proteins (Shahsavar *et al*., 2012). Furthermore, there are distinct conformational changes in the extracellular domain of glycine receptors between activation and desensitization (Wang and Lynch, 2011). Our previous studies have shown that 5-HT$_3$A receptors are constitutively internalized in HEK cells (Morton *et al*., 2011). It is possible that conformational changes due to agonist or antagonist binding in the 5-HT$_3$A receptors could lead to changes in the rate of constitutive internalization. Our data indicated that the constitutive internalization of 5-HT$_3$A receptors in HEK cells results in a near complete turnover of surface receptors in approximately 30 min at 37°C (Morton *et al*., 2011); therefore, even small alterations in receptor internalization could lead to significant changes in surface expression after 24 h. It remains unclear how any of these extracellular changes would affect the intracellular domains of the receptor leading to changes in internalization. However, the structure of the 5-HT$_3$A receptor is now available and may give insight into any structural differences between the ligand bound and unbound states (Hassaine *et al*., 2014) and if such structural changes could alter receptor internalization.

In conclusion, we demonstrate here that pre-incubation with agonists or morphine will up-regulate 5-HT$_3$A/BBS receptors. Furthermore, pre-incubation with agonist induced up-regulation of endogenous 5-HT$_3$A receptors in a neuroblastoma/dorsal root ganglia hybrid cell line. Agonist/antagonist up-regulation could have implications for patients using 5-HT selective re-uptake inhibitors for depression, ondansetron for post-operative nausea and emesis, or patients using morphine for chronic pain. However, further studies are needed to confirm the up-regulation in neurons and other cell types.

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Author contributions

R. A. M. substantially contributed to the concept or design of the experiments, acquisition, analysis, interpretation of data and drafting of the manuscript. D. T. B.-H. substantially contributed to the acquisition, analysis, interpretation of data and drafting of the manuscript. T. G. H. substantially contributed to the concept or design of the experiments, interpretation of data and drafting of the manuscript. D. M. L. substantially contributed to the concept or design of the experiments, interpretation of data and drafting of the manuscript.

Conflict of interest

None.

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