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Comparative desensitization of the human 5-HT_{2A} and 5-HT_{2C} receptors expressed in the human neuroblastoma cell line SH-SY5Y

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1 We have used previously characterized clones of the human neuroblastoma cell line, SH-SY5Y, constitutively expressing either the human 5-HT_{2A} or 5-HT_{2C} receptor to compare their desensitization profiles after exposure to 5-HT.

2 5-HT stimulated [^{3}H]-inositol phosphate ([^{3}H]-IP_x) production at both the 5-HT_{2C} ($pEC_{50} = 8.03 \pm 0.15$) and 5-HT_{2A} receptors ($pEC_{50} = 7.15 \pm 0.08$), with maximal responses occurring after exposure to 1 μM and 10 μM 5-HT, respectively.

3 Exposure of cells to maximally effective concentrations of 5-HT caused time- and concentration-dependent desensitization of [^{3}H]-IP_x formation. The 5-HT_{2A} response desensitized slower ($t_{1/2} = 110$ min) and with lower sensitivity than that of the 5-HT_{2C} receptor ($t_{1/2} = 12.5$ min). In each case, desensitization was blocked by co-administration of a specific receptor antagonist. Following exposure to 10 μM 5-HT for 2 h, both receptors exhibited extensive desensitization, with subsequent responses to 5-HT reduced by more than 80%.

4 5-HT stimulated Ins(1,4,5)P_3 production with a potency similar to that for [^{3}H]-IP_x production at each receptor. In both cases Ins(1,4,5)P_3 levels peaked rapidly then returned to basal level within a short time. This peak consistently occurred earlier for the 5-HT_{2C} receptor (5 s) than for the 5-HT_{2A} receptor (20 s).

5 Prior exposure of SH-SY5Y/5-HT_{2C} cells to 5-HT (1 μM/15 min) caused a significant decrease in the 5-HT-stimulated peak in Ins(1,4,5)P_3 levels whereas no such change occurred in SH-SY5Y/5-HT_{2A} cells following exposure to 10 μM 5-HT for 15 min.

6 These results indicate that the human 5-HT_{2A} and 5-HT_{2C} receptors both exhibit desensitization at the level of inositol phosphate formation when expressed in the same cellular environment, with the 5-HT_{2C} receptor being more sensitive to 5-HT-mediated desensitization than the 5-HT_{2A} receptor.

Keywords: 5-HT_{2A} receptor; 5-HT_{2C} receptor; human; desensitization; inositol phosphates; SH-SY5Y cell

Introduction

Desensitization defines the reduction in response seen after the continued stimulation of many biological systems (Lohse, 1993). Functional desensitization of G-protein coupled receptors is widespread, and in-depth studies of the β_2-adrenergic receptor in particular have delineated the roles of receptor phosphorylation, internalization and down-regulation in this process (Hausdorff *et al*., 1990). More recently, it has become evident that closely related receptors within a family may show differing desensitization characteristics. For instance, whilst the β_1- and β_2-adrenergic receptors are readily desensitized, the β_3 subtype is desensitization-resistant, a property conferred by the lack of available phosphorylation sites in its C-terminal tail (Bousquet-Melou *et al*., 1995; Liggett *et al*., 1993). Similarly, the α_2C4-receptor is less susceptible to desensitization than the highly homologous α_2C2- or α_2C10-adrenergic receptors (Eason & Liggett, 1992). In this study we have investigated differences in the short-term agonist regulation of the 5-HT_{2A} and 5-HT_{2C} receptors, two members of the 5-HT_2 receptor family.

The 5-hydroxytryptamine (5-HT) receptor family currently comprises 14 receptor subtypes which have been classified according to their structural homology, and functional and pharmacological characteristics (Martin & Humphrey, 1994). The 5-HT_2 receptor sub-family comprises the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, all of which belong to the G-protein coupled receptor superfamily and which appear to play a role in such diverse CNS functions as mood, appetite control, perception and anxiety (Cowen, 1991). These receptors show a high degree of homology at both the genetic and structural level. Functionally, all three receptors activate the G_q family of G-proteins to activate phospholipase C (PLC) and generate the second messenger molecules inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol.

This leads subsequently to release of calcium from intracellular stores and activation of protein kinase C, respectively (Martin & Humphrey, 1994). Previous studies have shown that 5-HT_2 receptor desensitization can occur both *in vivo* and *in vitro* at a functional level through mechanisms which may be analogous to those described for the β_2-adrenergic receptor including phosphorylation and down-regulation (Westphal *et al*., 1995; Van Huizen *et al*., 1993; Leysen *et al*., 1989; Rahman & Neuman, 1993). However, comparative functional data on 5-HT_2 receptor sub-type regulation within a common cellular environment is not available. In order to study these receptors in a comparative fashion, we generated stable clones of the human neuroblastoma cell line, SH-SY5Y, transfected to express the human 5-HT_{2A} or 5-HT_{2C} receptors (Newton *et al*., 1996). The receptors expressed in this cell line show typical pharmacology and function, and their expression in a human cell line of neuronal

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origin optimizes the degree to which the data may model the situation of neuronal cells *in vivo*. This may be of particular relevance in the study of receptor regulation, since the regulatory properties of receptors may be highly dependent upon the environment in which they are expressed (Grotweil & Sanders-Bush, 1994). In the present study, we have compared the desensitization of the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors at the level of second messenger production over short (<300 s) and longer (120 min) periods of stimulation. Our data indicate that the human 5-HT$_{2C}$ receptor is more susceptible to desensitization by its natural agonist, 5-HT, than the 5-HT$_{2A}$ receptor, when expressed in this cell line.

Methods

Materials

Tissue culture reagents and G418 (Geneticin) were obtained from ICN Flow Laboratories (U.K.) and foetal calf serum (whole and dialyzed) from Gibco (U.K.). 5-Hydroxytyptamine hydrochloride (5-HT) and carbamyl choline (carbachol, CCh) were obtained from Sigma (Poole, Dorset, U.K.). Mesulergine hydrochloride was supplied by Research Biochemicals International (U.S.A.), whilst ketanserin tartrate was a kind gift from Janssen Pharmaceuticals (U.K.). [^{3}H]-inositol 1,4,5-trisphosphate (34–50 Ci mmol^{-1}) and *myo*-[2-^{3}H]-inositol (15–20 Ci mmol^{-1}) were from Amersham International PLC (Amersham, U.K.). All other chemicals were of analytical grade and were obtained from either Sigma or B.D.H. (Dagenham, Essex, U.K.).

Cell culture and cell transfection

Clonal SH-SY5Y cell lines stably transfected with the cDNA for the human 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors were generated in this laboratory as previously described (Newton *et al*., 1996). Cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 480 μg ml^{-1} G418 to maintain selection pressure. Prior to all assays, cells were grown for 5 days in DMEM containing 5% dialyzed FCS (d-FCS).

Measurement of [^{3}H]-inositol phosphates ([^{3}H]-IP$_x$) formation

[^{3}H]-IP$_x$ formation was measured as described previously (Elliott *et al*., 1995). Briefly, cells were seeded onto 24-well tissue culture plates (150,000 to 200,000 cells per well) and allowed to equilibrate for 48 h in inositol-free DMEM containing 5% d-FCS, 2 mM glutamine and 1 μCi per well of *myo*-[2-^{3}H]-inositol. Where cells were exposed to desensitizing 5-HT, this was added during the equilibration period to minimize the effect of prolonged agonist exposure on the levels of [^{3}H]-phospholipids in the cell. In no experiment did exposure to 5-HT during this period alter the level of [^{3}H]-phospholipids in cell membranes as assessed from cellular chloroform extracts. By similar measurements, there was no difference in the level of [^{3}H]-inositol uptake and incorporation into [^{3}H]-phospholipids between cell lines. After labelling, cells were then washed for 2×5 min in inositol-free DMEM, before incubation with 10 mM lithium chloride (30 min/37°C) and subsequent addition of agonist. Cells were stimulated for 15 min, over which time period the response to all agonists remained linear. The reaction was terminated by removal of the medium and addition of ice-cold methanol. Cells were removed to a mix of methanol : chloroform : HCl (50 : 50 : 1) and a sample of the aqueous layer was transferred onto Dowex AG1-X8 columns (formate form) (Bio-Rad). After washing with 60 mM sodium formate/5 mM sodium tetraborate to remove [^{3}H]-inositol, [^{3}H]-inositol phosphates ([^{3}H]-IP$_x$) were eluted with 800 mM ammonium formate/100 mM formic acid. Samples were counted in 10 ml of scintillant (Ultima XR-Gold) and quantified. In addition a sample of the chloroform phase was removed, evaporated to dryness then counted to assess the incorporation of [^{3}H]-inositol into cell phospholipids. Results are expressed as a percentage conversion of [^{3}H]-inositol phospholipids to [^{3}H]-inositol phosphates. Samples were generated in quadruplicate within each experiment.

Measurement of Ins(1,4,5)P$_3$ mass

Cells were grown for 36 h on 24-well plates in DMEM supplemented with 5% d-FCS and 2 mM glutamine, then incubated in serum-free DMEM for 16 h prior to assay. This was replaced 30 min prior to assay with a modified Krebs/HEPES buffer (pH 7.4) (concentrations (mM); NaCl, 118, KCl 4.69, KH_2PO_4 1.20, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 25, $CaCl_2$ 1.3, D-glucose 10, HEPES 10)). Where cells were exposed to 5-HT, this was added during the 30 min equilibration period. Plates were floated in a 37°C water bath and the wells aspirated individually, before stimulation with 100 μl of buffer $\pm$ agonist; all results were corrected for a parallel stimulation with buffer alone. The reaction was terminated by addition of 100 μl 1 M trichloroacetic acid (TCA). For basal level determinations, 100 μl of 1 M TCA was added, followed by 100 μl of buffer $\pm$ agonist. Samples were generated in duplicate within each experiment. Protein samples were obtained by addition of 500 μl of 0.1 M NaOH to two wells per plate and concentrations were determined by the method of Peterson (1977). After incubation at 4°C for 30 min, samples were removed from wells and an equal volume of freon: tri-n-octylamine (1 : 1) was added. Samples were vortexed and centrifuged before removal of a sample which was neutralized with 25 mM $NaHCO_3$. Ins(1,4,5)P_3 levels were measured as described by Challiss *et al*. (1990). Briefly, samples were incubated on ice in 25 mM Tris/1 mM EDTA buffer (pH 8.0), with 3.6–4.0 nCi of authentic [^{3}H]-Ins(1,4,5)P_3 and $\sim$0.6 mg of bovine adrenocortical protein preparation. Samples were processed in parallel with standard samples (0–300 nM Ins(1,4,5)P_3) and non-specific binding determined in the presence of 10 μM Ins(1,4,5)P_3. After incubation on ice for 30 min, the reaction was terminated by rapid filtration over Whatman GF/B filters, the filters washed with 15 ml of washing buffer (25 mM Tris, 1 mM EDTA, 5 mM $NaHCO_3$, pH 8.0) and filter bound radioactivity assessed by liquid scintillation counting. Standard curves were estimated by weighted non-linear regression analysis fitted to a five parameter logistic equation (MS Exel 4.0) and sample Ins(1,4,5)P_3 levels interpolated from the curve.

Data analysis and statistics

Dose-response data was analysed by weighted non-linear regression fitted to the following three parameter logistic equation (Kaleidagraph 3.0):

$$E = \frac{E_{max}[A]}{EC_{50} + [A]} + \text{Basal}$$

where E represents the response, E_{max} the difference between maximal response and basal levels, A the concentration of

agonist and EC_{50} the concentration of agonist producing 50% maximal response.

Concentrations producing 50% desensitization were estimated visually from the graph. All other results are quoted as mean ± s.e.mean unless otherwise stated. Statistical comparisons were made using either Student's *t*-test or one- or two-way analysis of variance with *post-hoc* Dunnett's *t*-test.

Results

Characterization of [^{3}H]-IP$_x$ production in SH-SY5Y/5-HT$_{2A}$ and SH-SY5Y/5-HT$_{2C}$ cells

Phosphoinositide responses to 5-HT were investigated in clonal cell lines of the human neuroblastoma cell line SH-SY5Y stably transfected to express the human 5-HT$_{2A}$ receptor (B_{max} 450 fmol mg^{-1} protein) or the 5-HT$_{2C}$ receptor (B_{max} 550 fmol mg^{-1} protein in clone M and 110 fmol mg^{-1} protein in clone 24). Basal levels of [^{3}H]-IP$_x$ turnover were significantly higher in SH-SY5Y/5-HT$_{2A}$ cells and SH-SY5Y/5-HT$_{2C}$ clone M cells than non-transfected cells but not in the lower expressing SH-SY5Y/5-HT$_{2C}$ clone 24 cells. Both 5-HT and carbachol caused a dose-dependent increase in [^{3}H]-IP$_x$ production in all three cell lines, the parameters of which are detailed in Table 1. In line with other studies, 5-HT was a more potent agonist of [^{3}H]-IP$_x$ production in SH-SY5Y/5-HT$_{2C}$ cells than in SH-SY5Y/5-HT$_{2A}$ cells. Furthermore, 5-HT was also more potent at stimulating [^{3}H]-IP$_x$ production in the higher expressing SH-SY5Y/5-HT$_{2C}$ cells (clone M) compared to clone 24, although the maximal response observed in these cells was lower. Maximal effect of 5-HT was observed at 1 μM for SH-SY5Y/5-HT$_{2C}$ cells and 10 μM for SH-SY5Y/5-HT$_{2A}$ cells. The absolute magnitude of maximal [^{3}H]-IP$_x$ production in SH-SY5Y/5-HT$_{2A}$ cells was substantially greater than that seen in SH-SY5Y/5-HT$_{2C}$ cells. The potency of carbachol in stimulating [^{3}H]-IP$_x$ production was not significantly different in any of the transfects from that seen in untransfected cells. However, there was a small but significant decrease in maximal [^{3}H]-IP$_x$ production by carbachol in both SH-SY5Y/5-HT$_{2A}$ cells and the higher expressing SH-SY5Y/5-HT$_{2C}$ clone compared to the host cells.

Desensitization of 5-HT$_{2A}$- and 5-HT$_{2C}$-mediated [^{3}H]-IP$_x$ production

To investigate receptor desensitization in this system, dose-response curves to 5-HT were generated in control cells and in cells previously incubated with 5-HT. After exposure to a maximally effective concentration of 5-HT (1 μM) for 20 min, the magnitude of 5-HT-stimulated [^{3}H]-IP$_x$ production in SH-SY5Y/5-HT$_{2C}$ cells (clone 24) was significantly decreased

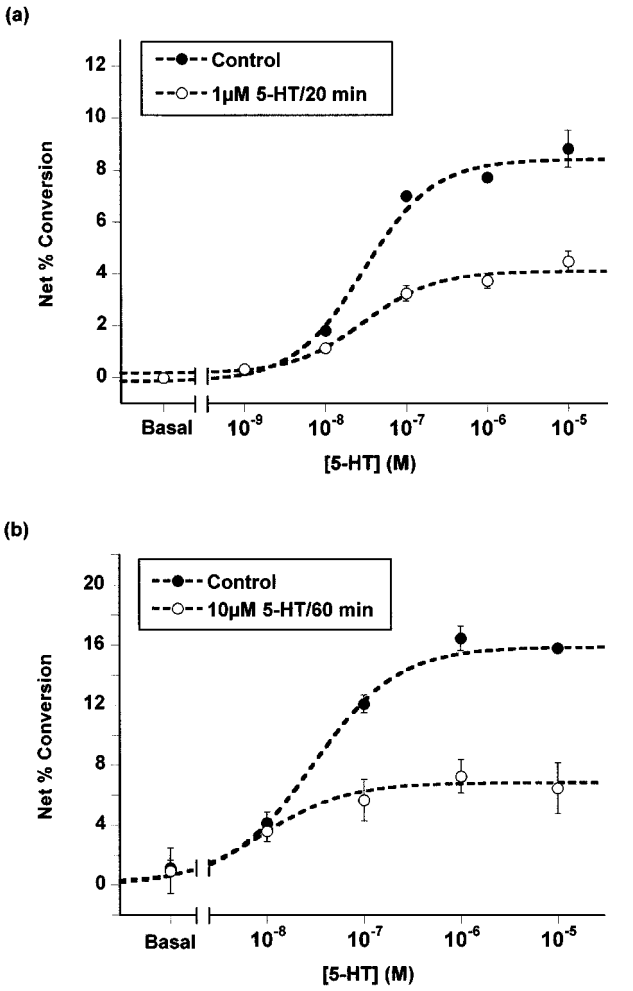

Figure 1 Effect of prior exposure to 5-HT on 5-HT-mediated [^{3}H]-inositol phosphate formation in (a) SH-SY5Y/5-HT$_{2C}$ (Clone 24) and (b) SH-SY5Y/5-HT$_{2A}$ cells. Cells were exposed to vehicle or 5-HT for 15 min (5-HT$_{2C}$) or 60 min (5-HT$_{2A}$) during the [^{3}H]-inositol labelling period, washed twice in DMEM, then the production of [^{3}H]-inositol phosphates in response to 0 to 10 μM 5-HT determined as described in Methods. Results are shown as mean ± s.e.mean of quadruplicate determinations within a single experiment representative of three performed. Curves were fitted according to a three-parameter logistical fit as described. Mean figures for basal, E_{max} and pEC_{50} of control and desensitized cells are given in the text.

Table 1 Characteristics of carbachol and 5-HT-mediated [^{3}H]-inositol phosphate production in native and transfected SH-SY5Y cell lines

		Basal	*Carbachol*		*5-HT*	
	n	*turnover (%)*	*pEC_{50}*	*E_{max} (%)*	*pEC_{50}*	*E_{max} (%)*
SH-SY5Y (Wild type)	3	2.56 ± 0.07	4.93 ± 0.02	28.3 ± 1.2	ND	ND
SH-SY5Y/5-HT$_{2A}$	5	6.45 ± 0.44**††	4.66 ± 0.11	16.7 ± 1.4**††	7.15 ± 0.08††§	21.0 ± 3.3††§
SH-SY5Y/5-HT$_{2C}$ (clone M)	3	5.26 ± 0.41**††	4.89 ± 0.02	18.2 ± 1.4†*	8.03 ± 0.15††	3.8 ± 0.6†
SH-SY5Y/5-HT$_{2C}$ (clone 24)	8	2.91 ± 0.15	4.89 ± 0.02	25.4 ± 1.0	7.53 ± 0.03	8.3 ± 1.0

After equilibrium labelling with [^{3}H]-myo-inositol, cells were stimulated with carbachol or 5-HT and total [^{3}H]-inositol phosphates measured as described in Methods. Data were obtained from concentration-response curves, fitted to a three parameter logistic equation, and expressed as the mean ± s.e.mean from 'n' independent determinations. Basal and E_{max} values are expressed as the percentage conversion of [^{3}H]-inositol phospholipids to [^{3}H]-inositol phosphates. pEC_{50} values are calculated as negative $\log_{10}$ of the molar concentration of ligand producing 50% of the E_{max}. ND = not detected. Statistical comparisons using Dunnet's *t*-test: *$P<0.05$, **$P<0.01$ vs SH-SY5Y (Wild Type); †$P<0.05$, ††$P<0.01$ vs clone 24; §$P<0.01$ vs clone M.

(E_{max} = 10.1 ± 1.3 and 3.1 ± 0.7%, mean ± s.e.mean, n = 3; P < 0.05, Student's t-test) (Figure 1a). The potency of 5-HT to stimulate [^{3}H]-IP$_x$ production was not significantly altered (pEC_{50} = 7.46 ± 0.07 and 7.29 ± 0.11) and there was a small but non-significant increase in basal turnover following agonist exposure. Similar experiments in SH-SY5Y/5-HT$_{2A}$ cells also showed a significant decrease in [^{3}H]-IP$_x$ production after exposure to an equivalent maximally effective concentration of 5-HT (10 μM) for 1 h, with no change in the pEC_{50} for stimulation (Figure 1b) (E_{max} = 24.0 ± 3.0 and 7.3 ± 0.1%, pEC_{50} = 7.34 ± 0.14 and 7.27 ± 0.15, control and 5-HT exposed, respectively, n = 3) although basal levels were also increased (6.2 ± 0.7 vs 32.4 ± 3.4%).

These data confirmed that 1 μM (5-HT$_{2C}$) and 10 μM 5-HT (5-HT$_{2A}$) remained maximally effective concentrations of 5-HT both before and after desensitization. In subsequent experiments, after 5-HT exposure, responses were determined as the difference between basal levels and the response to these maximally effective concentrations of 5-HT. The time course of desensitization was investigated in SH-SY5Y/5-HT$_{2C}$ cells after exposure to 1 μM 5-HT for between 10 and 120 min (Figure 2a). There was an initial rapid loss of response to 50% control after 12.5 min exposure to 5-HT, followed by a slower loss of response between 20 and 120 min, eventually reducing the response to approximately 20% control. Desensitization was mediated by the 5-HT$_{2C}$ receptor, since it was significantly reduced by inclusion of the 5-HT$_{2C}$ antagonist mesulergine (100 nM) (P < 0.001, two-way ANOVA). Similar results were obtained with the higher expressing SH-SY5Y/5-HT$_{2C}$ (clone M) cells. Desensitization of 5-HT$_{2A}$ receptor function was also time-dependent, but exhibited a much slower time course, with 50% desensitization occurring after 110 min exposure to 10 μM 5-HT (Figure 2b). Similarly, this desensitization was extensive at the highest 5-HT concentration and was prevented by inclusion of the 5-HT$_{2A}$ receptor antagonist, ketanserin (100 nM) (P < 0.01, two-way ANOVA). Exposure of the cells to the antagonists alone did not alter the subsequent response to 5-HT.

Desensitization of both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors by 5-HT was concentration-dependent, and was extensive for both receptors, reducing the subsequent response to 5-HT by over 80% at the highest concentrations tested. In SH-SY5Y/5-HT$_{2C}$ cells, exposure to 1 nM – 10 μM 5-HT for 2 h caused a concentration-dependent decrease in the resulting response to 1 μM 5-HT (Figure 3a), with reduction to 50% control by 0.2 μM and 0.1 μM 5-HT in clone 24 and clone M cells, respectively. In contrast, in SH-SY5Y/5-HT$_{2A}$ cells, [^{3}H]-IP$_x$ production was reduced to 50% control by a substantially higher concentration of 5-HT (2 μM) (Figure 3b).

In SH-SY5Y/5-HT$_{2C}$ cells, the response to 100 μM carbachol was not affected by exposure to 5-HT, indicating that the desensitization in this case was homologous (Figure 3a). In SH-SY5Y/5-HT$_{2A}$ cells, the response to carbachol showed a small but non-significant decrease after exposure to higher concentrations of 5-HT (Figure 3b).

Characterization of 5-HT$_{2A}$- and 5-HT$_{2C}$-mediated Ins(1,4,5)P$_3$ production

To investigate second messenger production and receptor desensitization over shorter time periods, 5-HT-stimulated Ins(1,4,5)P$_3$ production was measured by radioreceptor assay. Basal levels of Ins(1,4,5)P$_3$ were significantly higher in SH-SY5Y/5-HT$_{2A}$ cells than in SH-SY5Y/5-HT$_{2C}$ cells (26.6 ± 3.4 and 8.5 ± 0.7 pmol mg^{-1} protein, n = 9 and n = 11, respectively; P < 0.001). Both of these values were significantly different

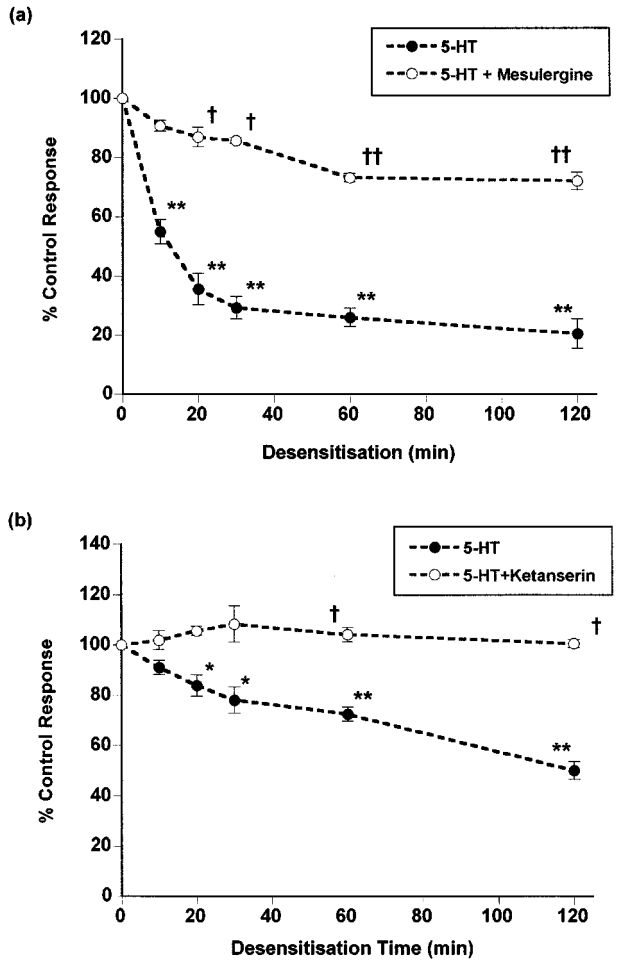

Figure 2 Time course of desensitization of 5-HT-mediated [^{3}H]-inositol phosphate formation in (a) SH-SY5Y/5-HT$_{2C}$ (Clone 24) and (b) SH-SY5Y/5-HT$_{2A}$ cells. Cells were exposed to either 1 μM 5-HT (5-HT$_{2C}$) or 10 μM 5-HT (5-HT$_{2A}$) in the presence or absence of a receptor antagonist for the times indicated during the [^{3}H]-inositol labelling period. Cells were then washed and the net [^{3}H]-inositol phosphate response to either 1 μM 5-HT (5-HT$_{2C}$) or 10 μM 5-HT (5-HT$_{2A}$) determined. Results, expressed as a percentage of the control response (cells exposed to vehicle only), are expressed as mean ± s.e.mean of either (a) n = 6 or (b) n = 5 independent experiments. In the case of experiments involving antagonist addition, mesulergine (100 nM) or ketanserin (100 nM) were added 30 min prior to the desensitizing 5-HT. In neither case did the antagonist alone affect the subsequent stimulation by 5-HT. *P < 0.05 and **P < 0.01 vs corresponding control response; †P < 0.05 and ††P < 0.01 vs corresponding response in the absence of antagonist (one-way ANOVA, followed by Dunnett's t-test).

(P < 0.05) from basal Ins(1,4,5)P$_3$ levels in native SH-SY5Y cells (16.3 ± 3.1 pmol mg^{-1} protein, n = 9). In both SH-SY5Y/5-HT$_{2A}$ and SH-SY5Y/5-HT$_{2C}$ (clone M) cells (using 1 μM and 10 μM 5-HT, respectively) Ins(1,4,5)P$_3$ levels rose rapidly, peaked, and returned to basal levels within 60 s (Figure 4). However, there were notable differences in the profiles seen for the two receptors, namely, Ins(1,4,5)P$_3$ levels peaked consistently earlier in SH-SY5Y/5-HT$_{2C}$ cells (5 s) compared to SH-SY5Y/5-HT$_{2A}$ cells (20 s); the peak response was larger in magnitude in SH-SY5Y/5-HT$_{2A}$ cells compared to SH-SY5Y/5-HT$_{2C}$ cells (122.3 ± 18.7 vs 54.8 ± 8.3 pmol mg^{-1} protein, n = 5, P < 0.01) and there was a secondary rise above basal levels seen after 300 s stimulation in SH-SY5Y/5-HT$_{2C}$ cells, which was not present in SH-SY5Y/5-HT$_{2A}$ cells. Concentration response curves performed at the time of peak response

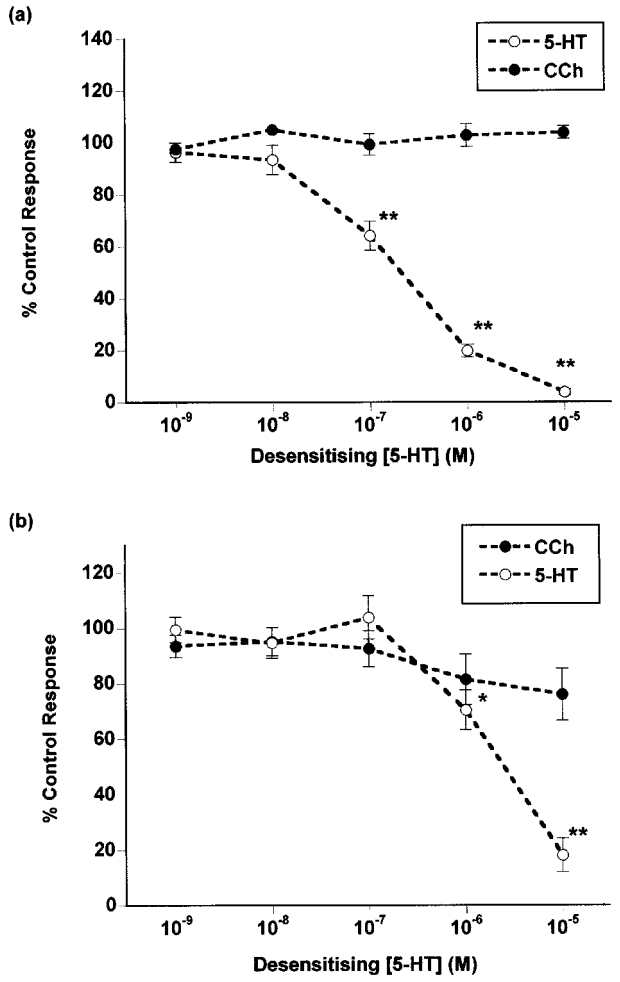

Figure 3 Concentration-dependent desensitization of 5-HT- but not carbachol-mediated [^{3}H]-inositol phosphate formation in (a) SH-SY5Y/5-HT$_{2C}$ (Clone 24) and (b) SH-SY5Y/ 5-HT$_{2A}$ cells after exposure to 5-HT. Cells were exposed to 0 to 10 μM 5-HT for 2 h during the [^{3}H]-inositol labelling period. Cells were then washed and the net [^{3}H]-inositol phosphate response to a maximally effective concentration of 5-HT (1 μM for 5-HT$_{2C}$, 10 μM for 5-HT$_{2A}$) or 100 μM carbachol (CCh) determined. Results are expressed as a percentage of the control response (cells exposed to vehicle only). Data are expressed as mean$\pm$s.e.mean of between three and six independent experiments. *$P<0.05$, **$P<0.01$ vs control response (one-way ANOVA followed by Dunett's t-test).

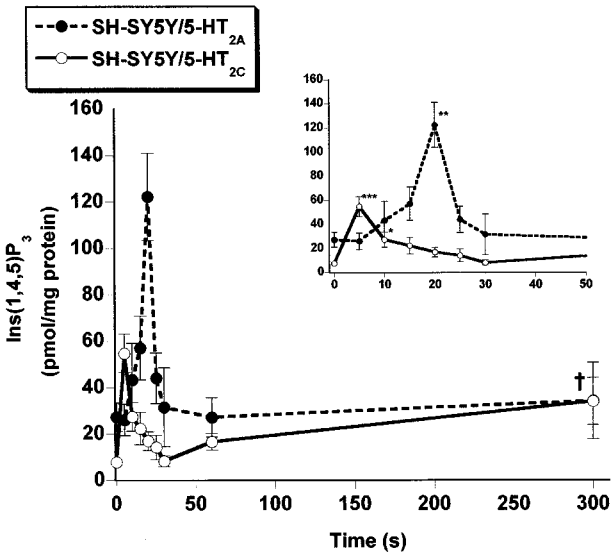

Figure 4 Time course of Ins(1,4,5)P$_3$ production in SH-SY5Y/5-HT$_{2C}$ (Clone M) cells and SH-SY5Y/5-HT$_{2A}$ cells. After a 30 min equilibration in Krebs/HEPES buffer, cells were washed and stimulated with either 1 μM 5-HT (5-HT$_{2C}$) or 10 μM 5-HT (5-HT$_{2A}$) for the times indicated, the reaction stopped and Ins(1,4,5)P$_3$ levels determined by radioreceptor assay. The inset shows the 0–60 s data in more detail. Results are shown as the mean$\pm$s.e.mean of five independent experiments, each performed as duplicates. *$P<0.05$ vs basal levels in 5-HT$_{2A}$ cells, †$P<0.05$ vs basal levels in 5-HT$_{2C}$ cells (one-way ANOVA followed by Dunett's t-test).

showed that 5-HT stimulated Ins(1,4,5)P$_3$ production with similar potency to [^{3}H]-IP$_x$ production in these cells, and that 1 μM and 10 μM 5-HT represented maximally effective concentrations for the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, respectively (pEC$_{50}$ = 7.55$\pm$0.02 and 7.93$\pm$0.02, n=3). These experiments also confirmed the larger maximal response seen in SH-SY5Y/5-HT$_{2A}$ cells compared to SH-SY5Y/5-HT$_{2C}$ cells (E$_{max}$ = 117.4$\pm$6.7 vs 44.1$\pm$2.7 pmol mg^{-1} protein, respectively). Untransfected cells showed no Ins(1,4,5)P$_3$ response to 5-HT at concentrations up to 100 μM.

Desensitization of 5-HT$_{2A}$- and 5-HT$_{2C}$-mediated Ins(1,4,5)P$_3$ production

As demonstrated above, prior exposure of SH-SY5Y/5-HT$_{2A}$ and SH-SY5Y/5-HT$_{2C}$ cells to 10 μM or 1 μM 5-HT, respectively, for 15 min caused 15% reduction of [^{3}H]-IP$_x$ production in SH-SY5Y/5-HT$_{2A}$ cells and 65% reduction in SH-SY5Y/5-HT$_{2C}$ cells (see Figure 2). In SH-SY5Y/5-HT$_{2C}$ cells, similar exposure to 1 μM 5-HT for 15 min caused a significant decrease in the peak Ins(1,4,5)P$_3$ response to 1 μM 5-HT (66.8$\pm$6.8 vs 21.7$\pm$5.2 fmol mg^{-1} protein, n=3, $P<0.01$), and a delay in the time to peak response from 5 s to 10 s (Figure 5a). In contrast, the magnitude of the peak response in SH-SY5Y/5-HT$_{2A}$ cells after prior exposure to 5-HT was not significantly altered (119.7$\pm$27.7 vs 120.6$\pm$8.6 fmol mg^{-1} protein, n=3) although the time to peak response was shortened from 20 s to 10 s (Figure 5b). In neither case were the basal levels of Ins(1,4,5)P$_3$ significantly altered by the 5-HT treatment.

Discussion

Investigation of the modulatory properties of human neuronal receptors *in situ* is clearly problematic and therefore such studies are generally carried out using appropriate animal or cell culture model systems. The transfection of human 5-HT$_2$ receptors into the human neuroblastoma cell line SH-SY5Y has previously been demonstrated to represent a reliable model, in terms of biochemical and electrophysiological profile (Newton *et al.*, 1996), which exhibits the potential for modulation by antagonists (Newton & Elliott, 1997). This study extends the earlier reports by demonstrating that continued exposure to the endogenous agonist 5-HT induces desensitization of phosphoinositide responses in both the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors.

Initial experiments confirmed the ability of 5-HT to cause a characteristic production of inositol phosphates in cells expressing both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. In line with previous studies, 5-HT was more potent agonist at the 5-HT$_{2C}$ receptor than at the 5-HT$_{2A}$ receptor (Berg *et al.*, 1994; Newton *et al.*, 1996), with EC$_{50}$ approximately 8 fold lower in

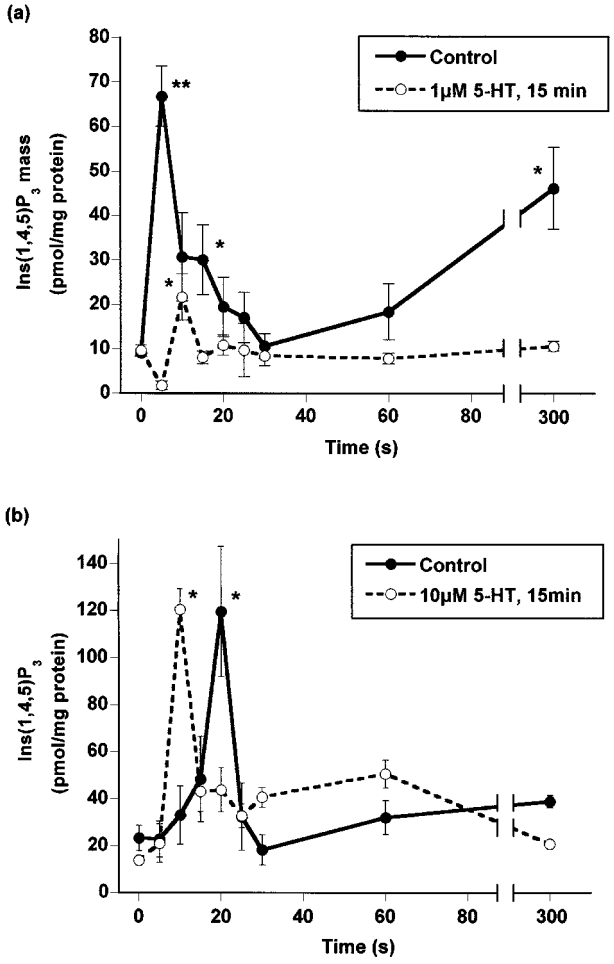

Figure 5 Effect of prior exposure to 5-HT on 5-HT-mediated Ins(1,4,5)P_3 production in (a) SH-SY5Y/5-HT$_{2C}$ (Clone M) and (b) SH-SY5Y/5-HT$_{2A}$ cells. SH-SY5Y/5-HT$_{2C}$ cells (a) or SH-SY5Y/5-HT$_{2A}$ cells (b) were exposed to either 1 μM or 10 μM 5-HT, respectively, or vehicle during the 15 min Krebs/HEPES equilibration period. Cells were subsequently washed three times and rechallenged with the same concentration of 5-HT for the times indicated, the reaction stopped and Ins(1,4,5)P_3 levels determined by radioreceptor assay as described in Methods. Results are shown as mean $\pm$ s.e.mean of three independent experiments, each performed in duplicate. $^*P<0.05$ and $^{**}P<0.01$ vs corresponding basal level (one-way ANOVA followed by Dunett's *t*-test).

cells with equivalent levels of receptor expression as measured by both inositol phosphate accumulation and direct Ins(1,4,5)P_3 measurement. 5-HT was also a more potent agonist at 5-HT$_{2C}$ receptors in the higher expressing clone M cell line, probably due to the existence of a small ($\sim$20%) receptor reserve in these cells which is not present in the lower expressing clone 24 cells (J.M. Elliott and R.A. Newton, unpublished observation). The basal level of inositol phosphate turnover was also greater in clone M than in clone 24 but the net maximal stimulation of [^{3}H]-IP$_x$ by 5-HT was greater in the lower expressing clone. When considered in terms of absolute [^{3}H]-IP$_x$ production, however, the maximal responses in the two clones were similar, suggesting that this second messenger system may play an important role in the mechanism of acute desensitization of the 5-HT$_{2C}$ receptor. The basal level of inositol phosphate turnover in cells expressing the 5-HT$_{2A}$ receptor was greater than non-transfected cells or 5-HT$_{2C}$ clone 24 but not clone M cells, whereas the maximal stimulation of [^{3}H]-IP$_x$ by 5-HT (both net and absolute) was substantially greater than either cell line expressing the 5-HT$_{2C}$ receptor. This suggests that the 5-HT$_{2A}$ receptor is not particularly sensitive to the level of inositol phosphates within the cell and hence that associated regulatory systems, such as protein kinase C, may not be involved in acute desensitization of the 5-HT$_{2A}$ receptor. Stimulation of [^{3}H]-IP$_x$ production by carbachol in all transfected cell lines was similar to that seen in native SH-SY5Y cells in our hands and to that described previously (Fisher *et al*., 1994), indicating that the introduction of exogenous receptors had not grossly altered the signal transduction machinery already present. However, the E_{max} for stimulation was somewhat reduced in both SH-SY5Y/5-HT$_{2A}$ cells and the higher expressing SH-SY5Y/5-HT$_{2C}$ cell line compared to native cells. This may be due to constitutive activation of the 5-HT$_2$ receptors, corresponding with the increased basal levels of inositol phosphates observed in these cell lines, modulating the activity of the muscarinic receptors through heterologous desensitization.

In both SH-SY5Y/5-HT$_{2A}$ and SH-SY5Y/5-HT$_{2C}$ cells, prior exposure to 5-HT caused a decrease in the E_{max} but not potency for subsequent 5-HT stimulation of [^{3}H]-IP$_x$ production. However, the rate of desensitization of 5-HT$_{2C}$ receptor was substantially quicker than the 5-HT$_{2A}$ receptor following equivalent levels of receptor stimulation. The time course for desensitization of each receptor was similar to that previously reported in independent studies. For instance, both the transfected hamster and human 5-HT$_{2A}$ receptors and the endogenous rat 5-HT$_{2A}$ receptor desensitized with a similar time course to that seen in SH-SY5Y/5-HT$_{2A}$ cells (Vouret-Cavriani *et al*., 1995; Van Huizen *et al*., 1993; Ivins & Molinoff, 1991).

Furthermore, desensitization of 5-HT$_{2C}$-mediated calcium-mobilization in transfected CHO cells followed a similar time course to that seen in SH-SY5Y/5-HT$_{2C}$ cells (Akiyoshi *et al*., 1995). This difference in the rate of desensitization could not be attributed to differences in the level of receptor expression, since the rate was still substantially faster for the 5-HT$_{2C}$ receptor in clone M, which expressed a similar number of receptors to SH-SY5Y/5-HT$_{2A}$ cells. Following prolonged exposure (2 h) to varying concentrations of 5-HT, the 5-HT$_{2C}$ receptor also showed approximately 10 fold greater sensitivity to desensitization than the 5-HT$_{2A}$ receptor. At high 5-HT concentrations, however, both receptors exhibited considerable desensitization, reducing agonist stimulation to less than 20% control response, indicating the capability for substantial modulation of functional activity of both the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. Desensitization in SH-SY5Y/5-HT$_{2A}$ cells was also associated with a significant increase in basal level of inositol phosphates, but this is unlikely to account for the subsequent decreased response to 5-HT since inclusion of the antagonist ketanserin did not prevent the increase in basal levels of [^{3}H]-IP$_x$ production, but did prevent the observed desensitization. Furthermore, exposure of both SH-SY5Y/5-HT$_{2C}$ and SH-SY5Y/5-HT$_{2A}$ cells to 5-HT did not significantly alter the subsequent response to carbachol, indicating the continued capability of the cell to hydrolyze inositol phospholipids.

The rapid rise and fall in Ins(1,4,5)P_3 levels after 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor stimulation seen in these cell lines in response to 5-HT is a general characteristic of receptors linked to phospholipase C (PLC) activation (Wojcikiewicz & Nahorski, 1993), and has previously been observed for the 5-HT$_{2A}$ receptor in uterine artery and the 5-HT$_{2C}$ receptor in CHO cells (Zhang & Hu, 1995; Akiyoshi *et al*., 1995). Studies of the muscarinic-M$_3$ receptor indicate that such a pattern of peak and fall in Ins(1,4,5)P_3 levels is due to desensitization at

the level of PLC activation correlated with increased receptor phosphorylation (Tobin *et al*., 1992; 1995). A similar profile for Ins(1,4,5)P_3 levels in SH-SY5Y/5-HT$_{2A}$ and SH-SY5Y/5-HT$_{2C}$ cells could also indicate modulation of PLC activation, possibly at the level of the receptor, with the earlier termination in 5-HT$_{2C}$-mediated Ins(1,4,5)P_3 production indicating a more rapid loss of function at this receptor compared to the 5-HT$_{2A}$ receptor. This hypothesis is supported by measurements of [^{3}H]-IP$_x$ accumulation over short time periods, which demonstrated a fall in the rate of [^{3}H]-IP$_x$ production, and hence PLC activity, after 10 s in SH-SY5Y/5-HT$_{2C}$ and 30 s in SH-SY5Y/5-HT$_{2A}$ cells (S.J. Briddon and J.M. Elliott, unpublished observations). Exposure of the cells to maximally effective concentrations of 5-HT for 15 min demonstrated 35% loss in the peak Ins(1,4,5)P_3 response mediated by the 5-HT$_{2C}$ receptor but no alteration in the magnitude of the 5-HT$_{2A}$ response. These results correspond well with those obtained measuring [^{3}H]-IP$_x$ accumulation after an equivalent exposure to 5-HT, confirming the greater sensitivity of the 5-HT$_{2C}$ receptor to desensitization by both methods of phosphoinositide analysis. Although several studies have assessed these receptors separately, this investigation represents the first direct biochemical comparison of desensitization of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors expressed in a common environment. At a more complex level, Darmain & Gerdes (1995) showed that administration of a single dose of the mixed 5-HT$_{2A/2C}$ agonist DOI to mice caused a substantial decrease in 5-HT$_{2C}$-receptor mediated behaviour to a subsequent dose of DOI, whilst having less effect on 5-HT$_{2A}$-receptor mediated behaviour, suggesting that our biochemical results may be applicable at a physiological level.

It is possible that differences in the rates of receptor desensitization result from differences in the mechanisms by which this process occurs. The biochemical mechanisms involved in rapid desensitization of G-protein coupled receptors generally involve receptor phosphorylation by a G-protein receptor kinase (GRK) or a second messenger-dependent kinase such as PKC, which lead to uncoupling of the receptor from its G-protein (Hausdorff *et al*., 1990). Desensitization can then lead to internalization of receptors into endosomes and, after longer periods of agonist stimulation, receptor down-regulation. Differences in desensitization mechanisms relating to 5-HT$_2$ receptors are indicated by studies showing that the 5-HT$_{2C}$ receptor expressed in fibroblasts becomes phosphorylated after desensitization (Barker & Sanders-Bush, 1993), whilst in contrast, the 5-HT$_{2A}$ receptor expressed in hamster fibroblasts was not phosphorylated after exposure to 5-HT, and removal of the regions containing likely phosphorylation sites did not affect receptor desensitization (Vouret-Cavriari *et al*., 1995). At the level of primary structure, differences in the desensitization profiles of the β-adrenoceptors have been attributed to differences in the number of potential receptor phosphorylation sites. The β_2-adrenoceptor, which contains several potential PKA and β-ARK phosphorylation sites, desensitizes more readily than the β_3-adrenoceptor which has far fewer (Bousquet-Melou *et al*., 1995). Similar comparisons between the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors indicate equivalent numbers of both serine and threonine residues, and potential PKC phosphorylation sites (Roth *et al*., 1990; Buck *et al*., 1991). However, these are only potential sites of phosphorylation and two studies have indicated that removal of this region of the 5-HT$_{2A}$ receptor does not affect its functional or desensitization characteristics (Buck *et al*., 1991; Vouret-Caviari *et al*., 1995). Similar studies with the 5-HT$_{2C}$ receptor may reveal the comparable importance of such sites to its desensitization.

In conclusion, the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors expressed in the human neuroblastoma cell line, SH-SY5Y, exhibit characteristic function and pharmacology, and provide a useful model to study receptor regulation (Newton & Elliott, 1997). Using two methods of functional analysis, the 5-HT$_{2C}$ receptor in this cell line demonstrated greater sensitivity to 5-HT-induced desensitization than the 5-HT$_{2A}$ receptor. Further studies will attempt to differentiate the mechanisms underlying such processes.

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