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Endogenous Serotonin-2A and -2C Receptors in Balb/c-3T3 Cells Revealed in Serotonin-Free Medium

DESENSITIZATION AND DOWN-REGULATION BY SEROTONIN

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ABSTRACT. We studied the endogenous expression of the serotonin-2A (5-hydroxytryptamine$_{2A}$, 5-HT$_{2A}$) 5-HT$_{2C}$, and a splice-variant of the 5-HT$_{2C}$ receptor in murine Balb/c-3T3 fibroblast cells that is revealed when these cells are maintained in medium containing 5-HT-free serum. RNA editing of the 5-HT$_{2C}$ receptor was exclusively at a single brain-specific site. Addition of 5-HT (EC_{50} = 23 ± 2.9 nM) induced an immediate release of calcium from an ionomycin-sensitive intracellular store by coupling to a pertussis toxin-insensitive pathway. The 5-HT-induced calcium mobilization displayed a 5-HT$_2$-like pharmacology, and ligand binding analyses indicated the presence of specific binding sites (27.5 ± 2 fmol/mg protein) with a 5-HT$_{2A}$-like pharmacology. Although the 5-HT$_{2A}$ receptor site was predominant, the smaller component of 5-HT$_{2C}$ receptors alone was sufficient to mediate a maximal calcium response. The 5-HT-induced increase in $[Ca^{2+}]_i$ was reversibly inhibited by >75% following a 12-hr pretreatment ($T_{1/2}$ = 2 hr) with 5-HT (EC_{50} = 400 nM). Extended treatment (24–96 hr) with 5-HT induced a complete functional desensitization that was associated with a partial (60%) reduction in 5-HT$_2$ receptor number, implicating both receptor down-regulation and post-receptor mechanisms in 5-HT-induced desensitization. Long-term (hours to days) treatment with 5-HT did not modulate DNA synthesis, cell proliferation, or transformation in Balb/c-3T3 cells. These results demonstrate that Balb/c-3T3 cells express endogenous 5-HT$_2$ receptors that are desensitized by the 5-HT present in normal serum, illustrating the importance of growth conditions in the identification of receptor responsiveness. The lack of proliferative response to 5-HT in Balb/c-3T3 suggests a putative role of desensitization as a "safety valve" to prevent abnormal cell growth during sustained 5-HT$_2$ receptor activation. BIOCHEM PHARMACOL **56**;10:1347–1357, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. serotonin; receptors; fibroblast; desensitization; down-regulation; cell proliferation

5-HT§ has been implicated in the control of various physiological functions, such as mood, sleep, appetite, pain, movement, and sexual drive [1–3]. These actions are mediated by a large family of 5-HT receptors that have been categorized into subclasses based on pharmacological properties, amino acid sequence, or signal transduction properties [4]. The 5-HT$_2$ subclass includes the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors. The 5-HT$_{2C}$ receptor is expressed predominantly in epithelial cells of the choroid plexus and to a lesser extent in neuronal populations of the hippocampus and cerebral cortex. The 5-HT$_{2B}$ receptor is present primarily in gastric mucosa, while the 5-HT$_{2A}$ receptor has the most wide-spread distribution, being found in platelets, smooth muscle, fibroblasts, mesangial cells, as well as specific brain regions [3–6].

The 5-HT$_2$ subclass of receptors shares extensive pharmacological and functional similarities, and determination of 5-HT$_2$ receptor subtype often requires RT–PCR cloning to provide unequivocal identification. The 5-HT$_2$ receptors couple to the PTx-insensitive G_q family of G proteins to stimulate PLC activity, which catalyzes the hydrolysis of phosphatidylinositol bisphosphate to diacylglycerol (DAG) and IP_3 [3, 4, 7]. Increase in IP_3 mobilizes calcium from intracellular stores to increase $[Ca^{2+}]_i$, whereas DAG activates PKC. These dual second messengers initiate a variety of biological responses in target cells, including enhanced secretion of hormones or neurotransmitters, smooth muscle contraction, and platelet activation [8]. The 5-HT$_{2C}$ receptor exhibits constitutive (agonist-independent) activity when over-expressed in transfected cells, but the significance of this phenomenon *in vivo* remains undetermined [9–11].

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§ *Abbreviations:* 5-HT, 5-hydroxytryptamine (serotonin); $[Ca^{2+}]_i$, cytosolic free calcium concentration; PTx, pertussis toxin; PLC, phospholipase C; PKC, protein kinase C; IP_3, inositol trisphosphate; FBS, fetal bovine serum; LSD, lysergic acid diethylamide; SSC, saline sodium citrate; DPAT, 8-hydroxy-2-(*N*,*N*-dipropylamino)1,2,3,4-tetrahydronaphthalene; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride; Fura-2/AM, Fura-2/acetoxymethyl ester; RT-PCR, reverse transcriptase–polymerase chain reaction; and THR, thrombin.

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Cellular responses mediated by 5-HT_{2A} and 5-HT_{2C} receptors are tightly regulated by receptor desensitization following exposure to an agonist [11–15]. Desensitization of these receptors involves both rapid events (uncoupling and sequestration) that occur within seconds to minutes [11, 14, 16, 17], and long-term changes (e.g. down-regulation) that occur in a time span of hours to days [18, 19]. Short-term agonist-induced desensitization could prevent over-stimulation of initial signaling events. Long-term desensitization is likely to regulate prolonged actions of the receptor on gene expression, which, in turn, may regulate events such as cell growth, differentiation, or motility. The regulation of the 5-HT_{2A} receptor in the platelets is a potential model of receptor dysregulation involved in alteration of mental status [7, 20, 21]. An understanding of 5-HT_2 receptor function and regulation in fibroblast cells may also be relevant to mechanisms of receptor regulation in the brain.

Previously, we and others had assumed that fibroblasts, like NIH-3T3 and Balb/c-3T3 cells, lack 5-HT receptors since no specific binding or response to 5-HT was detected in vector- or non-transfected cells [11, 14, 22–24]. However, the serum used to grow the cells contains 5-HT released during platelet aggregation [25, 26]. When NIH-3T3 [27] or Balb/c-3T3 (present study) cells are cultivated in medium containing 10% dialyzed FBS, which lacks 5-HT, the cells become responsive to 5-HT, which causes an increase in $[Ca^{2+}]_i$. In NIH-3T3 cells, the response is mediated by 5-HT_{2A} receptors that are remarkably sensitive to agonist-induced functional desensitization and down-regulation [27]. Balb/c-3T3 and NIH-3T3 cells are used frequently to study the oncogenicity of transfected genes. The sustained activation of 5-HT_2 receptors during maintenance in normal serum could complicate interpretation of oncogenic activity. The present studies were undertaken to: (1) identify 5-HT receptors endogenously expressed in Balb/c-3T3 cells; (2) investigate if 5-HT could induce receptor desensitization; and (3) examine whether prolonged activation of endogenous 5-HT receptors modulates Balb/c-3T3 cell growth.

MATERIALS AND METHODS

Materials

Balb/c-3T3 fibroblast cells were purchased from the American Type Culture Collection. The GF6 and PØ cell lines were provided by Dr. David Julius, UCSF. All reagents for cell culture and molecular biology were reagent grade obtained from GIBCO-BRL; the Sigma Chemical Co.; Molecular Probes, Inc.; or Calbiochem. [^{125}I]Succinyl cAMP and [^{125}I]−(+)LSD (2200 Ci/mmol) were from NEN. Ketanserin, mianserin, DOI, and 5-HT were from Research Biochemical Inc. (RBI), and DPAT was from Sigma. Mesulergine (from RBI) was a gift from Dr. Pierre Blier (McGill University). Radionucleotides [α^{32}P]dCTP and [α^{32}P]dATP (2200 Ci/mmol), HyBond-N membrane, and *Taq* DNA polymerase were from the Amersham Corp.

Cell Culture

Murine Balb/c-3T3 cells were propagated as a monolayer in Dulbecco's modified Eagle's medium (DMEM high glucose medium), supplemented with 10% heat-inactivated (65° for 10 min) FBS or dialyzed FBS, at 37° under a humidified atmosphere of 5% CO_2. Cells were passaged every 3 days by exposure to 0.05% trypsin/0.02% EDTA solution.

Measurement of $[Ca^{2+}]_i$

Balb/c-3T3 cells were grown in 100-mm petri dishes to 80% confluence, and medium was changed 12–16 hr prior to the assay. Cells were harvested by trypsinization and washed with HBBS buffer (118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 10 mM D-glucose, 20 mM HEPES, pH 7.2). Then the cells were resuspended in 1 mL of HBBS buffer containing a 2 μM concentration of membrane-permeant Fura-2/AM and incubated, with shaking, for 25 min at 37°, after which the cells were washed twice and resuspended in 2 mL of HBBS buffer. Change in fluorescence (R) was monitored using a Perkin–Elmer LS-50 spectroflurometer reading at λ_{EX} = 340/380 nm, λ_{EM} = 510 nm. Calibration was done by addition of 0.1% Triton X-100 and 2.0 mM Tris base to obtain R_{max} and 10 mM EGTA for R_{min}. Drugs were added directly to the cuvette in a volume of 10 μL (200-fold concentrated) as indicated in the figures.

RT-PCR

REVERSE TRANSCRIPTION. The cDNA was synthesized from Balb/c-3T3 poly(A^+) mRNA. Briefly, 1 μg of RNA was denatured at 70° for 10 min in the presence of 100 pmol random hexamers (Boehringer) and reverse transcribed in a total volume of 40 μL using *Taq* buffer, 200 U of reverse transcriptase (Superscript, GIBCO), and 1 mM dNTPs. The reaction proceeded for 1 hr at 37° before being heat-denatured at 95° for 15 min.

PCR AMPLIFICATION. Amplification from the reverse-transcribed Balb/c-3T3 mRNA was performed by "touch-down" PCR (MJ Research PTC-1000 thermal cycler) using degenerate oligonucleotides corresponding to nucleotide sequences at transmembrane domain (TM) III (Primer No. 1: 5′ - TCCATCATGCA{T/C}CTCTG{T/C}GCCAT - 3′) and TM VI (Primer No. 2: 5′-GT{G/A}ATGAA{G/A}AA {T/G/C}GGGCACCACAT-3′) of cloned 5-HT_2 receptors [27–29]. A 1-μL aliquot of cDNA was amplified with 2.5 U of *Taq* polymerase and 0.2 mM dNTPs in a cycling program that had an initial annealing temperature of 65° ramping downwards 1° per cycle for 15 cycles followed by 20 cycles with an annealing temperature of 50°. Two bands of 542/525 bp and 400 bp were isolated from agarose gels and were subcloned into pCR 2.1 (TA cloning, Invitrogen). Multiple individual plasmid clones were sequenced manually from several independent amplifications using the dideoxynucleotide termination reaction (Pharmacia).

RNA Preparation and Northern Blot Analysis

Poly(A$^+$) RNA was isolated from the Balb/c-3T3 fibroblast cell line, mouse brain tissues, and total RNA from NIH-3T3 cells transfected with the rat 5-HT$_{2A}$ (GF6) or 5-HT$_{2C}$ (PØ) receptor [30]. Poly(+) RNA was prepared with a QuickPrep Micro mRNA Purification Kit (Pharmacia). For the northern blot analysis, 20–40 μg of total RNA or 5–8 μg of poly(A$^+$) RNA was denatured in formaldehyde and formamide, electrophoresed on a 1.3% agarose-formaldehyde gel, blotted overnight onto nylon membrane, and fixed by baking at 80° for 2 hr under vacuum. The blots were probed with a ^{32}P-labeled fragment (corresponding to the region between TM III and VI) of the mouse 5-HT$_{2A}$ (N*) [27] or 5-HT$_{2C}$ receptor cDNA in 50% formamide–Denhardt's solution overnight at 42°, washed twice in 2× SSC, 1% SDS at room temperature for 15 min; twice in 0.2× SSC, 1% SDS at 65° for 15 min and exposed to Kodak XAR-5 film at −80° for 1 day to 1 week with intensifying screen, or to a PhosphorImager screen (Molecular Dynamics) for 16 hr [30].

Radioligand Binding

MEMBRANE PREPARATION. The cells were washed twice with PBS at room temperature, incubated in 1/5 TME buffer (1/5 × 50 mM Tris, pH 7.7, 10 mM $MgCl_2$, 0.5 mM EDTA) for 20 min at 4° and collected with fresh 1/5 TME buffer by scraping. After homogenization using a polytron (at 60 for 15 sec) and low speed centrifugation (1000 × *g* for 5 min at 4°) to pellet nuclei and cell debris, the supernatant containing cell membranes was pelleted (48,000 × *g* for 30 min at 4°), resuspended, and pelleted again, and finally resuspended in 50 mM Tris–HCl, pH 7.7. Freshly prepared membranes were used for binding assays. Membranes prepared for down-regulation experiments underwent more extensive washing. After low speed centrifugation, the supernatant was incubated for 15 min at 37° and then centrifuged (48,000 × *g* for 30 min at 4°), and the pellet was rehomogenized. The manipulation was repeated twice before finally resuspending the membrane in 50 mM Tris–HCl, pH 7.7, for binding [31].

BINDING ASSAY. Binding experiments were performed in triplicate in a total volume of 150 μL containing 50 μg membrane protein and appropriate concentrations of [^{125}I]LSD and experimental compounds. The reaction proceeded for 1 hr at 37° and was terminated using a Brandel Harvester by rapid filtration through glass fiber filters presoaked with 0.3% polyethylenimine, followed by three washes with ice-cold 50 mM Tris–HCl, pH 7.7. Specific binding was defined as that displaced by 10 μM mesulergine and represented less than 50% of the total binding. The radioactivity was quantified using a Cobra II autogamma counter (CANBERRA PACKARD). Binding data were analyzed using the programs LIGAND (version 3.0, ELSEVIER software) and Prism (version 2.0, Graphpad). The IC_{50} values used for pK_i determination were determined by non-linear regression analysis [32].

Measurement of DNA Synthesis and Cell Growth

PROLIFERATIVE CELLS. Balb/c-3T3 cells were grown on 24-well dishes until 80% confluence (20,000–25,000 cells/well) and then switched to the appropriate treatment for a period of 24 hr.

QUIESCENT CELLS. Cells grown as described above were maintained in serum-free medium for 48 hr after reaching confluence, and treatments were done in serum-free medium the following 24 hr.

[^{3}H]THYMIDINE INCORPORATION. Two methods of labelling were used for each assay: a *pulse assay* in which 1 μCi/well of [^{3}H]thymidine was added 4 hr before collection; and a *cumulative assay* in which [^{3}H]thymidine was added at the beginning of the treatment. Extraction and isotopic counting of genomic DNA were performed as described [24, 33].

CELL PROLIFERATION ASSAY. Balb/c-3T3 cells were plated on 6-well plates at a density of 40,000 cells/well and grown for 4 days in medium supplemented with 10% dialyzed FBS. The 5-HT was added daily at a concentration of 1 μM. Duplicate samples from each well were counted using a cell counter (Coulter).

SOFT AGAR GROWTH ASSAY. Balb/c-3T3 cells (500/well) were cultivated in 0.35% soft agar (35 mm dish) containing DMEM plus 10% dialyzed FBS for 4 weeks. Medium was replaced every 3 days, and 1 μM 5-HT was added daily. Colonies were counted in ten separate fields of view for each well [33].

RESULTS

Serotonin-Induced Increase in [Ca^{2+}]$_i$ in Balb/c-3T3 Cells

The 5-HT-induced change in $[Ca^{2+}]_i$ was measured in suspension of Fura 2-loaded Balb/c-3T3 cells cultivated in medium supplemented with 10% normal FBS (5-HT containing) or dialyzed FBS (5-HT free) (Fig. 1A). In multiple assays, no consistent difference in basal $[Ca^{2+}]_i$ was observed. Serotonin (5-HT, 10 μM) induced a 2.2 ± 0.4-fold increase in $[Ca^{2+}]_i$ that was observed only in cells that were maintained in dialyzed serum. Hence, for all further experiments, Balb/c-3T3 cells were grown in medium supplemented with dialyzed serum. The possibility that intracellular calcium stores were depleted in cells grown in normal FBS was excluded since THR (1 U/mL) and ATP (1–10 μM) induced robust (2- to 4-fold) increases in $[Ca^{2+}]_i$ (data not shown). The enhancement of $[Ca^{2+}]_i$ by 5-HT observed in Balb/c-3T3 cells was saturable and concentration dependent, with an EC_{50} value of 23 ± 2.9 nM (Fig. 1B).

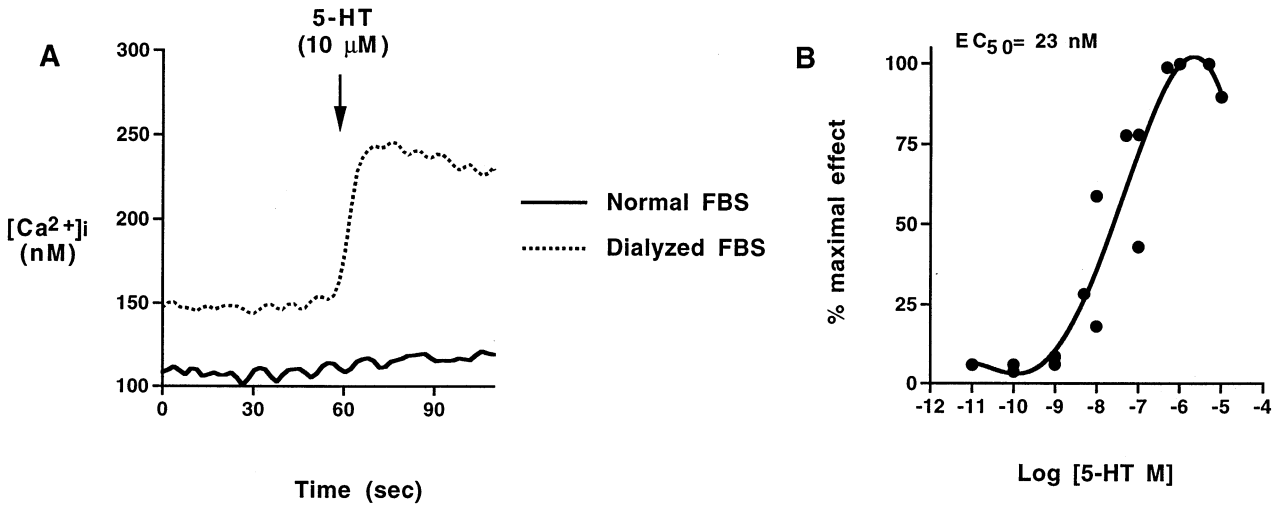

FIG. 1. Action of 5-HT on $[Ca^{2+}]_i$ in Balb/c-3T3 cells. (A) Effects if 5-HT on $[Ca^{2+}]_i$ in suspension of Balb/c-3T3 cells. The level of $[Ca^{2+}]_i$ versus time was measured in Balb/c-3T3 fibroblast cells loaded with Fura-2/AM grown in medium supplemented with 10% non-dialyzed FBS (———) or dialyzed FBS (- - - -). Serotonin (10 μM) was added as indicated. N.B.: All further $[Ca^{2+}]_i$ assays were performed in cells grown in 10% dialyzed serum unless otherwise mentioned. (B) Concentration-dependence of the 5-HT-induced increase in $[Ca^{2+}]_i$. The average 5-HT-induced change in peak $[Ca^{2+}]_i$ in Balb/c-3T3 cells is shown as percent maximal response.

The source of calcium for the 5-HT-mediated peak increase in $[Ca^{2+}]_i$ was investigated using excess EGTA (3 mM) to chelate extracellular calcium, and ionomycin (1 μM), a calcium ionophore that liberates calcium from intracellular stores [34]. The 5-HT-induced calcium response was maintained in the presence of EGTA (88.0 ± 13.6% of untreated cells; N = 3), but was abolished completely by ionomycin, suggesting that 5-HT induces release of intracellular calcium from ionomycin-sensitive intracellular stores. The 5-HT-induced calcium response was unaltered by a 16-hr pretreatment of the cells with 50 ng/mL of PTx (97.3 ± 10.3% of untreated cells; N = 3), although the THR (1 U/mL)-induced response was blocked by more than 50%. Over the concentration range of 0.1 to 10 μM, 5-HT produced no significant alteration in basal or forskolin (10 μM) stimulated cAMP level (data not shown). These experiments suggest that an endogenous 5-HT receptor, such as a 5-HT_2 receptor, mobilizes intracellular calcium in Balb/c-3T3 cells by coupling to a PTx-insensitive G_q protein.

The subtype of 5-HT receptor implicated in the 5-HT-induced change in $[Ca^{2+}]_i$ was examined further using saturating concentrations of selective 5-HT receptor agonists and antagonists (Table 1). The 5-$HT_{1A/7}$ receptor selective agonist DPAT (10 μM) lacked activity, whereas the 5-HT_2 receptor agonist DOI induced an increase in $[Ca^{2+}]_i$ that was smaller than with 5-HT (Table 1), consistent with its partial agonist activity [35]. The calcium response induced by 1 μM 5-HT was inhibited by 100 nM mianserin, and completely blocked by 100 nM mesulergine, consistent with involvement of a 5-HT_2 receptor. Inhibition of 5-HT-evoked increase in $[Ca^{2+}]_i$ was concentration dependent at 1, 10, and 100 nM mesulergine (data not shown). At a concentration that effectively blocks 5-HT_{2A} receptors in other systems [4, 27], ketanserin and spiperone were the least effective antagonists tested. Ketanserin blocked only partially the 5-HT (1 μM) induced increase in $[Ca^{2+}]_i$, while spiperone had no significant effect. The weak inhibitory activity of 5-HT_{2A} antagonists and the high potency of 5-HT (Fig. 1) suggest that the calcium response to 1 μM 5-HT is mediated, in part, by a 5-HT_{2C}-like receptor.

Molecular Identification of 5-HT Receptors in Balb/c-3T3 Cells

To identify the molecular species of 5-HT_2 receptor RNA that is expressed in Balb/c-3T3 cells, we utilized RT–PCR

TABLE 1. Pharmacology of the 5-HT-induced calcium response in Balb/c-3T3 cells

Agonists	% of 5-HT-evoked calcium response
DOI	39.8 ± 6.5(4)
DPAT	0 (3)

Antagonists	% Inhibition of calcium response
Ketanserin	33.8 ± 9.7 (4)
Mesulergine	96.6 ± 3.2 (4)
Mianserin	79.7 ± 8.4 (4)
Spiperone	4.9 ± 14.8(5)

For agonists, the change in $[Ca^{2+}]_i$ produced by a 10 μM concentration of each agonist was measured in Balb/c-3T3 cells. For antagonists, the effect of a 1-min pretreatment with various 5-HT receptor antagonists (100 μM) on the subsequent 5-HT (1 μM) induced increase in $[Ca^{2+}]_i$ was determined. The 5-HT-induced fold increase in $[Ca^{2+}]_i$ over basal (mean ± SEM) was calculated, and the values are shown as mean ± SD in percent inhibition versus untreated cells. The number of independent replicates is indicated in parentheses.

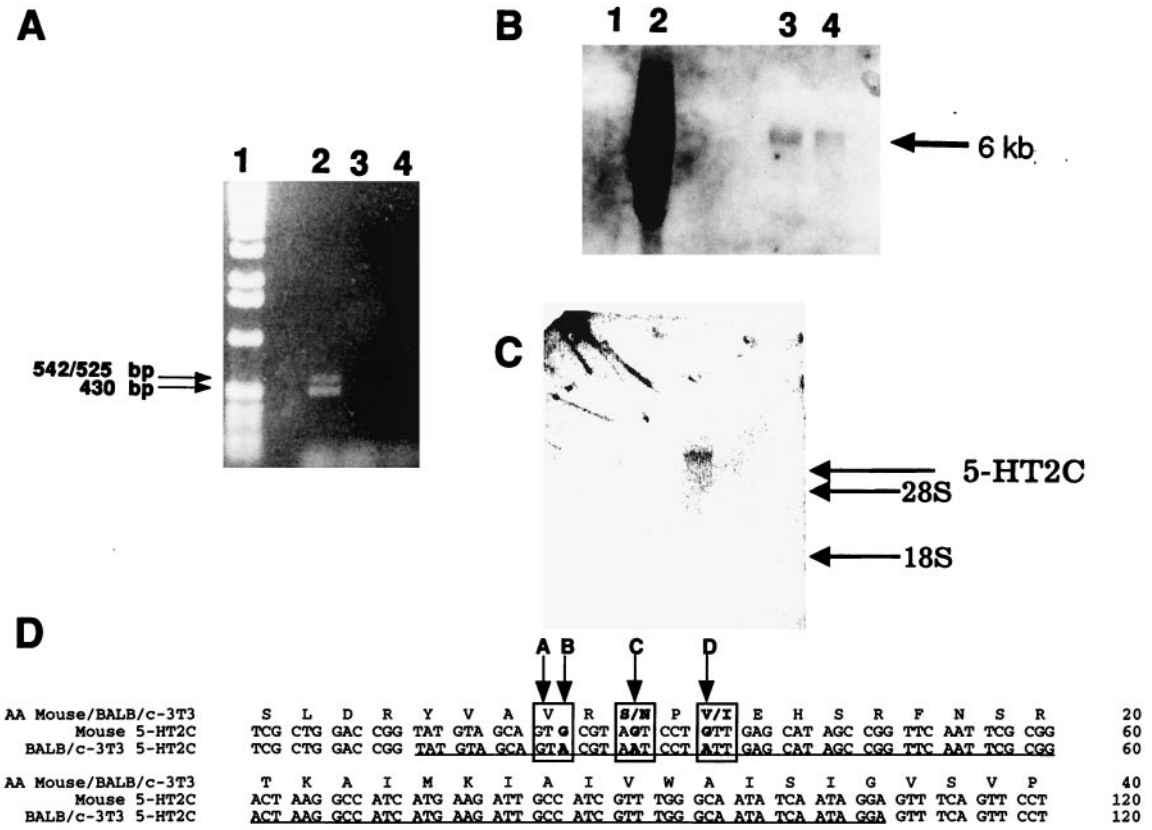

FIG. 2. Molecular identification of 5-HT receptors in Balb/c-3T3 cells. (A) RT-PCR analysis. Degenerate oligonucleotide primers derived from nucleotide sequences at the TM III and TM VI domains of cloned 5-HT$_2$ receptors cDNAs were used for RT–PCR amplification, and the products were electrophoresed and stained with ethidium bromide. Lane 1, molecular weight standards: lane 2, amplification of cDNA synthesized using mRNA from Balb/c-3T3 cells; lane 3, reverse transcriptase was omitted; and lane 4, no RNA template was included. Two specific DNA fragments (~550 and 450 bp) were amplified. (B) Northern blot analysis of total RNA (20–40 μg) from NIH-3T3 cells transfected wtih rat 5-HT$_{2C}$ (PØ, lane 1) or 5-HT$_{2A}$ (GF6, lane 2) receptor cDNA, of poly(A$^+$) mRNA from Balb/c-3T3 cells (5 μg, lane 3), and of mouse brain (5 μg, lane 4) probed with the cloned 542-bp fragment of mouse 5-HT$_{2A}$ receptor cDNA [27]. The major 5-HT$_{2A}$ receptor RNA species of 6 kb in size is indicated. (C) Northern blot analysis of 8 μg of poly(A$^+$) RNA from Balb/c-3T3 cells probed with the cloned 525-bp fragment of the 5-HT$_{2C}$ receptor cDNA (in panel A). (D) Nucleotide and amino acid sequences of the 5-HT$_{2C}$ receptor cDNA from mouse (fully-edited) and Balb/c-3T3 cells. Sites of RNA editing (from [40]) are indicated by arrows. Nucleotides that are spliced out of the short form 5-HT$_{2C}$ receptor splice-variant are underlined.

with degenerate oligonucleotide primers derived from sequences conserved among the three cloned rat 5-HT$_2$ receptor subtypes [28, 29]. As shown in Fig. 2A, no amplification products were observed in the absence of reverse transcriptase, indicating that there was no detectable contamination with genomic DNA. The RT–PCR of Balb/c-3T3 RNA yielded two specific bands (Fig. 2A) that consisted of three discrete DNA fragments of 542/525 bp (upper band) and 430 bp (lower band) in size. Sequence analysis of fragments obtained from several independent RT–PCR amplifications indicated, to our surprise, that the 542/525-bp band contained two specific DNA products that encoded the mouse 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor cDNAs, respectively. The 430-bp amplification product corresponded to a splice-variant of the 5-HT$_{2C}$ receptor. The splice-variant lacks the same 95 nucleotides identified in mouse, rat, and human cDNA clones (Fig. 2D) [36, 37].

The nucleotide sequence of the 5-HT$_{2A}$ receptor cDNA fragment identified in Balb/c-3T3 cells was identical to that of the previously cloned mouse 5-HT$_{2A}$ receptor cDNA (484–1026 bp) [38] with the exception of 5 nucleotide changes. The predicted amino acid sequences were identical except for a serine to proline substitution at position 298 in the third cytoplasmic loop of the 5-HT$_{2A}$ receptor. The 5-HT$_{2C}$ receptor sequence was identical to the corresponding region (998–1540 bp) of the cloned mouse brain 5-HT$_{2C}$ receptor cDNA with 3 nucleotide substitutions [39]. Recently, RNA editing has been demonstrated for the rat 5-HT$_{2C}$ receptor cDNA at the sites of nucleotide variation identified above [40]. The nucleotide sequence corresponds to RNA editing exclusively at the A site, which is found in brain but not in choroid plexus (Fig. 2D). Interestingly, the predicted amino acid changes in 5-HT$_{2C}$ receptor were a serine to asparagine shift at position 159 and a valine to isoleucine shift at position 161 in the second cytoplasmic loop. These substitutions generate a more active form of the receptor [40].

The expression of both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor

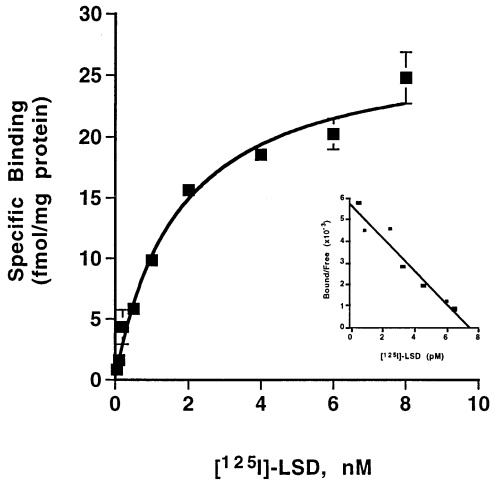

FIG. 3. Ligand binding studies of Balb/c-3T3 cell membranes using [^{125}I]LSD. Specific binding (total − non-specific binding) of [^{125}I]LSD was determined using 50 μg of membranes prepared from Balb/c-3T3 cells (see Materials and Methods). Non-specific binding was determined in the presence of 10 μM mesulergine. Data for specific binding are plotted as means ± SEM of triplicate samples from a representative experiment. Inset: Scatchard transformation of these data yielded a K_d = 1.3 nM, B_{max} = 23 fmol/mg protein.

RNA was examined further by northern blot analysis using total RNA isolated from Balb/c-3T3 cells, mouse brain cortex, and GF6 and PØ cells (NIH-3T3 cells transfected with the rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, respectively). A major 5-HT$_{2A}$ receptor RNA transcript of approximately 6 kb in size was detected in both Balb/c-3T3 cells and mouse brain cortex (Fig. 2B). No transcript was detected in RNA prepared from PØ cells, but multiple bands were present in the GF6 cells, demonstrating the specificity of the 5-HT$_{2A}$ probe. 5-HT$_{2C}$ receptor RNA was not detected in northern blot analysis of total RNA from Balb/c-3T3 cells. To detect 5-HT$_{2C}$ receptor RNA, poly(A$^+$) enriched RNA isolated from Balb/c-3T3 cells was probed with a fragment from the mouse 5-HT$_{2C}$ receptor cDNA. A doublet of about 4 kb was identified corresponding to the spliced and unspliced forms of the receptor RNA (Fig. 2C). No transcript was detected in RNA prepared from GF6 cells (data not shown), indicating 5-HT$_{2C}$ probe specificity. These results suggest that both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor mRNA transcripts are present in Balb/c-3T3 cells, although the former is more abundant.

Ligand Binding Studies

The specific binding of the 5-HT receptor ligand [^{125}I]LSD to crude membranes prepared from Balb/c-3T3 cells grown in 5-HT-free medium was used to generate saturation binding isotherms (Fig. 3). Scatchard analysis of specific [^{125}I]LSD binding from three independent experiments indicated a receptor density (B_{max}) of 27.5 ± 2 fmol/mg protein, with K_d = 1.7 ± 0.3 nM. Although the expression level of 5-HT receptors was low, the pharmacology of this binding site was characterized by competition assays using various concentrations of non-radioactive 5-HT ligands. The observed pK_i values were compared with published pK_i values at cloned 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors (Table 2). Ketanserin, mesulergine, mianserin, and spiperone potently inhibited [^{125}I]LSD binding with pK_i values ranging from −8.2 to −9.1, similar to values determined for the cloned 5-HT$_{2A}$ receptor. In accord with northern blots, the binding data favored the predominant expression of the 5-HT$_{2A}$ receptor subtype in Balb/c-3T3 cells. The two affinity states observed for 5-HT were consistent with binding to 5-HT$_{2C}$ (high affinity) and 5-HT$_{2A}$ (low affinity) sites, but possible contributions due to multiple affinity states of one receptor complicates this interpretation. The 5-HT$_{2A}$-selective antagonist ketanserin also displayed two affinity states, consistent with the presence of a small component (20%) of non-5-HT$_{2A}$ receptors, likely to represent the 5-HT$_{2C}$ receptor based on the functional pharmacology and molecular analysis.

Receptor Desensitization and Down-Regulation by 5-HT

The following experiments were done to test whether or not the 5-HT$_2$ receptors endogenously expressed in Balb/c-3T3 cells are desensitized by 5-HT. Upon preincubation of Balb/c-3T3 cells in medium supplemented with 1 μM 5-HT, the subsequent 5-HT-induced calcium response was reduced by 75% within 6–12 hr, with a half-time of about 2 hr (Fig. 4A). Response to 5-HT was blocked completely following 24–96 hr of treatment with 1 μM 5-HT, whereas ATP- or THR-induced responses were intact (data not shown). Balb/c-3T3 cells that were desensitized completely by 5-HT treatment completely recovered responsiveness to 5-HT within 12 hr of culture in 5-HT-free medium (Fig. 4B). The concentration dependence for 5-HT-induced desensitization generated an EC_{50} of 400 nM (Fig. 4C). Prolonged exposure to agonist often results in a decrease in the level of cell surface receptors. As shown in Fig. 4D, long-term (96 hr) treatment with 5-HT resulted in a 60% diminution of the number of cell membrane 5-HT$_2$ receptors, as indicated by a decrease in B_{max} ($B_{max(5\text{-}HT)}$) = 10.2

TABLE 2. Affinity of ligands for 5-HT$_2$ binding sites

Drug	Observed pK_i in Balb/c-3T3 cells	Predicted pK_i 5-HT$_{2A}$	Predicted pK_i 5-HT$_{2C}$
Serotonin	7.3 ± 0.3; 5.4 ± 0.6	5.5	7.5
DOI	7.8 ± 0.2	7.3	7.8
8-OH-DPAT	5.1 ± 0.5	5.0	5.2
Ketanserin	9.1 ± 0.1; 6.3 ± 1.4	8.9	7.0
Mesulergine	8.2 ± 0.1	8.5	ND*
Mianserin	8.2 ± 0.1	8.1	8.0
Spiperone	8.7 ± 0.1	8.8	5.9

The values tabulated represent the negative logarithm of the dissociation constant (pK_i) calculated from competition experiments and expressed as means ± SEM, N = 3. The predicted pK_i values of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors are as reported [1, 4, 29].

*ND = not determined.

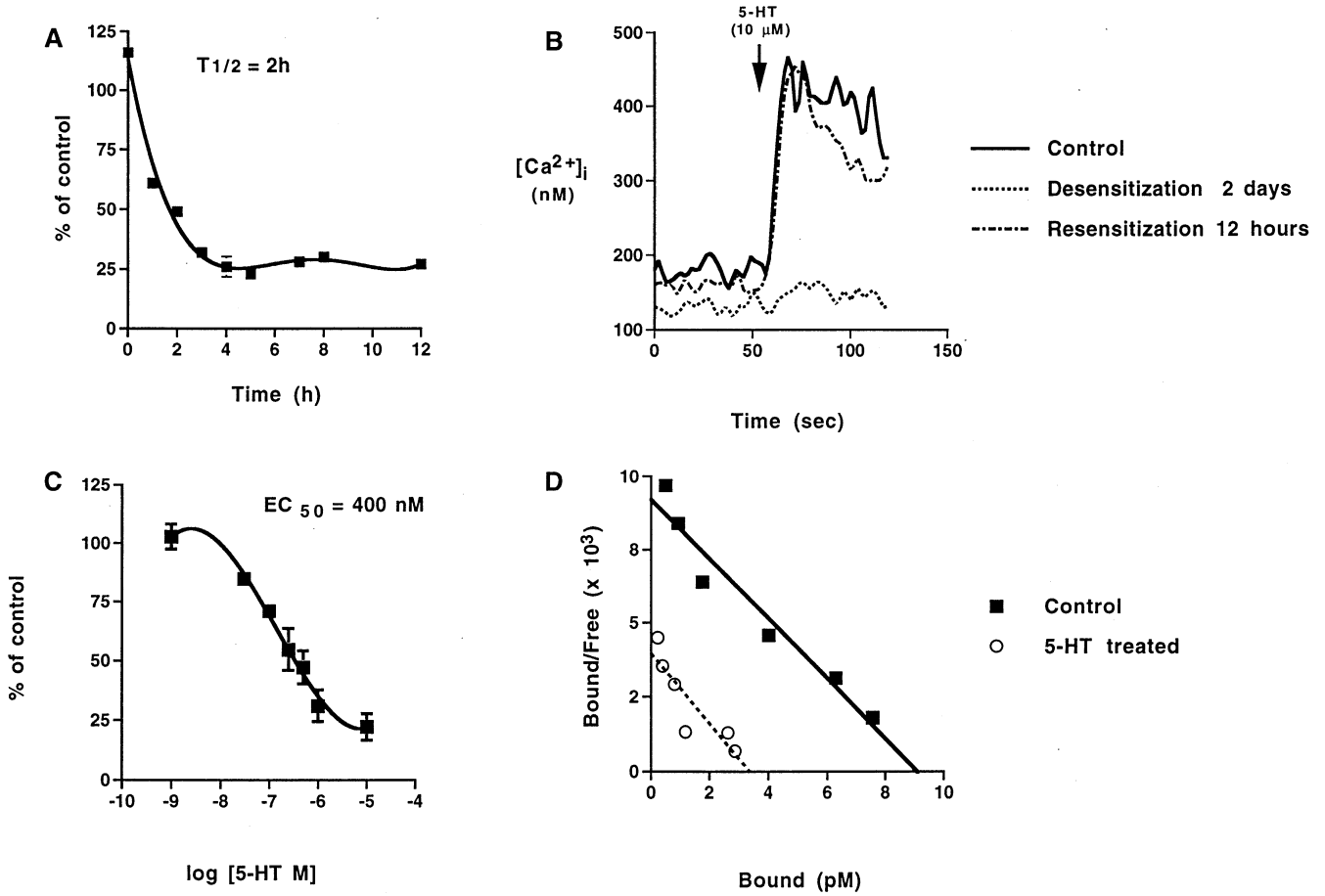

FIG. 4. Desnsitization/resensitization and down-regulation of 5-HT$_2$ receptors by 5-HT. (A) Functional desensitization of 5-HT$_2$ receptors by 5-HT. Change in $[Ca^{2+}]_i$ induced by 10 μM 5-HT was measured in subconfluent Balb/c-3T3 cells (one 10-cm dish/assay) cultivated in medium supplemented with 1 μM 5-HT for various periods of time. The results for (A) and (C) are shown as percent of control (cells not pretreated with 5-HT) in which 5-HT elicited a 2.2 ± 0.4-fold increase in $[Ca^{2+}]_i$. Data are expressed as means ± SEM of at least three separate experiments. (B) Recovery from desensitization. The graph shows the change in level of $[Ca^{2+}]_i$ (nM) induced by 10 μM 5-HT. Control (———), Balb/c-3T3 cells maintained in dialyzed serum. Balb/c-3T3 cells (one 10-cm dish/assay) were also grown in medium supplemented daily with 1 μM 5-HT for 2 days (- - - -) and then left to recover for 12 hr in 5-HT-free medium (-·-·-·-). These results are representative of two experiments. (C) Concentration dependence of 5-HT-induced desensitization. Balb/c-3T3 cells were grown for 4 hr in medium supplemented with different concentrations of 5-HT and assayed for $[Ca^{2+}]_i$ in response to 10 μM 5-HT. A concentration–response curve was generated from data of at least three independent experiments and plotted as mean ± SEM percent of control 5-HT response in untreated cells. (D) Representative Scatchard plot for specific [^{125}I]LSD binding to control Balb/c-3T3 cells (K_d = 0.99 nM; B_{max} = 26.7 fmol/mg protein) or cells preincubated with 5 μM 5-HT for 96 hr (K_d = 0.85 nM; B_{max} = 10.2 fmol/mg protein).

fmol/mg) compared with untreated cells ($B_{max(control)}$ = 26.7 fmol/mg). The receptor K_d was unaffected by prolonged treatment with 5-HT. Thus, the 5-HT$_2$ receptors endogenously expressed in Balb/c-3T3 cells are densensitized functionally by 5-HT, and this is associated with a partial down-regulation of receptor number.

Regulation of Cell Growth

The third objective of these studies was to examine the effect of long-term exposure to 5-HT on Balb/c-3T3 cell growth. The action of 1 μM 5-HT on [^{3}H]thymidine incorporation into acid-precipitable DNA was measured in proliferative (Fig. 5A) and quiescent (Fig. 5B) Balb/c-3T3 cells. In either condition, the rate of DNA synthesis over control was unaltered by 5-HT. Similarly, treatment with 5-HT for 4 days did not change the cell number compared with the untreated control (Fig. 5C). The growth of Balb/c-3T3 in soft agar in response to 5-HT was used to assay for anchorage-independent growth, a hallmark of transformed cells. In the presence or absence of 1 μM 5-HT, no anchorage-independent colonies were detected (Fig. 5D). Thus, the activation of endogenous 5-HT$_2$ receptors did not modulate the rate of DNA synthesis, cell proliferation, or transformation of Balb/c-3T3 cells.

DISCUSSION

5-HT$_2$ Receptors in Balb/c-3T3 Cells

By a combination of functional, pharmacological, and molecular analyses, we determined that Balb/c-3T3 fibro-

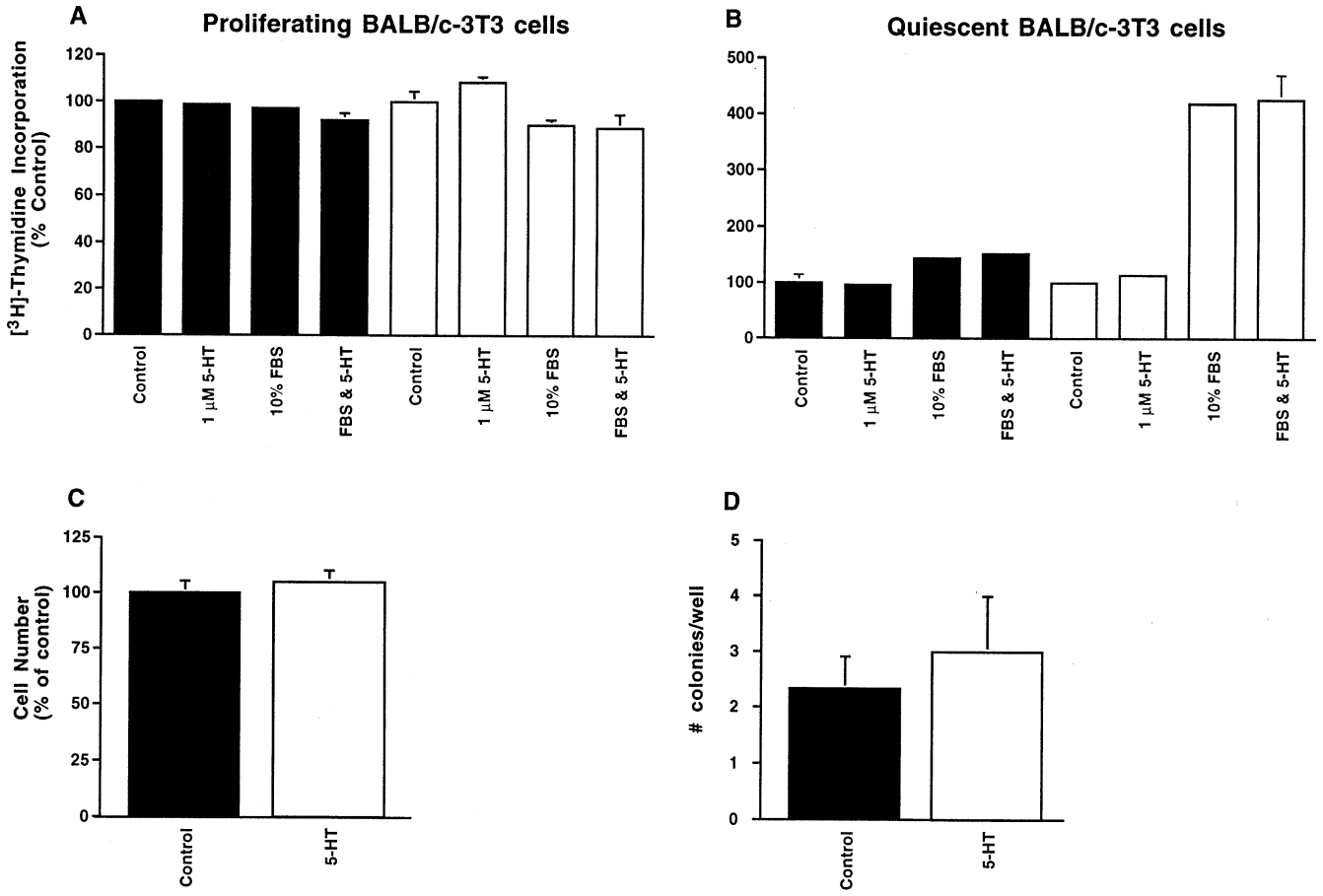

FIG. 5. Effect of 5-HT_2 receptor activation on Balb/c-3T3 cells growth and cellular transformation. The cumulative (filled bars) and pulse (open bars) incorporation of [^{3}H]thymidine into cellular DNA, as indicator of DNA synthesis, was measured in proliferating (A) and quiescent (B) Balb/c-3T3 cells plated in 24-well dishes (20,000–25,000 cells/well). The data are expressed as percent of control ± SEM of three experiments where 100% corresponds to mean control values of 242,000 cpm/well (cumulative assay) or 64,000 cpm/well (pulse assay) for proliferating cells (A) and 146,000 cpm/well (cumulative assay) or 19,000 cpm/well (pulse assay) for quiescent cells (B). As indicated in the figure, cells were treated or not treated with 1 μm 5-HT or 10% FBS in the appropriate medium for 24 hr. (C) Balb/c-3T3 cells were plated in 6-well dishes at an initial subconfluent density of 40,000 cells/well. Cells were treated daily with 1 μM 5-HT (open bar) or were untreated (filled bar). Values are means ± SEM of three experiments. (D) The ability of 5-HT to induce cellular transformation of Balb/c-3T3 cells was tested using a soft-agar growth assay. The number of colonies formed after a period of 4 weeks in the presence (open bar) or absence (filled bar) of 1 μM 5-HT was determined in triplicate samples (mean ± SEM) for each treatment.

blast cells endogenously express the 5-HT_{2A} receptor and two splice-variants of the 5-HT_{2C} receptor that are revealed when the cells are propagated in 5-HT-free medium. The lack of 5-HT response when these cells were maintained in normal serum may be due to the presence of platelet-derived 5-HT (0.2 to 40 μM), at concentrations sufficient to desensitize endogenous 5-HT_2 receptors [25, 26]. Removal of 5-HT from the serum, either by dialysis or charcoal treatment, resulted in a recovery of 5-HT-induced responses and receptor number.

As described previously in other tissues [36, 37], two splice-variants of the 5-HT_{2C} receptor were identified in Balb/c-3T3 cells. The shorter isoform of the 5-HT_{2C} receptor lacks 95 nucleotides in the second intracellular loop and TM IV domains, generating a frameshift in coding sequence that results in a premature stop codon and yields a truncated receptor that fails to bind serotonergic ligands. The short isoform has been identified in all tissues that express the 5-HT_{2C} receptor but with a tissue-specific expression ratio. In choroid plexus tumors, the ratio of splice-variant to full-length 5-HT_{2C} receptor RNA is greater than in normal tissue. The splice-variant is proposed to modulate tumorigenesis and cellular differentiation, but this remains unresolved. The Balb/c-3T3 cells could provide a model in which to examine what role, if any, this splice-variant might play in growth regulation and cellular transformation.

RNA editing has been demonstrated to occur at four adenosine residues located in exon 3, close to the alternately spliced intron of the 5-HT_{2C} receptor [40]. The variant expressed in Balb/c-3T3 cells was modified exclusively at the first adenosine (site A, Fig. 2D). This receptor variant couples more efficiently to PLC than the fully-edited form. This form of the receptor is expressed in mouse

brain, but is absent in choroid plexus. Thus, the Balb/c-3T3 cells may provide a model of RNA editing that more closely resembles the pattern in the brain.

The 5-HT-induced calcium response was not blocked by the 5-HT$_{2A}$ antagonists ketanserin or spiperone even though the 5-HT$_{2A}$ receptor appears to predominate based on ligand binding and northern blot analyses. This result suggests that the smaller number of 5-HT$_{2C}$ receptors expressed in Balb/c-3T3 cells is sufficient to induce a maximal calcium response, since the 5-HT-mediated calcium response was unaltered upon blockade of 5-HT$_{2A}$ receptors. The efficacy of 5-HT$_{2C}$ receptor signaling may reflect the 10-fold higher affinity of 5-HT$_{2C}$ over 5-HT$_{2A}$ receptors for 5-HT. Moreover, the edited variant of 5-HT$_{2C}$ receptor expressed in these cells has been shown to couple 10- to 15-fold more efficiently to PLC [40]. Thus, discrepancies between binding and functional pharmacology appear to result from the co-expression of known receptors, rather than from the presence of a previously uncharacterized receptor subtype.

Receptor desensitization

In Balb/c-3T3 cells, the 5-HT-induced calcium response was desensitized by 75% within several hours of pretreatment with 5-HT and was desensitized completely after 24 hr. The residual (25%) 5-HT response remaining after 4 hr of treatment appears to represent a slowly desensitizing pool of receptors that may correspond to 5-HT$_{2C}$ receptors, but was too small to assess pharmacologically. The EC_{50} of 5-HT-induced desensitization at 4 hr was similar to the EC_{50} observed for 5-HT$_{2A}$ receptor activation and desensitization. We have reported previously that in NIH-3T3 cells that express endogenously the 5-HT$_{2A}$ receptor, 5-HT-mediated desensitization had a similar EC_{50} (200 vs 400 nM) and was complete within 4 hr [27], suggesting that the 5-HT$_{2A}$ receptor can be desensitized completely within 4 hr.

Complete desensitization of the 5-HT-induced calcium response was associated with a 60% decrease in the number of binding sites at 96 hr. A decrease in 5-HT$_2$ receptor number could result from increased receptor degradation or decreased synthesis. Decreased receptor synthesis can be mediated by decreases in RNA transcription, mRNA stability, or translation of the receptor protein. Interestingly, transfected rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors in NIH-3T3 cells are partially desensitized by agonist, but no down-regulation of receptors was observed even after several days of exposure to agonist [11, 14, 41]. However, the 5-HT$_{2A}$ receptor that is expressed endogenously in NIH-3T3 cells undergoes agonist-mediated functional desensitization that is associated with a pronounced down-regulation of 5-HT$_{2A}$ receptor number and RNA [27]. Thus, the regulation of endogenously expressed receptors may involve both pre- and post-translational events.

Although decrease in receptor number (i.e. 60%) can account partly for the 5-HT-induced desensitization, other mechanisms must also contribute to the complete desensitization observed in Balb/c-3T3 cells. Phosphorylation events mediated by PKC are implicated in acute agonist-mediated receptor desensitization of the 5-HT$_{2A}$ receptor in platelets [42], rat cortical neurons [43], cerebellar granule cells [12], and in our preliminary studies in NIH-3T3 and Balb/c-3T3 cells. Recent studies using epitope-tagged 5-HT$_{2A}$ receptor and 5-HT$_{2A}$/thrombin chimeric receptor constructs transfected into CCL39 fibroblast cells [16] suggest that receptor sequestration, rather than receptor phosphorylation, is the primary mechanism of acute receptor desensitization of 5-HT$_{2A}$ receptors (and vice versa for THR receptors). Receptor sequestration, but not degradation or down-regulation, was observed also in NIH-3T3 cells transfected with 5-HT$_{2A}$ receptors [14, 17]. Although sequestration has been proposed as a desensitization mechanism for 5-HT$_{2A}$ receptors, the sequestered receptors can also undergo internalization leading to receptor degradation upon prolonged (hours) exposure to agonist. A sustained degradative pathway in combination with a reduction in receptor synthesis could produce the desensitization we observed. However, additional mechanisms have been described for other G protein-coupled receptors such as desensitization or depletion of downstream elements (e.g. G protein subunits, effectors) [44, 45].

The less abundant component of 5-HT receptors detected in Balb/c-3T3 cells was the 5-HT$_{2C}$ subtype. Evidence indicates that long-term treatment with 5-HT agonists induces 5-HT$_{2C}$ receptor desensitization in the choroid plexus [7]. In cell lines transfected with the 5-HT$_{2C}$ receptor (CHO and A9 cells), acute agonist-mediated functional desensitization of the receptor involves activation of PKC [15, 46]. More recently, agonist-induced phosphorylation of the 5-HT$_{2C}$ receptor in transfected NIH-3T3 cells was found to parallel functional receptor desensitization [11].

Cell Proliferation

Persistent activation of rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors transfected into NIH-3T3 cells enhances cell proliferation and mediates oncogenic transformation [22, 23, 26]. However, in Balb/c-3T3 cells, activation of the endogenous 5-HT$_2$ receptors neither promoted nor inhibited DNA synthesis, the rate of cell division, or transformation even in the presence of 10% dialyzed FBS. This lack of modulation of cell growth could be due to the lower level of expression of endogenous 5-HT$_2$ receptors compared with transfected receptors in NIH-3T3 cells. Yet the endogenous receptors produce a robust calcium response that is greater than 50% of the magnitude produced by activation of transfected receptors in NIH-3T3 cells (data not shown). Although it is possible that the larger magnitude of response in transfected cells plays a role in inducing proliferation, an alternate hypothesis is that desensitization and down-regulation of the endogenous 5-HT$_2$ receptors prevents sustained stimulation that could mediate cell prolif-

eration in fibroblasts. A similar hypothesis has been explored for the muscarinic-m3 receptor in CHO-K1 cells [47] and neurokinin receptor (NK-2) in Rat-1 fibroblast cells [48]. In cells in which 5-HT induces proliferation (e.g. mesangial, endothelial or smooth muscle [6, 49–51]), it remains to be determined whether the 5-HT_2 receptors present are desensitized or down-regulated by prolonged 5-HT treatment. Desensitization of 5-HT_2 receptors could provide a convenient shut-off mechanism to restrict aberrant 5-HT-induced cell proliferation.

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