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Functional Serotonin-2B Receptors Are Expressed by a Teratocarcinoma-Derived Cell Line during Serotoninergic Differentiation

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SUMMARY

Among immortalized teratocarcinoma-derived cells, the clone 1C11 is a committed precursor of the neuronal lineage. On day 2 of its serotoninergic differentiation, this clone expresses only one subtype of serotonin [5-hydroxytryptamine (5-HT)] receptor, which is functionally coupled to phosphatidylinositol hydrolysis. The identity of these receptors was established by comparing their properties with those of 5-HT_{2B} receptors expressed by $LMTK^-$ fibroblasts stably transfected with the recently cloned murine cDNA NP75 (LM5 cells). In both cell types, the analysis of (±)-1-(2,5-dimethoxy-4-[^{125}I]iodophenyl)-2-aminopropane HCl ([^{125}I]DOI) binding revealed the presence of a single class of sites, the affinity of which was 1 order of magnitude lower than that reported for 5-HT_{2A} receptors. In 1C11 cells differentiated for 2 days, as well as in LM5 cells, DOI binding was decreased by nonhydrolyzable analogs of GTP, indicating that the 5-HT_{2B} receptor is functionally coupled to a G protein. The DOI-induced increase of phosphoinositide hydrolysis, which was correlated with both GTPase activity and binding data, is mediated by a G_q protein. This work demonstrates that the 5-HT_{2B} receptor is functionally expressed before complete serotoninergic differentiation of 1C11 cells. The inducible 1C11 clone thus provides an *in vitro* model to investigate the possible role of the 5-HT_{2B} receptor in the expression of the serotoninergic phenotype.

The monoamine serotonin (5-HT) modulates a wide variety of important behavioral and physiological processes in both the central and peripheral nervous systems, such as sleep, anxiety, cognition and memory, perception, sexual behavior, appetite, cardiovascular function, smooth muscle response, and gastrointestinal contraction (1). Such numerous functions mainly result from interactions between 5-HT and various cell membrane receptors throughout the body. On the basis of their affinities for 5-HT, serotonin receptors were first classified into 5-HT_1 (1–10 nM) and 5-HT_2 (~1 μM) subclasses. Binding data using more selective ligands led to the distinction of two additional subclasses, 5-HT_3 and 5-HT_4. The pharmacological properties of these four subclasses are now well established, and the cDNAs for all of these receptors have been cloned (2). Moreover, the conservation of large amino acid motifs within the sequences of these receptors allowed the cloning of additional subclasses, i.e., 5-HT_5 (3), 5-HT_6 (4), and 5-HT_7 (5). The cDNA-deduced primary amino acid structures revealed that, whereas 5-HT_3 receptors are ligand-gated channel receptors, all other subclasses belong to the superfamily of seven-transmembrane domain receptors that interact with G proteins. In contrast to the well documented 5-HT_1 subtypes (5-HT_{1A} to 5-HT_{1F}) (2), the occurrence of distinct members in the 5-HT_2 subclass remained controversial until recently. Radioligand binding data suggested the existence of either two states (6–8) or two distinct subtypes (9, 10) of the 5-HT_2 receptor.

We cloned a mouse cDNA (NP75) encoding one member of the 5-HT_2 family that is expressed in the cardiovascular system, the gut, and the developing mammalian brain (11). The cDNA-deduced primary amino acid sequence of this receptor shares a high degree of homology with both the 5-HT_{2F} receptor cloned from rat stomach fundus (12, 13) and its human counterpart (14–17). The protein encoded by the

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PLC-β, phospholipase C-β; Bt_2cAMP, dibutyryl-cAMP; CCA, cyclohexane carboxylic acid; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; DMEM, Dulbecco's modified Eagle medium; IP_3, inositol trisphosphate; NAS, *N*-acetylserotonin; 1C11*d2 cells, 1C11 cells 2 days after addition of inducers; PCR, polymerase chain reaction; RT, reverse transcription; FCS, fetal calf serum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin.

NP75 cDNA was consensually named 5-HT$_{2B}$ (2) (previously 5-HT$_{2C}$) (11). The two other members of the 5-HT$_2$ subclass are 5-HT$_{2A}$ (previously 5-HT$_2$) (18) and 5-HT$_{2C}$ (previously 5-HT$_{1C}$) (19). Both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have been shown to be coupled to PLC-β activation, Ca^{2+} mobilization, and opening of Ca^{2+}-activated K^+ channels (2). Because 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors are coupled to phosphoinositide breakdown, it is tempting to speculate that the same second messenger may be used by the third member of the subclass, 5-HT$_{2B}$. In addition, there are so far no clear indications as to which members of the G protein family are coupled to the new 5-HT$_{2B}$ receptor.

The clone 1C11 was derived from the multipotent mouse embryonal carcinoma F$_9$ cell line through transformation by a recombinant plasmid, pK$_4$, carrying the simian virus 40 early region under the control of the adenovirus 5 E1A promoter (20). 1C11 cells have features of immortalized committed precursor cells of neuronal or neuroendocrine lineage. When cultured in the presence of Bt$_2$cAMP and CCA, 1C11 cells extend bipolar extensions, express neuron-associated markers, and within 4 days acquire a complete serotoninergic phenotype, namely the ability to take up, store, synthesize, and catabolize 5-HT (21). The switch from the undifferentiated committed 1C11 cell type to the 1C11* serotoninergic cell type occurs in >90% of the cell population. The potential of 1C11 cells for proliferation and differentiation led us to investigate whether 5-HT receptors were also induced upon this switch. Compared with transfected cells, neuronal cell lines expressing receptors allow studies under conditions closer to *in vivo* physiological conditions, mostly in terms of stoichiometry and pathways of G protein coupling, which can provide estimates of ligand affinity and agonist efficacy (22). Moreover, because of the inducibility of the described cell line, the possible role of such 5-HT receptors, upon the expression of the neuronal phenotype, could also be investigated.

We report here that the immortalized, inducible, 1C11 cell line expresses, upon differentiation, 5-HT$_{2B}$ receptors functionally coupled to G proteins and phosphoinositide breakdown. The identity of these receptors was established by comparison with 5-HT$_{2B}$ receptors overexpressed on membranes of mouse LMTK$^-$ fibroblasts stably transfected with NP75 cDNA (LM5 cells).

Experimental Procedures

Materials. CCA and Bt$_2$cAMP were from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). All other chemicals were reagent grade, purchased from commercial sources. Ketanserin, ritanserin, and setoperone were kindly provided by Janssen (Beerse, Belgium). ICS 205–930 and MDL 72222 were gifts from Sandoz (Basel, Switzerland) and Merell-Dow (Strasbourg, France), respectively. Other neurochemicals were from Research Biochemicals (Natick, MA). [^{125}I]DOI (81.4 TBq/mmol), [^{3}H]8-OH-DPAT (5.89 TBq/mmol), [^{3}H]ketanserin (2.22 TBq/mmol), and [^{3}H]quipazine (2.96 TBq/mmol) were from DuPont-New England Nuclear. [^{3}H]CP-96,501 (1.11 TBq/mmol) was a gift from Pfizer (Lenexa, KS), and [^{3}H]mesulergine (2.63TBq/mmol) was from Amersham. The avian myeloblastosis virus reverse transcriptase and *Thermus aquaticus* polymerase were purchased from Pharmacia and Perkin-Elmer Cetus, respectively.

Cell culture. The 1C11 clone was grown and induced to differentiate in the presence of 1 mM Bt$_2$cAMP and 0.05% CCA (21). Experiments were performed on undifferentiated 1C11 cells and 1C11*d2 cells (5×10^3 cells/cm^2). When sodium butyrate (1–5 mM) was added instead of Bt$_2$cAMP, the 1C11 precursor cells did not differentiate along the serotoninergic pathway.

Determination of cellular content of biogenic amines and related enzyme and transport activities. As reported previously (21), unconjugated 5-HT, histamine, and catecholamines (dopamine, norepinephrine, and epinephrine), as well as tryptophan hydroxylase (EC 1.14.16.4), tyrosine hydroxylase (EC 1.14.16.2), aromatic L-amino acid decarboxylase (EC 4.1.1.28), dopamine β-hydroxylase (EC 1.14.17.1), and monoamine oxidase (EC 1.4.3.4) type A and B activities, were measured radioenzymatically. Dopamine, norepinephrine, and 5-HT uptake experiments were also performed as reported previously (21).

Transfection and stable expression of the mouse 5-HT$_{2B}$ receptor. The NP75 cDNA was ligated into the *Eco*RI site of the mammalian expression vector pSG5 (11). After calcium phosphate co-transfection (23) of the fibroblast mouse cell line LMTK$^-$ with this plasmid together with PY3, carrying the gene for resistance to hygromycin (24) and antibiotic selection, clones were obtained by limiting dilution and LM5 was selected.

Membrane preparation. To prepare crude membranes for binding assays, 1C11, 1C11*d2, LMTK$^-$, and LM5 cells were washed twice with cold PBS and then harvested, with a rubber policeman, in 1.5 ml of PBS containing 1 μg/ml pepstatin, 1 μg/ml antipain, 15 μg/ml benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation, the resulting pellet was frozen at −70° before homogenization. The frozen cell pellet was thawed at 37°, resuspended in 10 ml of cold 4 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole buffer, pH 7.30, and centrifuged for 10 min at 5000 × g. The supernatant obtained from this centrifugation was collected, poured onto a 20% sucrose cushion, and then centrifuged for 90 min at 100,000 × g. The membrane-containing pellet was resuspended in 75 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole buffer, pH 7.30, for use in binding assays. Protein contents were determined using the bicinchoninic acid protein assay (Pierce, Chichester, UK).

Radioligand binding experiments. Bindings were performed either with intact cells or with crude membranes. For intact-cell experiments, cells were washed twice with DMEM. Binding experiments were performed at room temperature, with shaking. Assays were initiated by the addition of 100 μl of FCS-free DMEM containing either 0.1–10 nM [^{125}I]DOI, 0.5 nM [^{3}H]8-OH-DPAT, 0.3 nM [^{3}H]CP-96,501, 1 nM [^{3}H]mesulergine, 1 nM [^{3}H]ketanserin, or 1 nM [^{3}H]quipazine, with appropriate competing ligands. After a 30-min incubation, cells were washed twice with cold PBS and 2 ml of 1 N $HClO_4$ were added, 500 μl of which were counted for their radioactive content. For membrane experiments, the binding was initiated by the addition of 50 μl of 50 mM Tris buffer, pH 7.40, containing 0.1–10 nM [^{125}I]DOI and appropriate competing ligands, to 50 μl of membranes (20 μg of protein). A 30-min incubation period was followed by the addition of 5 ml of ice-cold 10 mM Tris buffer, pH 7.40. Samples were filtered on polyethyleneimine-treated filters (25) and counted. The specific binding was defined as the binding that was inhibited by 1 μM levels of either unlabeled ketanserin or unlabeled DOI.

Determination of GTPase activities. GTPase activities were measured by monitoring the release of [^{32}P]P_i from [γ-^{32}P]GTP as described previously (26), with minor modifications. The incubation medium (final volume, 100 μl) contained 10 mM $MgCl_2$, 5 mM phosphocreatine, 70 μl of creatine kinase, 0.2 mM ATP, 0.1 mM EGTA, 1 mg/ml bovine serum albumin, 50 mM Tris·HCl, pH 7.40, and the appropriate drugs. The reaction was initiated by addition of membranes. Test tubes were incubated in a water bath for 2 min at 20°. Unless otherwise indicated, 0.15 μM [γ-^{32}P]GTP was added for an additional 3-min incubation. The reaction was terminated by addition of 500 μl of cold 50 mM KH_2PO_4 buffer, pH 7.40, containing 5% (w/v) Norit-A charcoal. The tubes were vortex-mixed and centrifuged

at 2500 × *g* for 10 min, and 300 μl of supernatant were taken for β-scintillation counting. Reactions were performed in quadriplicate with blanks containing no added membranes. [^{32}P]P_i released from [γ-^{32}P]GTP in the absence of membranes represented 0.7–2% of the added [γ-^{32}P]GTP. GTPase activity is expressed as femtomoles of GTP hydrolyzed per milligram of protein per minute.

Extensively purified cholera and pertussis toxins (generous gifts from Pr. J. E. Alouf, Institut Pasteur, Paris, France) were added at a concentration (13 μg/ml) known to ensure complete ADP-ribosylation of their endogenous substrates $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha o}$. Polyclonal antisera raised against peptide sequences of $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q/11}$ (27), used at a dilution of 1/1000, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and NEN.

Determination of endogenous IP_3 levels. Cells were washed twice and incubated for various times with agonists and antagonists in FCS-free DMEM. At the end of the incubations, cells were washed twice in cold PBS and scraped with a rubber policeman. After centrifugation, 100 μl of PBS were added to the cell pellet and adjusted to 250 μl with 50 mM Tris·HCl, pH 7.40. Then, after addition of 250 μl of cold 1 N $HClO_4$, the mixture was incubated for 10 min at 4°.

Determination of endogenous cAMP levels. Cells were washed twice in FCS-free DMEM and incubated for 15 min at 37° with 100 μM isobutylmethylxanthine and test agents. The reaction was stopped by aspiration of the medium, followed by addition of 500 μl of ice-cold 95% ethanol/5% formic acid (1:1, v/v). After 1 hr at 4°, the ethanol phase was collected and lyophilized. cAMP was quantified using a iodinated radioimmunoassay kit (Pasteur Diagnostics, Paris, France). Despite the addition of 1 mM Bt_2cAMP to the 1C11 growth medium, the basal cAMP level was about the same in 1C11*d2, LM5, and mesodermal C1 cells (28) (about 300 pmol/mg of protein). 7β-[γ-(Morpholino)butyryl]forskolin at 1 μM typically yielded a 10-fold increase in cAMP levels.

Data analysis and statistics. Binding data were analyzed using the iterative nonlinear fitting software LIGAND 3.0 (29). Nonparametric statistical tests (30) were used. The chosen significance criterion was $p < 0.05$. All values are given as arithmetic means ± standard errors.

PCR experiments. A previously described classical procedure (11) was used for RNA extraction. For quantitative RT-PCR experiments, a standard 100-μl PCR buffer was used in the presence of 10 μg of total RNA. After denaturation, avian myeloblastosis virus reverse transcriptase (13 units) and *Thermus aquaticus* polymerase (5 units) were added, extension was performed at 50 ° for 15 min, and then a standard PCR protocol (31) was used. Samples were taken after 20, 25, and 30 cycles to ensure that the reaction was in the exponential phase of synthesis. We used, as an internal standard, primers corresponding to the mRNA of the mouse ribosomal elongation factor EF1A, amplified in the same reaction as the $5\text{-}HT_{2B}$ primers. All primers are located in different exons (11), to discriminate between DNA and RNA amplification.

Results

Characterization of the $5\text{-}HT_{2B}$ receptor binding evidenced in 1C11*d2 cells. After 2 days of induction with Bt_2cAMP and CCA, 1C11*d2 cells are already engaged in serotoninergic differentiation, because they have acquired the ability to synthesize and catabolize 5-HT in a specific manner (Table 1). However, the absence of any detectable 5-HT uptake (Table 1) shows that, compared with 1C11 cells induced for 4 days (17), 1C11*d2 cells are not yet fully differentiated. Radioligand binding studies were then carried out to detect 5-HT receptors on 1C11 cells during their serotoninergic differentiation. The absence of any detectable binding with 0.5 nM [^{3}H]8-OH-DPAT, 0.3 nM [^{3}H]CP-96,501, 1 nM [^{3}H]ketanserin, 1 nM [^{3}H]mesulergine, or 1 nM [^{3}H]quipazine

TABLE 1

Phenotype of undifferentiated 1C11 cells and 1C11*d2 cells

Values are means ± standard errors of five experiments.

Properties	1C11 cells	1C11*d2 cells
Bioamine contents (pmol/mg of protein)		
Histamine	<0.05	<0.05
Dopamine, norepinephrine, epinephrine	<0.05	<0.05
5-HT	8 ± 4	155 ± 12
Enzyme activities (pmol/mg of protein/min)		
Tyrosine hydroxylase	<0.3	<0.3
Tryptophan hydroxylase	<0.3	5 ± 1
Aromatic amino acid decarboxylase	5 ± 2	11 ± 2
Dopamine β-hydroxylase	<0.1	<0.1
Monoamine oxidase type A	<0.07	<0.07
Monoamine oxidase type B	<0.08	29 ± 2
Uptake (fmol/mg of protein/min)		
Dopamine, norepinephrine	<2	<2
5-HT	<2	<2
Binding (fmol/mg of protein)		
[^{3}H]8-OH-DPAT (0.5 nM)	<0.1	<0.1
[^{3}H]CP-96,501 (0.3 nM)	<0.1	<0.1
[^{3}H]Mesulergine (1 nM)	<0.1	<0.1
[^{3}H]Ketanserin (1 nM)	<0.1	<0.1
[^{125}I]DOI (1 nM)	<0.1	3.5 ± 0.8
[^{3}H]Quipazine (1 nM)	<0.1	<0.1

excluded the presence on 1C11*d2 cells of $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$, $5\text{-}HT_{2C}$, and $5\text{-}HT_3$ binding sites, respectively. The only positive binding (range of specific binding, 60–75%) was observed for a radiolabeled form of DOI (Table 1), one of the most potent $5\text{-}HT_2$ agonists. Steady state binding assays were thus performed with this ligand. In contrast to undifferentiated 1C11 cells, intact 1C11*d2 cells bound [^{125}I]DOI in a saturable manner with high affinity ($k_{+1} = 0.133 \times 10^7$ M^{-1} min^{-1}, $k_{-1} = 0.032$ min^{-1}) (Fig. 1a). The saturation of the DOI binding sites occurred above 40 nM [^{125}I]DOI (predicted K_d = 24 nM). The bound radioligand could be removed by further addition of 1 μM levels of either ketanserin or unlabeled DOI. The Rosenthal transformation of the data resulted in a linear Scatchard plot (Fig. 1b), indicating only one class of DOI binding sites, with an apparent B_{max} of 4.6 fmol of bound DOI/10^6 cells (2800 sites/cell) and an apparent K_d of 21.9 ± 2.3 nM (five experiments). This K_d value is

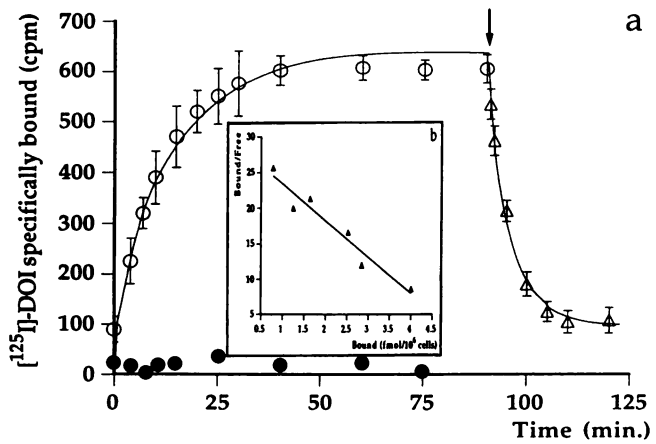

Fig. 1. Saturation (a) and Scatchard (b) plots of [^{125}I]DOI binding to undifferentiated 1C11 cells (●) and 1C11*d2 cells (○). Either unlabeled ketanserin (*vertical arrow*, △) (a) or unlabeled DOI (b) at 1 μM was used to define nonspecific binding. Data shown are means ± standard errors of five independent experiments.

significantly higher than that measured for the 5-HT$_{2A}$ receptor (K_d = 2.2 nM) (32) but is very close to those observed for mouse 5-HT$_{2B}$ receptor-transfected COS (K_d = 25.6 ± 0.5 nM) (11) and LM5 (K_d = 23.4 ± 0.3 nM, B_{max} = 224 ± 8 fmol/mg of protein) cells. The DOI binding sites present on 1C11*d2 cell membranes were further characterized through competition studies. Inhibition of [^{125}I]DOI binding by various drugs resulted in monophasic curves, which were best fit by a one-site model of binding (Fig. 2). Comparisons of the deduced inhibition constants (K_i values) (Table 2) with those obtained for LM5 cells or brain 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors (33) are shown in Fig. 3. Using the nonparametric Spearman test, a highly significant correlation was apparent between the K_i values calculated for 1C11*d2 and LM5 cells (r_s = 0.9571, $p < 0.0005$) (Fig. 3a). With either pig choroid plexus 5-HT$_{2C}$ (r_s = 0.5238, $p < 0.05$) (Fig. 3c) or mouse cortex 5-HT$_{2A}$ (r_s = 0.7429, $p < 0.01$) (Fig. 3b) K_i values, correlations were at least 1 order of magnitude less significant. Among the molecules tested, ritanserin exhibited the greatest affinity for the 5-HT$_{2B}$ DOI binding site and NAS was the molecule that discriminated best between 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors.

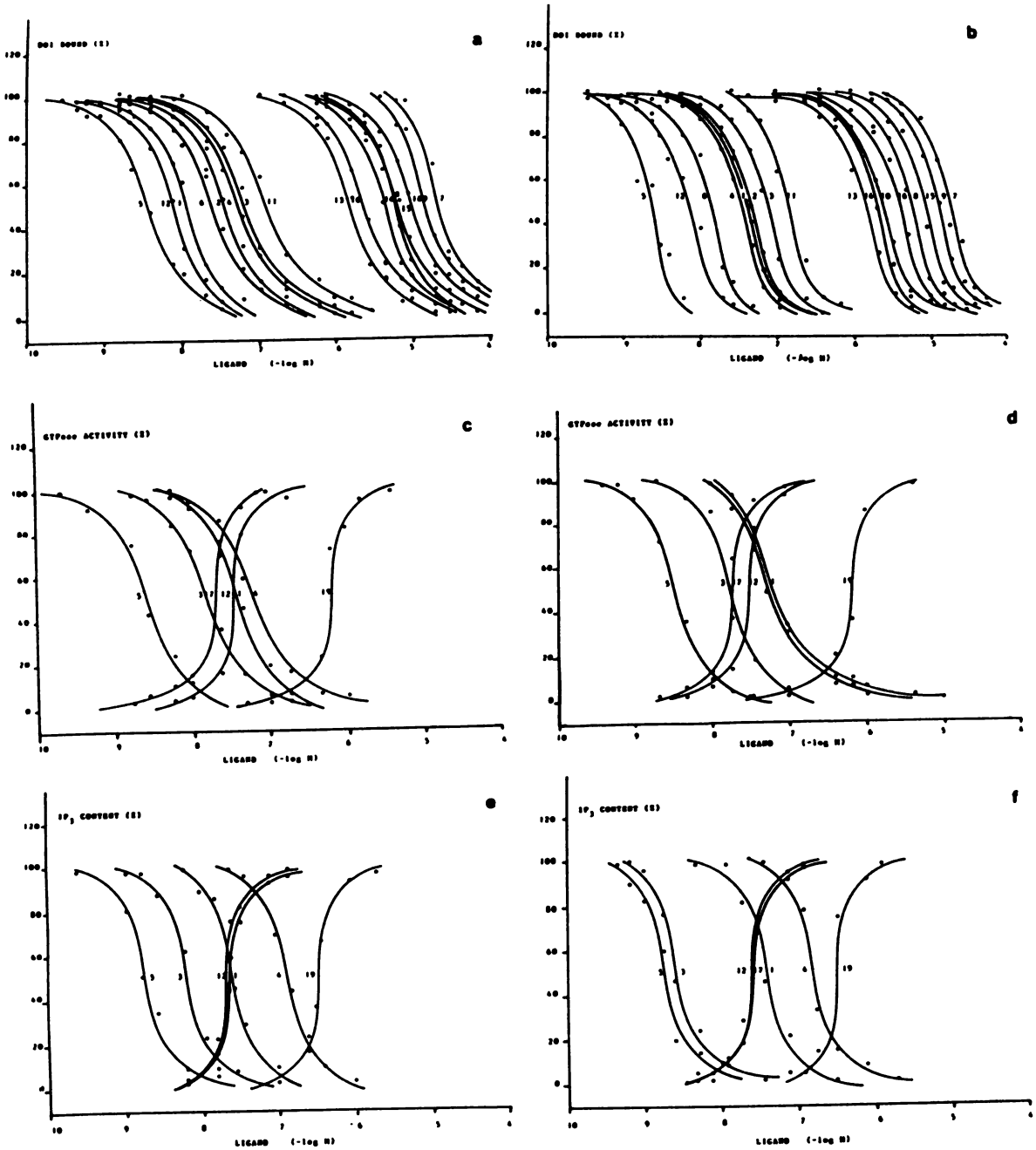

Fig. 2. Displacement of bound [^{125}I]DOI (a and b), stimulation and inhibition of GTPase activity (c and d), and IP$_3$ production (e and f) for 1C11*d2 cells (a, c, and e) and LM5 cells (b, d, and f). The results are from a typical experiment performed in triplicate. This figure depicts the effects at the indicated concentrations of competing drugs (**1**, methysergide; **2**, spiperone; **3**, setoperone; **4**, ketanserin; **5**, ritanserin; **6**, cyproheptadine; **7**, MDL 72222; **8**, quipazine; **9**, *N,N'*-dimethyl-5-methoxytryptamine; **10**, 1-methylserotonin; **11**, tryptamine; **12**, NAS; **13**, serotonin; **14**, 8-OH-DPAT; **15**, 2-methylserotonin; **16**, ICS 205–930; **17**, DOI; **19**, α-methylserotonin.

TABLE 2
Pharmacology of the mouse 5-HT$_{2B}$ receptors in LM5 cells and in 1C11*d2 cells

	5-HT$_{2B}$ DOI binding, pK_i		IP$_3$ production, pEC$_{50}$ or pK_i		GTPase activity, pEC$_{50}$ or pK_i	
	LM5	1C11*d2	LM5	1C11*d2	LM5	1C11*d2
1 Methysergide	7.86	7.36	7.56	7.35	7.35	7.22
2 Spiperone	7.38	7.32				
3 Setoperone	7.16	7.18	8.17	8.59	7.78	7.72
4 Ketanserin	7.24	7.41	6.83	6.75	7.20	7.26
5 Ritanserin	8.39	8.62	8.73	8.68	8.54	8.47
6 Cyproheptadine	7.58	7.81				
7 MDL 72222	4.67	4.78				
8 Quipazine	5.20	5.21				
9 *N,N'*-Dimethyl-5-methoxytryptamine	4.86	4.91				
10 1-Methylserotonin	5.63	5.62				
11 Tryptamine	6.87	6.83				
12 NAS	8.05	8.12	7.68	7.59	7.44	7.52
13 5-HT	5.76	5.80				
14 8-OH-DPAT	5.28	5.63				
15 2-Methylserotonin	5.20	5.11				
16 ICS 205–930	4.98	5.33				
17 DOI			7.63	7.58	7.72	7.68
19 α-Methylserotonin			6.49	6.42	6.20	6.17
Chlorpromazine	<4.0	<4.0				
Histamine	<4.0	<4.0				

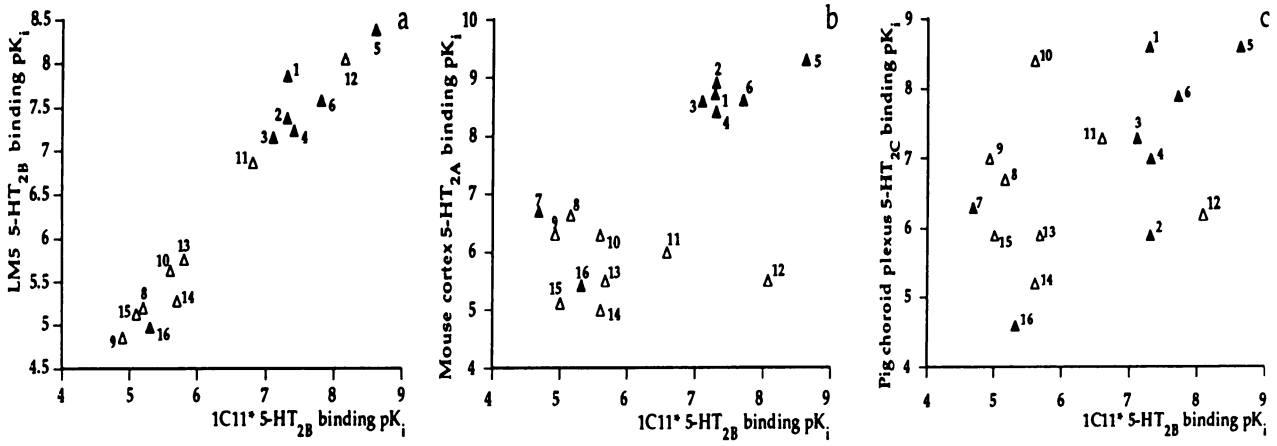

Fig. 3. Comparison of competition experiments for [^{125}I]DOI binding to 1C11*d2 cells and either [^{125}I]DOI binding to LM5 cells (a), [^{3}H]ketanserin binding to mouse cortex 5-HT$_{2A}$ receptors (29) (b), or [^{3}H]mesulergine binding to pig choroid plexus 5-HT$_{2C}$ receptors (29) (c). Reported values are the means of three independent experiments performed in triplicate. Eight to 10 different concentrations of each competing agonist (△) (**8**, quipazine; **9**, *N,N'*-dimethyl-5-methoxytryptamine; **10**, 1-methylserotonin; **11**, tryptamine; **12**, NAS; **13**, serotonin; **14**, 8-OH-DPAT; **15**, 2-methylserotonin) or antagonist (▲) (**1**, methysergide; **2**, spiperone; **3**, setoperone; **4**, ketanserin; **5**, ritanserin; **6**, cyproheptadine; **7**, MDL 72222; **16**, ICS 205–930) were used.

Coupling of the 5-HT$_{2B}$ receptor to G protein(s). A functional seven-transmembrane domain receptor, such as 5-HT$_{2B}$ (11), signals to cellular enzymes and/or ion channels through G protein heterotrimer(s), $G_{\alpha\beta\gamma}$. The addition of a nonhydrolyzable GTP analog that binds irreversibly to α subunits induces the loss of the G protein transition state and weakens agonist binding (34). Therefore, as a first step in assessing the functionality of the 1C11*d2 5-HT$_{2B}$ receptor, the effect of nonhydrolyzable GTP analogs on [^{125}I]DOI binding was studied. Compared with a basal value of 100%, corresponding to 4.6 and 211 fmol/mg of protein for 1C11*d2 and LM5 cells, respectively, the addition of 10 μM Gpp(NH)p or GTPγs reduced by about 90% [^{125}I]DOI binding in both cell lines [Gpp(NH)p, 8.3 ± 1.6% and 8.1 ± 1.8%; GTPγS, 6.7 ± 1.7% and 6.7 ± 2.2% for 1C11*d2 and LM5 cells, respectively; mean ± standard error, three experiments]. These data strongly suggest that the DOI binding sites expressed by 1C11*d2 and LM5 cells are coupled to G proteins.

Stimulation of the cell membrane GTPase activity by 5-HT$_2$ receptor agonists and antagonists. NAS caused a time-dependent and saturable (nearly 20 times the activity in the absence of agonist) stimulation of GTPase activity in 1C11*d2 cell membranes (Fig. 4, *left*). The Hill plot (not shown) of the dose-response curve for the NAS-induced GTPase stimulation indicated that the activation occurred through a first-order process, consistent with the expression of a single population of 5-HT$_{2B}$ receptors on the membranes of 1C11*d2 cells. Two other 5-HT$_2$ agonists, DOI and α-methylserotonin, were also able to increase the GTPase activities of both 1C11*d2 and LM5 cell membranes (Fig. 4, *right*). These stimulations and the abilities of some 5-HT receptor antagonists to reduce the GTPase stimulation induced by 100

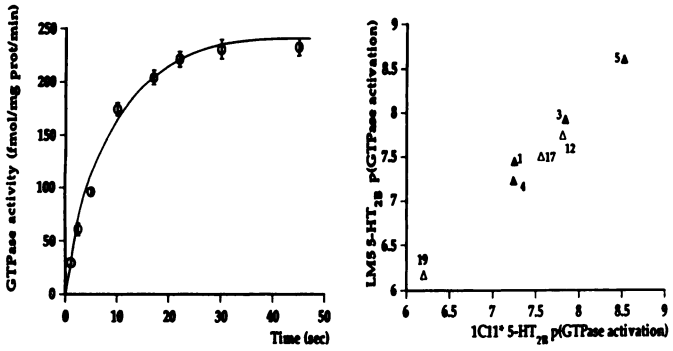

Fig. 4. Time-dependent stimulation of GTPase activity in 1C11*d2 cells by 100 nM NAS (*left*) and comparison of the agonist (**12**, NAS; **17**, DOI; **19**, α-methylserotonin)-induced stimulation of GTPase activity (△) and the antagonist (**1**, methysergide; **3**, setoperone; **4**, ketanserin; **5**, ritanserin)-dependent inhibition of GTPase activity stimulated by 100 nM DOI (▲) in 1C11*d2 cells and LM5 cells (*right*). The pEC$_{50}$ and pK_i values shown were calculated by nonlinear regression analysis from data obtained with six to eight different concentrations of each competing drug and are the mean of three experiments performed in quadruplicate.

nM DOI in membrane preparations from either 1C11*d2 or LM5 cells appeared to be correlated ($r_s = 0.9825$, $p < 0.05$) (Fig. 4, *right*).

Characterization of the G protein coupled to the 5-HT$_{2B}$ receptor. The addition to 1C11*d2 and LM5 cell membranes of either cholera toxin or pertussis toxin, which ADP-ribosylate the G_s and G_i/G_o α subunits, respectively, had no effect on DOI-induced GTPase stimulation (Table 3). Equally, preincubation with anti-$G_{\alpha i}$ or anti-$G_{\alpha s}$ antibodies had no effect. However, a 15-min preincubation of either 1C11*d2 or LM5 cell membranes with a polyclonal antibody raised against a peptide sequence of $G_{\alpha q/11}$ (27) markedly reduced the DOI-induced GTPase stimulation. These results clearly indicate that neither G_s nor G_i/G_o is involved in the functional coupling of the 5-HT$_{2B}$ receptor, and they strongly favor the involvement of a G_q protein in both cell lines.

Functional coupling to phosphoinositide hydrolysis. Its structural homology to both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors suggests that the 5-HT$_{2B}$ receptor might be functionally coupled to PLC-β. Indeed, the addition of the agonist DOI triggered a time-dependent and saturable accumulation of IP$_3$ by 1C11*d2 cells (Fig. 5a). The DOI concentration eliciting a half-maximal response was 27.1 ± 3.4 nM (four experiments).

TABLE 3

Effects of toxins and antibodies raised against peptide sequences of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ on DOI-induced cellular GTPase activity of 1C11*d2 cells and LM5 cells

Values are means ± standard errors of four experiments (100% = 121 and 1920 fmol/mg of protein/min for 1C11*d2 and LM5 cells, respectively).

	DOI-induced cellular GTPase activity	
	1C11*d2 cells	LM5 cells
	%	
Basal values	100	100
+Cholera toxin	93.8 ± 6.7	97.0 ± 2.8
+Pertussis toxin	84.7 ± 7.0	86.4 ± 9.4
+Nonimmune serum	92.1 ± 2.6	98.4 ± 3.2
+Anti-$G_{\alpha i}$ antibodies	95.3 ± 7.2	91.6 ± 2.9
+Anti-$G_{\alpha q}$ antibodies	2.3 ± 0.7	2.7 ± 0.8
+Anti-$G_{\alpha s}$ antibodies	92.5 ± 6.3	87.4 ± 5.1

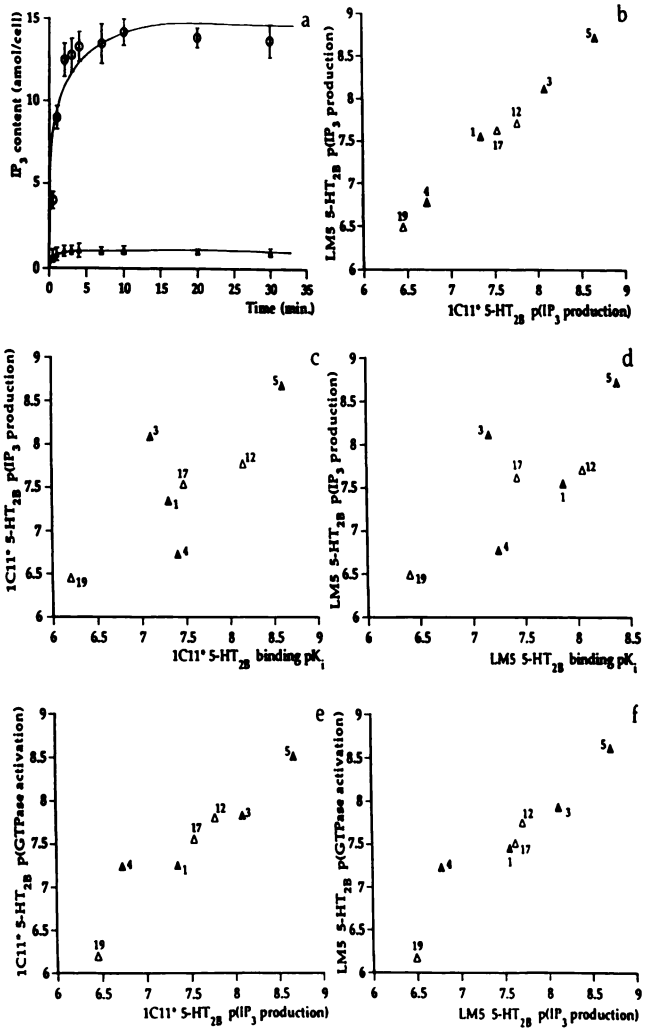

Fig. 5. Transduction pathway of the 5-HT$_{2B}$ receptors on 1C11*d2 cells and LM5 cells (four experiments). a, ○, Time-dependent stimulation of IP$_3$ production in 1C11*d2 cells by 100 nM DOI; ▲, basal values. b, Comparison of the agonist (**12**, NAS; **17**, DOI; **19**, α-methylserotonin)-induced stimulation of IP$_3$ production (△) and the antagonist (**1**, methysergide; **3**, setoperone; **4**, ketanserin; **5**, ritanserin)-dependent inhibition of IP$_3$ production stimulated by 100 nM DOI (▲) in 1C11*d2 and LM5 cells. The pEC$_{50}$ and pK_i values shown were calculated by nonlinear regression analysis from the data obtained with six to eight different concentrations of each competing drug and are the mean of three experiments performed in triplicate. c and d, Comparisons of agonist and antagonist effects on IP$_3$ production and [^{125}I]DOI binding in 1C11*d2 (c) and LM5 (d) cells. e and f, Comparisons of agonist and antagonist effects on IP$_3$ production and cell membrane GTPase activity in 1C11*d2 (e) and LM5 (f) cells.

A significant correlation ($r_s = 0.9852$, $p < 0.01$) was found between the effects (expressed as pEC$_{50}$ or pK_i values) (Table 2) of various drugs upon IP$_3$ production in 1C11*d2 and LM5 cells and their binding (Fig. 5b). This was also true for the pEC$_{50}$ or pK_i values for the effects of many 5-HT agonists and antagonists on IP$_3$ production and the values deduced from the 5-HT$_{2B}$ binding data [$r_s = 0.9643$, $p < 0.05$ for both 1C11*d2 (Fig. 5c) and LM5 (Fig. 5d) cells], with the exception of setoperone, one of the more specific 5-HT$_2$ antagonists (35), and, to a lesser extent, ketanserin, probably due to its complex pharmacological profile (36). The coupling of the 5-HT$_{2B}$ receptor to PLC-β by $G_{\alpha q}$ was finally assessed by the significant correlations found for both cell lines between the pEC$_{50}$

or pK_i values observed for the most discriminative drugs for IP_3 production and GTPase activation [r_s = 0.9650 and 0.9825 for 1C11*d2 (Fig. 5e) and LM5 (Fig. 5f) cells, respectively; $p < 0.05$ in both cases].

The NP75 cDNA encodes a protein containing both protein kinase C consensus phosphorylation sequences and several consensus sequences indicative of phosphorylation by protein kinase A. We therefore tested for agonist-induced stimulation of adenylate cyclase and for agonist-induced inhibition of 7β-[γ-(morpholino)butyryl]forskolin-induced activation of adenylate cyclase in both 1C11*d2 and LM5 cells. The cAMP levels of these two cell lines remained insensitive to the addition of agonists (data not shown), indicating that the 5-HT_{2B} receptor is actually coupled to phosphatidylinositol hydrolysis, without any apparent activation or inhibition of adenylate cyclase.

Expression of endogenous 5-HT_{2B} receptor transcripts in 1C11 cells. Amplimers specific for the 5-HT_{2B} receptor were used to amplify, by PCR, cDNA generated by RT of RNA from 1C11 cells. A PCR product of the expected size (900 base pairs) was obtained with RNA from 1C11*d2 cells, as well as with RNA derived from undifferentiated 1C11 cells (Fig. 6). Compared with the internal standard, the amounts of 5-HT_{2B} transcripts appeared similar in the two cases. Moreover, the same PCR product was also detected in 8.5-day mouse embryos (Fig. 6), confirming the early *in vivo* expression of the 5-HT_{2B} transcript.

Discussion

A clonal cell line that expresses at least a well characterized 5-HT receptor subtype is a prerequisite for identifying the pathway by which 5-HT exerts its physiological effects. Compared with transfected cells, which allow the dissection of receptor and transducer systems, cell lines that express receptors upon differentiation may address the problem of combining these findings into a new model, especially for receptor activation and agonist efficacy (22). Among the different immortalized teratocarcinoma-derived cells that have the properties of committed cells able to further differentiate along a restricted lineage (20, 28, 37), the 1C11 clone could be described as a serotoninergic cell line (21) after a 4-day induction with Bt_2cAMP and CCA. Two days after the addition of the inducers, 1C11*d2 cells are already engaged in the serotoninergic pathway but are not yet fully differentiated, because 5-HT uptake is not detectable whereas, in addition to a much higher 5-HT content, tryptophan hydroxylase and monoamine oxidase-B activities and [^{125}I]DOI binding sites have already become measurable, in contrast to the progenitor 1C11 cells (Table 1).

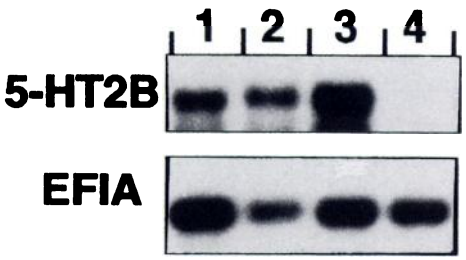

Fig. 6. RT-PCR analysis of 5-HT_{2B} expression. PCR experiments were performed with total RNA, using NP75- and EF1A-specific amplimers located in different exons to discriminate between DNA and RNA amplification. PCR products were detected by hybridization to ^{32}P-labeled primers different from the amplimers, after 30 cycles for 5-HT_{2B} (*5-HT2B*) or 20 cycles for EF1A (*EF1A*). *Lane 1*, result obtained for a 8.5-day mouse embryo; *lane 2*, undifferentiated 1C11 cells; *lane 3*, 1C11*d2 cells; *lane 4*, untransfected LMTK$^-$ cells.

The apparent DOI affinity is, however, too low to correspond to the canonical 5-HT_{2A} receptor (Fig. 1). Instead, the receptor present in 1C11*d2 cell membranes appears pharmacologically and functionally identical to the 5-HT_{2B} receptor encoded by the NP75 cDNA (11) and stably transfected in fibroblast LMTK$^-$ cells (yielding LM5 cells). The most significant results are (i) very similar K_d values for DOI, (ii) strikingly similar behaviors of agonists and antagonists in 1C11*d2 and LM5 cells upon [^{125}I]DOI binding, (iii) a pharmacological profile clearly distinct from those of 5-HT_{2A} and 5-HT_{2C} receptors, and (iv) functional coupling of the 5-HT_{2B} receptor, through a G_q protein, to phosphoinositide breakdown in both 1C11*d2 and LM5 cells. Incubation with various agonists led to increased cell IP_3 contents in both cases, and significant correlations were found between the relative potencies of various serotoninergic agents to affect the cell membrane GTPase activity and the cellular IP_3 production. These results confirm the functional link between G_q and PLC-β (38).

The present work therefore clearly establishes that the first 5-HT receptor appearing during the serotoninergic differentiation of the teratocarcinoma-derived 1C11 cell line belongs to the 5-HT_{2B} receptor subtype. The almost complete inhibition of agonist binding by nonhydrolyzable GTP analogs, including undetectable low affinity binding sites, might be due to the relatively small number of 5-HT_{2B} receptors expressed by 1C11*d2 cells. Another possible explanation, which remains to be explored, might be the atypical intrinsic activity that presently remains unique to the 5-HT_2 family of 5-HT receptors (39).

The signaling pathways activated by neurotransmitter receptors may modulate neuronal responses and regulate cell growth and differentiation (40). PLC activation and IP_3 production have been shown to be key components in triggering cell proliferation in response to neurotransmitters such as serotonin (40) or growth factors (41). Furthermore, it has been shown that some G protein-coupled receptors are able to potentiate the effect of growth factors (42). The expression of 5-HT_{2B} receptors in neural crest and heart regions (11, 43), where serotonin has been shown to be actively present (44), in early embryogenesis suggests that this receptor may have an autocrine function during the *in vivo* differentiation of serotoninergic neurons. Similarly, the 5-HT_{2B} receptor, detected as soon as day 2 of the 1C11 cell differentiation, may modulate the serotoninergic differentiation of 1C11 cells.

The precise mechanisms leading to the onset of a functional 5-HT_{2B} receptor during 1C11 serotoninergic differentiation remain to be understood. Both the 5-HT_{2B} receptor and its corresponding mRNA are expressed in 1C11*d2 cells, but the 5-HT_{2B} receptor mRNA was also detected in undifferentiated 1C11 cells, for which no DOI binding was detected (Table 1). These experiments, performed on total RNA, do not take into account the shortening of mRNAs, which affects the efficiency of mRNA translation (45). Even so, the presence of the 5-HT_{2B} receptor mRNA in both 1C11 and 1C11*d2 cells agrees with (i) the recent report showing that *N*-methyl-D-aspartate receptor type 1 mRNA is present in

both nerve growth factor-differentiated and undifferentiated rat pheochromocytoma PC-12 cells, whereas receptor protein is absent (46), and (ii) the data of Anderson and co-workers (47) showing that primary cultures of sympatho-adrenergic progenitors express mRNAs for several neuronal markers before their final cellular fate is chosen. We cannot exclude the possibility that the expression of the functional 5-HT$_{2B}$ receptor results from post-translational stabilization of the protein, although, as for the β_2-adrenergic receptor, the major impact of cAMP appears to be a long term modulation at the level of receptor mRNA (48).

Nevertheless, the primary effect of Bt$_2$cAMP on 1C11 cell differentiation remains to be understood. One highly probable effect of cAMP involves phosphorylation events. Direct phosphorylation at the receptor level seems unlikely, because the onset kinetics of serotoninergic markers take several days to be completed, but it should now be possible to examine whether 5-HT$_{2B}$ receptors participate in the processes that drive serotoninergic differentiation. More likely, the cAMP-dependent phosphorylation events may modify cellular effectors, such as transcription factors or cellular regulators, leading to the specific onset of the serotoninergic phenotype. In conclusion, the data reported here and previously (21) clearly show that 1C11 cells are differentiating cells that follow precise schedules, with ordered molecular switches, and specific pathways. The 1C11 cell line is therefore a valid *in vitro* model (i) to identify the intracellular target molecules related to 5-HT$_{2B}$ receptor activation and (ii) to further study the regulation of 5-HT$_{2B}$ receptor expression in serotoninergic differentiation.

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