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Sequential onset of three 5-HT receptors during the 5-hydroxytryptaminergic differentiation of the murine 1C11 cell line

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1 The murine 1C11 clone, which derives from a multipotential embryonal carcinoma cell line, has the features of a neuroectodermal precursor. When cultured in the presence of dibutyryl cyclic AMP, the 1C11 cells extend bipolar extensions and express neurone-associated markers. After 4 days, the resulting cells have acquired the ability to synthesize, take up, store and catabolize 5-hydroxytryptamine (5-HT). We have thus investigated the presence of 5-HT receptors during the 5-hydroxytryptaminergic differentiation of this inducible 1C11 cell line.

2 As shown by the binding of [^{125}I]-GTI and the CGS 12066-dependent inhibition of the forskolin-induced cyclic AMP production, functional 5-$HT_{1B/1D}$ receptors become expressed on day 2 of 1C11 cell differentiation. The density of these receptors remained unchanged until day 4.

3 The same holds true for the 5-HT_{2B} receptor, also identified by its pharmacological profile and its positive coupling to the phosphoinositide cascade.

4 On day 4 of 1C11 cell differentiation, a third 5-HT receptor, pharmacologically and functionally similar to 5-HT_{2A}, had become induced.

5 Strikingly, the amounts of each transcript encoding 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} receptor did not vary significantly during the time course of the 1C11 5-hydroxytryptaminergic differentiation.

6 The clone 1C11 may thus provide a useful *in vitro* model for studying regulation(s) between multiple G-linked receptors as well as the possible role of 5-HT upon the expression of a complete 5-hydroxytryptamine phenotype.

Keywords: 5-$HT_{1B/1D}$, 5-HT_{2A}, 5-HT_{2B} receptors; 5-hydroxytryptaminergic differentiation; 1C11 cells

Introduction

Numerous physiological, behavioral and cognitive functions involve interactions between the biogenic amine 5-hydroxytryptamine (5-HT) and specific receptors on the membrane of both neurones and non-neuronal cells (Wilkinson & Dourish, 1991). 5-HT receptors are at present divided into 7 classes, based upon their pharmacological profiles, cDNA-deduced primary sequences and signal transduction mechanisms (Hoyer *et al.*, 1994). With the exception of the ligand-gated channel 5-HT_3 receptor, all 5-HT receptors belong to the super-family of G-protein coupled receptors containing a predicted seven-transmembrane domain structure. The 5-HT_1 receptor class is subdivided into the 5-HT_{1A} (Fargin *et al.*, 1988), 5-HT_{1B} (5-$HT_{1D\beta}$) (Voigt *et al.*, 1991; Adham *et al.*, (1992); Bruinvels *et al.*, 1992; Hamblin *et al*, (1992); Maroteaux *et al*, (1992)), 5-HT_{1D} (5-$HT_{1D\alpha}$) (Weinshank *et al.*, 1992), 5-ht_{1E} (Zgombick *et al.*, 1992) and 5-ht_{1F} (Adham *et al.*, 1993) subtypes, all of which are negatively coupled to adenylate cyclase activity. Three 5-HT_2 receptor subtypes, each coupled to phosphatidylinositol-4,5-biphosphate hydrolysis, are at present defined: the 5-HT_{2A} (Pritchett *et al.*, 1988), 5-HT_{2C} (previously 5-HT_{1C}, Julius *et al.*, 1988), and 5-HT_{2B} (Foguet *et al.*, 1992; Kursar *et al.*, 1992) receptors. The 5-HT_4 (Gerald *et al.*, 1995), 5-ht_6 (Monsma *et al.*, 1993), and 5-ht_7 (Tsou *et al.*, 1994) receptors are positively coupled to adenylate cyclase. The transductional characteristics of the 5-ht_5 class, at present composed of two subtypes (5-ht_{5A} and 5-ht_{5B}, Matthes *et al.*, 1993), have not yet been established.

Such a diversity of 5-HT receptors makes difficult the elucidation of their precise roles in the various documented 5-HT-mediated functions and behaviours. In the central nervous system, 5-HT is released from a few 5-hydroxytryptaminergic neurones, the cell bodies of which form the raphe nuclei, where 5-HT_{1A} (Miquel *et al.*, 1991), 5-$HT_{1B/1D\beta}$ (Middlemiss, 1985; Engel *et al.*, 1986; Voigt *et al.*, 1991), and 5-HT_2 (Blue *et al.*, 1988; Wright *et al.*, 1995) receptor subtypes have been detected. However, it is not yet clear whether the same neurones or distinct subpopulations express these different receptor subtypes which behave as autoreceptors, contributing to the response of these neurones to external 5-HT. To address such a question, a first step would be the availability of a 5-hydroxytryptaminergic cell line expressing 5-HT receptors.

Immortalized precursor cells can be obtained from a multipotential embryonal carcinoma (EC) cell line transfected by a recombinant plasmid PK4 containing the early genes of SV40 under the control of the adenovirus E1A promoter (Kellermann & Kelly, 1986). The resulting immortalized cell lines have properties of committed stem cells, able to differentiate further along a restricted lineage (Kellermann *et al.*, 1987; 1990). Among them, the murine 1C11 clone, which derives from the F9-PK4 EC cell lines, has the features of a neuroectodermal precursor. Indeed, while maintaining a stable undifferentiated phenotype *in vitro*, almost 100% of the 1C11 cells extend bipolar extensions and express neurone-associated markers when cultured in the presence of dibutyryl cyclic AMP (db cyclic AMP) and cyclohexane carboxylic acid (CCA). After 4 days, the resulting 1C11* cells (1C11*d4 cells), which continue to divide, have acquired the ability to synthesize, take up, store and catabolize 5-HT (Buc-Caron *et al.*, 1990).

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We show here that, during the kinetics of the 5-hydroxytryptaminergic differentiation of the inducible 1C11 cell line, three different 5-HT receptors become detectable. The identity of each receptor is established by its pharmacological and transductional properties. Similarly to a 5-HT_{2B} receptor (Loric *et al.*, 1995), a 5-$HT_{1B/1D}$ receptor becomes expressed 2 days after the addition of db cyclic AMP and CCA. These two receptors remain functional until day 4 when a third 5-HT receptor, pharmacologically and functionally similar to 5-HT_{2A}, has become induced.

Methods

Cell culture

1C11 cells were grown and induced to differentiate in the presence of 1 mM db cyclic AMP and 0.05% cyclohexane carboxylic acid (CCA) (Buc-Caron *et al.*, 1990). Experiments were performed on undifferentiated 1C11 cells and 2 days (1C11*d2 cells, 5×10^3 cells cm^{-2}) or 4 days (1C11*d4 cells, 1.5×10^4 cells cm^{-2}) after addition of the inducers.

Membrane preparation

To prepare crude membranes for radioligand binding assays, 1C11, 1C11*d2 or 1C11*d4 cells were washed twice with ice-cold phosphate buffer saline (PBS), then harvested with a rubber policeman in 1.5 ml PBS containing 1 $\mu g\ ml^{-1}$ pepstatin, 1 $\mu g\ ml^{-1}$ antipain, 15 $\mu g\ ml^{-1}$ benzamidine and 0.1 mM phenylmethylsulphonyl fluoride. After centrifugation, the resulting pellet was frozen at -70 °C before homogenization. Frozen cell pellets were thawed at 37°C, resuspended with 10 ml of cold 4 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulphonyl 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 by the bicinchoninic acid protein assay (Pierce, Chichester, U.K.).

Radioligand binding experiments

These experiments were performed using either intact cells or crude membranes. For intact-cell experiments, cells were washed twice with Dulbecco's modified Eagles medium (DMEM). Binding experiments were carried out at room temperature (19–23°C) with shaking, in a total volume of 1 ml. Assays were initiated by the addition of 100 μl foetal calf serum (FCS)-free DMEM containing the radiolabelled ligand (up to 20 nM for saturation experiments) and the appropriate competing ligands. After a 30 min incubation, cells were washed twice with cold PBS and 2 ml of 1 N $HClO_4$ was added, 500 μl of which was counted in a γ (iodinated ligands) or β (tritiated ligands) scintillation counter (Packard, France). The specific binding was defined as the binding that was inhibited by 1 μM of homologous unlabelled ligands. It has long been recognized that the use of the cold form of the radioligand to define the level of nonspecific binding carries a greater risk of including non receptor binding in the definition of specific binding than when a structurally distinct compound is used. We nevertheless favoured this strategy in order to detect all 5-HT binding sites, including nonspecific ones, and thereafter to characterize them. The amounts of specific binding ranged from 42.3 to 76.8% for [^{3}H]-5-HT, [^{125}I]-(±)-1-(2,5-dimethoxy-4-iodophenyl-2 aminopropane ([^{125}I]-DOI), [^{125}I]-GTI, and [^{3}H]-ketanserin. 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 of radiolabelled ligand 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 and the radioactivity retained by the filters was determined as previously described (Loric *et al.*, 1995).

Data analysis and statistics

Binding data were analyzed by the iterative non-linear fitting software Prism (GraphPad, San Diego, CA, U.S.A.). This allowed the calculation of dissociation equilibrium constants (K_d) for saturation experiments, as well as inhibition constants (K_i) and Hill coefficients for displacement studies. Data points were fitted to a single or two-site models of binding, with goodness of fit assessed using an *F*-test. The statistical analysis on small groups used non-parametric tests (Siegel & Castellan, 1988) and the InStat software (GraphPad, San Diego, CA, U.S.A.). The chosen significance criterion was $P<0.01$. All values are given as means ± s.e.mean or 95% confidence intervals.

Determination of endogenous cyclic AMP levels

Cells were washed twice in FCS-free DMEM and incubated for 15 min at 37°C with 100 μM isobutylmethylxanthine and test agents. The reaction was stopped by aspiration of the medium, followed by addition of 500 μl ice-cold 95% ethanol/5% formic acid (1:1, v/v). After 1 h at 4°C, the ethanolic phase was collected and lyophilised. Cyclic AMP was quantified with an iodinated radioimmunoassay kit (cyclic AMP RIA kit, Pasteur Diagnostics, Paris, France). Despite the addition of 1 mM db cyclic AMP to the 1C11 growth medium, the basal cyclic AMP level (about 300 pmol mg^{-1} protein) was about the same in 1C11, 1C11*d2, 1C11*d4, and mesodermal C1 cells (Kellermann *et al.*, 1990). At a concentration of 1 μM 7β-[γ-(morpholino)butyryl]forskolin (FSK) typically yielded a 7 fold increase in cyclic AMP levels.

Determination of endogenous inositol 1,4,5-trisphosphate (IP_3) levels

Cells were washed twice and incubated for various times with agonists and antagonists (0.1–10 nM) in FCS-free DMEM. At the end of the incubation, cells were washed twice in cold PBS and scraped with a rubber policeman. After centrifugation, 100 μl of PBS was added to the cell pellet and adjusted to 250 μl with Tris HCl 50 mM, pH 7.40. Then, after addition of 250 μl of cold 1 N $HClO_4$, the mixture was incubated for 10 min at 4°C. After a 5 min centrifugation at 2000 *g* and 4°C, 400 μl of supernatant was added to 100 μl of 10 mM EDTA, mixed and 300 μl of tri-n-octylamine/1,1,2 trichlortrifluoroethane (1:1, v/v) was added. After thorough mixing and centrifugation at 2000 *g* for 5 min, 400 μl of the upper phase was removed and IP_3 was quantified with a tritiated radioimmunological kit (Amersham TRK1000 IP_3 kit, Les Ulis, Paris, France).

PCR amplification conditions

RNAs were purified from mouse embryos and 1C11* cells according to standard procedures as described previously (Loric *et al.*, 1992). For quantitative RT-PCR experiments, standard PCR buffer (50 μl) was used in the presence of 5 μg of total RNA. After denaturation, avian myeloblastosis virus reverse transcriptase (10 units) and *Thermus aquaticus* polymerase (5 units) were added, extension was performed at 50°C for 15 min, and then a standard PCR amplification protocol 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 ribosomal elongation factor EF1A, amplified in the same reaction as the specific amplimers: 5-HT_{1B}= TCGTCGGATATCACCTGTTGC and

TGGAACG CTTGTTTGAAGTCC, 5 - HT_{2A} = CAAT - ATCATGGCCGTCA and ATCCAGACAAACACATT - GAGCA, 5-HT_{2B} = AATGCTGGATGGGTCTCACA and TCTCCAGGAGTGTTTTGAGAGTG. The specificity of the amplified products was confirmed by hybridization with a ^{32}P-labelled specific oligonucleotide. When necessary, the RNA samples were treated by RNAse-free DNAse I in order to ensure the amplification of RNA and not of contaminating genomic DNA.

Materials

Dibutyryl cyclic AMP (db cyclic AMP) and cyclohexane carboxylic acid (CCA) were from Sigma-Aldrich Chimie (St-Quentin Fallavier, France). Ketanserin, 3-(1,2,5,6-tetrahydropyrid-4-yl)pyrolo[3,2-*b*]pyrid-5-one (CP 93129) and 3-tropanyl-3,5-dichlorobenzoate (MDL 72222) were gifts from Janssen (Beerse, Belgium), Pfizer (Lenexa, KS, U.S.A.), and Merell-Dow (Strasbourg, France) respectively. Other neurochemicals were from RBI (Natick, MA, U.S.A.). All other chemicals of the purest grade available were from classical commercial sources. [^{125}I]-DOI (81.4 TBq mmol^{-1}), [^{3}H]-8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]-8-OH-DPAT, 5.89 TBq mmol^{-1}), [^{3}H]-ketanserin (2.22 TBq mmol^{-1}), [^{3}H]-quipazine (2.96 TBq mmol^{-1}), and [^{3}H]-clozapine (3.19 TBq mmol^{-1}) were from DuPont-New England Nuclear (NEN). [^{3}H]-mesulergine (2.63 TBq mmol^{-1}), and [^{3}H]-[1-[2-(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl 1-methyl-1H-indole-3-carboxylate ([^{3}H]-GR 113808, 2.52 TBq mmol^{-1}) were from Amersham (Les Ulis, France). [^{125}I]-GTI (68.9 TBq mmol^{-1}) was from Immunotech (Marseille-Luminy, France). The reverse transcriptase enzyme and buffer were from Invitrogen (San Diego, CA, U.S.A.). The *Thermus aquaticus* polymerase was from Perkin Elmer-Cetus (Norwalk, CT, U.S.A.). PCR reactions were performed in a Perkin Elmer-Cetus (Norwalk, CT, U.S.A.) thermocycler.

Results

Several types of 5-HT binding sites are induced upon 1C11 5-hydroxytryptaminergic differentiation

To detect 5-HT binding sites on 1C11 cells during their 5-hydroxytryptaminergic differentiation, studies were first carried out with 10 nM [^{3}H]-5-HT, 1 nM [^{3}H]-quipazine and 10 nM [^{125}I]-DOI. At day 0, progenitor 1C11 cells did not show any detectable binding. It has been shown for several receptor systems that functional activity can be measured in the absence of radioligand binding. In similar binding conditions, at day 0, no functional 5-HT receptor activity was detectable by measuring endogenous IP_3 or the cyclic AMP level. In contrast, after four days of exposure to db cyclic AMP and CCA, the cells (1C11*d4) still did not bind [^{3}H]-quipazine, but bound significant amounts of [^{3}H]-5-HT and [^{125}I]-DOI. These preliminary results indicated the absence of 5-HT_3 binding sites and favoured the presence of both 5-HT_2-like (DOI binding) and either 5-HT_1-like, or/and 5-HT_4, 5-ht_5, 5-ht_6, 5-ht_7 binding sites (5-HT binding).

Two members of the 5-HT_2 receptor class become functionally expressed during the 5-hydroxytryptaminergic differentiation of the 1C11 cell line

In contrast to DOI binding, 1C11*d2 or 1C11*d4 cells were not able to bind 1 nM [^{3}H]-mesulergine. This strongly suggests the absence of 5-HT_{2C} binding sites and the presence of 5-HT_{2A} and/or 5-HT_{2B} binding sites. Steady-state binding of [^{125}I]-DOI, one of the most potent 5-HT_2 receptor agonists (Glennon *et al.*, 1988), was assayed during the time course of 1C11 cell differentiation. In contrast to undifferentiated 1C11 cells, 1C11*d2 and 1C11*d4 cells bound [^{125}I]-DOI in a saturable manner. The Rosenthal transformation of the data revealed one class of DOI binding sites at day 2 and two classes at day 4

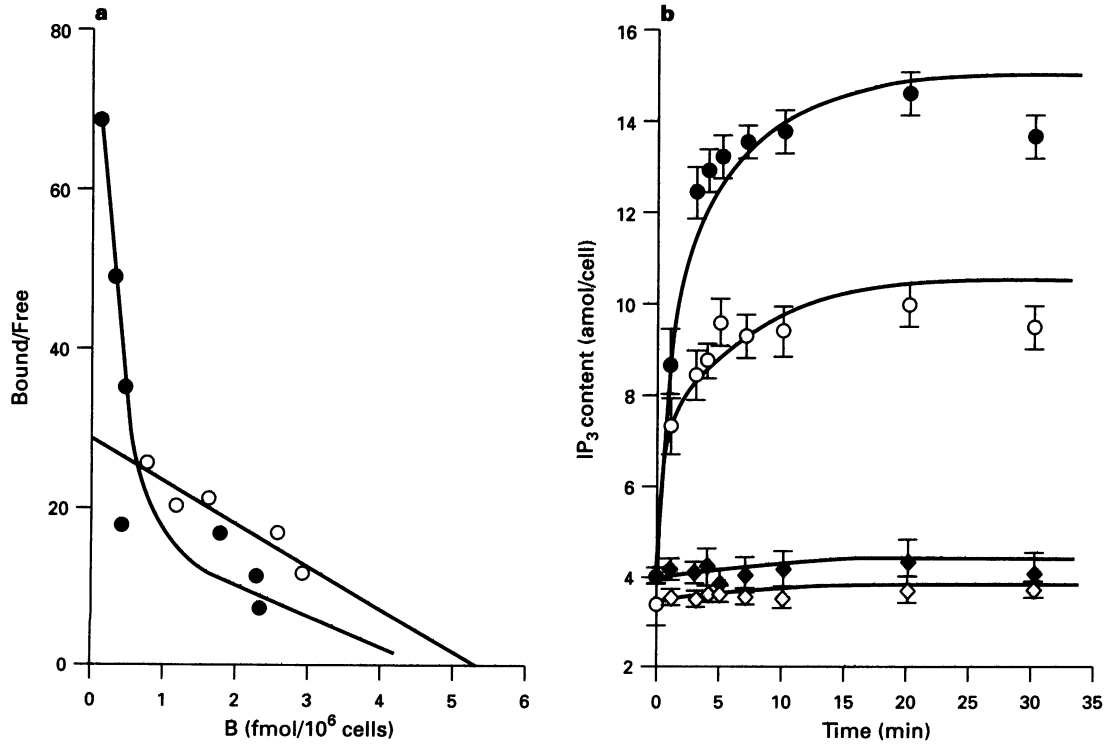


Figure 1 (a) Scatchard plot of [^{125}I]-DOI binding onto 1C11* cells at day 2 (○) and at day 4 (●) of their 5-hydroxytryptaminergic differentiation. Each curve represents the mean ± s.e.mean of tetraplicates from a single experiment representative of two others. (b) Basal concentrations (◇,◆) and 100 nM DOI-induced time-dependent IP_3 production in 1C11*d2 (○) and 1C11*d4 cells (●). Data shown are the mean ± s.e.mean of three independent experiments performed in triplicate.

(Figure 1a). The single class of DOI binding sites at day 2, (apparent $K_d = 21.9 \pm 2.3$ nM, 2100 sites per cell, Table 1) were previously shown to correspond to functional 5-HT_{2B} receptors (Loric *et al.*, 1995). At day 4, 1C11*d4 cells had acquired approximately 2500 DOI binding sites. The pharmacological profile (Table 2, Figure 2) and the number of 5-HT_{2B} sites (Table 1) did not vary between day 2 and day 4. A second class of DOI-binding sites with a significantly higher affinity (apparent $K_d = 0.85 \pm 0.1$ nM, Table 1) than the one associated to the single class present at day 2, was responsible for this increase of DOI binding sites per cell. The above K_d value, the binding of 1 nM [^{3}H]-ketanserin, and the pharmacological profile (Table 2, Figure 2) of this binding site are quite similar to the values previously reported for 5-HT_{2A} receptors (Engel *et al.*, 1986). About 400 5-HT_{2A} sites per 1C11*d4 cell can be deduced from the apparent B_{max} value (0.71 fmol per 10^6 cells) measured for this second class of DOI binding sites.

At day 2 and day 4 of differentiation, the addition of the agonist DOI triggered a time-dependent and saturable accumulation of IP_3 (Figure 1b). At day 4, the IP_3 production upon DOI addition was greater than on day 2. Since the apparent number of 5-HT_{2B} receptors did not increase between days 2 and 4 of 1C11* differentiation, the increase of IP_3 production might be attributed to the effect of DOI on the 5-HT_{2A} receptors which appeared between day 2 and day 4, assuming an unchanged coupling of 5-HT_{2B} to phospholipase C-β.

5-$HT_{1B/1D}$ receptors became functional upon 5-hydroxytryptaminergic differentiation of 1C11 cells

The addition of 10 nM 5-HT to 1C11*d2 or 1C11*d4 cells did not induce any increase in the intracellular cyclic AMP level. In contrast, the addition of 1 μM 7 β-[γ-(morpholino)butyryl]forskolin (FSK) increased the basal cyclic AMP level (240 pmol mg^{-1} protein) by about 7 fold (Figure 3). This FSK-induced cyclic AMP production was clearly inhibited by 5-HT with a apparent IC_{50} of 1.7 nM (95% confidence interval 1.5–1.8 nM), and a maximal inhibition of 85 (82–89)% occurring with 10^{-7} M 5-HT (Figure 3). Similarly to 5-HT, the addition of the 5-$HT_{1B/1D}$ receptor agonist, 7-trifluoromethyl-4(4-methyl-l-piperazinyl)-pyrolo (1,2-*a*)quinoxaline (CGS 12066) caused a smooth, monophasic (Hill slope = 0.99), inhibition of the FSK-induced cyclic AMP production with an apparent IC_{50} of 11.2 (10.9–11.4) nM and a maximal inhibition of 78 (74–82)% occurring with 10^{-6} M CGS 12066 (Figure 3). The absence of any 5-HT-induced increase in the intracellular cyclic AMP level clearly rules out the presence of functionally coupled 5-HT_4, 5-ht_6 and 5-ht_7 receptors on 1C11*d2 and 1C11*d4 cells. Indeed, at both times, neither [^{3}H]-GR 113808, the selective ligand of 5-HT_4 receptors (Gerald *et al.*, 1995), nor [^{3}H]-clozapine, used to label 5-ht_6 (Glatt *et al.*, 1995) binding sites, bind to the cells. The possibility remains for the presence on 1C11* cells of uncoupled 5-ht_7 binding sites or 5-ht_5 and/or 5-HT_1-like receptors, but no binding of [^{3}H]-8-OH-DPAT, the specific ligand of 5-HT_{1A} receptors, was detected.

As early as day 2 of their 5-hydroxytryptaminergic differentiation, 5-$HT_{1B/1D}$ sites are revealed on 1C11* cells by the binding of [^{125}I]-GTI, an agonist of 5-$HT_{1B/1D}$ sites (Boulenguez *et al.*, 1992). The apparent kinetic parameters are reported in Table 1. The apparent K_d value is in good agreement with that measured for rodent or non-rodent 5-$HT_{1B/1D\beta}$ receptors (Bruinvels *et al.*, 1992). The apparent B_{max} value enables calculation of approximately 1200 receptors per cell, for either 1C11*d2 or 1C11*d4 cells. The GTI-binding sites present on 1C11* cell membranes were further characterized by competition displacement studies (Table 3). The assayed compounds produced a monophasic displacement of radioligand. As shown in Table 3, all assayed drugs displayed similar binding properties at day 2 and day 4 of 1C11 5-hydroxytryptaminergic differentiation. Using the nonparametric Spearman rank test, highly significant correlations ($P < 0.001$; Table 4) were found between the pK_is calculated for 1C11* cells using [^{125}I]-GTI, and those reported either for

Table 2 Comparison of competition experiments between low and high [^{125}I]-DOI affinity binding sites onto 1C11* cells at day 4 of their differentiation

No.	*Drug*	*[^{125}I]-DOI binding to 1C11*d4 cells*	
		High affinity site	*Low affinity site*
1	5-CT	<4.0	6.57 ± 0.09
2	DHE	5.35 ± 0.12	6.45 ± 0.15
6	Methiothepin	8.76 ± 0.19	7.56 ± 0.16
8	Iodocyanopindolol	4.78 ± 0.15	4.80 ± 0.21
10	Yohimbine	5.67 ± 0.16	6.43 ± 0.23
11	Methysergide	8.61 ± 0.14	7.88 ± 0.09
12	8-OH-DPAT	4.85 ± 0.18	5.31 ± 0.18
17	Spiperone	8.85 ± 0.23	7.32 ± 0.23
18	Ketanserin	8.72 ± 0.14	7.43 ± 0.13
20	Mesulergine	8.36 ± 0.21	7.65 ± 0.26
21	Buspirone	6.44 ± 0.20	6.45 ± 0.08
22	MDL 72222	6.86 ± 0.16	4.44 ± 0.22
31	Tryptamine	5.78 ± 0.08	6.81 ± 0.18
39	N-acetyl 5-HT	5.56 ± 0.12	7.67 ± 0.14
40	Ritanserin	9.18 ± 0.22	8.18 ± 0.13
41	Pizotifen	7.72 ± 0.24	8.31 ± 0.14
42	α-Me 5-HT	7.24 ± 0.20	6.57 ± 0.17
43	Cyproheptadine	8.47 ± 0.17	7.34 ± 0.15
44	1-Me tryptamine	6.43 ± 0.21	5.48 ± 0.23
45	NN'-diMe 5-MT	6.22 ± 0.19	4.67 ± 0.27

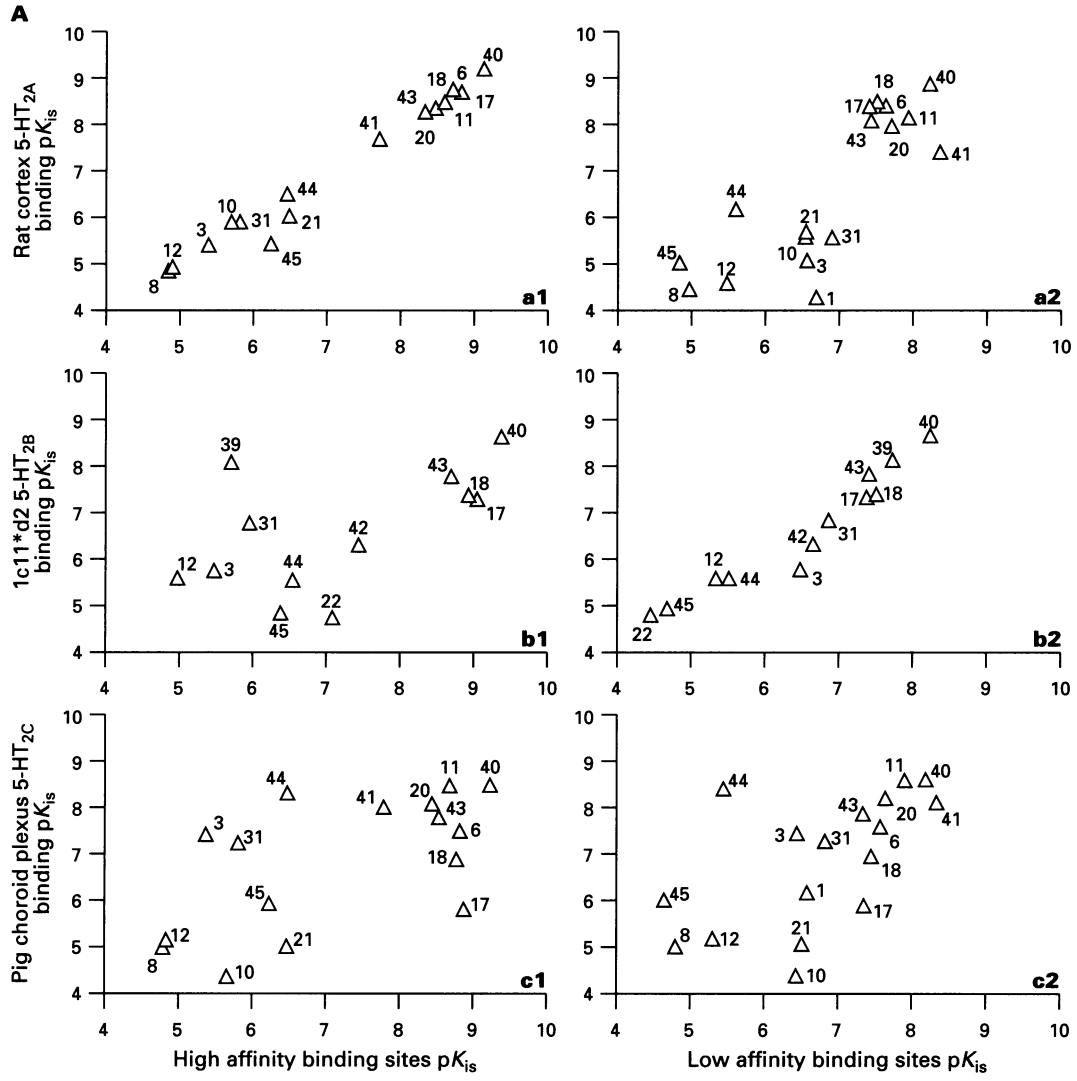
Reported values are the arithmetic mean ± s.e.mean of three independent experiments performed in triplicate. Eight to ten concentrations of each competing agonist or antagonist were used. 5-CT, 5-carboxamidotryptamine; DHE, dihydroergotamine.

Table 1 Binding parameters of [^{125}I]-GTI and [^{125}I]-DOI binding sites present on 1C11 cells at day 0 (progenitor cells), at day 2 (1C11*d2 cells) and day 4 (1C11*d4 cells) of their 5-hydroxytryptaminergic differentiation

		Progenitor 1C11 cells	*1C11*d2 cells*		*1C11*d4 cells*	
[^{125}I]-GTI binding (5-$HT_{1B/1D}$)						
K_d	(nM)	ND	0.53	(0.51–0.56)	0.59	(0.55–0.62)
B_{max}	(fmol mg^{-1} prot)		46.3	(36.4–55.2)	39.8	(31.7–48.3)
[^{125}I]-DOI binding (5-HT_{2B})						
K_d	(nM)	ND	21.9	(19.6–23.9)	21.8	(21.1–23.3)
B_{max}	(fmol mg^{-1} prot)		107.3	(95.0–119.6)	106.4	(95.5–117.3)
[^{125}I]-DOI binding (5-HT_{2A})						
K_d	(nM)	ND	ND		0.85	(0.76–0.97)
B_{max}	(fmol mg^{-1} prot)				15.2	(13.1–17.9)

Data shown are the mean and 95% confidence intervals of three experiments performed in triplicate (ND = not detected).

guinea-pig (Figure 4a) and human (Figure 4b) brain 5-$HT_{1D\beta}$ receptors with the same radioligand or for human 5-$HT_{1D\beta}$ receptors expressed in cDNA-transfected COS cells (Figure 4c) with [^{3}H]5-HT as radioligand. With pK_i values reported for 5-$HT_{1D\alpha}$, 5-ht_{1E}, 5-ht_{1F}, 5-ht_5 and 5-ht_7 receptors, correlations were less significant ($P>0.01$, Table 4). In addition to their



B

	[^{125}I]-DOI binding to 1C11*d4 cells			
	High affinity binding sites		*Low affinity binding sites*	
Rat cortex 5-HT_{2A}	rs = 0.978	P<0.0001	rs = 0.751	NS
1C11*d2 cells 5-HT_{2B}	rs = 0.440	NS	rs = 0.986	P<0.0001
Pig choroid plexus 5-HT_{2C}	rs = 0.525	NS	rs = 0.689	NS

Figure 2 (A) Comparison of competition experiments between [^{125}I]-DOI binding to 1C11* cells at day 4 of their 5-hydroxytryptaminergic differentiation and either [^{3}H]-ketanserin binding to rat cortex membranes (a1,a2), [^{125}I]-DOI binding to 1C11* cells at day 2 of their differentiation (b1,b2) or [^{3}H]-mesulergine binding to pig choroid plexus (c1,c2). The two binding sites (Figure 1a) were compared [high affinity binding site (K_d=0.85 nM for DOI) in column 1: a1,b1,c1; low affinity binding site (K_d=21.9 nM for DOI) in column 2: a2,b2,c2]. The following agonists and antagonists were tested: (1) 5-CT, (3) 5-HT, (6) methiothepin, (8) iodocyanopindolol, (9) sumatriptan, (10) yohimbine, (11) methysergide, (12) 8-OH-DPAT, (13) mianserin, (17) spiperone, (18) ketanserin, (20) mesulergine, (21) buspirone, (22) MDL 72222, (31) tryptamine, (39) N-acetyl-5-HT, (40) ritanserin, (41) pizotifen, (42) α-methyl 5-HT, (43) cyproheptadine, (44) 1-methyl tryptamine, (45) NN'-dimethyl tryptamine). These comparisons were performed using arithmetic means since all previous published data used this measure of location. (B) Two tailed Spearman rank correlations between pK_i values of various drugs for the two DOI binding sites observed onto 1C11*d4 cells and pK_i values for rat cortex 5-HT_{2A}, 1C11*d2 5-HT_{2B} and pig choroid plexus 5-HT_{2C} receptors. Data were analyzed using Instant software (GraphPad, San Diego, CA, U.S.A.). Statistical results were considered significant for $P<0.001$ (NS, not significant).

pharmacological profile, highly consistent with 5-$HT_{1B/1D}$ binding sites, these receptors appear, as soon as day 2 of 1C11 5-hydroxytryptaminergic differentiation, negatively coupled to adenylase cyclase (cf. above Figure 3). Indeed, the pK_i and pK_B values measured on 1C11* cells for the 5-HT_{1B} against CGS 12066 are in good agreement (Figure 3 and Table 3). Finally, the IP_3 content for 1C11*d2 or 1C11*d4 cells remained insensitive to the addition of CGS 12066.

Expression of endogenous 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} transcripts in 1C11 cells

Amplimers specific for each receptor subtype were used to PCR amplify cDNAs generated by reverse transcription of total RNA from 1C11 cells. PCR products of the expected sizes (Figure 5) were obtained with RNA from 1C11*d2 and 1C11*d4 cells as well as with RNA extracted from undifferentiated 1C11 cells. As compared to the interval standard, EF1A, the amounts of each transcript encoding 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} receptor respectively, did not vary significantly during the time course of 1C11 5-hydroxytryptaminergic differentiation. In addition, PCR products identical to those encoding 5-HT_{2B} and 5-HT_{1B} receptors were detected in 10 day old (ED10) mouse embryos (Figure 5), the former being already present at ED8. Since the expression of 5-HT_{2B} receptor was previously reported at ED8.5 in heart rudiments, neural fold and neural tube before its closure (Choi *et al.*, 1994), this points out the early *in vivo* expression of 5-HT_{1B} transcripts. In contrast, 5-HT_{2A} transcripts were detected either at ED8 or at ED10 only as a much fainter band than for the two other receptors (Figure 5), in agreement with previous reports showing the appearance of 5-HT_{2A} receptors in rat brain at the late prenatal and early postnatal periods (Morilak & Ciaranello, 1993).

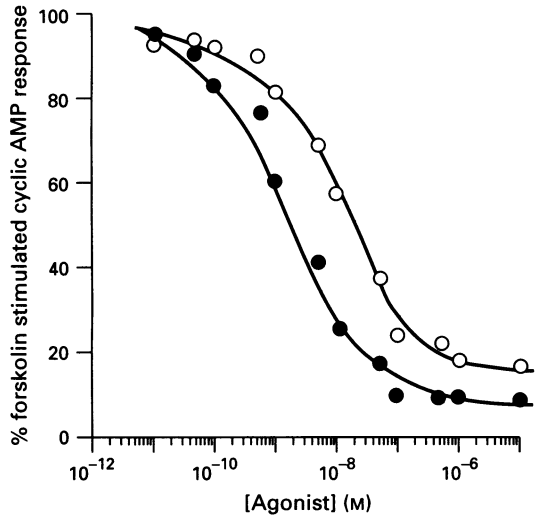


Figure 3 Inhibition of FSK-stimulated cyclic AMP production in 1C11*d2 cells by 5-HT (●) and CGS 12066 (○). Data were analyzed by computer-assisted non-linear regression analysis (Prism, GraphPad, San Diego, CA, U.S.A.). Each curve represents the mean of triplicates from a single experiment representative of two others. Similar data are obtained in 1C11*d4 cells.

Discussion

In most cases, cDNA-transfected heterologous cell lines are used to define the pharmacological properties and the possible

Table 3 Comparison of competition experiments between [^{125}I]-GTI onto 1C11* cells at day 2 and at day 4 of their differentiation

No.	*Drug*	*1C11*d2 cells*	*211C11*d4 cells*
1	5-CT	9.25±0.13	9.19±0.11
2	DHE	8.88±0.06	8.84±0.14
3	5-HT	8.76±0.14	8.81±0.08
4	Ergotamine	8.53±0.16	8.48±0.13
5	Metergoline	8.17±0.13	8.15±0.26
6	Methiothepin	7.86±0.10	7.89±0.09
7	CGS 12066	7.83±0.17	7.79±0.18
8	Iodocyanopindolol	7.44±0.08	7.42±0.10
9	Sumatriptan	7.71±0.12	7.74±0.05
10	Yohimbine	7.23±0.15	7.18±0.04
11	Methysergide	7.21±0.13	7.26±0.06
12	8-OH-DPAT	6.91±0.09	6.94±0.24
13	Mianserin	6.89±0.02	6.92±0.15
14	CP 93129	6.45±0.03	6.51±0.02
15	SCH 23390	6.42±0.18	6.37±0.10
16	(-)-Pindolol	5.88±0.08	5.90±0.10
17	Spiperone	5.83±0.15	5.87±0.02
18	Ketanserin	5.75±0.22	5.78±0.01
19	Ipsapirone	5.40±0.13	5.36±0.04
20	Mesulergine	5.18±0.17	5.20±0.18
21	Buspirone	4.54±0.21	4.48±0.07
22	MDL 72222	4.02±0.13	4.05±0.04

Reported values are arithmetic mean±s.e.mean of three independent experiments performed in triplicate. Eight to ten concentrations of each competing agonist or antagonist were used.

Table 4 Two tailed Spearman rank correlations between pK_i values of various drugs for 1C11*d2 cells 5-$HT_{1B/1D}$ receptor and pK_i values for guinea-pig cortex, rat brain and 1C11*d4 cells 5-$HT_{1B/1D}$, human 5-$HT_{1D\beta}$ and 5-$HT_{1D\alpha}$, mouse 5-ht_{1E}, 5-ht_{1F}, 5-ht_{5A}, 5-ht_{5B} and 5-ht_7 receptors

	Radioligand	*[^{125}I]-GTI binding sites on 1C11*day 2 cells*		*Number of drugs tested*	*Reference*
1C11*day 4 cells 5-$HT_{1B/1D}$	[^{125}I]-GTI	r_s=0.999	P<0.0001	22	
Guinea-pig cortex 5-$HT_{1D\beta}$	[^{125}I]-GTI	r_s=0.998	P<0.0001	21	(Bruinvels *et al.*, 1992)
Rat frontal cortex 5-HT_{1B}	[^{125}I]-ICYP	r_s=0.897	P<0.001	16	(Hoyer,1989)
Human caudate 5-$HT_{1D\beta}$	[^{125}I]-GTI	r_s=0.955	P<0.0001	22	(Bruinvels *et al.*, 1992)
Human 5-$HT_{1D\beta}$ in COS cells	[^{3}H]-5-HT	r_s=0.952	P<0.001	9	(Weinshank *et al.*, 1992)
Human 5-$HT_{1D\alpha}$ in COS cells	[^{3}H]-5-HT	r_s=0.856	P<0.01	9	(Weinshank *et al.*, 1992)
Mouse 5-ht_{1E} in HEK293 cells	[^{3}H]-5-HT	r_s=0.676	NS	12	(Zgombick *et al.*, 1992)
Mouse 5-ht_{1F} in LMTk$^-$ cells	[^{3}H]-5-HT	r_s=0.580	NS	12	(Adham *et al.*, 1993)
Mouse 5-ht_{5A} in COS cells	[^{125}I]-LSD	r_s=0.682	NS	13	(Matthes *et al.*, 1993)
Mouse 5-ht_{5B} in COS cells	[^{125}I]-LSD	r_s=0.558	NS	11	(Matthes *et al.*, 1993)
Mouse 5-ht_7 in COS cells	[^{3}H]-5-HT	r_s=0.591	NS	13	(Plassat *et al.*, 1993)

NS, not significant, P>0.01.

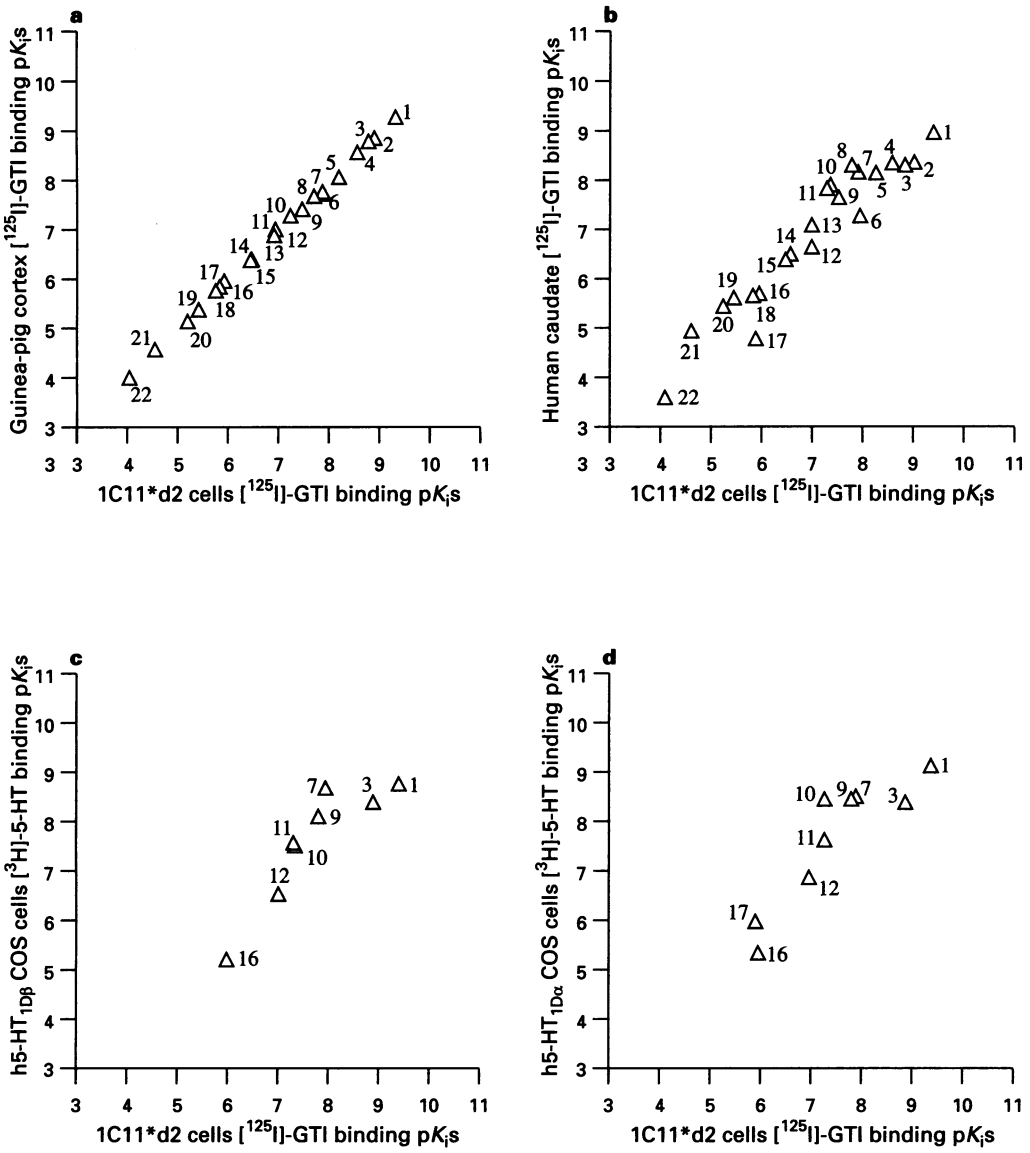


Figure 4 Comparison of competition experiments between [^{125}I]-GTI binding to 1C11* cells at day 2 of their 5-hydroxtryptaminergic differentiation and either [^{125}I]-GTI binding onto guinea-pig cortex membranes (8)(a), or [^{125}I]-GTI binding to human caudate membranes (8)(b), or [^{3}H]-5-HT binding to human 5-HT$_{1D\beta}$ transfected COS cells (9)(c) and onto human 5-HT$_{1D\alpha}$ transfected COS cells (9)(d). These comparisons were performed using arithmetic means since all previous published data used this measure of location. The following agonists and antagonists were tested: (1) 5-CT, (2) DHE, (3) 5-HT, (4) ergotamine, (5) metergoline, (6) methiothepin, (7) CGS 12066, (8) iodocyanopindolol, (9) sumatriptan, (10) yohimbine, (11) methysergide, (12) 8-OH-DPAT, (13) mianserin, (14) CP 93129, (15) SCH 23930, (16) (−)-pindolol, (17) spiperone, (18) ketanserin, (19) ipsapirone, (20) mesulergine, (21) buspirone, (22) MDL 72222.

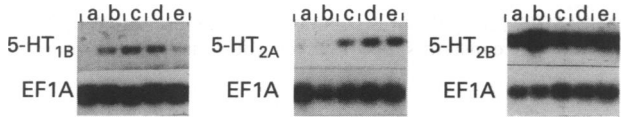


Figure 5 RT-PCR analysis of 5-HT$_{1B}$, 5-HT$_{2B}$ and 5-HT$_{2A}$ expression in mouse embryos (a = embryonic day 8 (ED8), b = ED10) and in 1C11 progenitor (c), 1C11*d2 (d) and 1C11*d4 (e) cells.

functional coupling(s) of one given receptor subtype. However, the ability to follow the induction of various endogenous receptors upon differentiation of a clonal cell line may yield additional information, such as the mechanism(s) regulating the expression of these receptors and the functional responses mediated by the activation of each receptor in such an integrated context.

Little is known about the ontogeny of the 5-hydroxytryptaminergic system or about the regulation of associated functions. Until recently, none of the *in vitro* model systems mimicking 5-hydroxytryptaminergic neurones exhibited a complete 5-hydroxytryptaminergic phenotype, i.e. 5-HT metabolism, storage, uptake and receptors. Indeed, blood platelets (Da Prada *et al.*, 1988) and the few cell lines, like human placental choriocarcinoma cell line (Ramamoorthy *et al.*, 1993), that have been used to study 5-HT uptake, do not synthesize 5-HT. However, the presence of 5-HT uptake, synthesis, and 5-HT$_{1A}$ receptors has now been reported in a 5-hydroxytryptaminergic cell line derived from rat embryonic raphé primary cultures (Eaton *et al.*, 1995).

The 1C11 clone is an immortalized teratocarcinoma-derived cell line that has properties of a neuroectodermal progenitor able to differentiate into 5-hydroxytryptaminergic cells after 4 days of induction in the presence of db cyclic AMP and CCA. During differentiation, the neurite outgrowth is paralleled by the expression of neural markers (N-CAM, synaptophysin, Met-enkephalin) and an ability to synthesize, catabolize, store

and take up 5-HT (Buc-Caron *et al.*, 1990). The present work extends the phenotypic characterization of this cell line and demonstrates that, in addition to 5-HT storage, metabolism, and transport, it acquires 5-$HT_{1B/1D}$, 5-HT_{2B}, and 5-HT_{2A} receptors at definite times during the course of its differentiation.

The pharmacological profile of [^{125}I]-GTI recognition sites present on 1C11*d2 or 1C11*d4 cells is fully comparable to that obtained with species homologues such as rodent 5-HT_{1B} or human 5-$HT_{1D\beta}$ (Figure 4). Indeed, these receptors have 91% homology, but are pharmacologically distinct. Although the origin of the cell line is murine and the mRNA is of 5-HT_{1B} type, the pharmacological profile described here appears more closely related to a 5-HT_{1D} receptor than to a 5-HT_{1B} one. The observed minor variations may be attributed either to species differences or/and to the different radioligands used in the various reported competition studies (Bruinvels *et al.*, 1992). The correlations observed between the pharmacological profile of 1C11* 5-$HT_{1B/1D}$ receptor and those of human 5-HT_{1D} receptors (Table 4) exhibit different *P* values. However, the absence of significant statistical difference between the two corresponding r_s does not allow to exclude the possibility that two 5-HT_{1D}-like receptors exist in the 1C11* cells. Anyhow, the addition of CGS 12066 to FSK-stimulated 1C11*d2 or 1C11*d4 cells produced a decrease of the intracellular cyclic AMP content. Thus, the 5-$HT_{1B/1D}$ receptors harboured by 1C11* cells become measurable and negatively coupled to adenylate cyclase before the completion of 1C11 5-hydroxytryptaminergic differentiation (i.e. at day 2).

In addition, the present data clearly indicate the simultaneous presence of two distinct receptors of the 5-HT_2 class on 1C11*d4 cells. According to the literature (Pritchett *et al.*, 1988; Loric *et al.*, 1992; Hoyer *et al.*, 1994), the apparent K_d measured for DOI binding at 5-HT_{2A} receptors of 1C11*d4 cells is ten fold lower than the apparent dissociation constant attributed to 5-HT_{2B} receptors already present on 1C11*d2 cells. Accordingly, the observed pharmacological profiles strongly correlate with those of 5-HT_{2A} and 5-HT_{2B} receptors (Figure 2). Finally, as compared to 1C11*d2 cells, the additional DOI-induced increase of IP_3 production observed for 1C11*d4 cells, when 5HT_{-2A} and 5-HT_{2B} receptors are co-expressed, suggests that both these receptors are functional and coupled to phosphoinositide hydrolysis.

Interestingly, these three 5-HT receptors do not appear at the same time, but at definite times during differentiation of 1C11 cells. Two days after the addition of db cyclic AMP and CCA, when intracellular 5-HT content and TPH activity have become measurable, 5-$HT_{1B/1D}$ and 5-HT_{2B} receptors are already functional. Whereas the capacity for 5-HT storage and synthesis still increases until day 4 (Buc-Caron *et.*, 1990; Loric *et al.*, 1995), the density of these two functional receptors does not vary. At day 4, the appearance of functional 5-HT_{2A} receptors coincides with the onset of an active 5-HT transport system.

Strikingly, the mRNAs encoding 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors are already present in 1C11 committed cells, despite the absence of any detectable binding site and functional 5-HT receptor activity on these cells. Moreover, the steady-state levels of the corresponding trancripts remain roughly constant along the 4 days of 1C11 5-hydroxytryptaminergic differentiation. Thus, the three 5-HT receptor genes appear to be transcriptionally active in the 1C11 precursor clone, although it still exhibits an immature phenotype, characterizing the stem cell state (Buc-Caron *et al.*, 1990). This agrees with the observations of Anderson's group showing that sympatho-adrenergic progenitors transcribe *in vivo* as well as *in vitro* (Vandenberg *et al.*, 1991) several neuronal markers prior to the choice of their final fate. The question may then arise whether these 5-HT receptors are triggered to be functional by *de novo* RNA translation, or post-translational maturation, or stabilization of pre-existing proteins.

In addition to its classical role as a neurotransmitter, 5-HT acts as a developmental regulatory signal (Lauder, 1993 for review) and has a trophic effect on the early development of 5-hydroxytryptaminergic neurones in the central nervous system (Lauder, 1990). The characterization of three 5-HT receptor subtypes co-expressed by 1C11*d4 cells is obviously a first step to the analysis of their role on 5-hydroxytryptaminergic functions. The presence of functional 5-$HT_{1B/1D}$ and 5-HT_{2B} receptors at day 2 of 1C11 5-hydroxytryptaminergic differentiation leads the cells competent to respond to 5-HT and may represent a critical step of commitment during the time course of 1C11 differentiation. In the case of both receptor types, available evidence indicates that they may act as autoreceptors modulating 5-hydroxytryptaminergic function: (i) 5-HT_{1B} transcripts are expressed *in vivo* by 5-hydroxytryptaminergic neurones of dorsal and median raphé nuclei, where a high density of 5-HT_{1B} binding sites has been detected; additionally, 5-HT_{1B} receptors were localized on 5-HT neurone terminals, and decreased brain 5-HT synthesis, metabolism or release have been observed following activation of the 5-HT_{1B} receptor subtype (Euvrard & Boissier 1980; Middlemiss, 1985; Engel *et al.*, 1986; Carlson *et al.*, 1987; Voigt *et al.*, 1991), (ii) the presence of 5-HT_{2B} receptors and of 5-HT during the early stages of mouse (Loric *et al.*, 1992; Choi *et al.*, 1994) and *Drosophila* (Colas *et al.*, 1995) embryogenesis strongly suggests that 5-HT developmental functions occur, at least partly, *via* receptors of the 5-HT_{2B} subtype; moreover, 5-HT_{2B} receptors are expressed in early mouse embryonic brain (Loric *et al.*, 1992; Choi *et al.*, 1994), where they may have an autocrine function in the differentiation of 5-hydroxytryptaminergic neurones; (iii) the onset of the 5-HT transporter in the 1C11 cell line appears to be down-regulated by 5-HT (J.M. Launay *et al.*, unpublished observations), favouring the idea that the external 5-HT concentration is able to modulate the 5-hydroxytryptaminergic differentiation of 1C11 cells; it should now be possible to examine whether 5-$HT_{1B/1D}$ or/and 5-HT_{2B} receptors, already functional at day 2 of 1C11 5-hydroxytryptaminergic differentiation, participate in this process.

In conclusion, the 1C11 clone appears to be the only inducible cell line which naturally expresses three well-characterized 5-HT receptor subtypes within a complete 5-hydroxytryptaminergic phenotype. 1C11 may thus represent a fruitful system to study the regulated expression of 5-HT receptors, and to define some mechanisms that integrate different signalling inputs mediated by 5-HT receptors to govern 5-hydroxytryptaminergic functions.

Note added in proof

This paper is dedicated to the memory of Mose Da Prada who died in April 1995 during the preparation of this manuscript. We greatly appreciate the constant support he generously lavished upon us.

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