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Different regulation of rat 5-HT_{2A} and rat 5-HT_{2C} receptors in NIH 3T3 cells upon exposure to 5-HT and pipamperone

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Abstract

The 5-HT_{2A} and 5-HT_{2C} receptors belong to the same subtype of the G-protein coupled receptor family and have several agonist and antagonist ligands in common. To gain more insight into the differences in the regulation of the two receptors, we studied the effect of agonist and antagonist pre-treatment on radioligand receptor binding and 5-HT-induced inositol phosphate formation on rat 5-HT_{2A} and rat 5-HT_{2C} receptors stable expressed in NIH 3T3 cells. We compared short (15 min) and prolonged (48 h) pre-treatment of the cells with the natural agonist, 5-HT and with the antagonist pipamperone, which can be readily washed out. The rat 5-HT_{2C} receptor showed an agonist-induced down-regulation (decrease in B_{max} of labelled agonist and antagonist binding) and desensitisation (decrease in 5-HT-induced inositol phosphate formation and potency of 5-HT). Antagonist pre-treatment induced an increase in rat 5-HT_{2C} receptor-mediated inositol phosphate formation as well as increased agonist and antagonist radioligand binding. These findings are consistent with the classical model of G-protein coupled receptor regulation. In contrast, the rat 5-HT_{2A} receptor expressed in the same host cell behaved differently, unlike the classical model. Pre-treatment with 5-HT for 15 min and 48 h did not change receptor levels measured by radioligand binding, but the signal transduction response (inositol phosphate formation) was significantly reduced. Pre-treatment with the antagonist pipamperone for 15 min and 48 h caused an increase in antagonist radioligand binding but a reduction in agonist radioligand binding and a decrease in inositol phosphate formation and potency of 5-HT. Hence, the rat 5-HT_{2A} receptor apparently undergoes agonist desensitisation without down-regulation of the total receptor number. Antagonist pre-treatment causes a paradoxical desensitisation, possibly by uncoupling of the receptor from G-proteins. The uncoupled receptor does not bind 5-HT in the nanomolar range but retains its antagonist binding properties. Paradoxical antagonist-induced desensitisation of rat 5-HT_{2A} receptors has also been observed in vivo.

Keywords: 5-HT_{2A} receptor, rat; 5-HT_{2C} receptor, rat; Desensitisation; Down-regulation; Up-regulation; Agonist binding site; Antagonist binding site; Inositol phosphate formation

1. Introduction

The 5-HT receptors, which couple via G-proteins to phospholipase C, are grouped in the 5-HT_2 receptor family. This receptor family currently consists of the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. The 5-HT_{2A} receptor corresponds to the original S_2 or 5-HT_2 receptor (Leysen et al., 1978; Peroutka et al., 1981), the 5-HT_{2B} receptor may relate to the originally defined "D" receptor, which was identified in functional tests using isolated fundus (Gaddum and Picavelli, 1957), the 5-HT_{2C} receptor was previously called the 5-HT_{1C} receptor (Pazos et al., 1984). The 5-HT_{2A} and 5-HT_{2C} receptors, sharing a number of characteristics including sequence and drug recognition, are widespread in the central nervous system and are believed to be involved in a variety of functions in the central nervous system (for review, see Hoyer et al., 1994).

Classical theories on receptor regulation predict that long-term receptor blockade or denervation cause a compensatory up-regulation of receptors, and conversely that persistent receptor activation causes receptor down-regulation. For the rat 5-HT_{2A} receptor, prior in vivo studies have uniformly demonstrated that sub-acute administration of agonists cause a rapid down-regulation of the receptors (Blackshear et al., 1986; Leysen et al., 1989), in line with

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the classical theory. However, a great number of studies reported a decrease in rat 5-HT_{2A} receptor density and functional responsiveness after long-term blockade by antagonists (Blackshear et al., 1983; Leysen et al., 1986). Similarly, in vivo studies of the regulation of the rat 5-HT_{2C} receptor have shown that the administration of quipazine, a serotonin receptor agonist, causes down-regulation of these receptors in rat choroid plexus (Sanders-Bush and Breeding, 1990), but antagonist treatment similarly resulted in a rat 5-HT_{2C} receptor decrease (Hietala et al., 1992; Sanders-Bush and Breeding, 1988).

In vitro, the cellular background in which rat 5-HT_{2A} receptors are expressed appears to determine how the receptors are regulated. Rat 5-HT_{2A} receptor agonists cause a down-regulation of rat 5-HT_{2A} receptors and a desensitisation of rat 5-HT_{2A} receptor-mediated phosphatidylinositol 4,5-biphosphate hydrolysis in P11 cells (Ivins and Molinoff, 1991) and in calf aortic smooth muscle cells (Pauwels et al., 1990). By contrast, Akiyoshi et al. (1993) found that chronic administration of the agonist 1-(2,5 dimethoxy-4-iodophenyl)-2-aminopropane (DOI) induced an up-regulation of rat 5-HT_{2A} receptors expressed in cerebellar granule cells. Similarly, Chen et al. (1995) showed an agonist-induced up-regulation of rat 5-HT_{2A} receptors expressed in cerebellar granule neurons. Grotewiel and Sanders-Bush (1994) stated that the effects of prolonged agonist exposure depend on the cell line used. Application of an antagonist to the rat 5-HT_{2A} receptor expressed in P11 cells did not alter the density of these receptors (Ferry et al., 1993). The effects of administration of agonists and antagonists in in vitro studies have also been described for rat 5-HT_{2C} receptors. Barker and Sanders-Bush (1993) showed that the level of rat 5-HT_{2C} receptor binding sites in epithelial cell cultures not only decreased after a 72-h treatment with (−)-1-(4-bromo-2,5 dimethoxyphenyl)-2-aminopropane (DOB), but was also reduced after exposure to mianserin for 72 h. Analogously, Akiyoshi et al. (1995) showed that the rat 5-HT_{2C} receptor expressed in Chinese hamster ovary cells (CHO) cells was rapidly desensitised by pre-exposure to 5-HT in a time- and concentration-dependent manner.

Taken together, the regulation of the 5-HT_{2A} and 5-HT_{2C} receptors has been incompletely studied and remains poorly understood, largely because of the complexity of neuronal networks, the heterogeneity of cell types and the sometimes unexpected and contradictory results in prior studies. The question could arise if the rat 5-HT_{2A} and rat 5-HT_{2C} receptor, are still differently regulated if both receptors are expressed to the same expression level in the same cellular background, cultured under the same cell conditions and treated with the same drugs. To compare the regulation of both receptors in the same cellular background in vitro, we used transfected NIH 3T3 cell lines, stably expressing the rat 5-HT_{2A} (Julius et al., 1990) or the rat 5-HT_{2C} receptor (Julius et al., 1988) as model system. Both cell lines have been previously characterised (Julius et al., 1988, 1990). Therefore, only a short study of the pharmacological profile was performed. The cells were cultured for different time periods in the presence of either 5-HT (agonist) or pipamperone (antagonist) and the receptor levels were investigated in membrane preparations using radioligand antagonist and agonist binding. In addition, the functional responsiveness of pre-treated cells to 5-HT was tested in inositol phosphate measurements.

2. Materials and methods

2.1. Cell culture

Two NIH 3T3 cell lines, stably transfected with the rat 5-HT_{2A} or rat 5-HT_{2C} receptor were cultured in Dulbecco's modified Eagle medium with 10% heat-inactivated calf serum (30 min at 56 °C). The selection medium for both cell lines was supplemented with 200 μg/ml G-418. Cells were grown in 175 cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Drug treatment

For drug treatment, both NIH 3T3 cell lines were cultured in Dulbecco's modified Eagle medium with 10% heat-inactivated dialysed calf serum (30 min at 56 °C; 5-HT concentration 8 nM) supplemented with the agonist 5-HT (10 μM) or the antagonist pipamperone (10 μM) for 15 min or 48 h. Untreated cells were grown in parallel. Cells were grown on 150 mm petri dishes at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell membrane preparation

After drug treatment, cells were washed twice with ice-cold phosphate-buffered saline, scraped from the tissue culture plates, suspended in 50 mM Tris–HCl, pH 7.4, and harvested by centrifugation (30 min at 50,000 × *g* at 4 °C). The cells were lysed in 5 mM hypotonic Tris–HCl, pH 7.4, homogenised with an Ultra Turrax homogeniser and membranes were collected by centrifugation (30 min at 50,000 × *g* at 4 °C) and resuspended in an appropriate volume of 50 mM Tris–HCl, pH 7.4. The preparation was kept on ice during the entire procedure.

2.4. Determination of membrane protein content

The amount of protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

2.5. Radioligand binding assay

Antagonist ligand concentration-binding isotherms on the rat 5-HT_{2A} and rat 5-HT_{2C} receptor were performed

with the antagonist [^{125}I]R93274 (125-iodine-*N*-[(3-*p*-fluorophenyl-1-propyl)-4-methyl-4-piperidinyl]-4-amino-5-iodo-2-methoxybenzamide) (Mertens et al., 1995) and the antagonist [^{3}H]mesulergine (Havlik and Peroutka, 1992), respectively. For binding studies with [^{125}I]R93274, membranes were thawed on ice and diluted in 50 mM Tris–HCl buffer, pH 7.5, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. For binding studies with [^{3}H]mesulergine, membranes were thawed on ice and suspended in 50 mM Tris–HCl buffer, pH 7.7, containing 4 mM $CaCl_2$.

Agonist ligand concentration-binding isotherms with [^{3}H]5-HT were performed with membranes thawed on ice and resupended in 50 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EGTA and 10 μM pargyline.

For the ligand concentration-binding studies on the rat 5-HT_{2A} receptor, non-specific binding of the radioligands was estimated in the presence of 10 μM of the 5-HT antagonist α-anilino-*N*-2-chlorphenoxypropylacetamidine hydrochloride monohydrate (BW 501). Incubations with the antagonist [^{125}I]R93274 were run for 60 min at 37 °C in a volume of 0.25 ml containing approximately 2.5 μg of membrane protein. Incubations with the agonist [^{3}H]5-HT were run for 30 min at 25 °C in a volume of 0.5 ml containing approximately 50 μg of membrane protein.

For the ligand binding studies on the rat 5-HT_{2C} receptor, non-specific binding of the radioligands was estimated in the presence of 10 μM ritanserin. NIH 3T3 cells expressing rat 5-HT_{2C} receptors were incubated with the antagonist [^{3}H]mesulergine for 30 min at 37 °C in a volume of 0.5 ml containing approximately 40 μg of membrane protein. Incubations with the agonist [^{3}H]5-HT were run for 30 min at 25 °C in a volume of 0.5 ml containing approximately 50 μg of membrane protein.

For ligand concentration binding isotherms, 10 concentrations of the radioligands in the range of the K_D were used. For competition binding experiments, at least 10 concentrations of compound, ranging from 10^{-5} to 10^{-10} M, were used to inhibit the binding of [^{125}I]R93274 (0.1 nM) or [^{3}H]mesulergine (1 nM), respectively.

The incubations were stopped by rapid filtration under suction over GF/B (pre-soaked in 0.1% polyethyleneimine) and GF/C Whatman glass-fibre filters for [^{3}H]- and [^{125}I]-radioligands, respectively, followed by three rinses with 3 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4. Filter-bound radioactivity was determined in a γ-counter ([^{125}I]-ligand) or by liquid scintillation counting ([^{3}H]-ligand).

Experiments were repeated independently for three to eight times.

2.6. Inositol phosphate formation

The NIH 3T3 cells expressing the rat 5-HT_{2A} or rat 5-HT_{2C} receptor were seeded at a density of 100,000–120,000/well in a 24-well plate. The cells were loaded for 24 h with 74 kBq [^{3}H]-inositol/well, ligand pre-treatment continued during the [^{3}H]-inositol loading of the cells. The cells were washed three times for 10 min with controlled salt solution (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 0.04 mM phenol red in 25 mM Tris–HCl buffer, pH 7.4, 37 °C) for removal of drugs used for cell pre-treatment. Thereafter, the cells were incubated for 30 min at 37 °C in controlled salt solution containing 1 μM paroxetin, 1 μM pargyline and 10 mM LiCl with seven rising 5-HT concentrations (in the range of 0.1 nM–100 μM). The incubation was stopped by addition of 0.1 ml of 1 N ice-cold perchloric acid, followed by neutralisation to pH 7.5 with 0.1 ml of 0.5 M ice-cold KOH/K_3PO_4, pH 13.5. When the $KClO_4$ precipitate was formed, plates were centrifuged for 5 min at $2000 \times g$. The inositol phosphates in the supernatant were separated from free inositol and phosphoinositides by ion exchange chromatography on AG-1X8 columns (Biorad). The eluted inositol phosphate fractions (3 ml) were counted in a β-counter with Ultima Flo MV (3.5 ml) as scintillation fluid.

2.7. Data analysis

Ligand concentration-binding isotherms (rectangular hyperbola) and sigmoid inhibition curves were calculated by non-linear regression analysis according to algorithms described by Oestreicher and Pinto (1987). The B_{max} (maximal number of binding sites) and K_D (the apparent equilibrium dissociation constant) values of the radioligand and the pIC_{50} (concentration that inhibits 50% of specific radioligand binding) values of the inhibitors were free parameters for the curve fitting. Sigmoidal response curves were prepared using the Graph Pad Prism program. The pEC_{50} (concentration that stimulates 50% of the maximal inositol phosphate formation) values of 5-HT were free parameters for the curve fitting.

Comparisons of the data were made using the Student's *t*-test (two-tailed) and the Statview software for the Macintosh.

2.8. Materials

The following materials were purchased from the sources indicated: Dulbecco's modified Eagle medium, calf serum and dialysed calf serum (GIBCO/BRL Life Technologies), plastic tissue culture flasks (Falcon), [^{3}H]mesulergine (≈ 2.96 TBq/mmol) and [^{3}H]5-HT (≈ 3.33 TBq/mmol) (Amersham Life Science), myo-[2-^{3}H(N)]-inositol (8.1 kBq/mmol) (New England Nuclear), protein assay kit (Bio-Rad Laboratories), GF/B and GF/C glass-fibre filters (Whatman), scintillation fluid (Packard). [^{125}I]R93274 (Mertens et al., 1995) (74 TBq/mmol) is a

radioligand of Janssen Pharmaceutica, synthesised at the V.U.B.-cyclotron. Solvents, buffers and salts were of analytical grade purity. Distilled and deionised water (Millipore, Milli-Q system) was used. Pipamperone, cisapride, ritanserin and R93274 are original compounds from Janssen Pharmaceutica. Methysergide was kindly donated by Sandoz Pharma. Ltd. 5-HT was purchased from Upjohn. Paroxetine and pargyline were purchased from Novo-Nordisk and Abbott Laboratories, respectively.

3. Results

3.1. Characterisation of rat 5-HT$_{2A}$ and rat 5-HT$_{2C}$ receptors expressed in NIH 3T3 cell lines

Membranes prepared from rat 5-HT$_{2A}$ and rat 5-HT$_{2C}$ receptor-expressing NIH 3T3 cell lines showed specific saturable [^{125}I]R93274 and [^{3}H]mesulergine antagonist binding to the rat 5-HT$_{2A}$ and rat 5-HT$_{2C}$ receptor, respectively. The ligand concentration-binding curves are shown in Figs. 1A and 2, respectively. Derived K_D- and B_{max}-values are listed in Table 1. Non-transfected NIH 3T3 cells showed no detectable specific radioligand binding (data not shown).

To estimate the portion of receptors coupled to G-proteins, i.e. the high affinity agonist binding site, [^{3}H]5-HT concentration-binding assays were performed (Figs. 1B and 2). Agonist binding to the rat 5-HT$_{2A}$ and rat 5-HT$_{2C}$ receptor showed a B_{max} about 52% and 57%, respectively, of the B_{max} for antagonist binding (Figs. 1 and 2; Table 1). [^{3}H]5-HT bound to both receptors at low nanomolar concentration (Table 1).

A series of serotonergic antagonists and agonists was tested for inhibition of [^{125}I]R93274 and [^{3}H]mesulergine binding. The pIC$_{50}$- and K_i-values and the Hill-coefficients are presented in Table 2.

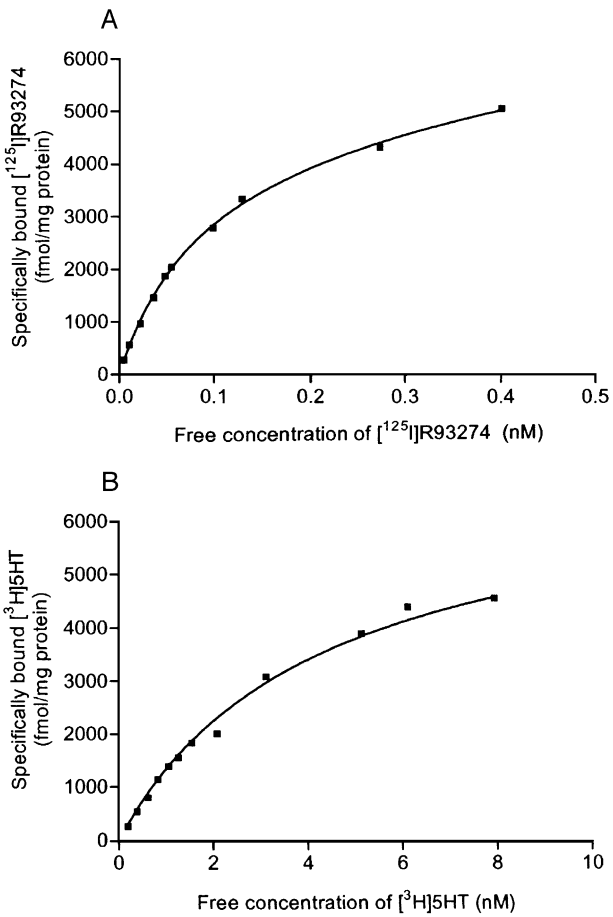

Fig. 1. Concentration-binding isotherms of (A) [^{125}I]R93274 (37 °C) and (B) [^{3}H]5-HT (25 °C) binding to membranes of rat 5-HT$_{2A}$-NIH 3T3 cells. Non-specific binding of the radioligands was estimated in the presence of 10 μM BW 501. Specific binding is presented as the mean of duplicate determinations. The data, representing a typical experiment out of nine ([^{125}I]R93274) or four ([^{3}H]5-HT) independent experiments, were best fitted to a one binding-site model. Mean values of the B_{max} and the K_D are summarised in Table 1.

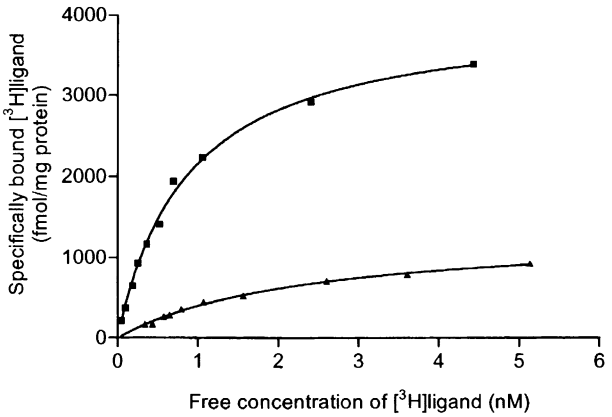

Fig. 2. Concentration-binding isotherms of [^{3}H]mesulergine (37 °C) (■) and [^{3}H]5-HT (25 °C) (▲) binding to membranes of rat 5-HT$_{2C}$-NIH 3T3 cells. Non-specific binding of the radioligands was estimated in the presence of 10 μM ritanserin. Specific binding is presented as the mean of duplicate determinations. The data, representing a typical experiment out of five ([^{3}H]mesulergine) or four ([^{3}H]5-HT) independent experiments, were best fitted to a one binding-site model. Mean values of the B_{max} and the K_D are given in Table 1.

Table 1
Binding parameters (K_D, B_{max}) for radioligands on rat 5-HT$_{2A}$ or rat 5-HT$_{2C}$ receptors stably expressed in NIH 3T3 cells. Antagonist assays ([^{125}I]R93274 or [^{3}H]mesulergine) were at 37 °C, agonist assays ([^{3}H]5-HT) at 25 °C

Transfected receptor	Radioligand	B_{max} (fmol/mg protein)	K_D (nM)	n
Rat 5-HT$_{2A}$	[^{125}I]R93274	3632 ± 982	0.12 ± 0.04	9
Rat 5-HT$_{2A}$	[^{3}H]5-HT	1881 ± 828	3 ± 2	4
Rat 5-HT$_{2C}$	[^{3}H]mesulergine	4754 ± 1556	2 ± 2	5
Rat 5-HT$_{2C}$	[^{3}H]5-HT	2728 ± 1344	1.6 ± 0.6	4

Mean values ± S.D. of four to nine independent experiments.

Table 2
pIC_{50}- and K_i-values and Hill-coefficients of various 5-HT agonists and antagonists for inhibition of [^{125}I]R93274 binding to rat 5-HT_{2A} receptors and for inhibition of [^{3}H]mesulergine binding to rat 5-HT_{2C} receptors expressed in NIH 3T3 cell lines

Compound	Rat 5-HT_{2A}			Rat 5-HT_{2C}		
	pIC_{50}	K_i	Hill-coefficient	pIC_{50}	K_i	Hill-coefficient
Cisapride	7.3 ± 0.2	25 ± 11 nM	1.2 ± 0.2	5.6 ± 0.1	1245 ± 301 nM	0.8 ± 0.2
Methysergide	7.7 ± 0.3	12 ± 7 nM	1.0 ± 0.2	8.6 ± 0.1	1.4 ± 0.4 nM	1.1 ± 0.1
Pipamperone	7.9 ± 0.2	7 ± 3 nM	1.00 ± 0.08	7.00 ± 0.07	54 ± 8 nM	1.0 ± 0.2
5-HT	5.24 ± 0.07	2888 ± 422 nM	0.8 ± 0.1	6.5 ± 0.1	184 ± 43 nM	0.8 ± 0.06
Mesulergine	7.39 ± 0.09	20 ± 4 nM	0.9 ± 0.2	8.7 ± 0.1	1.0 ± 0.3 nM	1.1 ± 0.08
Ritanserin	8.7 ± 0.2	1.0 ± 0.5 nM	1.2 ± 0.3	9.3 ± 0.1	0.29 ± 0.09 nM	1.3 ± 0.3
R93274	9.2 ± 0.1	0.34 ± 0.09 nM	1.1 ± 0.1	7.52 ± 0.04	16 ± 1 nM	1.03 ± 0.06

Mean values ± S.D. of three independent experiments.

3.2. Time-dependent effect of 5-HT pre-treatment on rat 5-HT_{2A} receptor binding sites

Membranes of NIH 3T3 cells expressing rat 5-HT_{2A} receptors were harvested after exposure of the cells for a short (15 min) or prolonged (48 h) time period to 10 μM 5-HT and used for ligand concentration-binding assays with [^{125}I]R93274 and [^{3}H]5-HT. B_{max}- and K_D-values derived from the curves are shown in Table 3 (schematic overview of findings in Table 4).

Short (15 min) and prolonged (48 h) pre-treatment with 5-HT caused no change in the number of antagonist or agonist binding sites. No significant shift in the K_D-values for [^{125}I]R93274 and [^{3}H]5-HT was observed.

3.3. Time-dependent effect of pipamperone pre-treatment on rat 5-HT_{2A} receptor binding sites

Membranes of NIH 3T3 cells expressing rat 5-HT_{2A} receptors were harvested after exposure of the cells for a short (15 min) or prolonged (48 h) time period to 10 μM pipamperone and used for ligand concentration-binding assays with [^{125}I]R93274 and [^{3}H]5-HT. B_{max}- and K_D-values derived from the curves are shown in Table 3 (schematic overview of findings in Table 4).

A pipamperone pre-treatment of 15 min caused an increase of 36% in B_{max}-levels of [^{125}I]R93274 binding. Prolonged exposure of 48 h led to an almost 3-fold increase in the number of antagonist binding sites. In contrast, the agonist binding sites were reduced by about 50% after short and prolonged pre-treatment with pipamperone. The K_D-value for [^{125}I]R93274 binding to rat 5-HT_{2A} receptors was unchanged after short and slightly increased after prolonged exposure to pipamperone. No significant shift in the K_D-values for [^{3}H]5-HT was observed.

3.4. Time-dependent effect of 5-HT pre-treatment on rat 5-HT_{2C} receptor binding sites

Membranes of NIH 3T3 cells expressing rat 5-HT_{2C} receptors were harvested after exposure of the cells for a short (15 min) or prolonged (48 h) time period to 10 μM 5-HT and used for ligand concentration-binding assays with [^{3}H]mesulergine and [^{3}H]5-HT. B_{max}- and K_D-values

Table 3
B_{max}-values (fmol/mg protein), K_D-values (nM) and percentage of G-protein-coupled receptors (% GPCR) determined from saturation binding of [^{125}I]R93274 and [^{3}H]5-HT to cell membrane preparations of rat 5-HT_{2A} receptor NIH 3T3 cells after short (15 min) and prolonged (48 h) exposure to 5-HT (10 μM) or pipamperone (10 μM)

Cell exposure	[^{125}I]R93274				[^{3}H]5-HT				% GPCR
	B_{max}	% of control	K_D	n	B_{max}	% of control	K_D	n	
Control	3600 ± 980	100	0.12 ± 0.04	9	1900 ± 830	100	3 ± 2	4	53
15 min to 5-HT	4500 ± 1800	120 ± 45	0.14 ± 0.06	9	2100 ± 780	120 ± 16	5 ± 2	4	47
48 h to 5-HT	4500 ± 2500	120 ± 46	0.17 ± 0.06	9	1800 ± 760	97 ± 32	4 ± 2	3	40
Control	3600 ± 1050	100	0.12 ± 0.04	9	1900 ± 830	100	3 ± 2	4	53
15 min to pipamperone	4700 ± 1600	140 ± 41[a]	0.3 ± 0.3	9	990 ± 501	40 ± 14[b]	3 ± 2	4	21
48 h to pipamperone	9200 ± 2070	270 ± 62[b]	0.3 ± 0.2[a]	9	950 ± 250	55 ± 14[b]	3 ± 2	4	10

Mean values ± S.D. of three to nine independent experiments are presented. Significant differences from controls (100%) according to Student's *t*-test (two-tailed) (no indication: not significant).

[a] $P < 0.05$.

[b] $P < 0.001$.

Table 4
Overview of the effects of short (15 min) and prolonged (48 h) exposure to 5-HT (10 μM) or pipamperone (10 μM) on the saturation binding to cell membrane preparations of rat 5-HT_{2A} or rat 5-HT_{2C} receptor NIH 3T3 cells and the 5-HT-induced inositol phosphate formation in intact rat 5-HT_{2A}-NIH 3T3 and rat 5-HT_{2C}-NIH 3T3 cells

Rat 5HT_{2A}-NIH 3T3							
Pre-treatment (10 μM)		[^{125}I]R93274 binding		[^{3}H]5-HT binding		5-HT-induced inositol phosphate formation	
		K_D	B_{max}	K_D	B_{max}	5-HT stimulation (x-fold over basal levels)	5-HT EC_{50}
		Observed changes versus no pretreatment					
5-HT	15 min	=	=	=	=	↓	=
	48 h	=	=	=	=	↓↓↓	↑↑↑
pipamperone	15 min	=	↑	=	↓↓↓	↓	↑↑↑
	48 h	↑	↑↑↑	=	↓↓↓	=	↑↑↑
Rat 5HT_{2C}-NIH 3T3							
Pre-treatment		[^{3}H]mesulergine binding		[^{3}H]5-HT binding		5-HT-induced inositol phosphate formation	
		K_D	B_{max}	K_D	B_{max}	5-HT stimulation (x-fold over basal levels)	5-HT EC_{50}
		Observed changes versus no pretreatment					
5-HT	15 min	=	=	=	=	=	=
	48 h	=	↓↓↓	=	↓↓	↓	↑↑↑
pipamperone	15 min	=	=	=	=	=	=
	48 h	=	↑	=	↑↑↑	↑	=

Observed changes are presented of K_D- and B_{max}-values determined from saturation binding of antagonistic ([^{125}I]R93274 and [^{3}H]mesulergine for rat 5-HT_{2A} and rat 5-HT_{2C} receptors, respectively) and agonistic ([^{3}H]5-HT) radioligands and of 5-HT stimulation (x-fold over basal levels) and EC_{50}-values. K_D- and B_{max}-values and 5-HT stimulation (x-fold over basal levels) that are significantly increased (↑) or decreased (↓) after drug treatment from the control-values according to Student's t-test (two-tailed) are indicated as follows: $P < 0.05$: ↑ or ↓, $P < 0.01$: ↑↑ or ↓↓, $P < 0.001$: ↑↑↑ or ↓↓↓ (=: not significant). The EC_{50}-values of 5-HT that are significantly increased (↑↑↑) or decreased (↓↓↓) from the control-values according to the point of showing no overlap of the 95% confidence intervals are indicated as follows: $P < 0.05$: ↑↑↑ or ↓↓↓ (=: not significant).

derived from the curves are shown in Table 5 (schematic overview of findings in Table 4).

After 15 min exposure to 5-HT, no consistent changes in the number of antagonist or agonist binding sites were detected. A prolonged exposure of 48 h to 5-HT caused a moderate reduction (about 30%) in B_{max}-levels of [^{3}H]mesulergine and [^{3}H]5-HT binding to the rat 5-HT_{2C} receptor. No significant shift in the K_D-values for [^{3}H]mesulergine and [^{3}H]5-HT was observed.

3.5. Time-dependent effect of pipamperone on rat 5-HT_{2C} receptor binding sites

Membranes of NIH 3T3 cells expressing rat 5-HT_{2C} receptors were harvested after exposure of the cells for a

Table 5
B_{max}-values (fmol/mg protein), K_D-values (nM) and percentage of G-protein-coupled receptors (% GPCR) determined from saturation binding of [^{3}H]mesulergine and [^{3}H]5-HT to cell membrane preparations of rat 5-HT_{2C} receptor NIH 3T3 cells after short (15 min) and prolonged (48 h) exposure to 5-HT (10 μM) or pipamperone (10 μM)

Cell exposure	[^{3}H]mesulergine				[^{3}H]5-HT				% GPCR
	B_{max}	% of control	K_D	n	B_{max}	% of control	K_D	n	
Control	4700 ± 1500	100	1.3 ± 0.3	5	2700 ± 1300	100	1.6 ± 0.6	4	57
15 min to 5-HT	4300 ± 2000	89 ± 12	1.2 ± 0.3	5	2700 ± 1800	85 ± 25	1.9 ± 0.7	4	61
48 h to 5-HT	3300 ± 1700	68 ± 12[a]	2 ± 2	4	2200 ± 1400	77 ± 11[b]	1.4 ± 0.08	4	65
Control	4900 ± 1700	100	1.4 ± 0.9	4	2900 ± 1600	100	1.6 ± 0.6	3	57
15 min to pipamperone	4900 ± 1800	100 ± 20	1.4 ± 0.3	4	3400 ± 1400	122 ± 20	3 ± 2	3	68
48 h to pipamperone	6900 ± 3000	140 ± 30[c]	2 ± 2	5	4500 ± 2500	155 ± 10[a]	2.1 ± 0.3	3	65

Mean values ± S.D. of three to nine independent experiments are presented. Significant differences from controls (100%) according to Student's t-test (two-tailed) (no indication: not significant).

[a] $P < 0.001$.

[b] $P < 0.01$

[c] $P < 0.05$.

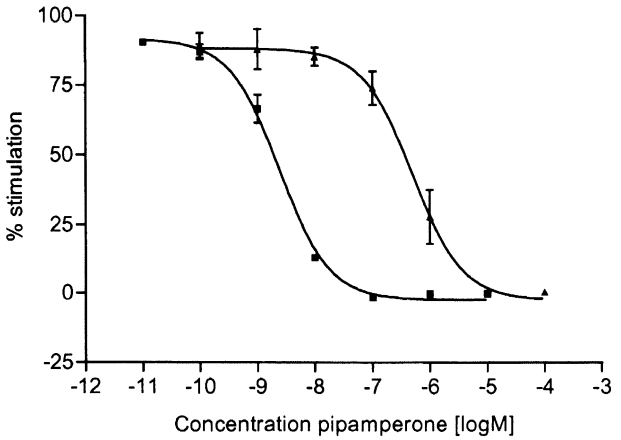

Fig. 3. Effect of pipamperone on the functional responsiveness to 5-HT of NIH 3T3 cell lines, stably expressing rat 5-HT_{2A} or rat 5-HT_{2C} receptors. 5-HT-induced inositol phosphate formation measured in the presence of increasing concentrations of pipamperone for 20 min after culturing the NIH 3T3 cell lines, stably expressing the rat 5-HT_{2A} (■) and rat 5-HT_{2C} receptors (▲), respectively, in 24-well plates. Cells, cultured in 24-well plates and loaded with 2 μCi [^{3}H]-inositol/well for 24 h, were incubated for 30 min with various concentrations of pipamperone, thereafter inositol phosphate formation by 5-HT (100 and 5 nM for rat 5-HT_{2A} and rat 5-HT_{2C} receptors, respectively) was measured as described under Section 2. The maximal response without pipamperone pre-treatment is defined as 100%.

short (15 min) or prolonged (48 h) time period to 10 μM pipamperone and used for ligand concentration-binding assays with [^{3}H]mesulergine and [^{3}H]5-HT. B_{max}- and K_D-values derived from the curves are shown in Table 5 (schematic overview of findings in Table 4).

A short (15 min) pre-treatment with pipamperone induced no consistent change in the number of antagonist or agonist binding sites. Prolonged (48 h) exposure caused a moderate increase of 39% and 55% in [^{3}H]mesulergine and [^{3}H]5-HT binding to rat 5-HT_{2C} receptors, respectively. No significant shift in the K_D-values for [^{3}H]mesulergine and [^{3}H]5-HT was observed.

3.6. Antagonism by pipamperone of the 5-HT-induced inositol phosphate formation mediated by rat 5-HT_{2A} or rat 5-HT_{2C} receptors expressed in NIH 3T3 cells

Antagonism by pipamperone on the inositol phosphate formation mediated by rat 5-HT_{2A} or rat 5-HT_{2C} receptors expressed in NIH 3T3 cells was evaluated by checking the effect on 5-HT (100 and 5 nM, respectively) stimulated phosphoinositide hydrolysis with increasing concentrations of pipamperone (in the range of 0.01 nM–10 μM; incubation time 20 min). Fig. 3 shows that pipamperone fully inhibited the 5-HT-induced inositol phosphate formation mediated by rat 5-HT_{2A} and rat 5-HT_{2C} receptors; derived K_i-values were 0.9 and 197 nM, respectively; full antagonism was reached at 100 nM and 10 μM, respectively.

3.7. Time-dependent effect of 5-HT and pipamperone on the 5-HT-induced inositol phosphate formation mediated by rat 5-HT_{2A} receptors expressed in NIH 3T3 cells

Measuring inositol phosphate formation monitored the rat 5-HT_{2A} receptor-mediated hydrolysis of phosphatidyl inositol in stably transfected NIH 3T3 cells. Activation of the rat 5-HT_{2A} receptor with 5-HT concentrations (seven concentrations in the range of 0.1 nM–100 μM) caused a 38-fold maximum increase in inositol phosphate levels relative to base line; the pEC_{50}-value of 5-HT was 7.35 ±

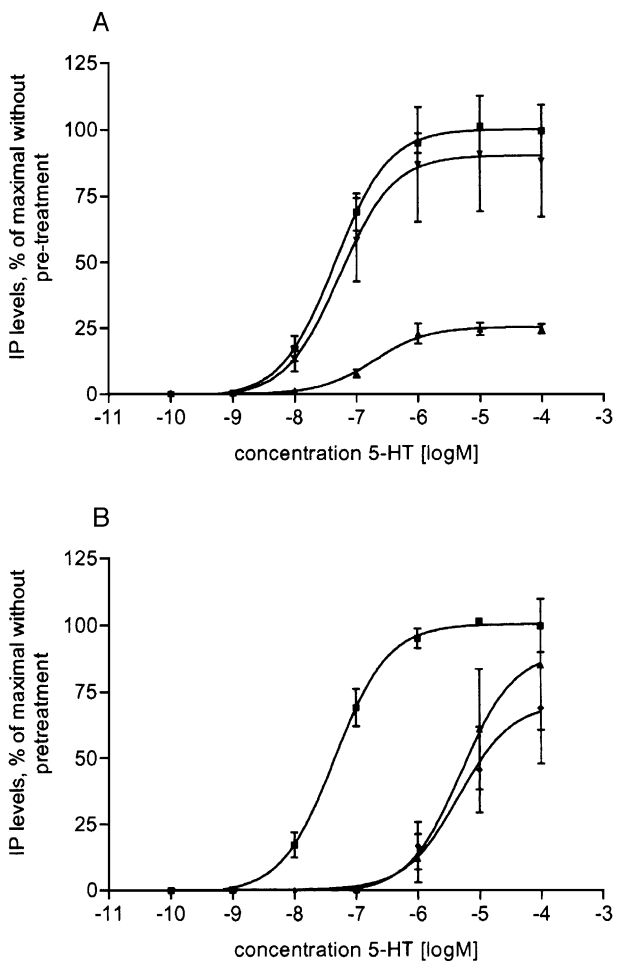

Fig. 4. Effect of 5-HT and pipamperone pre-treatment on the functional responsiveness to 5-HT of the NIH 3T3 cell line, stable expressing the rat 5-HT_{2A} receptor. (A) 5-HT-induced inositol phosphate formation in rat 5-HT_{2A}-NIH 3T3 without (■) or with pre-treatment for 15 min (▼) or 48 h (▲) with 5-HT. (B) 5-HT-induced inositol phosphate formation in rat 5-HT_{2A}-NIH 3T3 without (■) or with pre-treatment for 15 min (▼) or 48 h (▲) with pipamperone. Cells were cultured in 24-well plates and loaded with 2 μCi [^{3}H]-inositol/well for 24 h. After washing, cells were incubated for 30 min with various concentrations of 5-HT, thereafter inositol phosphate formation was measured as described under Section 2. The maximal response without pre-treatment is defined as 100%. 5-HT stimulation (*x*-fold over basal levels) reached by saturating 5-HT concentrations in each of the conditions, and the mean EC_{50} values are summarised in Table 6.

Table 6
5-HT-induced inositol phosphate formation in rat 5-HT_{2A}-NIH 3T3 and rat 5-HT_{2C}-NIH 3T3 cells after 15 min and 48 h pre-treatment of the cells with 5-HT or pipamperone

Transfected receptor	Rat $5HT_{2A}$-NIH 3T3			Rat $5HT_{2C}$-NIH 3T3		
Pre-treatment	Basal inositol phosphate levels (cpm)	5-HT stimulation (*x*-fold over basal levels)	pEC_{50} of 5-HT (M)	Basal inositol phosphate levels (cpm)	5-HT stimulation (*x*-fold over basal levels)	pEC_{50} of 5-HT (M)
None	4847 ± 1351	38 ± 2	7.35 ± 0.06	12,501 ± 1438	16 ± 5	8.59 ± 0.07
15 min 5-HT	8653 ± 5520	26 ± 6[a]	7.2 ± 0.3	20,558 ± 5895	14 ± 7	8.5 ± 0.2
48 h 5-HT	9520 ± 3660	6.4 ± 0.7[a]	6.7 ± 0.1[d]	10,362 ± 1654	8.3 ± 0.5[a]	7.6 ± 0.1[d]
15 min pipamperone	5028 ± 2585	31 ± 4[a]	5.3 ± 0.3[d]	11,106 ± 2364	21 ± 5	8.1 ± 0.3
48 h pipamperone	2989 ± 1198	41 ± 5	5.3 ± 0.3[d]	7458 ± 1764[a]	29 ± 13[a]	8.81 ± 0.08

Mean values ± S.D. of 5-HT stimulation (*x*-fold over basal levels) and pEC_{50}-values of 5-HT after short (15 min) and prolonged (48 h) exposure to 5-HT (10 μM) or pipamperone (10 μM) are presented ($n = 3$). Significant differences from controls in 5-HT stimulation (*x*-fold over basal levels) according to Student's *t*-test (two-tailed) are indicated as follows: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ (no indication: not significant). pEC_{50}-values of 5-HT that are significantly different from controls to the point of showing no overlap of the 95% confidence intervals are indicated as follows: [d]$P < 0.05$ (no indication: not significant).

0.06 (Fig. 4 and Table 6) (schematic overview of findings in Table 4).

Cells were pre-treated with 5-HT or pipamperone for 15 min or 48 h and 5-HT-induced inositol phosphate formation was measured (Fig. 4A,B and Tables 4 and 6). The 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) was 38. Short (15 min) exposure to 5-HT or pipamperone decreased the 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) mediated by rat 5-HT_{2A} receptors significantly to 26 and 31, respectively, whereas the 5-HT potency was unchanged or 100-fold decreased, respectively. After prolonged (48 h) pre-treatment with 5-HT or pipamperone, the 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) mediated by rat 5-HT_{2A} receptors was decreased to 6.4 or was not significantly changed, respectively, whereas the 5-HT potency was about 5- or 100-fold lower, respectively. Basal inositol phosphate levels were not significantly changed after short or prolonged exposure to 5-HT.

3.8. Time-dependent effect of 5-HT and pipamperone on the 5-HT-induced inositol phosphate formation mediated by rat 5-HT_{2C} receptors expressed in NIH 3T3 cells

The inositol phosphate formation mediated by rat 5-HT_{2C} receptors expressed in NIH 3T3 cells was evaluated by performing a 5-HT-stimulated phosphoinositide hydrolysis. 5-HT (seven concentrations in the range of 0.1 nM–10 μM) caused a maximal increase of 16-fold in inositol phosphate levels relative to base line, the pEC_{50} was 8.59 ± 0.07 (Fig. 5 and Table 6) (Schematic overview of findings in Table 4).

Cells were pre-treated with 5-HT or pipamperone for 15 min or 48 h and 5-HT-induced inositol phosphate formation was measured (Fig. 5A,B and Tables 4 and 6). The 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) was 16. Short (15 min) exposure to 5-HT or pipamperone did not change the 5-HT potency and the 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) mediated by rat 5-HT_{2C} receptors. After prolonged (48 h) pre-treatment with 5-HT or pipamperone, the 5-HT stimulated inositol phosphate formation (*x*-fold over basal levels) was decreased to 8.3 or increased to 29, respectively, whereas the 5-HT potency was about 10-fold lower or unchanged, respectively. Basal inositol phosphate levels were unchanged and significantly decreased after respectively short and prolonged exposure to pipamperone.

4. Discussion

The major finding of the present study is that both agonist and antagonist pre-treatment differently regulate the rat 5-HT_{2A} and rat 5-HT_{2C} receptors, expressed in the same cellular background. Consistent with classical models of G-protein-coupled receptor regulation, we found that the rat 5-HT_{2C} receptor is affected by an agonist-mediated desensitisation combined with a down-regulation, and an antagonist-induced up-regulation. On the other hand, our studies show that the rat 5-HT_{2A} receptor undergoes desensitisation of the functional response without down-regulation by agonist pre-treatment. Pre-treatment with an antagonist causes an increase in antagonist binding sites and a decrease in agonist binding sites as well as a decrease in 5-HT induced functional response.

The rat 5-HT_{2A} and rat 5-HT_{2C} receptors are unique among receptors coupled to guanine nucleotide binding proteins in that chronic treatment in vivo with agonists as well as antagonists was reported to decrease receptor density. The atypical regulation of rat 5-HT_2 receptors by antagonists in vivo may be a consequence of complexities inherent in the use of in vivo models. In vitro model systems are useful to examine receptor regulation because of the absence of complex variables that can hinder interpretation of the results in vivo (including drug disposition,

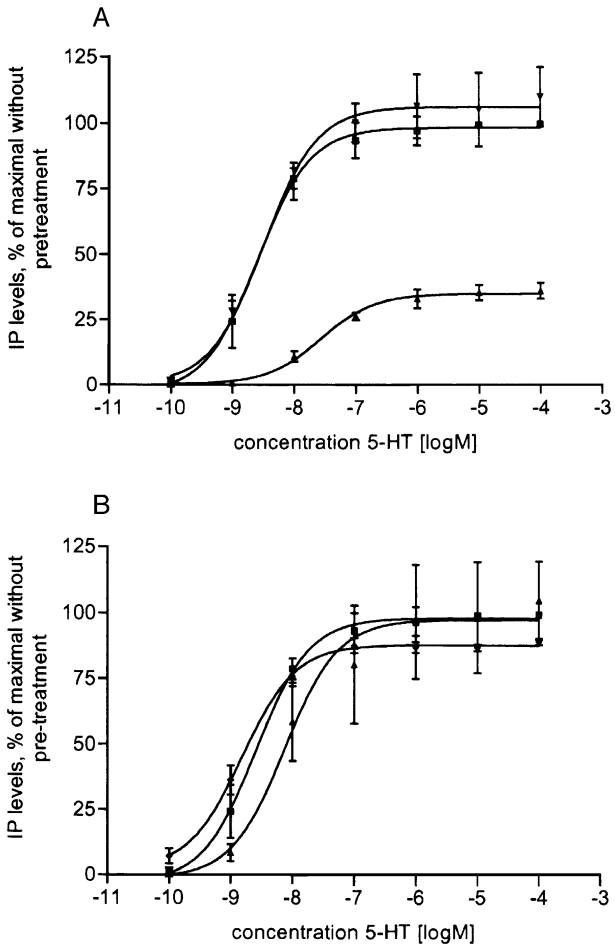

Fig. 5. Effect of 5-HT and pipamperone pre-treatment on the functional responsiveness to 5-HT of the NIH 3T3 cell line, stably expressing the rat 5-HT_{2C} receptor. (A) 5-HT-induced inositol phosphate formation in rat 5-HT_{2C}-NIH 3T3 without (■) or with pre-treatment for 15 min (▼) or 48 h (▲) with 5-HT. (B) 5-HT-induced inositol phosphate formation in rat 5-HT_{2C}-NIH 3T3 without (■) or with pre-treatment for 15 min (▼) or 48 h (▲) with pipamperone. Experiments were performed as described in the legend to Fig. 4 and under Section 2. The maximal response without pipamperone pre-treatment is defined as 100%. 5-HT stimulation (*x*-fold over basal levels) reached by saturating 5-HT concentrations in each of the conditions, and the mean EC_{50} values are summarised in Table 6.

drug metabolism, interacting neurons and multiple receptor subtypes). Inhibition of the autoregulatory response by interacting neurons may increase synaptic 5-HT levels, which can induce a down-regulation of postsynaptic receptors.

Therefore, we used two clonal cell lines, stably expressing the rat 5-HT_{2A} or rat 5-HT_{2C} receptor under the control of a constitutive promoter, to study drug interaction with both receptors in the same cellular background. These transfected cell lines are suitable for these experiments because transcriptional regulation does not to appear to play an important role in 5-HT_{2A} or 5-HT_{2C} receptor down-regulation (Roth and Ciaranello, 1991; Barker et al., 1994). In addition, Briddon et al. (1998) and Grotewiel and Sanders-Bush (1994) showed that differences in regulation properties are probably not due to differences in receptor density. Therefore, the impact of the higher absolute densities of both receptors in NIH 3T3 cells compared to the density in the brain tissue or some other cell lines used for in vitro regulation studies can be excluded. To study the effects of an antagonist on radioligand receptor binding and 5-HT-induced inositol phosphate formation in rat 5-HT_{2A} and rat 5-HT_{2C} receptors stably expressed in NIH 3T3 cells, we used pipamperone for three reasons. Firstly, pipamperone is an antagonist with an affinity for both the rat 5-HT_{2A} and rat 5-HT_{2C} receptors. Secondly, we showed in previous studies that pipamperone rapidly dissociates from both rat 5-HT_2 receptors (dissociation half time of 4.8 min) and that it inhibits antagonist binding to rat 5-HT_2 receptors in a competitive way (i.e. increased K_D-value, no change in B_{max}-value), indicating that pipamperone binding is reversible and easily washed off (Leysen et al., 1988). Thirdly, pipamperone has always been of clinical importance. Even recently, studies were published on the therapeutic use of pipamperone (Bont et al., 1998; Eikenboom et al., 1997; Schwaninger et al., 1998). It is therefore highly relevant to investigate the effects of prolonged treatment with pipamperone. We chose 10 μM 5-HT or pipamperone as the modulatory ligand concentration to investigate the agonist- and antagonist-mediated regulation of rat 5-HT_{2A} or 5-HT_{2C} receptors, to exclude the influence of differences in affinity and to use a saturating concentration to reach total receptor occupancy for both receptors.

4.1. Characterisation of the rat 5-HT_{2A} and rat 5-HT_{2C} receptors expressed in NIH 3T3 cells

We determined the pharmacological profile of the rat 5-HT_{2A} and rat 5-HT_{2C} receptors using ligand concentration-binding assays, competition binding experiments and inositol phosphate formation measurements (Figs. 1, 2 and 3; (Tables 1, 2, 4 and 6)). As illustrated by the data in Table 2, the ligand binding properties (measured using inhibition of radioactive antagonist binding) of the recombinant rat 5-HT_{2A} and rat 5-HT_{2C} receptors correspond nicely to the properties reported in studies using brain tissue (Leysen and Pauwels, 1990). For both receptors, agonist binding showed a lower B_{max} than antagonist binding. About 52% and 57% of the rat 5-HT_{2A} and rat 5-HT_{2C} receptor populations, respectively, is in an agonist high affinity, G-protein-coupled state. The K_D-values for [^{3}H]5-HT-binding to rat 5-HT_{2A} and rat 5-HT_{2C} receptors were 3 and 1.6 nM (in contrast to the K_i-value of 2900 and 180 nM for inhibition of antagonist binding, Table 2), respectively. Hence in the G-protein-coupled state both receptors bind 5-HT with nanomolar affinity, whereas the affinity for the uncoupled receptor is 1000-fold (rat 5-HT_{2A}) or 100-fold (rat 5-HT_{2C}) lower. To induce inositol

phosphate formation, 5-HT was relatively more potent at the rat 5-HT_{2C} than at the rat 5-HT_{2A} receptor (pEC_{50}-values of 8.50 and 7.25, respectively). The lower K_D- and K_i-values and higher pEC_{50}-values of 5-HT for the rat 5-HT_{2C} receptors compared to the rat 5-HT_{2A} receptors are consistent with other in vitro studies (Leonhardt et al., 1992).

We showed that pipamperone fully inhibits the 5-HT-induced inositol phosphate formation mediated by rat 5-HT_{2A} and rat 5-HT_{2C} receptors (K_i-values of 0.9 and 197 nM, respectively), indicating that pipamperone is an antagonist for both receptors. The antagonistic potency is in agreement with the binding affinity of pipamperone for the receptors (Table 2). [^{125}I]R93274 is an antagonist (Mertens et al., 1995) and [^{3}H]mesulergine is an antagonist (Havlik and Peroutka, 1992) for rat 5-HT_{2A} and rat 5-HT_{2C} receptors, respectively.

4.2. Time-dependent effect of the agonist 5-HT on the rat 5-HT_{2A} and rat 5-HT_{2C} receptors expressed in NIH 3T3 cells

To investigate the agonist-mediated regulation of the rat 5-HT_{2A} and rat 5-HT_{2C} receptors, both NIH 3T3 cell lines were cultured in the presence of 5-HT and used for ligand concentration-binding experiments and inositol phosphate formation measurements (schematic overview of findings in Table 4).

For the rat 5-HT_{2C} receptor, long-term pre-treatment with 5-HT resulted in significant reductions in the agonist and antagonist binding, the 5-HT stimulation (*x*-fold over basal levels) and pEC_{50}-values (i.e. decreased potency), whereas short exposure to 5-HT induced no clear effect. No significant shift in the K_D-values for [^{3}H]mesulergine and [^{3}H]5-HT was observed, indicating the absence of residual drug. The present study shows that rat 5-HT_{2C} receptors expressed in NIH 3T3 cells are desensitised and down-regulated after long-term exposure to 5-HT, whereas a 5-HT pre-treatment of 15 min did not desensitise or down-regulate the rat 5-HT_{2C} receptor. These results are in accordance with the classical theory of receptor regulation. Similar results have been obtained for the rat 5-HT_{2C} receptors in cultured epithelial cells (Akiyoshi et al., 1995), in CHO cells (Barker and Sanders-Bush, 1993) and for the human 5-HT_{2C} receptors in the human neuroblastoma cell line SH-SY5Y (Briddon et al., 1998).

On the other hand, agonists in NIH 3T3 cells differently regulate the rat 5-HT_{2A} receptor. Despite the functional responsiveness of the rat 5-HT_{2A} receptor to 5-HT being decreased (decreased pEC_{50}-values) after a prolonged incubation with 5-HT, we found, consistent with the results obtained by Grotewiel and Sanders-Bush (1994), no significant effect on B_{max}-levels of agonist and antagonist binding after chronic stimulation. This indicates that the shift in the serotonin dose–response curve and the decrease in the 5-HT stimulation (*x*-fold over basal levels) are independent of changes in total receptor density and of high affinity binding sites. Roth et al. (1995) demonstrated that rat 5-HT_{2A} receptor desensitisation could occur without down-regulation. Therefore, we conclude that the rat 5-HT_{2A} receptors are desensitised without down-regulation in NIH 3T3 cells after long-term exposure to 5-HT. A possible explanation for the apparent lack of rat 5-HT_{2A} receptor down-regulation in the transfected NIH 3T3 cells may be fast receptor synthesis and externalisation. However, it appears that the newly synthesised receptors in conditions where an agonist is present would not couple to G-proteins and hence do not give rise to functional responses. Similar to the rat 5-HT_{2C} receptors, a 5-HT pre-treatment of 15 min did not desensitise or down-regulate the rat 5-HT_{2A} receptors and no significant shift in the K_D-values for [^{125}I]R93274 and [^{3}H]5-HT binding was observed. Interestingly, Van Huizen et al. (1993) showed that the antagonist binding sites of the human 5-HT_{2A} receptor transfected into Swiss 3T3 cells were down-regulated by agonists. Taken together with our results, the type of agonist-induced modulation is not only cell line dependent (Grotewiel and Sanders-Bush, 1994), but may also vary according to the species of the receptor.

4.3. Time-dependent effect of the antagonist pipamperone on the rat 5-HT_{2A} and rat 5-HT_{2C} receptors expressed in NIH 3T3 cells

We performed ligand concentration-binding assays and inositol phosphate formation measurements to determine the time-dependent effect of the antagonist pipamperone on rat 5-HT_{2A}- and rat 5-HT_{2C}-NIH 3T3 cells (schematic overview of findings in Table 4).

Application of an antagonist at a receptor normally leads to supersensitivity and up-regulation of the receptor (Sanders-Bush, 1990). For the rat 5-HT_{2C} receptor, long-term exposure to pipamperone resulted in a significant increase in the B_{max}-levels of agonist and antagonist binding sites and in the 5-HT stimulation (*x*-fold over basal levels), whereas the potency of 5-HT (pEC_{50}) was unchanged. Residual pipamperone was absent in the radioligand binding assays or in the inositol phosphate formation measurements because no consistent changes in the K_D- and pEC_{50}-values were observed (Tables 5 and 6). There seems to be a tendency to a down-regulation of basal inositol phosphate levels after prolonged pre-treatment with pipamperone, possibly explained by an inverse agonist activity of pipamperone. It is to be noted that the decreased basal inositol phosphate levels after 48 h exposure to pipamperone affects the 5-HT stimulation (*x*-fold over basal levels) (Table 6). In contrast with our findings, rat 5-HT_{2C} receptors are down-regulated by mianserin in cultured epithelial cells (Barker and Sanders-Bush, 1993) and Sf9 cells (Labrecque et al., 1995). In addition, Newton and

Elliot (1997) demonstrated a mianserin-induced down-regulation of the human 5-HT_{2C} receptors expressed in the human neuroblastoma cell line SH-SY5Y. Inverse agonism of mianserin has been proposed as the basis of this antagonist-mediated down-regulation (Barker et al., 1994). Taken together, the antagonist-induced regulation of 5-HT_{2C} receptors may vary according to the used antagonist, host cell line and species of the receptor.

Antagonist pretreatment of rat 5-HT_{2A} receptor expressing cells appeared to affect the ratio of the G-protein-coupled and uncoupled rat 5-HT_{2A} receptor populations (Table 3). Short antagonist pre-treatment does not significantly increase the total number of (antagonist) binding sites, but profoundly reduces the agonist binding sites; as a result only 21% of the receptors remained in the G-protein-coupled state. Prolonged antagonist pre-treatment resulted in a substantial increase in the total number of antagonist binding sites, whereas the number of agonist binding sites was reduced; only 10% of the receptors was in a G-protein-coupled state. Hence, it appeared that the pipamperone pre-treatment shifts the receptor population in a G-protein-uncoupled state. It also induces increased receptor synthesis, but the newly synthesized receptor seems not to couple readily to the G-proteins. The observation that the K_D-value of [^{3}H]5-HT is unchanged, indicates that there is no residual pipamperone bound to the G-protein-coupled receptor. However, the K_D-value of the antagonist radioligand is 2.5 times increased which could point to some residual pipamperone bound to the uncoupled receptor. It should be noted that Newton and Elliot (1997) observed a mianserin-induced down-regulation of the human 5-HT_{2A} receptors expressed in the human neuroblastoma cell line SH-SY5Y. In addition, Rinaldi-Carmona et al. (1994) reported a down- and up-regulation of the rat 5-HT_{2A} receptors by the antagonists ketanserin and SR 46349B, respectively. Similar to the rat 5-HT_{2C} receptors, the antagonist-induced regulation of 5-HT_{2A} receptors may vary according to the used antagonist, host cell line and species of the receptor.

In conclusion, this is the first article where rat 5-HT_{2A} and rat 5-HT_{2C} receptor regulation by agonists and antagonists is compared in parallel using the same cell culture system and where the receptor properties are investigated by both radioactive agonist and antagonist binding and functional responses. The major finding of our in vitro study is that bothagonists and antagonists in vitro regulate rat 5-HT_{2A} and 5-HT_{2C} receptors differently. Our studies have illustrated that the 5-HT-and pipamperone-induced regulation of rat 5-HT_{2C} receptors expressed in NIH 3T3 cells is consistent with classical models of G-protein-coupled receptors. In contrast, the rat 5-HT_{2A} receptor is desensitised without down-regulation after chronic stimulation and after prolonged antagonist pretreatment, the receptor response is desensitized but the total receptor number is increased. This observation can be explained by an antagonist-induced uncoupling of the receptor from G-proteins accompanied by an increased receptor synthesis. The newly synthesised receptors appear not to couple readily to G-proteins, hence the reduced high affinity agonist binding. Our findings suggest that the rat 5-HT_{2A} and rat 5-HT_{2C} receptor differ markedly in their ability to couple to G-proteins to form a functionally active receptor. Compared to other published studies, our data reflect the importance of the cellular environment, the administered drug and the species of the receptor on the pattern of the in vitro regulation of the rat 5-HT_2 receptors.

Acknowledgements

Both NIH 3T3 cell lines, stably expressing the rat 5-HT_{2A} or rat 5-HT_{2C} receptor were the generous gift of Dr. David Julius (University of California, San Francisco, USA). We thank Martine Ercken and Paul Van Gompel for their helpful practical advice.

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