Serotonin 5-HT_{1B} and 5-HT_{1D} receptors form homodimers when expressed alone and heterodimers when co-expressed

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Abstract The serotonin (5-hydroxytryptamine (5-HT)) 1B and 1D receptor subtypes share a high amino acid sequence identity and have similar ligand binding properties. In this study, we demonstrate that both receptor subtypes exist as monomers and homodimers when expressed alone and as monomers and heterodimers when co-expressed. Gene expression studies have shown that there are brain regions where the 5-HT_{1B} and 5-HT_{1D} receptors are co-localized and where heterodimerization may occur physiologically. This is the first direct visualization of the physical association between G protein-coupled receptors of different subtypes.

Key words: G protein-coupled receptor; Receptor dimerization; Receptor oligomerization; Western blotting; Hetero-oligomerization

1. Introduction

The serotonin (5-hydroxytryptamine (5-HT)) receptors are a large and complex family of receptors [1]. Many more receptor subtypes have been discovered through molecular cloning than was predicted by pharmacological studies [2]. All 5-HT receptors, except those in the 5-HT_3 subfamily, are G protein-coupled receptors (GPCRs) and 5-HT-mediated signal transduction occurs through a variety of effectors. The diversity in the structure and function of 5-HT receptors has not only complicated the classification of these receptors, it has also created difficulties in understanding their physiological roles [2].

The 5-HT_1 receptors represent the largest subclass of 5-HT receptors and are defined in part by their preferential coupling to the $G_{i/o}$ class of G proteins [3]. Our group was the first to report the cloning of the human gene encoding one of these 5-HT_1 subtypes, the 5-HT_{1B} receptor [4]. Extensive studies of the 5-HT_{1B} receptor have been carried out. However, characterization of this receptor in vivo has been hindered due to the existence of the closely related 5-HT_{1D} receptor. The 5-HT_{1B} and 5-HT_{1D} receptors share ~68% amino acid sequence homology and are almost indistinguishable pharmacologically [5,6]. This similarity in the ligand binding properties has created problems in distinguishing the receptor subtypes in vivo and in establishing their respective physiological relevance [7].

Following the cloning of the 5-HT_{1B} receptor gene, we also examined signal transduction mechanisms and post-translational modifications of the 5-HT_{1B} receptor [8]. During these studies, it was observed that the 5-HT_{1B} receptor exists as monomers and homodimers. There is increasing evidence that all GPCRs form oligomeric structures. We have shown that heterologously expressed D1 and D2 dopamine receptors exist as homodimers [9,10] and, recently, we have demonstrated that D2 dopamine receptors exist as dimers in human and rat brain tissue [11]. Observations have been made suggesting that the M2 muscarinic [12], the β_2-adrenergic [13], the V2 vasopressin [13], the metabotropic glutamate [14], the H2 histamine [15], the δ-opioid [16], the D3 dopamine [17] and the Ca^{2+}-sensing [18] receptors also form dimers, demonstrating that oligomerization may be a universal aspect of GPCR biology.

In this study, we examined the dimeric states of the 5-HT_{1B} and 5-HT_{1D} receptors. We predicted that, like the 5-HT_{1B} receptor, the 5-HT_{1D} receptor would form dimers. In addition, we hypothesized that the 5-HT_{1B} and 5-HT_{1D} receptors would form heterodimers when co-expressed. The basis for our hypothesis was 2-fold. Firstly, while the precise site(s) of interaction between two monomeric GPCRs undergoing dimerization has not been elucidated, it has been postulated that receptor-receptor interactions between the transmembrane (TM) domains may be involved [13,19]. The homology between the 5-HT_{1B} and 5-HT_{1D} receptors in the TM domains is 77%. Therefore, we postulated that receptor-receptor interactions similar to those involved in homodimerization could occur between these two closely related 5-HT receptor subtypes. Secondly, there appears to be a large degree of overlap in the anatomical localization of the two receptors [20]. We were intrigued by the possibility that 5-HT_{1B} and 5-HT_{1D} receptors may be co-localized in vivo and that they may form heterodimers in these areas of co-expression.

In this investigation, in addition to demonstrating that both the 5-HT_{1B} and 5-HT_{1D} receptors form homodimers when expressed alone, we provide direct evidence of their heterodimerization, indicating a physical association of the 5-HT_{1B} receptor with the 5-HT_{1D} receptor.

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Abbreviations: 5-HT, 5-hydroxytryptamine; GPCR, G protein-coupled receptor; TM, transmembrane; PCR, polymerase chain reaction; 5-CT, 5-carboxamidotryptamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; K_i, inhibitory binding constant; GABA, γ-aminobutyric acid; $GABA_BR$, metabotropic GABA receptor

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2. Materials and methods

2.1. Construction of c-myc-tagged 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor baculoviruses

The 5-HT$_{1B}$ receptor cDNA and 5-HT$_{1D}$ receptor cDNA were modified using the Transformer Site Directed Mutagenesis kit (Clontech). A DNA sequence encoding an 11 residue c-myc epitope (EQKLISEEDLN) was inserted after the codon encoding the N-terminal start methionine. Recombinant baculovirus was created from the resulting c-myc-tagged receptor cDNA using the Bac-to-Bac kit (Life Technologies).

2.2. Cell culture

All culture media, antibiotics and supplements were purchased from Life Technologies. Sf9 cells were maintained at 27°C in Grace's Insect Media supplemented with 10% (v/v) fetal bovine serum, 0.5× antibiotic-antimycotic and 1% (v/v) Pluronic F-68, a surfactant. Suspension cultures were infected with baculovirus when the cell density was 1–3×10^6 cells/ml with a multiplicity of infection of approximately 5. Membranes were prepared from the cells 48 h after infection unless otherwise noted.

2.3. Membrane preparation

Cells were washed with phosphate-buffered saline, resuspended in hypotonic lysis buffer (5 mM Tris-HCl, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamide, 5 μg/ml soybean trypsin inhibitor, pH 7.4) and homogenized by Polytron (Brinkman). The homogenate was centrifuged to a pellet of unbroken cells and nuclei. The supernatant was collected and centrifuged at 40 000×g for 20 min and the resulting pellet was washed and resuspended in lysis buffer.

2.4. Radioligand binding assays

Saturation binding and competition binding assays were performed as previously described [8]. Briefly, for saturation experiments, 20–25 μg of membranes from receptor-expressing cells was incubated with increasing concentrations of [^{3}H]5-carboxamidotryptamine (5-CT) (NEN Life Sciences Products) or [^{3}H]GR-125743 (Amersham Pharmacia Biotech). The reaction volume was 0.5 ml and the binding buffer consisted of 50 mM Tris-HCl, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl and 120 mM NaCl with a pH of 7.4. Nonspecific binding was defined using 100 μM methysergide (RBI) or mesulergine (RBI).

For competition binding experiments, 20–25 μg of membranes was incubated with ∼1 nM [^{3}H]5-CT or ∼10 nM [^{3}H]GR-125743 and increasing concentrations of competing drugs.

For both assays, binding reactions were incubated at room temperature for 2 h to reach equilibrium. Bound radioligand was then isolated from free radioligand by rapid filtration through a Brandel 48 well harvester using Whatman GF/C filters. Data were analyzed using non-linear least-squares regression equations on the curve-fitting computer program Prism v2.01 (Graphpad).

2.5. Gel electrophoresis and immunoblotting

The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 10 or 12% pre-cast acrylamide gels (Novex) and transferred to nitrocellulose using a semi-dry transfer apparatus. The nitrocellulose was blocked using 10% (w/v) skim milk powder in Tris-buffered saline (TBS) and then incubated in 1% skim milk powder TBS containing the 9E10 antibody (Santa Cruz Biotechnology). The primary antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) and the Enhanced Chemiluminescent detection kit (Amersham). The relative intensities of bands detected by immunostaining were determined using reflective densitometry and the Gel Doc 1000 Video Documentation System and Molecular Analyst software (Bio-Rad).

3. Results

3.1. The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors formed homodimers when expressed alone

Immunoblot analysis of cells expressing the 5-HT$_{1B}$ receptor revealed bands with apparent molecular weights of ∼43 and ∼86 kDa, corresponding to 5-HT$_{1B}$ receptor monomers and dimers (Fig. 1, lane 1). In cells expressing the 5-HT$_{1D}$ receptor, bands with apparent molecular weights of ∼38 and ∼76 kDa representing 5-HT$_{1D}$ receptor monomers and dimers were immunodetected (Fig. 1, lane 2). 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor monomers are predicted by sequence analysis to be ∼43 and ∼38 kDa, respectively. The immunoreactive bands representing receptor monomers are close to their predicted molecular weight because proteins expressed using the baculovirus/Sf9 cell system are not subject to heavy glycosylation [21].

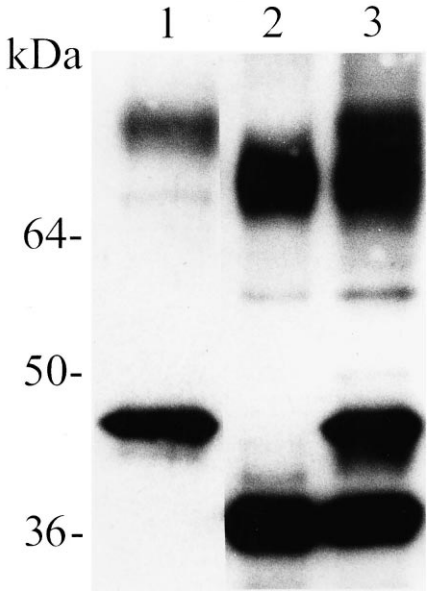

Fig. 1. Immunoblot analysis of membranes from Sf9 cells expressing the 5-HT$_{1B}$ receptor (lane 1) and the 5-HT$_{1D}$ receptor (lane 2) and of a mixture of both membranes (lane 3). Both receptors were c-myc epitope-tagged and the 9E10 monoclonal antibody was used for immunodetection. For each lane, 25 μg of protein was used. The immunoblot shown is representative of three independent experiments.

Membranes from cells expressing 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors were mixed, subjected to SDS-PAGE and immunoblotted. Proteins corresponding in molecular weight to 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor monomers and homodimers were immunodetected (Fig. 1, lane 3).

3.2. The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors formed heterodimers when co-expressed

Membranes from cells co-expressing the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors were immunoblotted (Fig. 2, lane 2). Immunoreactive bands corresponding to 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor monomers were detected. However, no band corresponding in molecular weight to either receptor homodimer was observed (Fig. 2, lane 2). An ∼80 kDa protein was immunodetected which, being intermediate in size, corresponded to 5-HT$_{1B}$ receptor/5-HT$_{1D}$ receptor heterodimer and which was not observed in cells expressing only one of the receptor subtypes (Fig. 2, lanes 1 and 3) or in the mixed membranes (Fig. 1, lane 3).

3.3. Ligand binding properties of cells co-expressing the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors

Saturation and competition binding assays were performed

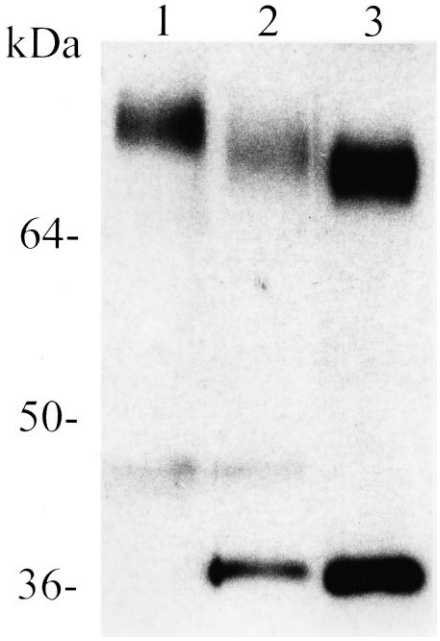

Fig. 2. Immunoblot analysis of membranes from Sf9 cells expressing the 5-HT_{1B} receptor alone (lane 1) and the 5-HT_{1D} receptor alone (lane 3) and membranes co-expressing both receptors (lane 2). Both receptors were c-myc epitope-tagged and the 9E10 monoclonal antibody was used for immunodetection. For each lane, 25 μg of protein was used. The immunoblot shown is representative of three independent experiments.

on membranes expressing the 5-HT_{1B} receptor alone, expressing the 5-HT_{1D} receptor alone and co-expressing both receptor subtypes. A mixture of membranes expressing only one subtype with membranes expressing only the other subtype was also tested. The affinity constants of the non-selective agonist 5-CT and the 5-HT_1 antagonist GR-125743 were calculated to be 1.2 and 2.6 nM, respectively, for the 5-HT_{1B} receptor and 0.9 and 2.8 nM, respectively, for the 5-HT_{1D} receptor. Co-expression of the receptor subtypes did not alter the affinity for 5-CT or GR-125743 (1.1 nM for 5-CT and 2.9 nM for GR-125743).

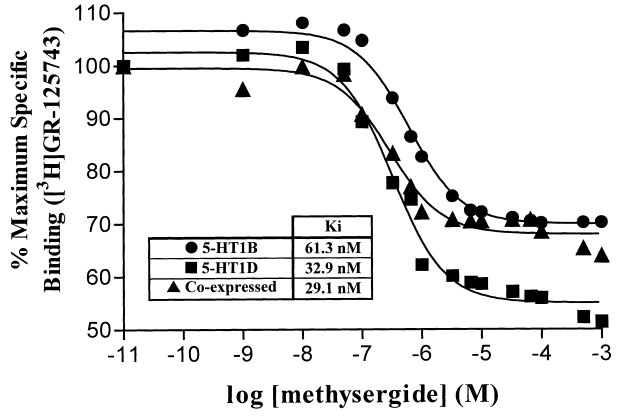

Fig. 3. Competition of [^{3}H]GR-125743 binding with methysergide in membranes prepared from Sf9 cells expressing the 5-HT_{1B} receptor (●) or the 5-HT_{1D} receptor (■) or co-expressing both receptors (▲). The inhibitory binding constants (K_i) calculated from the binding data are shown in the inset table.

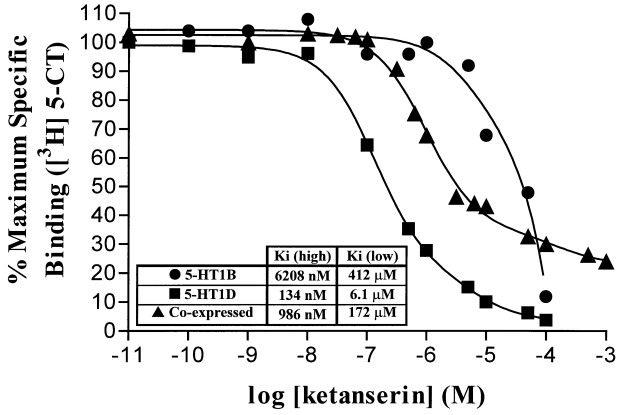

Fig. 4. Competition of [^{3}H]5-CT binding with ketanserin in membranes prepared from Sf9 cells expressing the 5-HT_{1B} receptor alone (●) or the 5-HT_{1D} receptor alone (■) or co-expressing both receptors (▲). The inhibitory binding constants (K_i) for the high and low affinity binding states calculated from the binding data are shown in the inset table.

Saturation binding with [^{3}H]ketanserin, an antagonist with a higher affinity for the 5-HT_{1D} receptor compared to 5-HT_{1B} receptor, was also performed. As expected, the affinity of ketanserin for the 5-HT_{1D} receptor (2.8 nM) was slightly higher than for the 5-HT_{1B} receptor (9.2 nM). However, no significant change in ketanserin affinity was seen in membranes co-expressing both receptors (9.0 nM).

In competition binding assays, [^{3}H]GR-125743 was displaced by the non-selective antagonist methysergide (Fig. 3) and [^{3}H]5-CT was displaced by ketanserin (Fig. 4). The inhibitory binding constant (K_i) of methysergide was almost identical for the 5-HT_{1B} receptor compared to the 5-HT_{1D} receptor. For the co-expressed 5-HT_{1B}/5-HT_{1D} receptors, the K_i value was not different from that of the receptors expressed alone.

Two affinity states were detected for ketanserin competition of [^{3}H]5-CT for the 5-HT_{1B} receptor, 5-HT_{1D} receptor and the co-expressed receptors (Fig. 4). The K_i values for the high and low affinity states were both approximately one log unit higher for the 5-HT_{1D} receptor compared to 5-HT_{1B} receptor. For membranes co-expressing the receptors, analysis of a four state model was not performed. However, when the binding data were subjected to a two state analysis, the high and low affinity state inhibitory constants approximated an average of constants for the two receptors expressed alone.

4. Discussion

We have shown that the serotonin 5-HT_{1B} and 5-HT_{1D} receptors form homodimers when expressed alone and heterodimers when co-expressed. This represents a direct demonstration of the physical association between two GPCR subtypes. No heterodimers were observed when membranes expressing one receptor subtype were mixed with membranes expressing only the other, indicating that heterodimerization in cells may require a specific cellular mechanism and does not result from non-specific aggregation of the receptors.

Interestingly, when the 5-HT_{1B} and 5-HT_{1D} receptors were co-expressed, no homodimers were observed. This suggests that, when co-expressed, the receptors favor the heterodimeric conformation. It has been shown that there are regions of the

brain where both of these 5-HT receptor subtypes occur together and there are regions in which expression of only one subtype occurs. For example, the 5-HT_{1B} and 5-HT_{1D} receptors are found to be both expressed in the cerebral cortex, the olfactory tubercle and the dorsal raphe nucleus [20]. The 5-HT_{1B} receptor is expressed without the 5-HT_{1D} receptor in the anterior caudate putamen, hypothalamus, thalamus and hippocampus and the 5-HT_{1D} receptor is expressed in the trigeminal nucleus and parts of the cerebellum, regions where the 5-HT_{1B} receptor does not occur [20]. This raises the possibility that two receptor subtypes could result in three populations of receptor complexes: two homodimeric and one heterodimeric.

The concept of hetero-oligomeric arrangements between closely related receptors may be novel within the GPCR family, however, the formation of 'hybrid' receptors among hormone receptors and growth factor receptors is commonly accepted as a mechanism for increasing the diversity of cellular responses to extracellular signals. The existence of hybrid receptors between the insulin-like growth factor receptor and the insulin receptor, between subtypes of the platelet-derived growth factor receptors and between the T3 thyroid hormone receptor and the retinoic acid receptor has been well-established (reviewed in [22]).

Our ligand binding experiments revealed no significant differences between ligand affinity profiles in membranes expressing one of the 5-HT_{1B} or 5-HT_{1D} receptors alone and membranes co-expressing both 5-HT receptor subtypes. This finding differs from our observations in the D2 dopamine receptor. We have shown that dimers of the D2 dopamine receptor have different binding characteristics than receptor monomers [11,23], indicating that the association of two GPCRs may result in an alteration of the existing ligand binding pockets or the creation of novel binding sites. Our observations with the 5-HT_{1B} and 5-HT_{1D} receptors suggest that a novel or altered binding pocket did not result from heterodimerization. Since our binding experiments indicated no differences between co-expressed 5-HT_{1B} and 5-HT_{1D} receptors and the receptors expressed alone, it is possible that heterodimers of these closely related receptor subtypes do not possess novel binding characteristics but may differ in another functional aspect related to receptor structure.

Interestingly, suggestions of cross-talk among serotonin receptor subtypes and between serotonin receptors and other receptors have recently been made [24–26]. While current thinking on this matter speculates that this type of association between different receptors occurs at the effector level, our data suggest the novel possibility that cross-talk may be at the level of the receptor as well.

During the course of our work, hetero-oligomerization between the two known metabotropic γ-aminobutyric acid (GABA) receptor subtypes $GABA_BR1$ and $GABA_BR2$ was reported [27–30]. However, the conclusion that $GABA_BR1$ and $GABA_BR2$ form hetero-oligomers was made without direct visualization of heterodimeric protein complexes. Furthermore, while the $GABA_B$ receptors are GPCRs, they are members of a distinct subfamily sometimes referred to as the family C [31]. The 5-HT GPCRs are members of the rhodopsin-like GPCRs or family A [32].

In conclusion, this study represents the first direct visualization of GPCR heterodimerization and the first demonstration that rhodopsin-like GPCRs form heterodimers. While the precise role of homodimeric and heterodimeric 5-HT_{1B} and 5-HT_{1D} receptors remains to be elucidated, it is possible that the phenomenon may be exploited therapeutically. For instance, it has been shown that dimeric serotonin ligands are more potent than their monomeric equivalents at the 5-HT_{1B} and 5-HT_{1D} receptors [33,34]. It will be interesting to determine if heterodimerization occurs among other serotonin receptor subtypes and other GPCRs resulting in a greater diversity of the GPCR function than that predicted by the number of cloned receptor genes.

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References

[1] Uphouse, L. (1997) Neurosci. Biobehav. Rev. 21, 679–698.
[2] Hoyer, D. and Martin, G.R. (1996) Behav. Brain Res. 73, 263–268.
[3] Gerhardt, C.C. and van Heerikhuizen, H. (1997) Eur. J. Pharmacol. 334, 1–23.
[4] Jin, H., Oksenberg, D., Ashkenazi, A., Peroutka, S.J., Duncan, A.M., Rozmahel, R., Yang, Y., Mengod, G., Palacios, J.M. and O'Dowd, B.F. (1992) J. Biol. Chem. 267, 5735–5738.
[5] Weinshank, R.L., Zgombick, J.M., Macchi, M.J., Branchek, T.A. and Hartig, P.R. (1992) Proc. Natl. Acad. Sci. USA 89, 3630–3634.
[6] Hartig, P.R., Branchek, T.A. and Weinshank, R.L. (1992) Trends Pharmacol. Sci. 13, 152–159.
[7] Pauwels, P.J. (1997) Gen. Pharmacol. 29, 293–303.
[8] Ng, G.Y., George, S.R., Zastawny, R.L., Caron, M., Bouvier, M., Dennis, M. and O'Dowd, B.F. (1993) Biochemistry 32, 11727–11733.
[9] Ng, G.Y., Varghese, G., Chung, H.T., Trogadis, J., Seeman, P., O'Dowd, B.F. and George, S.R. (1997) Endocrinology 138, 4199–4206.
[10] George, S.R., Lee, S.P., Varghese, G., Zeman, P.R., Seeman, P., Ng, G.Y. and O'Dowd, B.F. (1998) J. Biol. Chem. 273, 30244–30248.
[11] Zawarynski, P., Tallerico, T., Seeman, P., Lee, S.P., O'Dowd, B.F. and George, S.R. (1998) FEBS Lett. 441, 383–386.
[12] Maggio, R., Vogel, Z. and Wess, J. (1993) Proc. Natl. Acad. Sci. USA 90, 3103–3107.
[13] Hebert, T.E., Moffett, S., Morello, J.P., Loisel, T.P., Bichet, D.G., Barret, C. and Bouvier, M. (1996) J. Biol. Chem. 271, 16384–16392.
[14] Romano, C., Yang, W.L. and O'Malley, K.L. (1996) J. Biol. Chem. 271, 28612–28616.
[15] Fukushima, Y., Asano, T., Saitoh, T., Anai, M., Funaki, M., Ogihara, T., Katagiri, H., Matsuhashi, N., Yazaki, Y. and Sugano, K. (1997) FEBS Lett. 409, 283–286.
[16] Cvejic, S. and Devi, L.A. (1997) J. Biol. Chem. 272, 26959–26964.
[17] Nimchinsky, E.A., Hof, P.R., Janssen, W.G., Morrison, J.H. and Schmauss, C. (1997) J. Biol. Chem. 272, 29229–29237.
[18] Bai, M., Trivedi, S. and Brown, E.M. (1998) J. Biol. Chem. 273, 23605–23610.
[19] Gouldson, P.R., Snell, C.R. and Reynolds, C.A. (1997) J. Med. Chem. 40, 3871–3886.
[20] Bonaventure, P., Voorn, P., Luyten, W.H., Jurzak, M., Schotte, A. and Leysen, J.E. (1998) Neuroscience 82, 469–484.
[21] Jarvis, D.L. (1997) in: The Baculoviruses (Miller, L.K., Ed.), pp. 389–431, Plenum Press, New York.
[22] Jacobs, S. and Moxham, C. (1991) New Biol. 3, 110–115.
[23] Ng, G.Y., O'Dowd, B.F., Lee, S.P., Chung, H.T., Brann, M.R., Seeman, P. and George, S.R. (1996) Biochem. Biophys. Res. Commun. 227, 200–204.
[24] Martin, G.R., Eglen, R.M., Hamblin, M.W., Hoyer, D. and Yocca, F. (1998) Trends Pharmacol. Sci. 19, 2–4.

[25] Martin, G.R., MacLennan, S.J., Maxwell, M. and Smith, R.R. (1998) Ann. N.Y. Acad. Sci. 861, 251–252.
[26] Berg, K.A., Maayani, S. and Clarke, W.P. (1998) Ann. N.Y. Acad. Sci. 861, 111–120.
[27] Jones, K.A., Borowsky, B., Tamm, J.A., Craig, D.A., Durkin, M.M., Dai, M., Yao, W.J., Johnson, M., Gunwaldsen, C., Huang, L.Y., Tang, C., Shen, Q., Salon, J.A., Morse, K., Laz, T., Smith, K.E., Nagarathnam, D., Noble, S.A., Branchek, T.A. and Gerald, C. (1998) Nature 396, 674–679.
[28] Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A. and Bettler, B. (1998) Nature 396, 683–687.
[29] White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M. and Marshall, F.H. (1998) Nature 396, 679–682.
[30] Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A. and Kornau, H.C. (1999) Science 283, 74–77.
[31] http://www.gcrdb.uthscsa.edu/fc_intro.html
[32] http://www.gcrdb.uthscsa.edu/fa_intro.html
[33] Halazy, S., Perez, M., Fourrier, C., Pallard, I., Pauwels, P.J., Palmier, C., John, G.W., Valentin, J.P., Bonnafous, R. and Martinez, J. (1996) J. Med. Chem. 39, 4920–4927.
[34] Perez, M., Pauwels, P.J., Fourrier, C., Chopin, P., Valentin, J.P., John, G.W., Marien, M. and Halazy, S. (1998) Bioorg. Med. Chem. Lett. 8, 675–680.