Expression of functional mouse 5-HT_{5A} serotonin receptor in the methylotrophic yeast *Pichia pastoris*: pharmacological characterization and localization

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Abstract The methylotrophic yeast *Pichia pastoris* was tested for heterologous expression of the mouse 5-HT_{5A} receptor. Three different expression plasmids were constructed where the cDNA of the receptor was cloned under the transcriptional control of the highly inducible promotor of the *P. pastoris* alcohol oxidase 1 (AOX1) gen. The expression plasmids differed with respect to the signal sequences used for N-terminal fusion. In two cases the coding region was additionally fused to the c-*myc* tag to permit immunological detection of the receptor. Expression of functional receptor after transformation of strain GS115 was detected by radioligand binding using [^{3}H]LSD. The construct with the best expression levels in strain GS115 was used for transformation of the protease deficient strain SMD1163. Here, the expression level was 2–8 times higher. Whole cells as well as crude membrane preparations of recombinant clones showed saturable binding of [^{3}H]LSD with a K_d of ≈1.9 nM. Receptor concentrations of ≈22 pmol/mg membrane protein revealed the potential of the *P. pastoris* expression system for high level expression of membrane proteins. The pharmacological properties were comparable to those reported for the receptor expressed in mammalian systems.

Key words: *Pichia pastoris*; Membrane protein; Heterologous expression; Mouse 5-HT_{5A} receptor; Receptor affinity; Immunogold electron microscopy

1. Introduction

The methylotrophic yeast *Pichia pastoris* has been developed as a host for the efficient production of foreign proteins [1]. Interest in the *Pichia pastoris* expression system has grown, since this organism has the potential for high level expression and rapid growth to very high cell densities on inexpensive media [2,3]. A number of genes have already been heterologously expressed using the promoter of the alcohol oxidase I (*AOXI*) gene of *Pichia pastoris*. *AOXI* is the first enzyme in the methanol-utilization pathway and is strongly regulated. The enzyme is undetectable when cells are cultured on carbon sources such as glucose, glycerol or ethanol, but upon induction by addition of methanol it can constitute up to 30% of total soluble protein [4]. Stringent regulation of the *AOX1* is mainly achieved at the transcriptional level [5] and therefore, the promoter of the *AOXI* gene has been used as a powerful tool for the heterologous expression of genes which might otherwise be toxic to growing cells. In some cases, heterologous expression yielded up to several grams of the desired recombinant protein per liter of culture [6]. Although the *P. pastoris* system has been successfully used for the production of many soluble proteins [7,8,9,10], reports on the successful heterologous expression of membrane proteins in this system are lacking.

The mouse 5-HT_{5A} receptor belongs to the large family of G-protein-coupled receptors which is of great pharmacological relevance. The receptor gene was cloned and characterized previously [11]. The pharmacological profile of the receptor and sequence comparisons led to the assumption that the 5-HT_{5A} receptor, together with the 5-HT_{5B} receptor, defines a new subtype of serotonin receptors [12]. By in situ hybridization experiments expression of the 5-HT_{5A} receptor gene could be detected only in different parts of the CNS (cerebral cortex, olfactory bulb, hippocampus, etc.) but not outside the CNS [11]. Until now, there has been a lack of knowledge concerning the effector pathway of the 5-HT_5 receptors.

Like the majority of medical and pharmacological relevant membrane proteins, the 5-HT_{5A} receptor is found naturally in only very small quantities. Therefore, overexpression of such proteins in a heterologous system and subsequent purification of the protein to homogeneity is a prerequisite for biophysical as well as structural studies [13]. The search for good expression systems for membrane proteins proved to be difficult, as protein yields are often poor and therefore purification tends not to be worthwhile. As the 5-HT_{5A} receptor could be functionally expressed in insect cells via the baculovirus expression system [14], as well as in the yeast *S. cervisiae* [15], we used this receptor to test the *P. pastoris* system as a tool for membrane protein expression.

2. Materials and methods

2.1. Strains and transformations

E. coli strain XL-1 Blue (Stratagene) was used for transformation [16] and propagation of the recombinant plasmids. *Pichia pastoris* strain GS115 (his4) and *Pichia pastoris* strain SMD1163 (his4, pep4, pbr) (Invitrogen, San Diego) were used in the expression study. The strain SMD1163 bears a protease deficency. Yeast transformation was performed according to the spheroplast method described in the Manual Version 3.0 of the Pichia Expression Kit (Invitrogen, San Diego).

2.2. Recombinant DNA technology and construction of the expression plasmid

Plasmid constructions were performed using established techniques [16,17] and cloning steps were ensured by DNA sequencing [18].

2.2.1. pHIL-D2-5-HT_{5A}. The 5-HT_{5A} gene was isolated as a ≈1.15 kbp *Eco*RI fragment from the baculovirus transfer vector pVL935-HT_{5A} [14] and ligated into the *Eco*RI site of vector pHIL-D2 (Invitrogen, San Diego).

2.2.2. pHIL-S1-5-HT_{5A}myc. The 5-HT_{5A} gene together with the se-

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Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamine.

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quence coding for the *c-myc* [19] epitope at its 5'-end, was isolated as a ≈1.15 kbp *Bgl*II/*Bam*HI fragment from the baculovirus transfer vector pVLMelMyc5-HT$_{5A}$ [14], and ligated into the *Bam*HI side of vector pHIL-S1 (Invitrogen, San Diego). The reading frame between the *PHO1* secretion signal of the vector and the c-*myc* epitope was corrected by opening the *Xho*I side and a fill-in reaction with Klenow polymerase. The *PHO1* signal sequence in vector pHIL-S1 was derived from the *P. pastoris* acid phosphatase [9].

2.2.3. pPIC9-5-HT$_{5A}$myc. The 5-HT$_{5A}$ gene, together with the sequence coding for the c-*myc* epitope already fused to the 5' end of the receptor, was excised as a ≈1.15 kb *Bgl*II/*Eco*RI fragment from the plasmid pVLMelMyc5-HT$_{5A}$ [14]. The reading frame was corrected by fill-in of the *Bgl*II side. The fragment was ligated in the pPIC9 vektor (Invitrogen, San Diego) using the *Sna*BI and *Eco*RI side. This cloning step leads to an in-frame fusion of the c-*myc* tagged 5-HT$_{5A}$ receptor gene to the α-factor signal sequence of *S. cervisiae*, which is encoded by the pPIC9 vector.

In all resulting expression plasmids, the receptor gene was under the transcriptional control of the *AOX1* promoter. The vectors were linearized before transformation with *Not*I (pHIL-D2-5-HT$_{5A}$) or *Bgl*II (pHIL-S1-5-HT$_{5A}$myc, pPIC9-5-HT$_{5A}$myc) to get Mut^s (slow methanol utilization) transformants and with *Sal*I to get Mut$^+$ (same methanol utilization as wild-type) transformants.

2.3. Yeast cultures

P. pastoris strains were maintained and transformed as described in the Manual Version 3.0 of the Pichia Expression Kit (Invitrogen, San Diego). The Mut phenotype of recombinant clones was determined by patching His$^-$ transformants on minimal dextrose (1.34% yeast nitrogen base with ammonium sulfate and without amino acids; 4×10^{-5}% biotin and 1% dextrose) and minimal methanol (0.5% methanol instead of dextrose) plates. In Mut^s transformants the *AOX1* gene is replaced by the vector during transformation, resulting in strongly retarded growth on methanol.

2.4. Isolation of crude membranes from P. pastoris *cells*

Cells were harvested 24–30 h after induction by centrifugation, washed once with ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol), and resuspended to an OD_{600} of about 130 in breaking buffer supplemented with protease inhibitors (0.5 mg/ml Pefabloc, 0.5 μg/ml Leupeptin, 0.7 μg/ml Pepstatin). When preparing membranes from small cultures, an equal volume of glassbeads (Mesh 400–600; Sigma) was added to the suspension, and cells were broken by vigorous vortexing at 4°C for 5 min, with cooling on ice of the suspension every 30 s. When performing a large scale membrane preparation, cells were broken using a disintegrator. Intact cells and other crude particles were subsequently separated from the membrane suspension by a brief centrifugation step (700 × *g*, 10 min). Membranes were pelleted at 100,000 × *g* and 4°C for 30 min, and resuspended in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA plus protease inhibitors (1/100 to 1/50 volume of the initial culture). Aliquots were snap-frozen and stored at −75°C for further analysis. The protein concentration of the membrane preparation was determined using the BCA method (Pierce, Rockford, IL).

2.5. Radioligand binding assay

Saturation and competition binding studies were performed as described previously [20]. The serotonin-specific ligand [^{3}H]LSD was used in all studies (spec. act. 2.5–3.1 TBq/mmol; DuPont-NEN). Non-specific binding was determined in the presence of 1 mM serotonin. K_d-and B_{max}-values were calculated by computer-aided non-linear regression analysis of the binding isotherms under equilibrium conditions. Competition displacement was performed in the presence of [^{3}H]LSD at a final concentration of 1.6 nM and variable concentrations of the unlabeled drugs. For initial screening of recombinant clones, $2–12 \times 10^6$ whole cells of the respective clones were incubated as described before. In this case a ligand concentration of 13 nM [^{3}H]LSD was used.

2.6. Fixation and embedding for immunogold electron microscopy

Transformed cells of *P. pastoris* were fixed, embedded and cut as described previously [20]. Thin sections were incubated with the primary monoclonal antibody 9E10 (2 h, diluted 1:2000) directed against the c-*myc* tag. Goat anti-mouse antibodies (2 h) coupled to gold particles (Amersham Buchler, Braunschweig) were used as secondary antibodies. After silver enhancement the final analysis was performed in the electron microscope CM12 (Philips, Eindhoven, The Netherlands).

3. Results

3.1. Vector constructions for the heterologous expression of the mouse 5-HT$_{5A}$ receptor in P. pastoris

Three different plasmids for the heterologous expression of the 5-HT$_{5A}$ receptor were constructed (Fig. 1A). In plasmid pHIL-D2-5-HT$_{5A}$, the 5-HT$_{5A}$ cDNA has been cloned without modifications, whereas the other two constructs (pHIL-S1-5-HT$_{5A}$myc; pPIC9-5-HT$_{5A}$myc) bore the coding region for a c-*myc* tagged 5-HT$_{5A}$ receptor fused to two different signal sequences. The use of signal sequences has been reported to enhance the expression of membrane receptors [21], and therefore we tested two different signal sequences for their ability to enhance expression of the receptor. The *PHO1* signal sequence derived from the *P. pastoris* acid phosphatase [9] was utilized in the expression plasmid pHIL-S1-5-HT$_{5A}$myc, whereas vector pPIC9-myc5-HT$_{5A}$ contained the *S. cervisiae* α-factor signal sequence. The two signal sequences used in the different vectors have totally different properties. In contrast to classical signal sequences like the *PHO1* signal sequences, the 89 amino acids spanning the α-factor signal sequence from *S. cervisiae* are not removed from the α-factor in the endoplasmic reticulum like other signal peptides, but within the Golgi by the dibasic *KEX2* protease [22,23]. As has been reported previously, this cleavage also occurs efficiently in the heterologous surrounding of *P. pastoris* [10]. Nevertheless, both constructions, after cleavage of the signal sequences, should allow the immunological detection of the heterologously produced protein which has been fused to the c-*myc* epitope. As mentioned above, the three vectors were linearized prior to transformation *P. pastoris* strain GS115 to obtain recombinant clones with different phenotypes concerning methanol utilization. When the vectors pHIL-D2-5-HT$_{5A}$, pHIL-S1-5-HT$_{5A}$myc and pPIC9-5-HT$_{5A}$myc were linearized with *Not*I or *Bgl*II, respectively, about 10% of His$^+$ transformants showed a Mut^s phenotype. After *Sal*I-digestion of the vectors prior to transformation, nearly all His$^+$ clones revealed a Mut$^+$ phenotype.

3.2. Expression levels reached with the different transformed clones

The expression levels of functional 5-HT$_{5A}$ receptor in recombinant GS115 cells were significantly different for each of the three vectors as revealed by radioligand binding with the serotonergic ligand [^{3}H]LSD on whole cells (Fig. 1B). The expression varied from clone to clone bearing the same expression casette, but no significant difference between clones with Mut^s and those with Mut$^+$ phenotype could be detected. In general, the expression of the receptor was found to be best 24–40 h after induction with methanol. Recombinant clones derived after transformation with plasmid pPIC9-5-HT$_{5A}$myc showed the highest receptor densities (Fig. 1B), indicating that the α-factor signal sequence improved expression in comparison to the construct with the *PHO1* signal sequence. Therefore, plasmid pPIC9-5-HT$_{5A}$myc was used for transformation of *P. pastoris* strain SMD1163. This strain bears mutations in two protease genes whereas strain GS115 is wild-type in respect to its protease content. Ten recombinant clones with a Mut$^+$ phenotype subsequently were analysed for receptor expression. Two

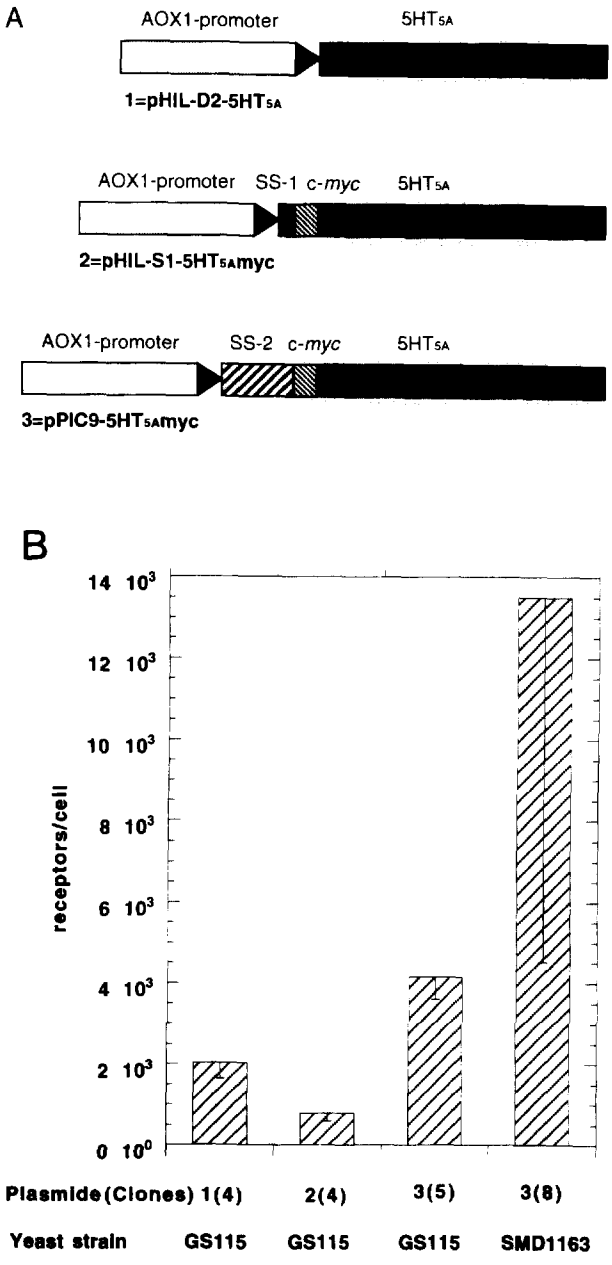

Fig. 1. (A) Expression plasmids constructed for heterologous expression of the mouse 5-HT_{5A} receptor in *P. pastoris*. 5'AOX1, promotor of *P. pastoris* alcoholoxidase 1 gen; SS-1, coding region for signal sequence from *P. pastoris* acide phosphatase; SS-2, coding region for signal sequence from *S. cerevisiae* mating type factor α; c-*myc*, coding region for the c-*myc* tag; 5-HT_{5A}, c-DNA of the mouse 5-HT_{5A} receptor. (B) Receptor densities achieved using this three expression plasmids with the third used in two different *P. pastoris* strains. Standard deviations are indicated. The numbers in brackets indicate how many clones were analysed for receptor expression.

recombinant clones exhibited only low receptor expression levels, whereas the other clones showed a 2–8 times higher receptor number per cell than the corresponding GS115-clones (Fig. 1B). One clone which showed reproducible high expression level (22,000–32,000 receptors/cell, 24–48 h after induction) was selected for further pharmacological characterization.

3.3. *Pharmacological characterization of the 5-HT_{5A} receptor produced in* P. pastoris

Neurotransmitter receptors are characterized by their ability to bind specific ligands with high affinities. Pharmacological classification of a given receptor results finally from the ranking order of binding potency for certain ligands. In order to establish the functional integrity of the 5-HT_{5A} receptor heterologously expressed in *P. pastoris*, ligand binding affinities were determined. Transformed yeast cells as well as membranes prepared here from were tested by radioligand binding assay using the serotonergic agonist [^{3}H]LSD. No endogenous expression of [^{3}H]LSD binding sites could be detected in *P. pastoris* clones which had been transformed with the control plasmid. Crude membranes of the yeast strain SMD1163 which had been transformed with pPIC9-5-HT_{5A}myc exhibited specific saturable binding of [^{3}H]LSD with a K_d of 1.9 ± 0.26 nM (Fig. 2), and specific saturable binding of [^{3}H]LSD could also be measured with whole cells. Scatchard transformation (Fig. 2) of the data obtained from the experiments on membranes indicated that the binding of [^{3}H]LSD to the mouse 5-HT_{5A} receptor expressed in *P. pastoris* was best described by a model assuming a single class of equivalent and independent binding sites. The expression level (B_{max}) in the recombinant SMD1163 clone was determined to be ≈22 pmol/mg of membrane protein.

To determine the pharmacological profile of the heterologously expressed 5-HT_{5A} receptor, bound [^{3}H]LSD was displaced with various serotonergic drugs (Fig. 3). These compounds displayed the following rank order of potencies: ergotamine > 5-CT > methysergide > 5-HT > lysergic acid > bufotenine > yohimbine (Table 1), which is comparable to the data obtained for the 5-HT_{5A} receptor expressed in Cos-7 cells [11]. None of the competition curves showed a biphasic profile (Fig. 3), indicating that [^{3}H]LSD binding sites have a homogenous affinity to the above-mentioned drugs.

3.4. *Cellular localization of the 5-HT_{5A} receptor heterologously produced in* P. pastoris

For cellular localization of the heterologously produced receptor, recombinant yeast cells were fixed about 25 h after induction and embedded in plastic. Cells were analysed by the postembedding immunogold staining method. The monoclonal

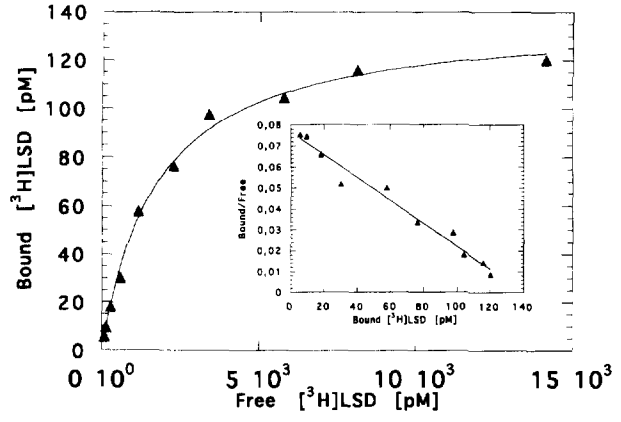

Fig. 2. Saturation isotherms of [^{3}H]LSD binding to membranes prepared from *P. pastoris* strain SMD1163 transformed with pPIC9-5-HT_{5A}myc. (Inset) Scatchard transformation of the data. Data are representative for three independent experiments with similar results with each point beeing measured in triplicate.

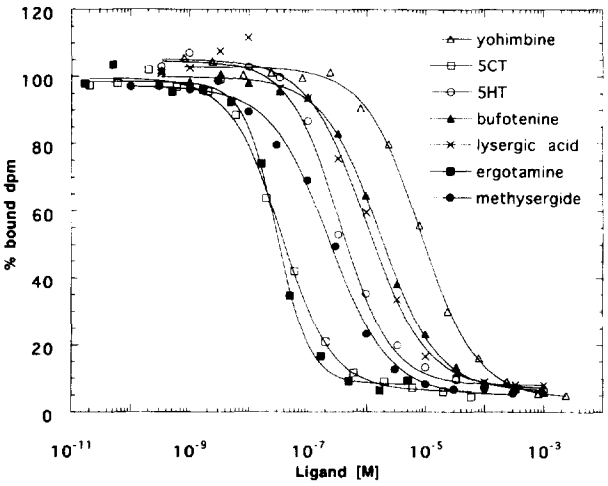

Fig. 3. Pharmacological profile of the 5-HT_{5A} receptor heterologously expressed in recombinant *P. pastoris* strain SMD1163 Plasmid pPIC9-5-HT_{5A}myc was used for transformation of *P. pastoris* strain SMD1163. Competitive binding to the membranes was performed at a final [^{3}H]LSD concentration of 1.6 nM. The 100% value corresponds to specific binding in absence of any competitor. the 0% value corresponds to the non-specific binding. Data are representative for two independent experiments, with each point being measured in duplicate. 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine.

antibody 9E10 which specifically reacts against the c-*myc* epitope fused to the receptor in combination with a gold-labeled anti-mouse serum was applied to thin sections obtained from the embedded cells. Immunostaining of *P. pastoris* cells which expressed a 5-HT_{5A} receptor that had not been c-*myc* tagged, only revealed background labeling, i.e. at the appropriate antibody dilution (9E10; 1:2000) almost no labeling can be seen (Fig. 4A). Specific staining of different cell compartments, however, was observed over cells which produced the c-*myc* tagged serotonin receptor. Gold particles could be observed over the rough endoplasmatic reticulum over cytoplasmic membrane vesicles and the vacuole. No strong distinct labeling occured over the plasma membrane.

4. Discussion

Considering the medical and pharmacological importance of the G-protein coupled receptor family, a three-dimensional structure of one of these receptors would have a great impact. Attempts to study receptor function and structure have been hampered by the absence of pure populations of receptor. Natural sources cannot provide the amount of protein needed for initial crystallization attempts and in addition they represent complex mixtures of cell types and also receptor subtypes which do not allow the purification of the receptor to homogeneity. The way to overcome this problem is to express the receptor gene of interest in a suitable expression system thus allowing the production of a homogeneous population of receptor protein. Expression levels of membrane proteins are usually poor in comparison to the expression levels which can be reached with soluble proteins. Therefore, large amounts of biomass are a prerequisite for purification attempts. Yeasts that grow fast on inexpensive media are in this task an interesting alternative to expression for example in insect cells using the baculovirus system or in mammalian cells. The best-characterized yeast *S. cervisiae* has been successfully used in this field, and reports about receptor expression in *S. pombe* are also available [24,25]. In the case of soluble proteins, the heterologous expression in *P. pastoris* often resulted in higher expression levels than in *S. cerevisiae* [26].

Here, we tested the *P. pastoris* system as a tool for the expression of the 5-HT_{5A} serotonin receptor. Three vector constructs from which the 5-HT_{5A}-receptor alone as well as in fusion to two different signal sequences could be functionally expressed, were examined. The signal sequences in this two constructs should enforce proper insertion into the ER membrane. The construct with the *PHOI* signal sequence resulted in even lower expression levels as the construct with no modifications at the N-terminus as revealed by radioligand binding assays. The plasmid construct using the *S. cerevisiae* α-factor signal sequence resulted in the highest expression level in *P. pastoris* strain GS115. This strain used for transformation and initial characterization of all constructs, is wild-type in respect to its protease content. The value of protease deficient strains for membrane protein expression in *S. cervisiae* has been described previously [20]. Therefore, plasmid pPIC9-5-HT_{5A}myc was used for further characterization of the heterologously expressed 5-HT_{5A} receptor in the protease deficient *P. pastoris* strain SMD1163. Whole cells as well as membranes prepared here from, exhibited saturable binding for [^{3}H]LSD, a potent serotonergic agonist. Bound [^{3}H]LSD could be displaced by a variety of unlabeled serotonin analogs. It should be noted, however, that the $K_d$of ≈ 1.9 nM and the K_i's for the different competitors differ from published values determined in transiently transfected mammalian cells by an order of magnitude. As has been discussed previously, a possible explanation for this discrepancy could be the lipid composition of the membranes surrounding the receptor molecules [20]. Nevertheless, the pharmacological profile is virtually identical to the profile obtained after expression of the receptor in *S. cerevisiae* [15]. The receptor density of maximally ≈30,000 receptors per recombinant *P. pastoris* cell corresponds to a value of more than 1 mg receptor/l of yeast culture. In comparison, the endogenous *STE2* receptor of *S. cerevisiae* had approximately 3000–8000 copies/cell [27], whereas the heterologously produced mACh-receptor subtype m1 and the D_{2S} receptor had approximately 2–40 copies/cell and 500–1000 copies/cell, respectively [20,28]. The receptor level reached in membranes of

Table 1
Pharmacological profile of the $5HT_{5A}$ receptor heterologously expressed in *P. pastoris* strain SMD1163 transformed with pPIC9-$5HT_{5A}$myc

Ligand	K_i (M)
Ergotamine	$3.8 \pm 0.5 \times 10^{-8}$
5-Carboxamidotryptamine (5-CT)	$5.5 \pm 0.6 \times 10^{-8}$
Methysergide	$5.0 \pm 2.4 \times 10^{-7}$
5-Hydroxytryptamine (5-HT)	$1.1 \pm 0.8 \times 10^{-6}$
Lysergic acid	$2.0 \pm 0.8 \times 10^{-6}$
Bufotenine	$2.9 \pm 0.6 \times 10^{-6}$
Yohimbine	$1.2 \pm 0.1 \times 10^{-5}$

K_i values were calculated from the IC_{50} obtained in the competition experiments depicted in Fig. 3. They were calculated from the IC_{50} values according to the equation $K_i = IC_{50}(1 + C/K_d)$, where C is the concentration of [^{3}H]LSD and K_d (1.9 nM) the equilibrium dissociation constant of [^{3}H]LSD for the heterologously expressed $5HT_{5A}$ receptor (see text).

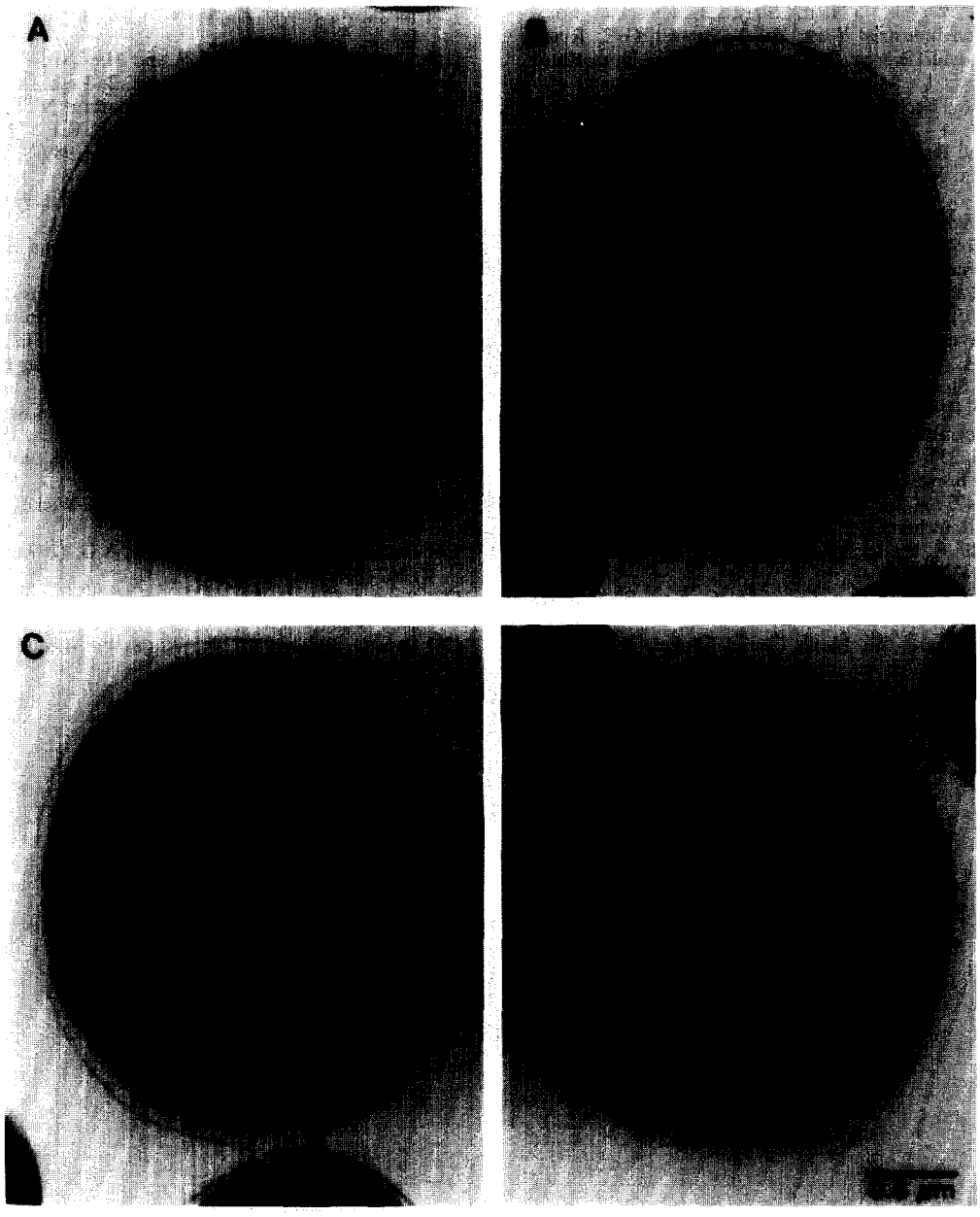

Fig. 4. Cellular localization of the heterologously expressed 5-HT_{5A} receptor by immunogold labeling Post-embedding immunogold staining of ultrathin sections from recombinant *P. pastoris* strain SMD1163 cells either transformed with plasmid pHIL-D2-5-HT_{5A} (A) or plasmid pPIC9-5-HT_{5A}myc (B,C,D). M, mitochondrium; N, nucleus; V, vacuole.

recombinant *P. pastoris* (≈22 pmol/mg membrane protein) is 14 times higher than the levels reported for transfected mammalian cells (1.6 pmol/mg membrane protein; [11]), about 1.4 times higher than the level reached in *S. cerevisiae* [15], and is in the range that can be reached in insect cells after infection with recombinant baculovirus (≥30 pmol/mg membrane protein; [14]). Localization studies of the epitope-tagged receptor revealed that it was localized mainly in the internal membrane compartments of the yeast cells. Almost no labeling occurred over the plasma membrane, although the receptor is a natural resident of the plasma membrane.

In conclusion, these results indicate that the *Pichia pastoris* expression system might be of great value for the overexpression of membrane proteins. The mouse 5-HT_{5A} receptor heterologously expressed in this yeast is biologically active and suitable for further biochemical, pharmacological and biophysical studies.

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