Pharmacological Reports
2011, 63, 45–53
ISSN 1734-1140

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Differences between human wild-type and C23S variant 5-HT$_{2C}$ receptors in inverse agonist-induced resensitization

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Abstract:
The aim of this study was to analyze functional properties of the naturally occurring C23S variant of the human 5-HT$_{2C}$ receptor. In HEK293 cells transiently expressing the unedited forms of the variant receptor (VR) or the wild-type receptor (WTR), surface expression was determined by [^{3}H]mesulergine binding to membrane fragments. Function was examined by an aequorin luminescence-based Ca^{2+} assay. Surface expression of the VR was 116% of that of the WTR. The 5-HT-induced increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), and its inhibition by the inverse agonist SB 206553 did not differ between VR- or WTR-expressing cells. Preexposure of VR- or WTR-expressing cells to 0.5 μM 5-HT (3 min–4.5 h) led to a practically identical time course and extent in the reduction of the 5-HT-induced increase in $[Ca^{2+}]_i$. In contrast, prolonged preexposure to the inverse agonist SB 206553 (1 μM) elevated the 5-HT-induced increase in $[Ca^{2+}]_i$ for both isoreceptors. A preexposure time of 4.5 h was necessary to significantly elevate the Ca^{2+} response of the WTR, but the VR produced this elevation within 1 h with virtually no further effect after 4.5 h of preexposure. In conclusion, prolonged preexposure to 5-HT caused equally rapid and strong desensitization of both isoreceptors. The different time course of SB 206553-induced resensitization of the two isoreceptors might be therapeutically relevant for drugs exhibiting inverse agonist properties at 5-HT$_{2C}$ receptors, such as atypical antipsychotics and certain antidepressants.

Key words:
aequorin, calcium, 5-HT$_{2C}$ receptor, C23S variant, receptor desensitization, inverse agonist, SB 206553, HEK293 cells

Abbreviations: SNP – single nucleotide polymorphism, VR – C23S variant receptor, WTR – wild-type receptor

Introduction

5-HT$_{2C}$ receptors are widely distributed throughout the central nervous system, including areas involved in Parkinson's disease and psychiatric disorders, such as schizophrenia and drug dependence [18] (for general 5-HT receptor reviews see [4, 16]). 5-HT$_{2C}$ receptors located on inhibitory GABAergic interneurons in the ventral tegmental area and the striatum exhibit inhibitory effects on limbic and striatal dopamine pathways. As a consequence, inhibition of 5-HT$_{2C}$ receptors leads to increased dopamine release, which is relevant for the therapy of the above mentioned diseases [17]. However, direct excitatory control of dopaminergic neurons is also discussed [5].

5-HT$_{2C}$ receptors are heterogeneous due to alternative splicing, giving rise to three different receptor

isoforms [10, 44], and the propensity of transcripts to undergo adenosine-to-inosine mRNA editing. The latter causes amino acid substitutions within the second intracellular loop of the receptor [9]. This leads to diverse tissue-specific isoforms which differ with regard to their constitutive activity, a typical property of 5-HT_{2C} receptors (for review see [2]). The unedited receptor (INI isoform) exhibits pronounced constitutive activity, whereas the partially- and fully-edited forms show intermediate and minimal constitutive activities, respectively [22]. This is interesting with regard to the fact that many atypical antipsychotics (e.g., clozapine and olanzapine) and some antidepressants (e.g., mianserin and mirtazapine) act as inverse agonists at these receptors and are thereby able to counteract constitutive activity [3, 11, 23].

In addition, single-nucleotide polymorphisms (SNPs) in the 5-HT_{2C} receptor gene might be relevant with regard to disease susceptibility or inter-individual variations in drug responses. Several SNPs have been found in the promoter region that might influence receptor expression levels [38]. A SNP in the coding region of the gene, leading to substitution of cysteine 23 to serine (C23S) in the 5-HT_{2C} receptor (frequency of the S23 encoding allele in unrelated Caucasians, 0.13) [27], has been found to be associated with neuropsychological diseases such as eating disorders [24, 45], unipolar and bipolar depression [29] or chronic hospitalization of schizophrenic individuals [40]. It has also been found to be associated with extrapyramidal adverse effects of antipsychotic drugs [21] and an altered response to clozapine [41].

Data obtained as a result of a search for consequences of the C23S exchange on 5-HT_{2C} receptor function were not completely consistent. Whereas Okada et al. [36] reported on slightly reduced affinities of 5-HT_{2C} receptor ligands to the variant receptor (VR) and on a higher constitutive activity of the VR, compared to the wild-type receptor (WTR), Fentress et al. [15] did not find any difference in the functional properties of the two isoreceptors. Further research was highly desirable, not only because of the inconsistencies described, but even more so because of the peculiarity of the location of the C23S exchange in the N-terminal domain of the 5-HT_{2C} receptor. Naturally occurring amino acid exchanges within the N-terminal domains of the human 5-HT_{1A} receptor (G22S [39]) and the β_2-adrenoceptor (R16G [19, 20, 33] or G27E [8]) have been causally related to a modified downregulation and/or desensitization in response to prolonged exposure to a relevant agonist. Furthermore, Okada et al. [36] already proposed that the C23S mutation might lead to increased receptor desensitization.

In the context of the latter considerations, the main aim of the present study was to determine whether the desensitization by prolonged exposure to a 5-HT_{2C} receptor agonist (here 5-HT) is modified by the C23S mutation. A logical extension of that topic was based on the hypothesis that constitutive activity by itself might lead to desensitization. Thus, prolonged treatment with 5-HT_{2C} receptor inverse agonists (here SB 206553), by attenuating constitutive activation of desensitization mechanisms, would permit resensitization of the system. This, in turn, would lead to increased responsiveness to classical agonists (see also [6]). In fact, this phenomenon has not only been demonstrated in receptor systems with artificially high levels of constitutive activity [28, 37] but also in systems with low receptor expression levels [6]. The functional consequences of a prolonged exposure of the two 5-HT_{2C} isoreceptors to inverse agonists have not been assessed in previous studies. Therefore, we aimed at obtaining basic evidence for such a resensitization of the unedited 5-HT_{2C} INI receptor and at examining whether the VR and WTR differ in this respect.

Materials and Methods

Chemicals and drugs

Coelenterazine *h* (Nanolight; Pinetop, AZ, USA), 5-hydroxytryptamine creatinine sulfate (5-HT, serotonin) and ketanserin tartrate (Sigma; Munich, Germany), mianserin hydrochloride (Organon; Oss, Holland), SB 206553 hydrochloride (5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole) (Tocris Bioscience; Ellisville, MO, USA), [^{3}H]mesulergine (specific activity 84 Ci/mmol) (GE Healthcare; Munich, Germany).

Isolation of the human 5-HT_{2C} receptor coding sequence and site-directed mutagenesis

Total human adult brain RNA (Clontech; Heidelberg, Germany) was used as the template for cDNA synthesis using random hexamer primers and Superscript II

reverse transcriptase (Invitrogen; Karlsruhe, Germany). Oligonucleotide primers based on the human 5-HT$_{2C}$ sequence (GenBank accession number U49516.1; sense: ATGGTGAACCTGAGGAATGC, antisense: TCACACACTGCTAATCCTTTC) were used to amplify 5-HT$_{2C}$ cDNA (WTR). The resulting fragment was subcloned into the pCR2.1 vector (Invitrogen; Karlsruhe, Germany), excised with BamHI/XhoI and subcloned into the mammalian expression vector pcDNA3 (Invitrogen; Karlsruhe, Germany).

Site-directed mutagenesis was performed to introduce the C23S mutation (VR) using the "QuikChange Site-Directed Mutagenesis" kit (Stratagene; La Jolla, CA, USA). The following primers were used (mutated nucleotides underlined): sense: GCCTATTGGTTTGGCAATCTGATATTTCTGTGAGC, antisense: GGCTCACAGAAATATCAGATTGCCAAACCAATAGGC. The fidelity of the cDNA sequences was verified by sequencing. The isolated 5-HT$_{2C}$ cDNA sequence corresponds to the unedited (INI) isoform.

Cell culture and transfection

HEK 293 cells (ATCC; Manassas, VA, USA) were grown as monolayers in DMEM/Ham's F12 (1:1) (Sigma; Munich, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories; Pasching, Austria) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cells were seeded in either 75-cm^2 or 25-cm^2 culture flasks in DMEM/Ham's F12 (1:1) + 10% dialyzed FCS (PAA Laboratories) for transient transfection on the following day. Cells were transfected with the WTR- or the VR cDNA using the liposome-based TransIT-293 Transfection Reagent (Mobitec, Göttingen, Germany) to transport either 15 µg or 5 µg of plasmid DNA depending on the use of 75- or 25-cm^2 flasks, respectively. For aequorin assays, cells were additionally transfected with apoaequorin cDNA, which was originally derived from cytAEQ/pcDNA1 (Molecular Probes – Invitrogen; Karlsruhe, Germany) and was subcloned into HindIII/XbaI-digested pcDNA 3.1/zeo(+) (Invitrogen). The portions of cDNAs used were as follows: 67% apoaequorin cDNA and 33% receptor cDNA. For experiments with prolonged preexposure to 5-HT (0.5 µM) or SB 206553 (1 µM), medium was replaced 24 h after transfection with medium containing 5% dialyzed FCS and, if relevant, 0.5 µM 5-HT and 0.1 mM ascorbic acid. Cells were used 48 h post transfection.

Membrane preparation and radioligand binding assay

Transiently transfected cells from a 75-cm^2 culture flask were used for preparation of crude membranes as previously described [13, 34]. For saturation experiments, 5 µg of membranes were incubated in triplicate with six increasing concentrations of the potent 5-HT$_{2C}$ inverse agonist [^{3}H]mesulergine (0.1–30 nM) in a final reaction volume of 0.5 ml. After an incubation time of 60 min at room temperature, incubation mixes were filtered through GF/B-filters (Whatman, Kent, UK) using a cell harvester (Brandel; Unterföhring, Germany) and washed three times with 2 ml of ice-cold buffer. Radioactivity was measured in a liquid scintillation counter (Beckman; Fullerton, CA, USA). Specific binding was determined as the fraction of [^{3}H]mesulergine that could be displaced by 30.5 µM of the inverse agonist ketanserin.

Aequorin luminescence assay

The aequorin assay for estimation of 5-HT-induced (0.001–100 µM) increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed as previously described [13, 43]. Harvested cells were loaded with 5 µM coelenterazine *h* for 2.5 h at room temperature. Suspensions of cells in assay buffer were used for luminometric measurements in a Centro LB 960 luminometer (Berthold; Bad Wildbad, Germany). Luminescence was recorded from 5 s prior until 30 s after autoinjection of 5-HT at a sampling rate of 2 Hz.

For determination of the potency of SB 206553 in counteracting the increase in $[Ca^{2+}]_i$ induced by 10 µM 5-HT, cells were preincubated with SB 206553 (concentrations ranging from 0.3 to 300 µM) for 10 min. When cell suspension aliquots were exposed for prolonged periods to 5-HT (0.5 µM) or SB 206553 (1 µM), these drugs were added at the relevant time during the coelenterazine *h* loading period. In the case of overnight exposure (19 h) with 5-HT, preincubation was continued after cell harvesting. For removal of the drugs under study prior to the assay, cells were washed three times with 1.5 ml assay buffer. Determination of 5-HT (100 µM)-induced transients in $[Ca^{2+}]_i$ began 30 min after removal of the incubation medium containing coelenterazine *h* and the drug.

At the end of those experiments, in which 5-HT (100 µM)-induced maximum responses were recorded, cells were lysed with Triton X-100 0.1%

(v/v) and $CaCl_2$ 50 mM. Under these conditions, remaining aequorin luminescence was recorded to obtain the maximum possible Ca^{2+} response.

Data analysis

Relative light units (RLU) for 5-HT-induced increases of $[Ca^{2+}]_i$ have been obtained by subtraction of baseline luminescence from the 5-HT-induced peak maximum luminescence. In 5-HT maximum response experiments, the peak luminescence (RLU_{peak}) was normalized against total aequorin luminescence (RLU_{max}) after cell lysis to control for differences in transfection efficiency and cell number [$RLU_{peak}/(RLU_{peak} + RLU_{max})$]. The concentration-response curves and saturation binding curves, the corresponding constants pEC_{50} and pIC_{50} and the binding constants B_{max} and K_d were calculated by means of GraphPad Prism 5.0 (GraphPad Software Inc.; San Diego, CA, USA). The pIC_{50} values were converted to K_i values using the equation of Cheng and Prusoff [12]. Data are presented as the means ± SEM. Statistical analysis was performed with Student's *t*-test and, when appropriate, two-way repeated measures ANOVA followed by Bonferroni's post-test. Differences were considered significant at $p < 0.05$.

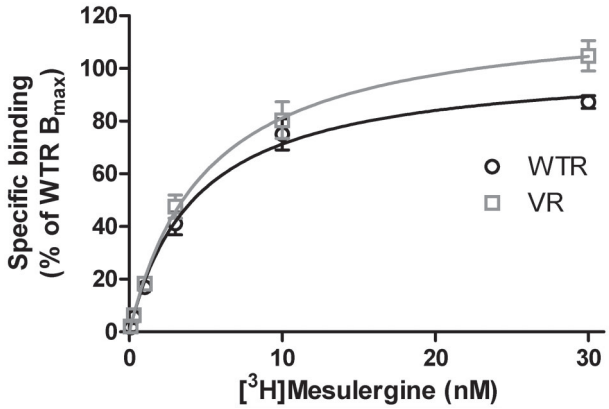

Fig. 1. [^{3}H]Mesulergine (0.1–30 nM) saturation binding to membranes of HEK293 cells transiently expressing either the 5-HT_{2C} wild-type receptor (WTR) or the C23S variant receptor (VR). Shown are the means ± SEM of four independent experiments with each isoreceptor performed in triplicate. Non-specific binding was determined in the presence of 30.5 μM ketanserin. Data are expressed as percentage of the B_{max} at the WTR receptor. B_{max} was significantly higher for the VR than for the WTR ($p < 0.05$)

Results

[^{3}H]Mesulergine binding

Saturation binding experiments on membranes with the 5-HT_{2C} receptor inverse agonist [^{3}H]mesulergine [3, 6] revealed a single binding site both on the WTR and the C23S VR transiently expressed in HEK293 cells (Fig. 1). The B_{max} amounted to 7.80 ± 0.46 pmol/mg protein and 9.07 ± 0.20 pmol/mg protein, respectively. Thus, the C23S mutation led to increased membrane expression of the VR of 116.2 ± 2.6%, compared to the WTR ($p < 0.05$). However, the K_d values of [^{3}H]mesulergine were similar for both receptor isoforms (3.47 ± 0.25 nM and 3.99 ± 0.50 nM, respectively).

5-HT-induced increase in $[Ca^{2+}]_i$ and interaction with SB 206553

Cells expressing the VR did not differ from those expressing the WTR, in that the physiological agonist 5-HT (0.001–100 μM) caused a concentration-dependent increase in aequorin luminescence reflecting elevation of $[Ca^{2+}]_i$ (Fig. 2a). The corresponding concentration-response curves were characterized by practically identical pEC_{50} (EC_{50}) values of 6.74 ± 0.13 (182 nM) and 6.73 ± 0.15 (186 nM), respectively. Although the VR was expressed at a higher density on the cell surface (Fig. 1), the 5-HT (100 μM)-induced maximum Ca^{2+} response did not significantly differ from that for the WTR (90.4 ± 11.9 %; n = 11).

The 5-HT_{2C} receptor inverse agonist SB 206553 (0.3–300 μM) concentration-dependently inhibited the 5-HT (10 μM)-induced luminescence signal of cells expressing the WTR or the VR (Fig. 2b) with similar pIC_{50} (K_i) values of 5.21 ± 0.18 (6.17 μM) and 5.25 ± 0.20 (5.62 μM), respectively.

Effects of prolonged exposure to 5-HT

Cells expressing the VR did not differ from cells expressing the WTR in that a 3 min preexposure to 0.5 μM 5-HT approximately halved the 5-HT (100 μM)-evoked increase in $[Ca^{2+}]_i$. Up to a preexposure time of 19 h, this was followed in both cell types by a tendency towards further gradual decline (virtually negligible in size) to about 40% of the signal without preexposure (Fig. 3a).

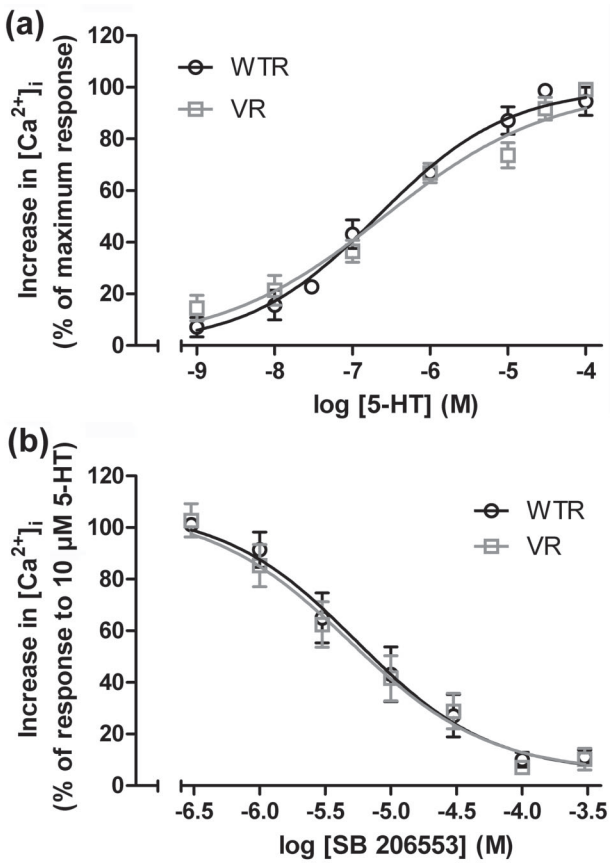

Fig. 2. 5-HT-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and interaction with the inverse agonist SB 206553 of HEK293 cells transiently expressing either the 5-HT$_{2C}$ wild-type receptor (WTR) or the C23S variant receptor (VR). 5-HT-induced (0.001–100 µM) aequorin luminescence in response to increased $[Ca^{2+}]_i$ was measured in coelenterazine *h*-loaded cells transiently expressing apoaequorin and either the WTR or the VR. (**a**) Concentration-dependent 5-HT-induced increases in $[Ca^{2+}]_i$ are expressed as percentages of the respective maximal peak response. Shown are the means ± SEM of six or seven independent experiments. (**b**) Inhibition of the 5-HT (10 µM)-induced Ca^{2+} response by increasing concentrations (0.3–300 µM) of the inverse agonist SB 206553. SB 206553 was present 10 min before and during 5-HT application. Data are expressed as percentages of the 5-HT responses in the absence of the inverse agonist (means ± SEM of five independent experiments)

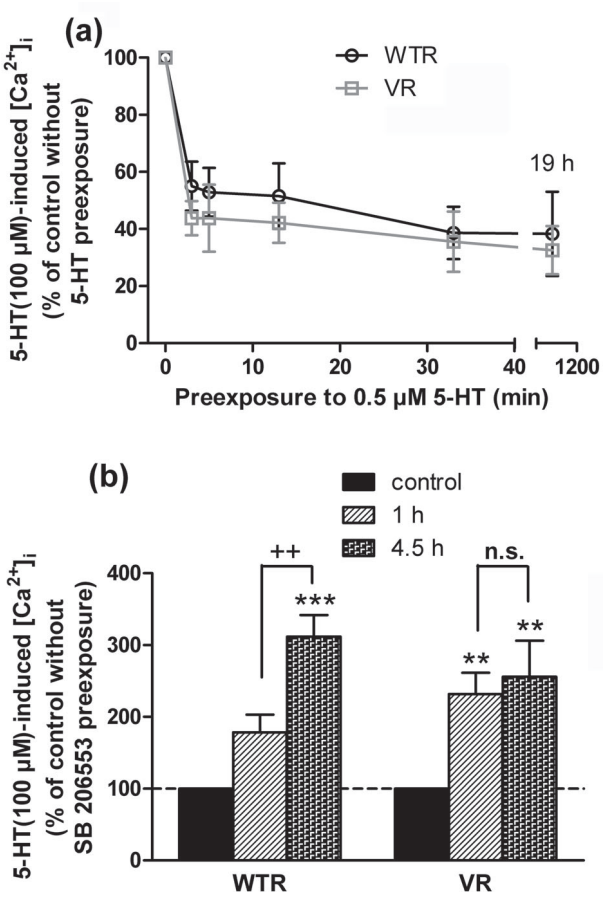

Fig. 3. Effect of prolonged preexposure to 5-HT or SB 206553 on the 5-HT (100 µM)-induced increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in intact HEK293 cells transiently expressing either the 5-HT$_{2C}$ wild-type receptor (WTR) or the C23S variant receptor (VR). Prior to the Ca^{2+} assay, cells were washed three times with 1.5 ml assay buffer. Determination of $[Ca^{2+}]_i$ started 30 min after removal of the incubation medium containing coelenterazine *h* and the respective drug. (**a**) Time courses of 5-HT (0.5 µM)-induced increases of $[Ca^{2+}]_i$ responses in WTR- and VR cells. Data (the means ± SEM of four or five independent experiments) are expressed as percentages of the respective responses without pretreatment. (**b**) Elevation in 5-HT-evoked $[Ca^{2+}]_i$ following 1 h or 4.5 h of preexposure to 1 µM SB 206553. Data (the means ± SEM of seven independent experiments) are expressed as percentages of the respective responses without SB 206553 preexposure (control). Significant differences compared to control (** $p < 0.01$, *** $p < 0.001$) or between corresponding means (++ $p < 0.01$; n.s. – not significant) (two-way repeated measures ANOVA followed by Bonferroni's post-test)

Effects of prolonged exposure to the inverse agonist SB 206553

Prolonged exposure (for up to 4.5 h) to 1 µM SB 206553 of cells expressing either the WTR or the VR led to an approximately three-fold elevation of the 5-HT (100 µM)-induced increase in $[Ca^{2+}]_i$ (Fig. 3b). In the case of the VR, such an increase already occurred after 1 h of preexposure and remained practically on the same level until 4.5 h (Fig. 3b). In contrast, in the case of the WTR, 4.5 h of preexposure was necessary to produce a significant elevation of the 5-HT-induced increase in $[Ca^{2+}]_i$. The extension of the preexposure time from 1 to 4.5 h led to a 133% elevation of the 5-HT-induced Ca^{2+} response of cells expressing the WTR. In contrast, in the case of the VR, there was no significant further increase of the 5-HT-induced Ca^{2+} signal (Fig. 3b).

Discussion

These data prove that the system of coelenterazine *h*-loaded HEK293 cells co-transfected with apoaequorin and either the 5-HT_{2C} WTR- or VR cDNA is suitable for functional characterization of 5-HT_{2C} receptors by high-throughput luminometric measurement of $[Ca^{2+}]_i$. The following findings support this contention. HEK293 cells have been chosen as heterologous expression system because they do not natively express 5-HT receptors, as previously shown by our group [43]. Activation of recombinant 5-HT_{2C} receptors in HEK293 cells initiates phospholipase C-mediated accumulation of IP_3 [14], which in turn leads to Ca^{2+} mobilization from intracellular stores and, as a consequence, to an increase in $[Ca^{2+}]_i$. Therefore, agonistic or antagonistic properties of 5-HT_{2C} receptor ligands can be estimated by aequorin bioluminescence-based measurement of changes in $[Ca^{2+}]_i$.

Our [^{3}H]mesulergine saturation-binding experiments on membranes of transiently transfected cells revealed the same K_d values for the WTR and the VR as reported previously [36]. However, in contrast to previous studies, we found the B_{max} for [^{3}H]mesulergine at the VR to be significantly increased to 116%, compared to that at the WTR. Amino acid 23 is located in the so-called 'extra' hydrophobic domain of the extracellular N-terminus, which is a special feature of some 5-HT receptors. A mutated mouse 5-HT_{2C} receptor lacking this domain exhibited an increased surface expression, compared to that of the non-mutated receptor [25]. Hence, it is tempting to speculate that the altered structure of the extracellular hydrophobic domain due to the C23S exchange may lead to the enhanced cell surface expression of the VR found here. A recent study revealed that overexpression of 5-HT_{2C} receptors in the forebrain leads to elevated anxiety and hypoactivity in transgenic mice [26]. Thus, an increased surface expression of mutant 5-HT_{2C} receptors might increase the susceptibility of carriers to psychiatric disorders.

In agreement with the results of two previous studies [15, 27], the concentration-response curves recorded here for the 5-HT-evoked increase in $[Ca^{2+}]_i$ were virtually identical for the WTR and its variant. This was different from the results reported in another study, in which an unusually steep concentration-response curve of 5-HT at the VR was observed [36]. The reason for this discrepancy is unknown but the use of a different cell line might play a role. The lack of difference in 5-HT-induced maximum Ca^{2+} response between the VR and the WTR found here, in spite of the increased surface expression of the VR, also cannot be explained. The inhibitory potency of the inverse agonist SB 206553 in counteracting the 5-HT-induced increase in $[Ca^{2+}]_i$ was practically identical at the WTR and the VR. Both our study and that of Fentress et al. [15] revealed identity of VR and WTR with respect to ligand potencies or affinities. This implicates that amino acid 23 of the 5-HT_{2C} receptor is not involved in ligand binding and that the conformation of the ligand binding site is not changed by the C23S mutation.

Following our working hypothesis, we analyzed whether the well-known desensitization of the WTR by prolonged exposure to a 5-HT_{2C} receptor agonist is modified by the C23S mutation. This possibility can be denied, because cells expressing the VR did not differ from cells expressing the WTR in desensitization after 3 min to 19 h preexposure to 5-HT. For both isoreceptors, 19 h preexposure to 5-HT led to a reduction of the 5-HT-evoked increase in $[Ca^{2+}]_i$ to a level of approximately 40% of the corresponding response without preexposure. This desensitization by approximately 60% is in the range of the 60–80% reduction shown in previous studies for the 5-HT_{2C} receptor expressed in other cell lines [1, 7, 35, 42]. The time course of desensitization was somewhat faster than previously reported, most likely due to differences in the cellular milieu in which the receptors are expressed and to the different experimental setups.

In view of the pronounced constitutive activity of the unedited 5-HT_{2C} INI receptor [11, 15] expressed in HEK293 cells in this study, the prototypical inverse agonist SB 206553 [2, 6] should reduce basal 5-HT_{2C} receptor activity. As a consequence, prolonged treatment with this compound should counteract constitutive desensitization thereby leading to resensitization of the 5-HT_{2C} receptor. This resensitization would then be observed as an enhanced responsiveness to classical agonists, such as 5-HT, in our experiments (see Introduction). Indeed, prolonged preexposure of cells expressing the WTR or the VR to SB 206553 led to an elevation of the 5-HT-induced increase in $[Ca^{2+}]_i$. In the case of the WTR, 4.5 h of preexposure were necessary to produce a significant elevation of the 5-HT-evoked increase in $[Ca^{2+}]_i$ compared to the response without preexposure. In contrast, in the case of the VR, such an elevation in evoked Ca^{2+} response

already occurred after 1 h with virtually no further increase at 4.5 h of preexposure.

From these results, it may be concluded that prolonged exposure of both 5-HT_{2C} isoreceptors to an inverse agonist increases receptor responsiveness to classical agonists such as 5-HT. This enhanced responsiveness occurs earlier in cells (and presumably also in individuals) expressing the VR than in those expressing the WTR.

The mechanisms that mediate resensitization, i.e., elevation of agonist-induced Ca^{2+} response after prolonged pretreatment with inverse agonists, may be an increased responsiveness towards the IP_3 signaling pathway, an increased cell surface expression or both [2]. Two studies demonstrated increased membrane insertion of recombinant 5-HT_{2C} receptors in HEK293 cells after SB 206553 pretreatment, which is presumably due to recovery from constitutive internalization [11, 31]. On the other hand, Berg et al. [6] demonstrated that the increased IP_3 accumulation after pretreatment of CHO cells heterologously expressing 5-HT_{2C} receptors was not caused by receptor upregulation at the cell surface but by an increased level of $G\alpha_q$ protein.

The different time course of resensitization of the two isoreceptors caused by SB 206553 pretreatment may imply differential responses to inverse agonists *in vivo*. The assumption for physiological relevance is the existence of constitutive activity of 5-HT_{2C} receptors *in vivo*, which indeed has recently been shown in several animal studies (reviewed in [2, 5]). Thus, individuals carrying the C23S variant might exhibit an altered responsiveness towards some antidepressants, such as mianserin and mirtazapine, and atypical antipsychotics, such as clozapine and olanzapine, which act as inverse agonists at 5-HT_{2C} receptors [3, 11, 23]. Our results are consistent with a genetic study that revealed a significant association of the C23S variant with the clozapine response in schizophrenic patients. More subjects with the serine allele than subjects with the cysteine allele were responders [41]. However, this association could not be replicated in two following studies [30, 32].

Taken together, the following main conclusions can be drawn from our data. Prolonged exposure with the classical agonist 5-HT did not result in a difference between cells expressing the 5-HT_{2C} VR or WTR with respect to desensitization. In contrast, prolonged treatment of cells expressing the WTR or the VR with the inverse agonist SB 206553 leads to resensitization of the following receptor-mediated increase in $[Ca^{2+}]_i$. Such a prolonged preexposure to an inverse agonist is assumed to reduce the constitutive activity of the 5-HT_{2C} receptor, thereby increasing receptor responsiveness to classical agonists. This enhanced responsiveness occurs earlier in cells (and probably individuals) expressing the VR. The different time course of resensitization of the two isoreceptors may be therapeutically relevant with regard to drugs exhibiting inverse agonist properties at 5-HT_{2C} receptors, such as atypical antipsychotics and some antidepressants.

Acknowledgments:
This work was supported by the Deutsche Forschungsgemeinschaft. We thank Gundula Hesse and Natalie Lobes for skillful technical assistance.

References:

1. Akiyoshi J, Nishizono A, Yamada K, Nagayama H, Mifune K, Fujii I: Rapid desensitization of serotonin 5-HT2C receptor-stimulated intracellular calcium mobilization in CHO cells transfected with cloned human 5-HT2C receptors. J Neurochem, 1995, 64, 2473–2479.
2. Aloyo VJ, Berg KA, Spampinato U, Clarke WP, Harvey JA: Current status of inverse agonism at serotonin$_{2A}$ (5-HT_{2A}) and 5-HT_{2C} receptors. Pharmacol Ther, 2009, 121, 160–173.
3. Barker EL, Westphal RS, Schmidt D, Sanders-Bush E: Constitutively active 5-hydroxytryptamine$_{2C}$ receptors reveal novel inverse agonist activity of receptor ligands. J Biol Chem, 1994, 269, 11687–11690.
4. Barnes NM, Sharp T: A review of central 5-HT receptors and their function. Neuropharmacology, 1999, 38, 1083–1152.
5. Berg KA, Clarke WP, Cunningham KA, Spampinato U: Fine-tuning serotonin$_{2c}$ receptor function in the brain: molecular and functional implications. Neuropharmacology, 2008, 55, 969–976.
6. Berg KA, Stout BD, Cropper JD, Maayani S, Clarke WP: Novel actions of inverse agonists on 5-HT_{2C} receptor systems. Mol Pharmacol, 1999, 55, 863–872.
7. Briddon SJ, Leslie RA, Elliott JM: Comparative desensitization of the human 5-HT_{2A} and 5-HT_{2C} receptors expressed in the human neuroblastoma cell line SH-SY5Y. Br J Pharmacol, 1998, 125, 727–734.
8. Bruck H, Leineweber K, Buscher R, Ulrich A, Radke J, Insel PA, Brodde OE: The Gln27Glu beta2-adrenoceptor polymorphism slows the onset of desensitization of cardiac functional responses in vivo. Pharmacogenetics, 2003, 13, 59–66.
9. Burns CM, Chu H, Rueter SM, Hutchinson LK, Canton H, Sanders-Bush E, Emeson RB: Regulation of serotonin-2C receptor G-protein coupling by RNA editing. Nature, 1997, 387, 303–308.

10. Canton H, Emeson RB, Barker EL, Backstrom JR, Lu JT, Chang MS, Sanders-Bush E: Identification, molecular cloning, and distribution of a short variant of the 5-hydroxytryptamine2C receptor produced by alternative splicing. Mol Pharmacol, 1996, 50, 799–807.
11. Chanrion B, Mannoury la Cour C, Gavarini S, Seimandi M, Vincent L, Pujol JF, Bockaert J et al.: Inverse agonist and neutral antagonist actions of antidepressants at recombinant and native 5-hydroxytryptamine$_{2C}$ receptors: differential modulation of cell surface expression and signal transduction. Mol Pharmacol, 2008, 73, 748–757.
12. Cheng Y, Prusoff WH: Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol, 1973, 22, 3099–3108.
13. Combrink S, Kostanian A, Walstab J, Barann M, Brüss M, Göthert M, Bönisch H: Characterization of the naturally occurring Arg344His variant of the human 5-HT$_{3A}$ receptor. Pharmacol Rep, 2009, 61, 785–797.
14. Conn PJ, Sanders-Bush E, Hoffman BJ, Hartig PR: A unique serotonin receptor in choroid plexus is linked to phosphatidylinositol turnover. Proc Natl Acad Sci USA, 1986, 83, 4086–4088.
15. Fentress HM, Grinde E, Mazurkiewicz JE, Backstrom JR, Herrick-Davis K, Sanders-Bush E: Pharmacological properties of the Cys23Ser single nucleotide polymorphism in human 5-HT$_{2C}$ receptor isoforms. Pharmacogenomics J, 2005, 5, 244–254.
16. Filip M, Bader M: Overview on 5-HT receptors and their role in physiology and pathology of the central nervous system. Pharmacol Rep, 2009, 61, 761–777.
17. Fink KB, Göthert M: 5-HT receptor regulation of neurotransmitter release. Pharmacol Rev, 2007, 59, 360–417.
18. Giorgetti M, Tecott LH: Contributions of 5-HT$_{2C}$ receptors to multiple actions of central serotonin systems. Eur J Pharmacol, 2004, 488, 1–9.
19. Green SA, Turki J, Bejarano P, Hall IP, Liggett SB: Influence of beta 2-adrenergic receptor genotypes on signal transduction in human airway smooth muscle cells. Am J Respir Cell Mol Biol, 1995, 13, 25–33.
20. Green SA, Turki J, Innis M, Liggett SB: Amino-terminal polymorphisms of the human β_2-adrenergic receptor impart distinct agonist-promoted regulatory properties. Biochemistry, 1994, 33, 9414–9419.
21. Gunes A, Dahl ML, Spina E, Scordo MG: Further evidence for the association between 5-HT$_{2C}$ receptor gene polymorphisms and extrapyramidal side effects in male schizophrenic patients. Eur J Clin Pharmacol, 2008, 64, 477–482.
22. Herrick-Davis K, Grinde E, Niswender CM: Serotonin 5-HT$_{2C}$ receptor RNA editing alters receptor basal activity: implications for serotonergic signal transduction. J Neurochem, 1999, 73, 1711–1717.
23. Herrick-Davis K, Grinde E, Teitler M: Inverse agonist activity of atypical antipsychotic drugs at human 5-hydroxytryptamine$_{2C}$ receptors. J Pharmacol Exp Ther, 2000, 295, 226–232.
24. Hu X, Giotakis O, Li T, Karwautz A, Treasure J, Collier DA: Association of the 5-HT$_{2C}$ gene with susceptibility and minimum body mass index in anorexia nervosa. Neuroreport, 2003, 14, 781–783.
25. Hurley JH, Bloem LJ, Pavalko F, Liu J, Tian M, Simon JR, Yu L: Structure-function studies of the eighth hydrophobic domain of a serotonin receptor. J Neurochem, 1999, 72, 413–421.
26. Kimura A, Stevenson PL, Carter RN, Maccoll G, French KL, Paul Simons J, Al-Shawi R et al.: Overexpression of 5-HT$_{2C}$ receptors in forebrain leads to elevated anxiety and hypoactivity. Eur J Neurosci, 2009, 30, 299–306.
27. Lappalainen J, Zhang L, Dean M, Oz M, Ozaki N, Yu DH, Virkkunen M et al.: Identification, expression, and pharmacology of a Cys23-Ser23 substitution in the human 5-HT$_{2C}$ receptor gene (HTR2C). Genomics, 1995, 27, 274–279.
28. Lee TW, Cotecchia S, Milligan G: Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1B}-adrenoceptor by ligands that act as inverse agonists. Biochem J, 1997, 325, 733–739.
29. Lerer B, Macciardi F, Segman RH, Adolfsson R, Blackwood D, Blairy S, Del Favero J et al.: Variability of 5-HT$_{2C}$ receptor cys23ser polymorphism among European populations and vulnerability to affective disorder. Mol Psychiatry, 2001, 6, 579–585.
30. Malhotra AK, Goldman D, Ozaki N, Rooney W, Clifton A, Buchanan RW, Breier A, Pickar D: Clozapine response and the 5HT$_{2C}$ Cys23Ser polymorphism. Neuroreport, 1996, 7, 2100–2102.
31. Marion S, Weiner DM, Caron MG: RNA editing induces variation in desensitization and trafficking of 5-hydroxytryptamine 2c receptor isoforms. J Biol Chem, 2004, 279, 2945–2954.
32. Masellis M, Basile V, Meltzer HY, Lieberman JA, Sevy S, Macciardi FM, Cola P et al.: Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients. Neuropsychopharmacology, 1998, 19, 123–132.
33. Moore PE, Laporte JD, Abraham JH, Schwartzman IN, Yandava CN, Silverman ES, Drazen JM et al.: Polymorphism of the β_2-adrenergic receptor gene and desensitization in human airway smooth muscle. Am J Respir Crit Care Med, 2000, 162, 2117–2124.
34. Niesler B, Walstab J, Combrink S, Möller D, Kapeller J, Rietdorf J, Bönisch H et al.: Characterization of the novel human serotonin receptor subunits 5-HT$_{3C}$, 5-HT$_{3D}$, and 5-HT$_{3E}$. Mol Pharmacol, 2007, 72, 8–17.
35. Oekelen DV, Jurzak M, Van de Wiel D, Van Hecke G, Luyten WH, Leysen JE: Different regulation of rat 5-HT$_{2A}$ and rat 5-HT$_{2C}$ receptors in NIH 3T3 cells upon exposure to 5-HT and pipamperone. Eur J Pharmacol, 2001, 425, 21–32.
36. Okada M, Northup JK, Ozaki N, Russell JT, Linnoila M, Goldman D: Modification of human 5-HT$_{2C}$ receptor function by Cys23Ser, an abundant, naturally occurring amino-acid substitution. Mol Psychiatry, 2004, 9, 55–64.
37. Pei G, Samama P, Lohse M, Wang M, Codina J, Lefkowitz RJ: A constitutively active mutant β_2-adrenergic receptor is constitutively desensitized and phosphorylated. Proc Natl Acad Sci USA, 1994, 91, 2699–2702.
38. Reynolds GP, Templeman LA, Zhang ZJ: The role of 5-HT$_{2C}$ receptor polymorphisms in the pharmacogenetics of antipsychotic drug treatment. Prog Neuropsychopharmacol Biol Psychiatry, 2005, 29, 1021–1028.

39. Rotondo A, Nielsen DA, Nakhai B, Hulihan-Giblin B, Bolos A, Goldman D: Agonist-promoted down-regulation and functional desensitization in two naturally occurring variants of the human serotonin$_{1A}$ receptor. Neuropsychopharmacology, 1997, 17, 18–26.
40. Segman RH, Ebstein RP, Heresco-Levy U, Gorfine M, Avnon M, Gur E, Nemanov L, Lerer B: Schizophrenia, chronic hospitalization and the 5-HT$_{2C}$ receptor gene. Psychiatr Genet, 1997, 7, 75–78.
41. Sodhi MS, Arranz MJ, Curtis D, Ball DM, Sham P, Roberts GW, Price J et al.: Association between clozapine response and allelic variation in the 5-HT$_{2C}$ receptor gene. Neuroreport, 1995, 7, 169–172.
42. Stout BD, Clarke WP, Berg KA: Rapid desensitization of the serotonin$_{2C}$ receptor system: effector pathway and agonist dependence. J Pharmacol Exp Ther, 2002, 302, 957–962.
43. Walstab J, Combrink S, Brüss M, Göthert M, Niesler B, Bönisch H: Aequorin luminescence-based assay for 5-hydroxytryptamine (serotonin) type 3 receptor characterization. Anal Biochem, 2007, 368, 185–192.
44. Wang Q, O'Brien PJ, Chen CX, Cho DS, Murray JM, Nishikura K: Altered G protein-coupling functions of RNA editing isoform and splicing variant serotonin2C receptors. J Neurochem, 2000, 74, 1290–1300.
45. Westberg L, Bah J, Rastam M, Gillberg C, Wentz E, Melke J, Hellstrand M, Eriksson E: Association between a polymorphism of the 5-HT2C receptor and weight loss in teenage girls. Neuropsychopharmacology, 2002, 26, 789–793.

Received: March 27, 2010; **in the revised form:** July 26, 2010; **accepted:** August 20, 2010.