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# Characterization of the naturally occurring Arg344His variant of the human 5-$HT_{3A}$ receptor

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**Abstract:**
The present study aimed at examining the function and pharmacological properties of the naturally occurring Arg344His variant of the human 5-$HT_{3A}$ receptor, identified in a schizophrenic patient. In intact human embryonic kidney (HEK) 293 cells expressing the wild-type (WT) or the variant receptor, the function was analyzed by indirect measurement of agonist-induced $Ca^{2+}$ current through the 5-$HT_{3A}$ receptor channel by an aequorin luminescence-based $Ca^{2+}$ assay. In cell membrane patches cation currents were determined electrophysiologically including technically demanding single channel analyses. The pharmacological properties were analyzed by [$^3$H]GR65630 binding to cell membrane fragments. The density of [$^3$H]GR65630 binding sites in cells expressing the variant receptor was reduced to 55% of that in cells expressing the WT receptor, which, however, was not accompanied by an analogous decrease in 5-HT-induced $Ca^{2+}$ influx through the receptor channel. However, the single channel analysis suggests an increase in single receptor channel mean open time (which is known to be subject of many variables) but not in unitary current amplitude. Radioligand competition experiments revealed that the affinity of five 5-$HT_3$ receptor agonists and four antagonists for the variant receptor did not differ from that for the WT receptor. In conclusion, the variant receptor resembles the WT receptor in that it forms functional homopentameric 5-$HT_{3A}$ receptors with identical pharmacological properties. In view of the lack of reduction in $Ca^{2+}$ flux through the variant receptor channels in spite of the decrease in its density on the cell membrane, the increase in single receptor channel mean open time appears to compensate for the reduction in variant receptor density.

**Key words:**
serotonin receptor, human 5-$HT_{3A}$ receptor, variant Arg344His receptor, [$^3$H]GR65630 binding, patch clamp studies, aequorin luminescence $Ca^{2+}$ assay, HEK293 cells

**Abbreviations:** HEK – human embryonic kidney, h5-$HT_{3A}$ – human 5-$HT_{3A}$, 5-HT – 5-hydroxytryptamine (serotonin), WT – wild-type

## Introduction

Among the seven major families of currently known serotonin (5-hydroxytryptamine, 5-HT) receptors, the 5-$HT_3$ receptor is the only ligand-gated ion channel belonging to the cys-loop family, whereas all others are members of the superfamily of G-protein coupled receptors [14]. The 5-$HT_3$ receptor is a pentameric channel, which is almost exclusively expressed in neurons. Upon activation, it becomes permeable to $Na^+$, $K^+$ and $Ca^{2+}$ ions. Binding of 5-HT leads to a fast excitatory response of the respective neuron. Like other ion channels of the cys-loop superfamily, each of the five 5-$HT_3$ receptor subunits comprises

four transmembrane domains, among which the second ones form the channel pore. The N- and C-termini are located extracellularly and the N-terminus has been identified as the principal ligand-binding region [33]. At present, five different subunits, 5-$HT_{3A,B,C,D,E}$ [2, 10, 26–28], have been identified and characterized. The diversity of the human (h) 5-$HT_3$ receptor is further increased by two h5-$HT_{3A}$ subunit splice variants which modify the 5-HT response of the receptor when co-expressed with the 5-$HT_{3A}$ subunit [5]. Only the 5-$HT_{3A}$ subunit is able to form functional homopentameric receptors whereas the other subunits are modulatory and can form functional heteromeric receptors with the 5-$HT_{3A}$ subunit [3, 10, 28].

5-$HT_3$ receptors occur not only in different brain regions, including the amygdala, hippocampus and the caudate nucleus, but also on peripheral neurons of the autonomic nervous system [19] where they stimulate neurotransmitter release [12] and on immune cells [11]. They play a role, e.g., in the vomiting reflex and perception of pain and they are involved in the pathogenesis of various diseases such as schizophrenia, depression, fibromyalgia and irritable bowel syndrome. Single nucleotide polymorphism leading to the exchange of a single amino acid of a receptor protein has been shown to modify the functional and pharmacological properties of recombinant and/or native 5-HT receptors [6, 7, 13, 21, 22, 30–32].

The 5-$HT_{3A}$ subunit gene has been screened for variations in schizophrenic and bipolar affective patients. In each of two schizophrenic patients, a rare missense mutation, Pro391Arg in one case and Arg344His in the other, has been identified in the large second cytoplasmatic loop of the receptor protein [29]. Although statistical evaluation revealed no association of these mutations with this disease, it does not exclude that the pharmacological and/or functional properties of the mutated receptor may be modified.

However, the pharmacological properties of the Pro391Arg variant of the recombinant 5-$HT_{3A}$ receptor did not differ from those of the wild-type (WT) receptor [23, 24, 36]. Its functional properties were also found not to be altered [23, 24] or, at most, a very slight decrease in potency of 5-HT in activating inward current through the variant receptor channel was observed [36].

In the case of the Arg344His variant, the pharmacological and functional properties were also not found to be altered compared to the WT receptor [9, 23, 36; the first reference represents our preliminary report of some of the present data at the 2004 Society for Neuroscience Meeting]. However, regarding the receptor density on the cell surface, these results are not consistent. Either a reduced surface expression of the variant of about 50% compared to that at the WT receptor was found [9, 23] or the two isoforms did not differ in receptor density [36]. Surprisingly, in the former study [23] the maximal agonist-induced inward current was not different between variant and WT receptor, thus not reflecting the reduced number of variant receptor channels in the cell membrane.

In two of the studies [23, 36] determination of the pharmacological properties of the 5-$HT_{3A}$ Arg344His receptor was based on the affinities of only 5-HT, three 5-$HT_3$ receptor antagonists applied in the radioligand competition experiments and the two radioligands ([$^3$H]GR65630 and [$^3$H]granisetron, respectively) used in the studies. With such a low number of affinity data, at least minor differences in pharmacological properties of the two receptor isoforms could have been overlooked. This possibility can be derived from the pharmacological properties of the Phe124Cys variant of the h5-$HT_{1B}$ receptor at which not only virtually identical but also higher and lower affinities of a high number of ligands were found [22].

Taking these points into account, the aims of this study on recombinant h5-$HT_{3A}$ isoreceptors expressed in human embryonic kidney (HEK) 293 cells were: 1) to determine the affinities of a higher number of 5-$HT_3$ receptor ligands to provide a more comprehensive pattern of affinities which forms the pharmacological fingerprint of the Arg344His variant compared to the WT receptor; 2) to re-evaluate whether or not the variant is expressed at lower density than the WT receptor; and if so 3) to examine whether a corresponding reduction in maximal agonist-induced current does not occur; and 4) to make an attempt to identify the reasons underlying such a discrepancy.

## Materials and Methods

### Materials

5-Hydroxytryptamine creatinine sulfate (5-HT), *meta*-chlorophenylbiguanide (mCPBG), 2-methyl-5-

hydroxytryptamine (2-Me-5-HT), phenylbiguanide (PBG) and quipazine were obtained from Sigma (Taufkirchen, Germany). D-tubocurarine was from ICN (MP Biochemicals, Heidelberg, Germany). Azasetron (former designation: Y-25130) was purchased from Biotrend (Cologne, Germany). Ondansetron hydrochloride and tropisetron were kind gifts from GlaxoSmithKline and Novartis, respectively. 3-Tropanyl-3,5-dichloro-benzoate (MDL72222) was obtained from RBI/Sigma (Taufkirchen, Germany) and [$^3$H]-3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone ([$^3$H]GR65630, specific activity 75 Ci/mmol) was from PerkinElmer (Rodgau-Jügesheim, Germany).

### Expression constructs and site-directed mutagenesis

The 5-$HT_{3A}$ receptor subunit cDNA (GenBank accession no. AJ003079) subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Karlsruhe, Germany) under control of the human cytomegalovirus promoter was used to introduce the 344-His variation with the "Quick change" site-directed mutagenesis system (Stratagene, La Jolla, California, USA). The following sense and antisense primers were used: sense: 5'-cctgcttggctgcAtcacctggttctg-3'; antisense: 5'-cagaaccaggtgaTgcagccaagcagg-3'. The 5-$HT_{3A}$ subunit in which three arginine residues were exchanged by their 5-$HT_{3B}$ subunit counterparts (Arg432Gln, Arg436Asp, Arg440Ala), named 5-$HT_{3A}$ QDA [20], in the pcDNA3 expression vector was a kind gift of J. P. Dilger. The 344-His variation was also introduced in this 5-$HT_{3A}$ QDA subunit, yielding 5-$HT_{3A}$ QDA Arg344His. After transformation of *E. coli* and plasmid preparation, both strands of the cDNA clone with the desired mutation were sequenced by automated dideoxynucleotide sequencing on a Licor L4200 sequencer (MWG Biotech Inc., High Point, North Carolina, USA) by using IRD800 end-labeled primers and the "deaza GTP cycle sequencing kit" to confirm that no other than the desired nucleotide exchange occurred. The apoaequorin cDNA (GeneBank accession number L29571) was originally derived from cytAEQ/pcDNA1 (Molecular Probes) and subcloned into *Hind*III/*Xba*I-digested pcDNA 3.1/zeo (+) (Invitrogen, Karlsruhe, Germany).

### Cell culture and transfection

HEK293 cells were grown as monolayers in DMEM /Ham's F12 (1:1) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere at 37°C containing 5% $CO_2$.

Stable transfection (for radioligand saturation/competition experiments and electrophysiological single channel recordings) as well as transient transfection (for radioligand saturation experiments and aequorin luminescence assays) with either the 5-$HT_{3A}$ wild-type or the 5-$HT_{3A}$ Arg344His subunit cDNAs (with and without the QDA-exchange; see above expression constructs) were performed by lipofection with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For aequorin luminescence assays of intracellular $Ca^{2+}$ transients, cells were co-transfected with the respective receptor subunit cDNA and the apoaequorin cDNA (1:1). Transient transfections for electrophysiological experiments were carried out by modified calcium phosphate precipitation [8]. Transiently transfected cells were used 48 h post transfection.

Stably transfected cells were selected by the addition of geneticin (G418, 800 µg/ml) to the culture medium. The medium was changed every second day and, after occurrence of single cell colonies they were separated by means of cloning cylinders (Sigma, Taufkirchen, Germany). Single colonies were further subcultured in 24-well plates (BD Falcon, Heidelberg, Germany) until confluence. About 20 colonies from each transfection experiment were tested for stable expression of the particular cDNA by determination of [$^{14}$C]guanidinium influx through the 5-$HT_3$ receptor channel into the cells [24] and of binding of the selective 5-$HT_3$ receptor antagonist [$^3$H]GR65630 to membranes (see below). Colonies with the highest expression were used for further experiments.

### Radioligand binding studies on plasma membranes

Plasma membranes were prepared from stably or transiently transfected HEK293 cells grown in 175-cm$^2$ cell culture flasks. Cells were washed once with ice cold buffer (Hepes, 5 mM; $MgCl_2$, 0.5 mM; ethyleneguaninetetraacetic acid (EGTA), 0.5 mM; ascorbic acid, 0.1 mM; phenylmethylsulfonyl fluoride (PMSF), 0.3 mM; pH 7.4). Then, the cells were harvested with

5 ml of this buffer using a rubber policeman. The cell suspension was homogenized on ice with a Polytron homogenizer and cell debris was pelleted by centrifugation for 5 min at 1000 × g. The supernatant was diluted to 40 ml with buffer (see above) and membranes were pelleted by centrifugation for 25 min at 40,000 × g at 4°C. The resulting pellet was washed twice with 40 ml buffer and again pelleted by centrifugation at 40,000 × g for 25 min. The final pellet was resuspended in 1 ml of the "extracellular solution" (ECS) as used in patch clamp experiments: NaCl 150 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1mM, Hepes 10 mM, KCl 5.6 mM, pH 7.4, and stored at –80°C until usage.

For radioligand binding experiments the membranes were diluted in ECS to a final concentration of 50 µg membrane protein/ml. In competition experiments with unlabeled 5-$HT_3$ receptor agonists and antagonists, membranes (20 µg protein) were incubated with 1 nM [$^3$H]GR65630 with or without the drug under study for 60 min at room temperature in a final volume of 500 µl. In saturation experiments, membranes of transiently or stably transfected cells were incubated with eight concentrations of [$^3$H]GR65630 (0.07–4.8 nM) under the conditions described above. Incubation mixes were filtered through polyethyleneimine (0.3%)-coated GF/B filters (Whatman, Kent, UK) using a Brandel cell harvester and washed three times with 2 ml of ice cold ECS. Radioactivity was measured in a liquid scintillation counter (Beckman Instruments Inc., Fullerton, California, USA). Specific binding was determined as the fraction of [$^3$H]GR65630 which could be displaced by 100 µM MDL72222.

### Radioligand binding studies on whole cells in suspension

After washing and harvesting transiently transfected cells as described in the previous subsection, $5 \times 10^4$ cells, diluted in ECS containing 5.55 mM D-glucose were incubated with increasing concentrations of [$^3$H]GR65630 (0.03–3 nM) for 30 min at room temperature. Half of the cells were permeabilized with 0.1% saponin before the assay for determination of intracellular binding sites. An aliquot of the cell suspension was used for determination of protein concentration after cell lysis with 0.05 % Triton-X100 according to the method of Lowry et al. [25]. For further details including subsequent steps, see previous subsection.

### Aequorin luminescence assay of intracellular $Ca^{2+}$ transients

$Ca^{2+}$ transients were measured by means of a newly developed aequorin bioluminescence – based $Ca^{2+}$ assay [37]. HEK293 cells transiently expressing apoaequorin and either 5-$HT_{3A}$ WT receptors or the Arg344His variant were tested for differences in increases in intracellular $Ca^{2+}$ concentrations. In brief, cells co-transfected with apoaequorin, were collected from 75-$cm^2$ cell culture flasks, pelleted by centrifugation and resuspended in 1.5 ml DMEM/Ham's F12 + 0.1 % BSA. Cell suspension was supplemented with 5 µM coelenterazine *h* (Nanolight, Pinetop, AZ, USA) and incubated for 3 h at room temperature in the dark. Before the experiment, cells were harvested by centrifugation, resuspended in assay buffer (NaCl 150 mM, KCl 5.6 mM, $CaCl_2$ 1.8 mM, Hepes 10 mM, D-glucose 20 mM) at a density of $2.5–3.5 \times 10^6$ cells/ml and incubated for 20 min at room temperature.

After measuring of baseline luminescence, 20 µl of the agonist solution was injected to 80 µl of the cell suspension in 96-well plates and light emission was measured in a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany) at a sampling rate of 2 Hz. For antagonist experiments cells were incubated with the respective antagonist for 15 min before starting the experiment. In the case of 5-HT maximum responses, remaining aequorin luminescence after measurement of the agonist-induced light signal was determined by cell lysis with 50 mM $CaCl_2$ and 0.1% (v/v) Triton X-100 and recording luminescence.

### Electrophysiological experiments

Responses to 5-HT (30 µM) in excised outside-out patches of transiently transfected HEK293 cells were measured in the voltage-clamp mode using a fast solution exchange system. Before starting patch-clamp recordings, the culture medium was replaced by ECS (see above) containing 20 mM D-glucose. D-Glucose was omitted from the extracellular solution used for superfusion of the excised patches. Patch pipettes with resistances of 3–6 mΩ were filled with 'intracellular' solution containing (in mM): KCl 140; EGTA 10; $MgCl_2$ 5; Hepes 10; pH 7.4. Experiments were performed at room temperature (20–23°C). A multichannel solution exchange system (RSC 200; Bio-Logic, Grenoble, France) offering a total exchange

time of less than 2 ms was used. The data were low-pass filtered at 1000 Hz and digitized at 2000 Hz; for further details, see [1].

For single channel recordings evoked by 30 μM 5-HT HEK293 cells either stably expressing the 5-$HT_{3A}$ QDA or the 5-$HT_{3A}$ QDA Arg344His receptor (see expression constructs) were used.

### Data analysis

Peak values for the concentration-response curves in the aequorin assay were obtained by subtraction of baseline luminescence from the agonist-induced maximum peak luminescence. In the case of maximal 5-HT responses, peak luminescence ($RLU_{peak}$) was normalized against total aequorin luminescence ($RLU_{max}$) after cell lysis in order to control for differences in transfection efficiency and cell number ($RLU_{peak}/RLU_{peak} + RLU_{max}$).

The concentration-response curves in the aequorin luminescence assay and in the competition radioligand binding experiments, the saturation binding curves as well as the corresponding constants $EC_{50}$, $IC_{50}$, Hill slope and the binding constants $K_d$ and $B_{max}$ were calculated by means of GraphPad Prism 4.0. Data analysis of the electrophysiological experiments was performed with pClamp 6 software (Axon). GraphPad Prism 4.0 software was used to create graphics.

### Statistical analysis

Results are given as the means ± SEM, unless stated otherwise. For comparison of the mean values, Student's *t*-test was applied; $p < 0.05$ was considered statistically significant. Linear regression analysis was carried out to examine whether a significant correlation exists between the $IC_{50}$ values of 5-$HT_3$ receptor ligands at the 5-$HT_{3A}$ WT and variant receptor.

## Results

### [$^3$H]GR65630 binding

In membranes of HEK293 cells transiently transfected with the cDNA of either the WT or the Arg344His variant of the h5-$HT_{3A}$ receptor subunit, saturation experiments with [$^3$H]GR65630, a 5-$HT_3$ receptor antagonist, revealed a single binding site for each of the two isoforms. The maximum number of binding sites was 44% lower ($p < 0.001$) in the membranes expressing the variant compared to the WT receptor (Fig. 1A): $B_{max}$ amounted to 2.6 ± 0.2 and 4.7 ± 0.2 pmol/mg protein, respectively. The $K_d$ values were 0.31 ± 0.10 and 0.24 ± 0.04 nM, respectively, which did not significantly differ from each other. Similar $K_d$ values (0.22 ± 0.07 and 0.50 ± 0.07 nM, respectively) were obtained in saturation experiments on membranes of cells stably transfected with the cDNA of the Arg344His variant or the WT of the h5-$HT_{3A}$ receptor subunit (n = 3 experiments in duplicate for each receptor isoform). However, the variant receptor was characterized by a much lower $B_{max}$ value than the WT receptor (0.68 ± 0.05 compared to 9.76 ± 0.46 pmol/mg protein, respectively; $p < 0.001$). In this context it should be kept in mind that in these eperiments the cell clones with the highest receptor density were selected for both receptor isoforms since the aim of these experiments was not to determine a change in the extent of expression caused by the mutation, but to measure the affinity or potency of ligands at both receptor isoforms as exactly as possible.

Saturation binding experiments with [$^3$H]GR65630 were also performed in intact cells transiently transfected with either the cDNA of the WT or the variant receptor subunit to further establish the density of the Arg344His variant on the cell surface. The $K_d$ values for the WT and the variant receptor were in a slightly lower range than in the experiments in membrane fragments and amounted to 0.09 ± 0.01 nM and 0.11 ± 0.01 nM, respectively. The $B_{max}$ values for the WT and the variant receptor were 3.32 ± 0.40 and 1.72 ± 0.33 pmol/mg protein, respectively (Fig. 1B). Thus, the percentage of $B_{max}$ in intact cells expressing the Arg344His variant accounted for only 52% of that in cells expressing the WT receptor, confirming the results obtained from binding to membrane fragments.

In the same set of experiments we determined the density of surface plus intracellular [$^3$H]GR65630 binding sites by permeabilizing the cells with saponin. In fact, part of the binding sites were found to be located intracellularly as has previously been shown by Ilegems et al. [18] for the mouse 5-$HT_{3A}$ receptor. The percentage of [$^3$H]GR65630 binding sites on the

cell surface related to their total number in permeabilized cells was not significantly different between cells expressing the WT or the variant receptor (69.8 ± 8.2% and 73.9 ± 3.6%, respectively) (Fig. 1B).

Competition binding experiments of 1 nM [$^3$H]GR65630 with nine 5-HT$_3$ receptor ligands were carried out with membranes of stably transfected cells. All 5-HT$_3$ receptor agonists and antagonists included in the study induced a complete monophasic inhibition of the specific binding of the radioligand to both isoforms of the receptor (Fig. 2A, B). The rank order of potency of the ligands, as represented by the pIC$_{50}$ values, did virtually not differ between the receptor isoforms (Fig. 2 A, B; Tab. 1): rank order of agonists: quipazine > mCPBG = 5-HT > 2-Me-5-HT > PBG; rank order of antagonists: tropisetron = azasetron = ondansetron > d-tubocurarine. Regression analysis revealed a highly significant correlation ($p < 0.0001$) between the WT and the variant h5-HT$_{3A}$ receptor regarding the potencies of these ligands in in-

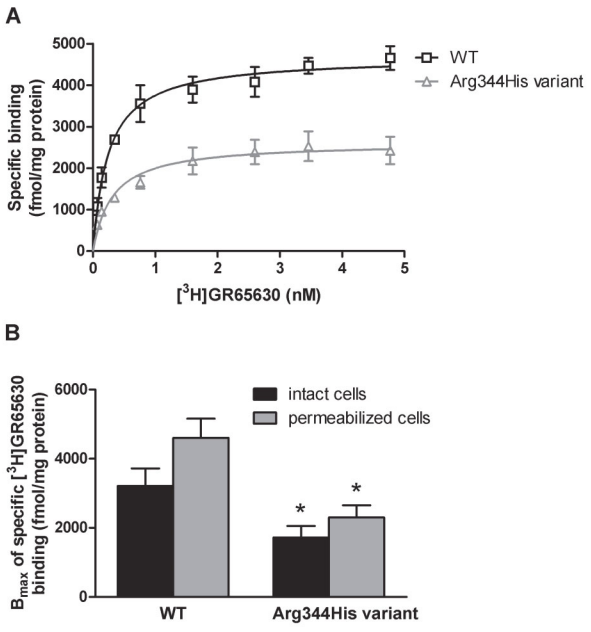



**Fig. 1.** [$^3$H]GR65630 saturation binding to HEK293 cell membranes (**A**) or cells in suspension (**B**) transiently expressing either the wild-type (WT) or the Arg344His variant of the human (h)5-HT$_{3A}$ receptor. **A)** Saturation curves for specific [$^3$H]GR65630 binding (0.07–4.8 nM) in HEK293 cell membranes expressing either the 5-HT$_{3A}$ WT or the 5-HT$_{3A}$ Arg344His variant. Nonspecific binding in the presence of 100 $\mu$M MDL72222 amounted to less than 10% of overall binding. Shown are the means ± SEM of three separate membrane preparations in each group. **B)** B$_{max}$ values of specific [$^3$H]GR65630 binding (0.03–3 nM) in HEK293 cells in suspension expressing either the 5-HT$_{3A}$ WT or the 5-HT$_{3A}$ Arg344His variant; binding experiments were carried out in intact or saponin (0.1%)-permeabilized cells in parallel (*, significantly different from corresponding WT receptor, $p < 0.05$). The number of [$^3$H]GR65630 binding sites on the cell surface (intact cells) related to their total number in permeabilized cells was not significantly different between cells expressing the WT or the variant receptor (69.8 ± 8.2% and 73.9 ± 3.6%, respectively). Bars represent the means ± SEM of three separate experiments in each group

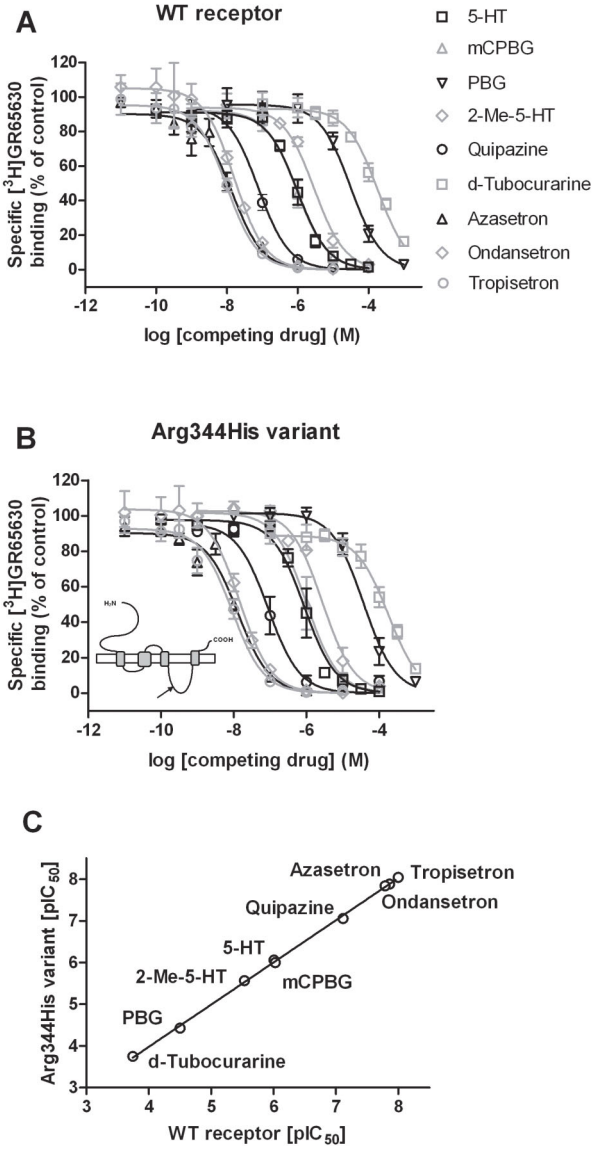



**Fig. 2.** Inhibition of [$^3$H]GR65630 binding to membranes of HEK293 cells stably expressing either (**A**) the wild-type (WT) or (**B**) the 5-HT$_{3A}$ Arg344His receptor. Nonspecific binding in the presence of 100 $\mu$M MDL72222 was lower than 10 % of overall binding. Reaction mixtures contained 1 nM [$^3$H]GR65630 and increasing concentrations of the competing unlabelled 5-HT$_3$ receptor agonists or antagonists. Shown are the inhibition curves for the means ± SEM of at least three experiments carried out in duplicate. **B, inset)** Schematic representation of the membrane topology of the 5-HT$_{3A}$ receptor subunit; the arrow indicates the approximate location of the Arg344His exchange in the second cytoplasmatic loop. **C)** Comparison of the mean pIC$_{50}$ values calculated from the curves shown in (**A**) and (**B**) and listed in Table 1. mCPBG, *meta*-chlorophenylbiguanide; PBG, phenylbiguanide; 2-Me-5-HT, 2-methyl-5-HT

**Tab. 1.** p$IC_{50}$ (the means ± SEM) and, in brackets, the mean $IC_{50}$ values for the inhibition of 1 nM [$^3$H]GR65630 binding to membranes of HEK293 cells stably expressing either the wild-type (WT) 5-$HT_{3A}$ receptor or its Arg344His variant

| | WT p$IC_{50}$($IC_{50}$; μM) | Arg344His p$IC_{50}$ ($IC_{50}$; μM) |
|---|---|---|
| Agonist | | |
| Quipazine | 7.11 ± 0.09 (0.08) | 7.05 ± 0.10 (0.09) |
| mCPBG | 6.03 ± 0.07 (0.94) | 6.00 ± 0.10 (1.00) |
| 5-HT | 6.01 ± 0.05 (0.98) | 6.06 ± 0.07 (0.87) |
| 2-Me-5-HT | 5.53 ± 0.08 (2.93) | 5.56 ± 0.07 (2.73) |
| PBG | 4.50 ± 0.12 (31.4) | 4.43 ± 0.09 (37.2) |
| Antagonist | | |
| Tropisetron | 8.00 ± 0.11 (0.01) | 8.04 ± 0.08 (0.01) |
| Azasetron | 7.86 ± 0.10 (0.01) | 7.88 ± 0.08 (0.01) |
| Ondansetron | 7.79 ± 0.08 (0.02) | 7.84 ± 0.08 (0.01) |
| d-Tubocurarine | 3.74 ± 0.06 (181) | 3.75 ± 0.07 (178) |

Values were calculated from the concentration-response curves shown in Fig. 2 A,B (at least three separate experiments in duplicate). mCPBG – *meta*-chlorophenylbiguanide, 2-Me-5-HT – 2-methyl-5-HT, PBG – phenylbiguanide

hibiting specific binding of [$^3$H]GR65630 (Fig. 2C; Tab. 1); both the slope of the regression line (0.98) and the regression coefficient (r = 0.99) were close to unity.

### Aequorin luminescence assays of intracellular $Ca^{2+}$ transients

Aequorin assays are based on the ability of the photoprotein aequorin to act as an indicator of intracellular $Ca^{2+}$ transients (for further details, see [37]). All aequorin assays were carried out with HEK293 cells transiently co-transfected with the cDNA of apoaequorin and either the cDNA of the 5-$HT_{3A}$ WT receptor subunit or the cDNA of the Arg344His variant subunit. Not only 5-HT, but also the selective 5-$HT_3$ receptor agonist mCPBG induced increases in $[Ca^{2+}]_i$ in HEK293 cells transiently co-transfected with apoaequorin and one of the receptor isoforms. Analysis of concentration-response curves of the two agonists revealed no difference in the potencies of these ligands between the two receptor isoforms (Fig. 3A, B; Tab. 2). Also the maximum increases in $[Ca^{2+}]_i$ induced by 10 μM 5-HT did not differ between the WT and the variant receptor (Fig. 3E). The 5-$HT_3$ receptor antagonists ondansetron and azasetron

**Tab. 2.** p$EC_{50}$ values, Hill coefficients of concentration-response curves and maximum peak effects of 5-$HT_3$ receptor agonists in inducing $Ca^{2+}$influx ($E_{max}$) in intact HEK293 cells transiently expressing either the wild-type (WT) or the Arg344His variant of the 5-$HT_{3A}$ receptor as well as p$IC_{50}$ values of 5-$HT_3$ antagonists against the peak $Ca^{2+}$ influx in response to 10 μM 5-HT (Means ± SEM). In brackets the mean $EC_{50}$ and $IC_{50}$ values, respectively, are given

| | WT receptor | | | Arg344His variant | | |
|---|---|---|---|---|---|---|
| | Hill coefficient | p$EC_{50}$ ($EC_{50}$; μM) | $E_{max}$ (% of WT) | Hill coefficient | p$EC_{50}$ ($EC_{50}$; μM) | $E_{max}$ (% of WT) |
| Agonists | | | | | | |
| 5-HT | 2.88 ± 0.92 | 5.81 ± 0.05 (1.58) | 100.0 ± 13.3 | 3.30 ± 0.72 | 5.85 ± 0.05 (1.46) | 101.4 8.6 |
| mCPBG | 2.73 ± 0.06 | 5.81 ± 0.03 (1.56) | | 3.27 ± 0.21 | 5.83 ± 0.02 (1.47) | |
| | | p$IC_{50}$ (mean $IC_{50;}$ nM) | | | p$IC_{50}$ (mean $IC_{50}$; nM) | |
| Antagonists | | | | | | |
| Azasetron | | 9.13 ± 0.01 (0.75) | | | 9.15 ± 0.08 (0.73) | |
| Ondansetron | | 9.29 ± 0.11 (0.56) | | | 9.35 ± 0.01 (0.45) | |

Values represent the mean of the (at least three separate) experiments shown in Figure 3. $E_{max}$ values were normalized by dividing the agonist-induced peak luminescence by the overall peak luminescence and expressed as percentage of mean $E_{max}$ in WT receptor. mCPBG – *meta*-chlorophenylbiguanide

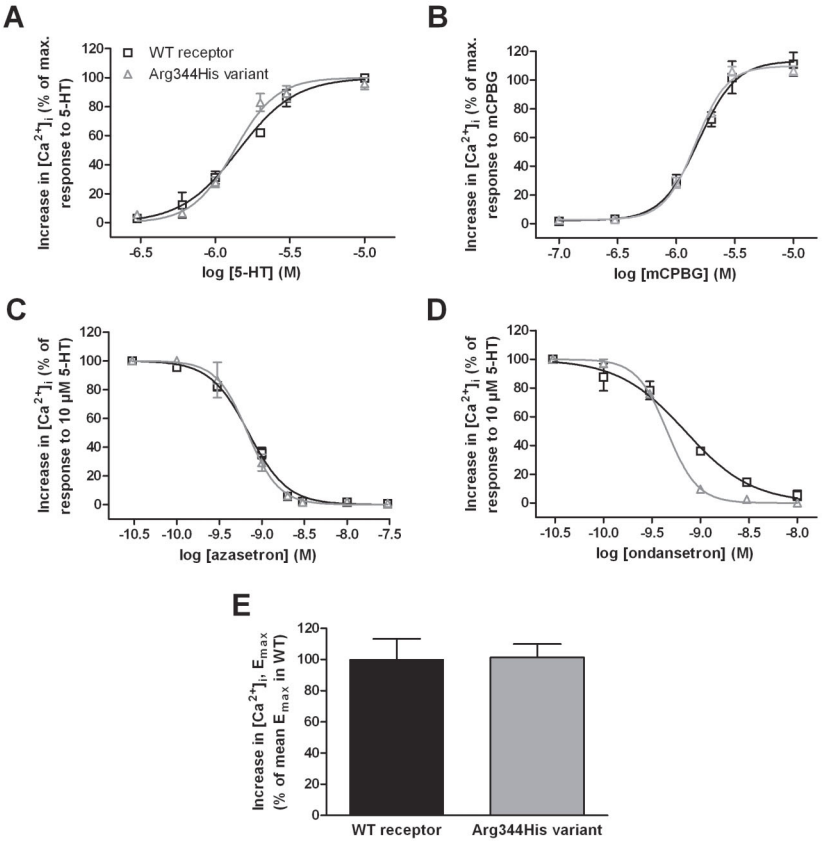


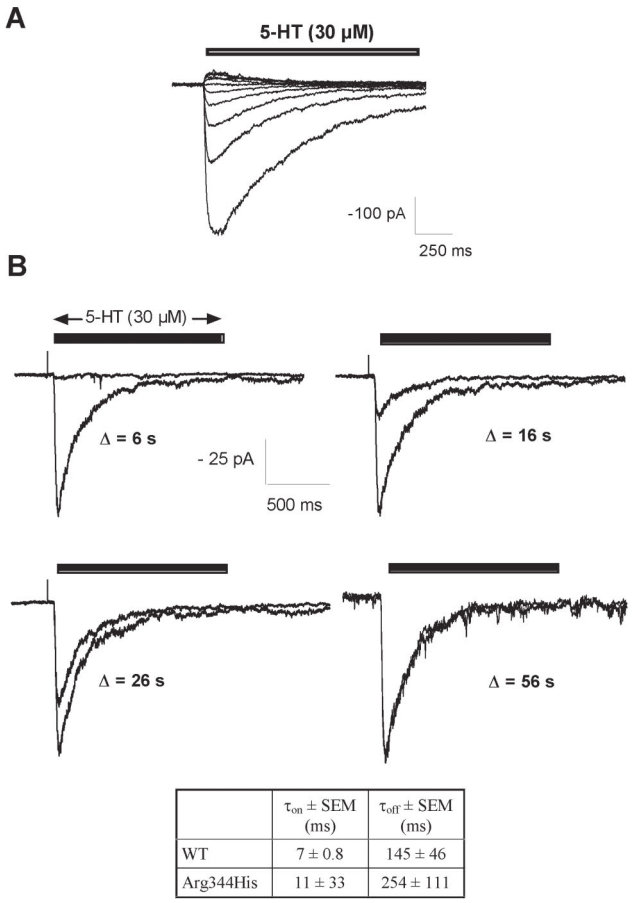

**Fig. 3.** Concentration-response curves (**A, B**) for 5-$HT_3$ receptor agonists in inducing increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and (**C, D**) for 5-$HT_3$ receptor antagonists in inhibiting the 5-HT (10 µM)- induced increase in ($[Ca^{2+}]_i$) as well as (**E**) maximum peak responses ($E_{max}$) to 5-HT (10 $\mu$M) determined in HEK293 cells transiently expressing either the wild-type (WT) or the Arg344His variant of the 5-$HT_{3A}$ receptor. An increase in $[Ca^{2+}]_i$-induced aequorin luminescence in response to agonist injection was measured in coelenterazine *h*-loaded cells transiently expressing apoaequorin and either the 5-$HT_{3A}$ receptor or its variant. An increase in ($[Ca^{2+}]_i$) evoked by increasing concentrations of (**A**) 5-HT or (**B**) mCPBG is expressed as percentage of the agonist maximal peak response. Inhibition of 5-HT (10 $\mu$M)-induced increase in ($[Ca^{2+}]_i$) by increasing concentrations of (**C**) azasetron and (**D**) ondansetron (present 15 min before and during 5-HT application) is expressed as percentage of the 5-HT (10 µM) response in the absence of the antagonist. (**E**) Maximum peak increase in ($[Ca^{2+}]_i$) in response to 5-HT (10$\mu$M) in cells expressing either the wild-type or the variant receptor is expressed as percentage of mean $E_{max}$ in wild-type receptor. Responses were normalized by dividing the agonist-induced peak luminescence ($RLU_{peak}$) by the overall aequorin peak luminescence ($RLU_{max}$, i.e. the agonist-induced peak luminescence plus the peak luminescence after cell lysis with $CaCl_2$ and Triton X-100). Shown are the means ± SEM of three to seven independent experiments. mCPBG, *meta*-chlorophenylbiguanide

concentration-dependently inhibited the increases in $[Ca^{2+}]_i$ induced by 10 µM 5-HT in both cell types. Comparison of the concentration-response curves of these antagonists showed only negligible differences in the $pIC_{50}$ values for the WT and the variant receptor (Fig. 3 C, D; Tab. 2).

### Electrophysiological experiments

At negative potential (–100 mV), rapid application of 30 µM 5-HT for 2 s induced transient inward currents in outside-out patches from HEK293 cells transiently transfected with the cDNA of either the WT or the Arg344His variant of the h5-$HT_{3A}$ receptor subunit. This is shown in Figure 4A for a representative experiment on a patch from a cell expressing the Arg344His variant. The current-voltage relationship was characterized by a pronounced inward rectification and was virtually identical to results obtained at the WT receptor [1]. Further characteristics of the currents through the WT receptor were a rapid monophasic activation and a slower monophasic decay (Fig. 4, inset). The onset and decay of currents through the Arg344His variant were not significantly different from those of the WT receptor, although a tendency towards slower time constants was found for this series of experiments (Fig. 4, inset). Similar to the results for the h5-$HT_{3A}$ WT receptor [1], the Arg344His variant showed a complete recovery from 5-HT-induced desensitization after 60 s wash (Fig. 4B).

Multiple attempts failed to record 5-HT-induced single channel currents in outside-out patches from cells transfected with the cDNA of the 5-$HT_{3A}$ WT receptor or its Arg344His variant. The reason is the extremely low single channel conductance of the homopentameric 5-$HT_{3A}$ receptor of 0.4 pS [4, 17] which was not detectable in our experiments. Therefore, we continued and strengthened our efforts by applying the 5-$HT_{3A}$ QDA mutant in which three arginine residues in the so-called "MA-stretch" of the large cyto-




| | $\tau_{on}$ ± SEM (ms) | $\tau_{off}$ ± SEM (ms) |
|---|---|---|
| WT | 7 ± 0.8 | 145 ± 46 |
| Arg344His | 11 ± 33 | 254 ± 111 |

**Fig. 4.** 5-HT (30 µM)-induced currents in a representative outside-out patch from a HEK293 cell transiently expressing the Arg344His receptor variant. **A)** Current/voltage relationship. Shown are the currents in response to 5-HT recorded from one patch. The potential was varied between –100 mV (bottom trace) and +100 mV (top trace). The large difference in amplitude between the bottom and top trace is typical for inward-rectification (for details see [1]). **B)** 5-HT double-pulse (voltage clamp condition: –100 mV) sequences on the same patch with increasing time intervals (△) between the pulses. As can be seen, a complete recovery from 5-HT-induced desensitization is observed after 1 min. Inset: Current onset ($\tau_{on}$) and decay ($\tau_{off}$) of WT and Arg344His variant (the means ± SEM of seven patches each)

plasmatic loop were replaced by their 5-HT$_{3B}$ counterparts. This 5-HT$_{3A}$ receptor mutant was found to exhibit a 28-fold increased single channel conductance [20]. Even in this system, we were able to record 5-HT-induced single channel currents only in eight patches (from 116 attempts; in each of these patches mean single channel open duration and current amplitude was based on the evaluation of 10–30 events) from cells expressing the 5-HT$_{3A}$ QDA (five patches from 56 attempts) or the 5-HT$_{3A}$ QDA Arg344His receptor (three patches from 60 attempts). The 5-HT-induced unitary current through the 5-HT$_{3A}$ QDA Arg344His receptor exhibited a prolongation of the mean open time compared to the corresponding current through the 5-HT$_{3A}$ QDA receptor (50.0 ± 10.5 ms [n = 3] *vs.* 21.1 ± 3.4 ms [n = 5], $p < 0.05$; however, see a note of caution in the Discussion). In contrast, the unitary current amplitudes did not differ between the two receptor isoforms (5-HT$_{3A}$ QDA Arg344His: 3.7 ± 0.2 pA (n = 3) and 5-HT$_{3A}$ QDA: 3.8 ± 0.1 pA (n = 5).

## Discussion

The main aim of the present study was to examine whether the point mutation leading to the amino acid exchange arginine against histidine at position 344 of the h5-HT$_{3A}$ receptor subunit is associated with changes in the pharmacological and/or functional properties of recombinant homomeric h5-HT$_{3A}$ receptors.

The most striking result was that in the saturation radioligand binding experiments on HEK293 cells transiently transfected with the respective h5-HT$_{3A}$ receptor subunit cDNA, $B_{max}$ at the Arg344His variant determined in cell membrane fragments or intact cells amounted to about only 55% of the wild-type receptor, indicating that the density of the variant receptor in the cell membrane was reduced by almost 50%. This finding is consistent with that of Krzywkowski et al. [23]. The missing reduction in surface expression of the Arg344His variant compared to the WT receptor in another study [36] may be due to the use of the

mouse 5-$HT_{3A}$ receptor cDNA. Several of the following possibilities have to be considered as explanation(s) for the decrease in membrane density of the variant receptor. The expression or stability of the corresponding mRNA or of the variant receptor protein may be lower. As a further alternative, the integration of the variant receptor into the cell membrane may be disturbed. We found that the percentage of [$^3$H]GR65630 binding sites expressed in the cell membrane, related to the total number of binding sites in permeabilized cells was not significantly different between the WT and the variant receptor. This result, which was also obtained in another study [23], rather argues against the possibility of an impaired integration of the variant receptor into the cell membrane. Finally, a combination of these possibilities has to be taken into consideration. Very recently, a region in the large cytoplasmatic loop of the 5-$HT_{3A}$ subunit has been identified to be essential for the indirect modulation of the 5-$HT_3$ receptor by protein kinase C [35]. The stimulation of this kinase has been demonstrated to cause an increased surface expression of 5-$HT_{3A}$ receptors by an indirect mechanism involving the F-actin cytoskeleton [34]. Since arginine 344 is located in direct neighborhood of this region, an influence of the Arg344His exchange on the extent of the modulation of 5-$HT_{3A}$ receptor expression by protein kinase C is conceivable. However, the elucidation of the mechanisms underlying the decrease in surface [$^3$H]GR65630 binding sites of variant compared to WT receptors was beyond the scope of this investigation.

As a consequence of the decreased expression, one would expect that the maximum stimulant effect of 5-$HT_3$ receptor agonists including the endogenous ligand 5-HT in the functional experiments should be decreased. However, as will be discussed below, this was not the case, thus, confirming the findings of another study [23] and clarifying the third open question within the aims. The possible reasons underlying this inconsistent result with respect to binding sites *vs.* ion currents will be briefly touched upon.

In contrast to this decrease in density of the mutant Arg344His receptor compared to the WT receptor, the radioligand competition binding experiments on stably transfected HEK293 cells revealed that the potencies of five 5-$HT_3$ receptor agonists and four 5-$HT_3$ receptor antagonists in inhibiting binding of the radioligand to the Arg344His and the WT receptor were not different. This finding indicates that the affinities of the 5-$HT_3$ receptor ligands for the orthosteric 5-$HT_3$ binding domain did not differ between both 5-$HT_3$ receptor isoforms. Accordingly, the rank order of potencies of the 5-$HT_3$ receptor agonists and antagonists was virtually identical at the variant and WT receptor. Thus, a more detailed and comprehensive pharmacological fingerprint of the variant compared to the WT receptor, for which was asked in the aims of the study, could be provided.

In order to analyze in more detail the function of the h5-$HT_{3A}$ variant and WT receptor receptors, i.e. influx of cations such as $Na^+$ and $Ca^{2+}$ ions *via* the 5-$HT_{3A}$ receptor channel in response to activation of the receptor, not only electrophysiological methods but also a new aequorin luminescence-based technique suitable to determine intracellular $Ca^{2+}$ transients was applied [37]. Activation of the 5-$HT_{3A}$ receptors by agonists (e.g., 5-HT or mCPBG) leads to an increase in intracellular $Ca^{2+}$ transients which are due to $Ca^{2+}$ influx *via* the 5-$HT_{3A}$ receptor channel. The present functional studies revealed that the Arg344His variant of the 5-$HT_{3A}$ receptor resembled the WT receptor in that it forms functional 5-$HT_{3A}$ receptor channels. The concentration-response curves of 5-$HT_3$ receptor ligands for their influence on $Ca^{2+}$ influx into the cells basically reflected the results of the competition binding studies: the potencies of the agonists investigated (5-HT, mCPBG) to induce $Ca^{2+}$ influx and antagonists (azasetron, ondansetron) to counteract the $Ca^{2+}$ influx evoked by 5-HT exhibited no significant difference between the WT and the variant receptor.

However, as already mentioned above, the decreased density of the variant compared to the WT receptor was surprisingly not accompanied by an analogous decrease in maximum 5-HT-induced $Ca^{2+}$ influx. A possible reason for this discrepancy among others would be that a decrease in density of the 5-$HT_{3A}$ Arg344His receptor may be compensated by an increase in channel open duration and/or probability. 5-$HT_{3A}$ receptor channel conductance is well known to be anomalously low in the sub-pico-Siemens range [4, 17] and, in agreement with this, we failed to record single channel traces in electrophysiological experiments on excised outside-out patches from the cell membrane of HEK293 cells expressing h5-$HT_{3A}$ WT and variant receptors. Basically, the so-called "MA-stretch" of the large second cytoplasmatic loop has been shown to influence the characteristics of single 5-$HT_3$ receptor channels. Thus, it was

found that replacement of three arginine residues within the cytoplasmatic loop by their 5-HT$_{3B}$ subunit counterparts, yielding the 5-HT$_{3A}$ QDA receptor, markedly increases single channel conductance [20]. Even with this, 5-HT$_{3A}$ QDA mutant single channel events could be recorded in only eight patches expressing the 5-HT$_{3A}$ QDA or the 5-HT$_{3A}$ QDA Arg344His receptor (n = 5 and 3, respectively). One of the shortcomings of the application of the 5-HT$_{3A}$ QDA receptor is that is has not yet been studied with respect to differences in pharmacological properties compared to the 5-HT$_{3A}$ WT receptor. However, the influence of mutations in the large cytoplasmic loop which is located far away from the orthosteric ligand binding site in the extracellular N-terminus is not likely; this contention is supported by the lack of differences in the pharmacological properties of the 5-HT$_{3A}$ wild-type receptor and its Arg344His variant found here. The observation from our single channel recordings was an increased mean open time of single 5-HT$_{3A}$ QDA Arg344His receptor channels compared to that of 5-HT$_{3A}$ QDA WT receptors which supports the possibility of a prolonged open time of the Arg344His variant receptor. Thus, the effect of a decreased density, i.e. decreased current, could be compensated by increased single channel open time. However, in view of the only borderline statistical significance, a note of caution should be added to this interpretation. The reason is that the open time of the channel is very variable and is, among other factors, dependent on temperature.

In the context of potential changes in the characteristics of currents through the variant 5-HT$_{3A}$ receptor compared to the WT receptor, the tendency towards a slower decay of the currents induced by 5-HT found here should be kept in mind. A slower decay, e.g., due to a change in the onset of desensitization, would be compatible with an increase in channel open duration. In fact, arginine residues in the 5-HT$_{3A}$ subunit have been shown to exert an impact on the function especially the desensitization kinetics of the 5-HT$_{3A}$ receptor [15, 16], which makes an influence of arginine 344 on the 5-HT$_{3A}$ channel properties conceivable. For example, Hu et al. [15] demonstrated, by exchanging the arginine residue in position 440 of the large cytoplasmatic loop of 5-HT$_{3A}$ with other amino acids, that the desensitization rate decreases with increasing hydrophobicity of the amino acid in this position. Considering the Arg344His variant, the exchange of the polar arginine against the more hydrophobic histidine would be in line with these data.

Taken together, the present electrophysiological investigation provides at least preliminary answers to the question for the mechanism underlying the discrepancy between the decrease in variant receptor density and the lack of functional consequences. More clear-cut answers to this question can be provided by a separate study exclusively devoted to this problem. However, our experiments dealing with the disclosure of the mechanisms underlying the above-mentioned discrepancies in the results at least pave the way for a resolution of the problem.

In conclusion, the present investigation on transfected HEK293 cells revealed that the Arg344His mutation of the h5-HT$_{3A}$ receptor, although not involved in genetic predisposition to schizophrenia, leads to a decreased density of the variant compared to the WT receptor. This decrease is not accompanied by a reduction in ion flux through the variant receptor channels. This lack of effect appears to be compensated by an increase in single channel open duration of the variant receptor. Such a compensatory mechanism has been observed in single 5-HT$_{3A}$ QDA Arg344His channels compared to 5-HT$_{3A}$ QDA receptors, in which three arginine residues in the large cytoplasmatic loop of the subunit have been exchanged by their 5-HT$_{3B}$ counterparts.

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## References:

1. Barann M, Meder W, Dorner Z, Brüss M, Bönisch H, Göthert M, Urban BW: Recombinant human 5-HT$_{3A}$ receptors in outside-out patches of HEK 293 cells: basic properties and barbiturate effects. Naunyn Schmiedebergs Arch Pharmacol, 2000, 362, 255–265.
2. Belelli D, Balcarek JM, Hope AG, Peters JA, Lambert JJ, Blackburn TP: Cloning and functional expression of a human 5-hydroxytryptamine type 3AS receptor subunit. Mol Pharmacol, 1995, 48, 1054–1062.
3. Boyd GW, Low P, Dunlop JI, Robertson LA, Vardy A, Lambert JJ, Peters JA, Connolly CN: Assembly and cell surface expression of homomeric and heteromeric 5-HT3 receptors: the role of oligomerization and chaperone proteins. Mol Cell Neurosci, 2002, 21, 38–50.

4. Brown AM, Hope AG, Lambert JJ, Peters JA: Ion permeation and conduction in a human recombinant 5-$HT_3$ receptor subunit (h5-$HT_{3A}$). J Physiol, 1998, 507, 653–665.
5. Brüss M, Barann M, Hayer-Zillgen M, Eucker T, Göthert M, Bönisch H: Modified 5-$HT_{3A}$ receptor function by co-expression of alternatively spliced human 5-$HT_{3A}$ receptor isoforms. Naunyn Schmiedebergs Arch Pharmacol, 2000, 362, 392–401.
6. Brüss M, Bönisch H, Bühlen M, Nöthen MM, Propping P, Göthert M: Modified ligand binding to the naturally occurring Cys-124 variant of the human serotonin 5-$HT_{1B}$ receptor. Pharmacogenetics, 1999, 9, 95–102.
7. Brüss M, Kostanian A, Bönisch H, Göthert M: The naturally occurring Arg219Leu variant of the human 5-$HT_{1A}$ receptor: impairment of signal transduction. Pharmacogenet Genomics, 2005, 15, 257–264.
8. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol, 1987, 7, 2745–2752.
9. Combrink S, Kostanian A, Barann M, Bönisch H, Göthert M, Brüss M: Functional characterization of the naturally occurring Arg344His variant of the human 5-$HT_{3A}$ receptor. Abstract (No. 626.6) to the annual Meeting of the Society for Neuroscience, San Diego, 2004.
10. Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG, Kirkness EF: The 5-$HT_{3B}$ subunit is a major determinant of serotonin-receptor function. Nature, 1999, 397, 359–363.
11. Fiebich BL, Akundi RS, Seidel M, Geyer V, Haus U, Müller W, Stratz T, Candelario-Jalil E: Expression of 5-$HT_{3A}$ receptors in cells of the immune system. Scand J Rheumatol, 2004, 33, Suppl 119, 9–11.
12. Fink KB, Göthert M: 5-HT receptor regulation of neurotransmitter release. Pharmacol Rev, 2007, 59, 360–417.
13. Göthert M, Propping P, Bönisch H, Brüss M, Nöthen MM: Genetic variation in human 5-HT receptors: potential pathogenetic and pharmacological role. Ann NY Acad Sci, 1998, 861, 26–30.
14. Hoyer D, Hannon JP, Martin GR: Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav, 2002, 71, 533–554.
15. Hu XQ, Sun H, Peoples RW, Hong R, Zhang L: An interaction involving an arginine residue in the cytoplasmic domain of the 5-$HT_{3A}$ receptor contributes to receptor desensitization mechanism. J Biol Chem, 2006, 281, 21781–21788.
16. Hu XQ, Zhang L, Stewart RR, Weight FF: Arginine 222 in the pre-transmembrane domain 1 of 5-$HT_{3A}$ receptors links agonist binding to channel gating. J Biol Chem, 2003, 278, 46583–46589.
17. Hussy N, Lukas W, Jones KA: Functional properties of a cloned 5-hydroxytryptamine ionotropic receptor subunit: comparison with native mouse receptors. J Physiol, 1994, 481 ( Pt 2), 311–323.
18. Ilegems E, Pick HM, Deluz C, Kellenberger S, Vogel H: Noninvasive imaging of 5-$HT_3$ receptor trafficking in live cells: from biosynthesis to endocytosis. J Biol Chem, 2004, 279, 53346–53352.
19. Jackson MB, Yakel JL: The 5-$HT_3$ receptor channel. Annu Rev Physiol, 1995, 57, 447–468.
20. Kelley SP, Dunlop JI, Kirkness EF, Lambert JJ, Peters JA: A cytoplasmic region determines single-channel conductance in 5-$HT_3$ receptors. Nature, 2003, 424, 321–324.
21. Kiel S, Bönisch H, Brüss M, Göthert M: Impairment of signal transduction in response to stimulation of the naturally occurring Pro279Leu variant of the h5-HT7(a) receptor. Pharmacogenetics, 2003, 13, 119–126.
22. Kiel S, Brüss M, Bönisch H, Göthert M: Pharmacological properties of the naturally occurring Phe-124-Cys variant of the human 5-$HT_{1B}$ receptor: changes in ligand binding, G-protein coupling and second messenger formation. Pharmacogenetics, 2000, 10, 655–666.
23. Krzywkowski K, Jensen AA, Connolly CN, Bräuner-Osborne H: Naturally occurring variations in the human 5-$HT_{3A}$ gene profoundly impact 5-$HT_3$ receptor function and expression. Pharmacogenet Genomics, 2007, 17, 255–266.
24. Kurzwelly D, Barann M, Kostanian A, Combrink S, Bönisch H, Göthert M, Brüss M: Pharmacological and electrophysiological properties of the naturally occurring Pro391Arg variant of the human 5-$HT_{3A}$ receptor. Pharmacogenetics, 2004, 14, 165–172.
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem, 1951, 193, 265–275.
26. Miyake A, Mochizuki S, Takemoto Y, Akuzawa S: Molecular cloning of human 5-hydroxytryptamine3 receptor: heterogeneity in distribution and function among species. Mol Pharmacol, 1995, 48, 407–416.
27. Niesler B, Frank B, Kapeller J, Rappold GA: Cloning, physical mapping and expression analysis of the human 5-$HT_3$ serotonin receptor-like genes HTR3C, HTR3D and HTR3E. Gene, 2003, 310, 101–111.
28. 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-HT3C, 5-HT3D, and 5-HT3E. Mol Pharmacol, 2007, 72, 8–17.
29. Niesler B, Weiss B, Fischer C, Nöthen MM, Propping P, Bondy B, Rietschel M et al.: Serotonin receptor gene HTR3A variants in schizophrenic and bipolar affective patients. Pharmacogenetics, 2001, 11, 21–27.
30. Nöthen MM, Rietschel M, Erdmann J, Oberlander H, Möller HJ, Nober D, Propping P: Genetic variation of the 5-$HT_{2A}$ receptor and response to clozapine. Lancet, 1995, 346, 908–909.
31. Ozaki N, Manji H, Lubierman V, Lu SJ, Lappalainen J, Rosenthal NE, Goldman D: A naturally occurring amino acid substitution of the human serotonin 5-$HT_{2A}$ receptor influences amplitude and timing of intracellular calcium mobilization. J Neurochem, 1997, 68, 2186–2193.
32. 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 serotonin1A receptor. Neuropsychopharmacology, 1997, 17, 18–26.
33. Spier AD, Lummis SC: Immunological characterization of 5-$HT_3$ receptor transmembrane topology. J Mol Neurosci, 2002, 18, 169–178.
34. Sun H, Hu XQ, Moradel EM, Weight FF, Zhang L: Modulation of 5-$HT_3$ receptor-mediated response and

trafficking by activation of protein kinase C. J Biol Chem, 2003, 278, 34150–34157.
35. Sun H, Xiong W, Lovinger DM, Zhang L: Molecular determinants of PKC modulation of 5-HT$_{3A}$ receptor function and trafficking. Abstract to the annual Meeting of the Society for Neuroscience, Washington, 2008.
36. Thompson AJ, Sullivan NL, Lummis SC: Characterization of 5-HT$_3$ receptor mutations identified in schizophrenic patients. J Mol Neurosci, 2006, 30, 273–281.
37. 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.

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