Identification of a Domain which Affects Kinetics and Antagonistic Potency of Clozapine at 5-HT<sub>3</sub> Receptors

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**Abstract**

The widely used atypical antipsychotic clozapine is a potent competitive antagonist at 5-HT<sub>3</sub> receptors which may contribute to its unique psychopharmacological profile. Clozapine binds to 5-HT<sub>3</sub> receptors of various species. However, the structural requirements of the respective binding site for clozapine remain to be determined. Differences in the primary sequences within the 5-HT<sub>3A</sub> receptor gene in schizophrenic patients may result in an alteration of the antipsychotic potency and/or the side effect profile of clozapine. To determine these structural requirements we constructed chimeras with different 5-HT<sub>3A</sub> receptor sequences of murine and human origin and expressed these mutants in human embryonic kidney (HEK) 293 cells. Clozapine antagonises recombinant mouse 5-HT<sub>3A</sub> receptors with higher potency compared to recombinant human 5-HT<sub>3A</sub> receptors. 5-HT activation curves and clozapine inhibition curves yielded the parameters EC<sub>50</sub> and IC<sub>50</sub> for all receptors tested in the range of 0.6–2.7 μM and 1.5–83.3 nM, respectively. The use of the Cheng-Prusoff equation to calculate the dissociation constant K<sub>0</sub> values for clozapine revealed that an extracellular sequence (length 86 aa) close to the transmembrane domain M1 strongly determines the binding affinity of clozapine. K<sub>0</sub> values of clozapine were significantly lower (0.3–1.1 nM) for receptors containing the murine sequence and higher when compared with receptors containing the respective human sequence (5.8–13.4 nM). Thus, individual differences in the primary sequence of 5-HT<sub>3</sub> receptors may be crucial for the antipsychotic potency and/or the side effect profile of clozapine.

**Introduction**

Schizophrenia is a severe psychiatric illness with hallucinations, delusions, poverty of thought and emotions, social withdrawal and cognitive deficits as leading symptoms. A dysregulation of the dopaminergic neurotransmitter system plays an important role in the pathophysiology of schizophrenia. However, current research indicates additional dysfunctions of glutamatergic, GABAergic and also serotonergic (5-HT) neurotransmission [1,2]. Most antipsychotic agents antagonise the actions of endogenous dopamine at type 2 dopamine (D<sub>2</sub>) receptors in the brain. In contrast, the widely used atypical antipsychotic clozapine has a relatively poor affinity to D<sub>2</sub> receptors, but exerts also antagonistic effects at histamine receptors, muscarinic acetylcholine receptors, α-adrenoceptors and serotonin receptors [3,4]. Within the 5-HT receptor subtypes clozapine is a potent antagonist at 5-HT<sub>2A</sub>, 5-HT<sub>3A</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors [5].

The dopamine hypothesis of schizophrenia suggests an enhanced mesolimbic activity of dopaminergic neurotransmission [6,7]. Behavioural, neurochemical and electrophysiological investigations indicate that 5-HT<sub>3</sub> receptors modulate dopaminergic activity in mesolimbic and nigrostriatal pathways [8,9]. 5-HT<sub>3</sub> receptor activation enhanced dopamine release from slices of rat nucleus accumbens [10], striatum [11,12], and increased the activity of dopaminergic neurons in the ventral tegmental area [13]. These data suggest that 5-HT<sub>3</sub> receptor antagonists could mimic certain inhibitory effects of antipsychotic drugs. It may therefore be assumed that the antagonistic effects of clozapine mediated via 5-HT<sub>3</sub> receptors might contribute to its antipsychotic potential.

Functional 5-HT<sub>3</sub> receptors can only be formed by 5-HT<sub>3A</sub> subunits, alone or in combination with the 5-HT<sub>3B</sub> subunit [14]. The functional antagonism of antipsychotics at the 5-HT<sub>3A</sub> receptor may have important physiological implications. In the CNS, the functional properties of presynaptic 5-HT<sub>3A</sub> receptors may differ from those of postsynaptic 5-HT<sub>3A</sub> receptors. Presynaptic 5-HT<sub>3A</sub> receptors are responsible for the elevation of intracellular Ca<sup>2+</sup> and modulate the release of several neurotransmitters such as glutamate, dopamine, GABA, norepinephrine and 5-HT [15,16,17]. Postsynaptic 5-HT<sub>3A</sub> receptors mediate fast synaptic neurotransmission in the CNS [18,19]. The reduction of these Na<sup>+</sup> and Ca<sup>2+</sup> fluxes by antipsychotics may be involved in their inhibitory effect on neuronal discharge activity, and modulation of postsynaptic 5-HT<sub>3A</sub> receptors could alter learning and memory processes [17,20,21,22].

The primary amino acid sequence of the receptor determines the affinity of agonists or antagonists for the specific binding site. The effects of the competitive 5-HT<sub>3A</sub> receptor antagonist clozapine are affected by either changes in the primary sequences
of the 5-HT$_{3A}$ receptor gene encoding for the binding site or by modulation of the binding affinity of the endogenous agonist 5-HT to the receptor. It is therefore possible that variations in the 5-HT$_{3}$ receptor gene of schizophrenic patients may result in an alteration of the antipsychotic potency and/or the side effect profile of clozapine.

Functional antagonistic properties of the atypical antipsychotic clozapine have previously been reported for recombinant mouse 5-HT$_{3A}$ receptors with even higher potency (IC$_{50}$ = 10 nM; [23,24]) compared to recombinant human 5-HT$_{3A}$ receptors (IC$_{50}$ = 680 nM; [5]). To investigate the structural domains involved in the ligand recognition site for clozapine and activation and deactivation kinetics of 5-HT$_{3A}$ receptors we constructed 5 different receptor chimeras consisting of different murine and human sequences. The antagonistic effects of clozapine and those of 5-HT on receptor kinetics were tested by monitoring cation currents in HEK 293 cells with cotransfected GFP as an expression marker.

Materials and Methods

Cell culture

Native human embryonic kidney cells (HEK 293 cells) were purchased (German collection of cell cultures, Braunschweig, Germany) and HEK 293 cells stably expressing the human 5-HT$_{3A}$ receptor [25] or the murine 5-HT$_{3A}$ receptor, respectively, were grown as previously described [5].

Transfection

cDNAs encoding the human 5-HT$_{3A}$ subunit (nucleotides 217–1663, GenBank accession no. D49394), and chimeras were cloned into pCDM8 plasmid vectors [14], the murine 5-HT$_{3A}$ subunit was cloned into a pCDM8Xl plasmid vector. HEK 293 cells were stably transfected with plasmids containing cDNA for the human 5-HT$_{3A}$ or with cDNAs for the murine 5-HT$_{3A}$ subunits. Chimeric 5-HT$_{3A}$ receptor subunits or the P391R mutant carrying an intracellular mutation [26] were transiently transfected. A plasmid (pCDM8, prK5) encoding for the cDNA of green fluorescent protein (GFP) as an expression marker was co-transfected. Exponentially growing HEK 293 cells (2×10$^6$ cells) were transfected with chimeric or P391R DNA and GFP DNA by electroporation (BTX Electroporation System, Electro Cell Manipulator 600, San Diego, CA). Cells were harvested 12–18 h before transfection. After harvesting a 20×100 mm culture dish, the cells were resuspended in an electroporation buffer (975 μL, distilled H$_2$O containing (in mM) 50 K$_2$HPO$_4$, 20 K$^+$-acetate, pH 7.35) and a magnesium-sulfate solution (25 μL distilled water containing 1 M MgSO$_4$, pH 6.7) before transfection plasmids containing cDNAs for the 5-HT$_{3A}$ receptor subunits (5 μg) and for GFP (3 μg) were added to the cell suspension. Electroporation was performed at 300 V and 1 mF with a pulse time of 30–45 ms. Transfected cells were replaced in 10×35 mm culture dishes with supplemented medium and incubated (5% CO$_2$, 95% air, and 100% relative humidity, 37°C) for 12–18 h before the experiments. After the incubation period, 5–30% of the transfected cells expressed GFP, which is soluble in the cytoplasm, and more than 50% of the green fluorescent cells yielded 5-HT$_{3}$-induced inward currents. The kinetics of 5-HT$_{3}$ receptor-mediated currents in HEK 293 cells with cotransfected GFP were identical to those in preparations without GFP cDNA co-transfection.

Construction of unique restriction sites in the human and mouse 5-HT$_{3}$ receptor

To create unique restriction sites we introduced a BstEII site in the human 5-HT$_{3}$ receptor subunit [27], corresponding to the BstEII site in the mouse 5-HT$_{3}$ receptor mRNA at position 531 [28]. Additionally, in the murine 5-HT$_{3}$ receptor subunit a SgrA1 restriction site was introduced corresponding to the SgrA1 site in the human 5-HT$_{3}$ receptor mRNA at position 953 and an XhoI multicloning site was introduced in the mouse 5-HT$_{3}$ receptor at position 1541 corresponding to the XhoI multicloning site in the human gene. Mutations were performed with the QuikChange Site-directed Mutagenesis Kit (Stratagene, USA). All mutations were silent.

Construction of chimeric receptors

To construct chimeric receptors the cDNAs of both the human and the murine receptor subunits were digested with BstEII and HindIII, SgrA1 and HindIII, SgrA1 and BstEII, SgrA1 and XhoI, BstEII and XhoI, respectively. The digestion products were resolved on 1% or 1.5% agarose gels. The resulting small murine digestion fragments were subcloned in the corresponding human vector fragments. The chimeric cDNAs were sequenced on both strands to verify integrity of the mutants.

Structure of 5-HT$_{3}$ receptor chimeras composed of human and murine 5-HT$_{3A}$ sequences

5-HT$_{3A}$ receptors are pentameric assemblies of subunits consisting of extracellular, transmembrane, and cytoplasmic domains [29]. To investigate the molecular determinants for the differences in receptor kinetics, affinity and antagonistic potency of clozapine we constructed different chimeric receptors between human and murine 5-HT$_{3}$ receptor sequences. We created five chimeric receptors (Fig. 1 and 2), which contained the sequence between the amino terminus (= restriction site HindIII) and restriction site of BstEII (defined as sequence 1) and the sequence between restriction site BstEII and SgrA1 (defined as sequence 2). Sequence 1 and 2 together belong to the extracellular domain and form the ligand binding site. The sequence between restriction site of SgrA1 and the carboxy-terminal domain (= restriction site XhoI; defined as sequence 3; Fig. 2) belongs to the transmembrane and cytoplasmic domain. For clarity, human, murine receptors and chimeras were indicated as a combination of the numbers for the three different sequences where human sequences are marked in bold and murine sequences are marked in italics. The prefix “H”, “M” and “C” indicates human, murine and chimeric receptors, respectively: human 5-HT$_{3}$ receptor = H1123, murine 5-HT$_{3}$ receptor = M/23, chimeras are either C123, C123, C2/23, C/23 or C123. Fig. 1A shows the different receptor chimeras and the respective sequences consisting of human and mouse 5-HT$_{3}$ receptor subunits.

Concentration clamp recordings

5-HT$_{3}$-induced inward Na$^+$ currents were recorded from lifted HEK cells transiently transfected with the human, murine 5-HT$_{3A}$ receptor and chimeras in the whole-cell voltage clamp configuration under visual control using an inverted microscope (Zeiss, Jena, Germany) as previously described [30]. Cells were kept in a bath solution containing 140 mM NaCl, 2.8 mM KCl, and 10 mM HEPES, pH 7.2. Patch electrodes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a horizontal pipette puller (Zeiss Instruments, Augsburg, Germany) to yield pipettes with a resistance of 3–6 MΩ. Pipettes were filled with a solution containing 130 mM CsCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 2 mM ATP, 0.2 mM Tris-GTP, 10 mM glucose, 10 mM HEPES, and 10 mM EGTA, pH 7.2. After the whole-cell configuration was established, the cells were lifted from the glass substrate and
10 μM 5-HT were applied using a fast superfusion device. We applied these concentrations since 10 μM 5-HT were used for the determination of the IC50 value for the inhibition of the 5-HT response by psychopharmacological drugs in our previous study [5], which was in the low micromolar range. For control experiments a piezo translator-driven double-barrelled application pipette was used to expose the lifted cell either to 5-HT-free or 5-HT-containing solution. A 2 s 5-HT pulse was delivered every 90 s. The stock solutions (10 mM or 10 μM) of clozapine were diluted with bath solution to the desired concentration. To control for any possible confounding solvent effects, currents were recorded with 0.1% ethanol in 5-HT-free or 5-HT-containing solutions. Current signals were recorded at a holding potential of -250 mV with an EPC-9 amplifier (Heka, Lamprecht, Germany) and were analysed using the Heka 8.5 PulseFit and IgorPro v. 5.04B (Wavemetrics, Lake Oswego, OR, USA) software on a Power Macintosh G3 computer. In experiments with clozapine, only results from stable cells entered the final analysis, that is, showing at least 50% recovery of responses to 5-HT following the removal of drugs. In some cells, recovery was not 100% because of rundown (see frequent activation experiments). To compensate for this effect the % antagonism at each concentration was based on both the control and the recovery current by assuming a linear time course for the rundown. Data are shown as mean ± SEM. Measurements were performed as independent experiments relative to control and recovery.

Dose-response curves and the respective EC50 and IC50 values were calculated by the four parameter logistic equation for agonists: \( I = \frac{I_{\text{max}} \cdot \text{agonist}}{\text{agonist} + EC50} \) and for antagonists: \( I = I_{\text{max}} \cdot \text{antagonist} \cdot \text{agonist} \). The corrected binding affinity of clozapine Ka was calculated using the Cheng-Prusoff equation [31]: \( Ka = IC50/(1+\text{agonist}/EC50) \). A full dose-response curve was determined from every cell and the EC50 and IC50 values with the respective Hill coefficients were calculated. These values from each single dose-response curve were averaged thereafter. Thus, the reported means ± SEM result from different cells after averaging. For the figures of the dose-response curves we fitted the curve according to the average value for each respective concentration. Because the charge represents the most appropriate measure for receptor activation the IC50 and EC50 values for charge entered the Cheng-Prusoff equation for Ka analysis.

Figure 1. Amino acid sequence of cloned cDNA encoding the human and mouse 5-HT3A receptor channel subunit. Marked in red: mismatches of the amino acid sequence. Marked in green: Restriction sites for BstEII and SgrA1 representing switching points of the chimeric receptors. C-C: Cys-loop. M1–M4 transmembrane segments.

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activations, the values of the last 5 5-HT applications (22nd–26th application) entered statistical analysis. A value of \( p < 0.05 \) was considered as statistically significant. Statistical analysis was performed using the SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

### Chemicals and Drugs

Clozapine was purchased from Sigma (Munich, Germany) and stock solutions (10 mM) were prepared in pure ethanol. Thus, the maximum ethanol concentration in experiments using drug concentrations of 10 mM was less than 0.1%. Serotonin was purchased from Sigma and dissolved in water.

### Results

**Rundown kinetics of recombinant human and murine 5-HT\(_3\) receptors after multiple activation**

5-HT\(_3\) receptor activity is very sensitive to the presence of external Ca\(^{2+}\) as currents decline when Ca\(^{2+}\) concentration increases [32]. Therefore, 5-HT-activated currents were recorded in a HEPES-buffered Ca\(^{2+}\)-free solution. First, we characterized the kinetics of H\(_{123}\) and M\(_{123}\) (see also Table 1). At a holding potential of \(-50\) mV, 5-HT (10 \(\mu\)M) applied for 2 s evoked inward currents which rose (H\(_{123}\): \(t_{\text{on}} = 20.1 \pm 1.5\) ms, \(n = 38\); M\(_{123}\): \(t_{\text{on}} = 28.5 \pm 1.6\) ms, \(n = 36\); Fig. 3A) to a peak of 3806.1 \(\pm\) 272.5 pA and 340.3 \(\pm\) 30.3 pA for H\(_{123}\) and M\(_{123}\), respectively, and induced an incomplete receptor desensitization (H\(_{123}\): \(t_{\text{des1}} = 1879.7 \pm 164.3\) ms, \(t_{\text{des2}} = 1359.4 \pm 161.7\) ms; M\(_{123}\): \(t_{\text{des1}} = 2647.6 \pm 132.9\) ms, \(t_{\text{des2}} = 2514.8 \pm 148.3\) ms; Fig. 3A). Because of these characteristics of receptor desensitization, true steady-state responses could not be determined. Hence, steady-state currents have been defined as the last 10 ms of 5-HT application (see also [5,25]). After the removal of 5-HT, receptor currents deactivated completely with a time constant of \(t_{\text{off}} = 1977.0 \pm 117.7\) ms and \(t_{\text{off}} = 5937.4 \pm 730.1\) ms for H\(_{123}\) and M\(_{123}\), respectively (Fig. 3A).

The frequent activation of human 5-HT\(_3\) receptors is accompanied by constant rundown kinetics as previously shown [5]. In the present study, multiple 5-HT applications (26 applications within 40 min) reduced peak currents through H\(_{123}\) to 74% (\(n = 4\)), accelerated \(t_{\text{des1}}\) and \(t_{\text{des2}}\) to 64% and 73%, respectively, and reduced the steady-state current to 37% (Fig. 3B, C). Rundown kinetics of M\(_{123}\) are significantly less pronounced (after 26 applications, peak currents were reduced to 84%, plateau currents to 76% and \(t_{\text{des1}}\) and \(t_{\text{des2}}\) were even slowed down to 110% and 109%, respectively; Fig. 3B, C). Since rundown affects plateau currents and desensitization of H\(_{123}\) more effectively than those of M\(_{123}\), the calculation of charge thus represents a very sensitive parameter for receptor activity. Fig. 3B (right) demonstrates the strong rundown of H\(_{123}\) charge to 42% of control in comparison to the reduction of M\(_{123}\) charge to 72%. The parameter charge is therefore a suitable tool to investigate the molecular determinants for the differences in receptor kinetics by constructing human/murine receptor chimeras.

In the present study, we found a 99.4% recovery for human and a 97.9% recovery for murine receptors of the peak amplitude when two 5-HT applications for 2 sec were separated by a 25 sec interval (data not shown). These results are consistent with previous reports [33,34] which found a nearly complete recovery after 25 to 60 sec. Thus, using an interval of 90 sec between two
5-HT applications as in the present study should not affect receptor desensitization and prevent consecutive accumulation of desensitization. Furthermore, a similar rundown was obtained for both human and murine 5-HT3 receptors after only two 5-HT applications separated by a 40 min interval (data not shown). As such, the rundown cannot be simply attributed to an enhancement of receptor desensitization induced by multiple 5-HT applications.

Rundown kinetics for chimeras

All chimeras tested produced functional currents upon 5-HT activation. However, the currents through the different receptor chimeras showed a strong variation in kinetics (Table 1) and rundown (Fig. 4A, B, C; Table 2, 3). Whereas these differences were only marginally for peak currents (except for C12 and C23 where amplitudes were significantly reduced to 58% and 58% of control, respectively), analysis of charge showed more pronounced variation between all receptor types (Fig. 4B, C). Concomitantly, charge variations between the different receptor types displayed a good correlation to kinetic parameters such as desensitization, plateau current and deactivation (Fig. 4C). The M123 receptor currents were less affected by multiple 5-HT applications, showing only a minor rundown. Statistical analysis revealed that the charge of all other receptor types was strongly reduced in comparison to M123. No significant difference could be found between either C123, H123, C12 and C23, C123, C23, respectively. However, each of the receptor types C12, C123 and C23 showed a significantly reduced charge compared to C123, H123 and C123.

Functional antagonistic properties of the atypical antipsychotic clozapine against H123 and M123 currents

Human and murine 5-HT3 receptors showed almost identical affinity to 5-HT [25] which was also confirmed in the present study. Peak amplitude and charge of currents through H123 and M123 were concentration-dependently increased (Fig. 5A). Clozapine antagonised 5-HT-activated currents through human and murine 5-HT3 receptors with different potencies [3,24]. In the present study, clozapine was significantly more potent against M123 whereas the peak amplitude and charge of H123 were reduced less effectively (Fig. 5B). As such, the structural domains involved in the ligand recognition for clozapine can be identified by human/murine chimeras.

5-HT-induced currents through chimeric receptors

All chimeric receptors were dose-dependently activated by 5-HT with an EC50 in the range of 1.22 μM to 4.92 μM and 0.65 μM to 2.76 μM for peak and charge, respectively (Fig. 6A, B; Table 4, 5). For each dose-response curve values were normalized to the responses induced by 300 nM 5-HT. The EC50 for the peak current through the chimera C123 significantly differed from all other receptor types, that from C123 only from M123 and C23. The EC50 for the peak current for C123, M123, C23, C123 and H123 were comparable with regard to the EC50 for charge: C123 and C23 showed the lowest affinity for 5-HT with an equal EC50 which differed significantly from those of all other receptor types. The dose-response curves for 5-HT of C123, C23, M123, H123 and C123 yielded a similar EC50 with no significant difference (Fig. 6 A, B).

Antagonistic properties of clozapine against chimeric and mutant receptors

All 5-HT-induced currents through chimeric receptors were dose-dependently reduced by clozapine in a competitive manner, however, with different potencies (Fig. 7 and 8; Table 6, 7). Clozapine was most potent against 5-HT-induced currents of C123 receptors and showed lowest affinity to antagonise currents through H123 receptors (Fig. 7 and 8). Interestingly, clozapine exerts higher antagonistic potencies against those receptors carrying the murine sequence 2, whereas clozapine antagonism was less potent against receptors with a corresponding human sequence.

Recently, a single point mutation in the cytoplasmic domain of the 5-HT3 has been identified in individuals diagnosed with schizophrenia [35]. To investigate whether a mutation located at the intracellular site of the receptor affects competitive antagonism, we tested the pharmacological potency of clozapine against the human P391R mutant [26]. Interestingly, clozapine reduced 5-HT-evoked currents through this transiently transfected receptor with higher potency as for H123 (IC50 for peak: 4.81 ± 0.18 nM (Hill = −1.35)); charge: 5.91 ± 0.1 nM (Hill = 1.21); Fig 7 and 8).
Figure 3. Rundown kinetics of recombinant human and murine 5-HT$_3$A receptors after multiple activation. (A) Representative current traces for human (left) and murine (right) receptors showing currents after the first and 26th application of 5-HT. Records were obtained from the same cell. (B) Repeated 5-HT applications (26 applications within 40 min) reduced peak currents (left) and charge (right) through H$_{123}$ (filled circles) and M$_{123}$ (open circles) differently. Since rundown affects plateau currents and desensitization of H$_{123}$ more effectively than those of M$_{123}$, the calculation of charge thus represents a very sensitive parameter for receptor activity. (C) Bar diagram showing the change in plateau currents, $\tau_{\text{des}1}$ and $\tau_{\text{des}2}$ after 22 to 26 5-HT applications (mean ± SEM). Values were normalized to current kinetics evoked by the first application of 5-HT.

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Figure 4. (A) Rundown kinetics for chimeras. Representative current traces for human, murine receptors and chimeras showing currents after the first and 26th application of 5-HT. Records were obtained from the same cell. All chimeras tested produced functional currents upon 5-HT activation. However, the currents through the different receptor chimeras showed a strong variation in kinetics and rundown. (B) Repeated 5-HT-applications (26 applications within 40 min) reduced peak currents (left) and charge (right). Differences were only marginally for peak currents, whereas analysis of charge showed more pronounced variation between all receptor types. (C) Charge variations between the different receptor types displayed a good correlation to kinetic parameters such as desensitization, plateau current and deactivation.

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Kb for all receptors were obtained with the Cheng-Prusoff estimate close to the transmembrane domain M1 which strongly activates 5-HT3 receptors. Clozapine inhibition curves were also obtained using the Cheng-Prusoff equation.

**Table 2. Differences in rundown with regard to peak amplitude after multiple activation for human, murine and chimeric receptors.**

| receptor | M123 | C123 | C12 | H123 | C12J | C2/3 | C12J |
|----------|------|------|------|------|------|------|------|
| M123     |      |      |      |      |      |      |      |
| C123     |      |      |      |      |      |      |      |
| C12      |      |      |      |      |      |      |      |
| H123     |      |      |      |      |      |      |      |
| C12J     |      |      |      |      |      |      |      |
| C2/3     |      |      |      |      |      |      |      |
| C12J     |      |      |      |      |      |      |      |

Significant differences (p<0.05, ANOVA) are indicated by black circles. doi:10.1371/journal.pone.0006715.t002

**Discussion**

In the present study we generated five chimeras with different 5-HT3A receptor sequences of murine and human origin to determine the structural basis for clozapine binding. Analysis of 5-HT3 activation curves and clozapine binding curves using the Cheng-Prusoff equation revealed an extracellular sequence (length 86 aa) close to the transmembrane domain M1 which strongly determines the binding affinity of clozapine. These results suggest that genetic variations within this sequence of the 5-HT3 receptor gene may contribute to the antipsychotic potency and/or the side effect profile of clozapine under clinical conditions.

There is now good evidence that both homomeric (5-HT3A) and heteromeric (5-HT3AB) 5-HT3 receptor isoforms exist in brain and peripheral neuronal tissue [9,36]. However, electrophysiological and immunohistochemical evidence indicates that the majority of native 5-HT3 receptor complexes do not contain the 5-HT3B subunit [36,37]; for review see [38]. Moreover, the 5-HT3B subunit does not contribute to the ligand-binding site [39]. Therefore, we focussed only on homomeric 5-HT3A receptors in the present study.

The amino acid sequence of the human 5-HT3A receptor displays 85% identity with the mouse subunit [40]. In contrast to guinea pig receptors, murine and human 5-HT3 receptors exhibit a somewhat similar pharmacological profile [25]. The human and the mouse receptor differ only marginally in their affinity to the natural ligand 5-HT [14,25,33,34] which could also be confirmed in the present study. However, both homo-oligomeric receptor types show remarkable differences with regard to receptor kinetics and clozapine affinity. Human 5-HT3A receptors are characterised by a lower charge transfer due to faster desensitization and deactivation kinetics and a more pronounced receptor desensitization (see also [25]). Furthermore, repeated activation of human 5-HT3A receptors produced a marked decline in charge transfer. Currents through mouse receptors are less affected. These differences in rundown kinetics can be best explained by a considerable acceleration of desensitization kinetics and plateau currents for human 5-HT3A receptors, whereas these parameters are only marginally affected in mouse 5-HT3A receptors. Moreover, with regard to pharmacology, the competitive antagonist clozapine more potently inhibits currents through mouse 5-HT3A receptors which is reflected by a 10-fold smaller IC50 value than that obtained for human 5-HT3A receptors (see also [5,24]).

These functional and pharmacological differences may be valuable for locating sequences important for rundown kinetics induced by repeated receptor activation and for clozapine binding affinity by creating human/mouse 5-HT3A receptor chimeras. The extracellular domain, which forms the ligand binding site [32,41], appears to be crucial for these functions. In the present study we therefore constructed three chimeric receptors (C123, C2/3, C12J) with an extracellular domain combined of human and murine sequences allowing a more detailed mapping of determinants of the agonist and antagonist binding site.

The frequent activation of 5-HT3A receptors is accompanied by constant rundown kinetics reflected by a pronounced acceleration of tdes1 and tdes2 and a strong reduction of the steady-state current. Although the peak amplitude also decreased after multiple 5-HT applications, this effect is less prominent. Thus, the charge transfer is the most suitable parameter for assessing receptor activity.

Analysis of rundown kinetics revealed a significantly smaller decline in charge transfer for murine receptors compared to human and all chimeric receptors. This decline is predominantly due to a pronounced receptor desensitization after repeated activation, as plateau currents are reduced to a similar degree. Since human and murine receptors recovered almost completely when two 5-HT applications were separated by a 25 sec interval (data not shown), the use of a 90 sec interval should not affect receptor desensitization and should prevent consecutive accumulation of desensitization. Interestingly, a similar rundown is observed for both human and murine 5-HT3 receptors after only two 5-HT applications separated by a 40 min interval. Thus, the rundown cannot be simply attributed to an enhancement of receptor desensitization induced by multiple 5-HT applications.

The analysis of the kinetic and rundown characteristics of all chimeras did not unravel a clear sequence correlation. Single

**Table 3. Differences in rundown with regard to charge after multiple activation for human, murine and chimeric receptors.**

| receptor | M123 | C123 | C12 | H123 | C12J | C2/3 | C12J |
|----------|------|------|------|------|------|------|------|
| M123     |      |      |      |      |      |      |      |
| C123     |      |      |      |      |      |      |      |
| C12      |      |      |      |      |      |      |      |
| H123     |      |      |      |      |      |      |      |
| C12J     |      |      |      |      |      |      |      |
| C2/3     |      |      |      |      |      |      |      |
| C12J     |      |      |      |      |      |      |      |

Significant differences (p<0.05, ANOVA) are indicated by black dots. doi:10.1371/journal.pone.0006715.t003
activation of C123 and C123 produced receptor kinetics similar to murine receptors, whereas repeated activation of these chimeras induced strong rundown kinetics. Conversely, likewise murine receptors, the chimera C123 showed only a marginal reduction in charge transfer after repeated activation but kinetics similar to human receptors. These results indicate that the molecular determinants responsible for rundown kinetics and for receptor desensitization are not associated with the same protein segment.

5-HT activates human and mouse 5-HT3A receptors with similar potency suggesting that the EC50 values for chimeras should not differ significantly. However, when considering the EC50 for charge and peak, 5-HT was significantly more potent in activating the chimeras C123 and C123 compared to C123 and C123. A plausible explanation for this inconsistency might be that the extracellular domain is not the single determinant for agonist affinity. This hypothesis is supported by a recent investigation demonstrating that a mutation in the cytoplasmic domain (P391R) can also cause alterations in agonist binding [26]. Moreover, irrespective of sequence composition, desensitization and deactivation parameters of chimeric receptors were constantly slower than the fast human receptor kinetics (see table 1). These observations cannot be explained by a simple sequence-to-function correlation. There is evidence that the extracellular domain determines agonist binding [32,41] whereas the cytoplasmic domain of the 5-HT3A receptor contributes to a receptor desensitization mechanism [25,42,43,44]. Concerning the molecular parameters for agonist affinity, receptor kinetics and rundown, our data rather favour the hypothesis of an involvement of the tertiary and quaternary structure of the whole receptor molecule than a restricted structural domain [32]. It is likely that the
successful spatial coupling of the neurotransmitter binding site to the ion channel and cytoplasmic domain is crucial for mediating 5-HT binding, kinetics and rundown properties. This assumption is supported by the fact that a P391R point mutation in the cytoplasmic domain of the 5-HT3 receptor affects the agonist binding site by increasing the EC_{50} for 5-HT [35,26]. Since the rapid desensitization of 5-HT 3A receptors during sustained activation [45,46,47,48] has great importance for synaptic regulation [16], alterations of the tertiary and quaternary structure may also have implications in the pathophysiology of schizophrenia.

Clozapine potently antagonises murine [24] and human 5-HT3A receptors [5] with a 10-fold higher affinity against murine receptors. The evaluation of the antagonistic potency of clozapine against chimeric receptors revealed IC_{50} values below the IC_{50} for human receptors and for C123 and C123 IC_{50} levels even below those of murine receptors. As clozapine is a competitive antagonist, calculation of true dissociation constant K_b values for each receptor needs the consideration of the specific 5-HT affinity. Estimation of K_b values for clozapine using the Cheng-Prusoff relationship revealed that sequence 2 of the extracellular ligand binding site (length 86 aa) close to the transmembrane domain M1 strongly determines the binding affinity of clozapine. When chimeric receptors contain the murine sequence 2 (C123, C12, C123), their K_b values for clozapine affinity were similar to the K_b for murine receptors and significantly lower (0.3–1.1 nM) compared to the chimeras C123, C123 containing the human sequence 2 with K_b values of 5.8 and 13.4 nM, respectively.

In contrast to other 5-HT receptors, the HTR3A gene shows a relatively high variability in the coding region, and it is possible that approximately 1% of schizophrenic patients carry 5-HTR3A mutations [35]. Approximately 30–60% of all schizophrenic patients fail to respond to typical antipsychotics [49] and hence, clozapine may be a valuable treatment alternative. The concentrations of clozapine in the cerebrospinal fluid under therapeutical conditions range from 70–130 nM. Genetic variations in the primary sequence of 5-HT3 receptors may be crucial for the antipsychotic potency and/or the side effect profile of clozapine in that they may determine the antagonistic properties against this ligand-gated ion channel. Recently, a missense mutation P391R residing in the highly conserved cytoplasmatic region has been found, which probably only occurs in schizophrenic patients [35].

![Figure 6](image.png)

**Figure 6.** (A, B) 5-HT-induced currents through chimeric receptors. All chimeric receptors were dose-dependently activated by 5-HT. For each dose-response curve values were normalized to the responses induced by 300 μM 5-HT. Dose-response curves for amplitude (A) and charge (B).

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**Table 4.** Potency of serotonin for human, mouse and chimeric 5-HT3A receptors against peak amplitude.

| receptor | C123 | M123 | C23 | C12 | H123 | C123 | C123 |
|----------|------|------|-----|-----|------|------|------|
| C123     | 1.22±0.20 | 1.40±0.10 | 1.91±0.26 | 1.91±0.16 | 2.27±0.18 | 3.07±0.32 | 4.92±0.19 |
| M123     | 1.40±0.10 | 1.91±0.26 | 1.91±0.16 | 2.27±0.18 | 3.07±0.32 | 4.92±0.19 |
| C23      | 1.22±0.20 | 1.40±0.10 | 1.91±0.26 | 1.91±0.16 | 2.27±0.18 | 3.07±0.32 | 4.92±0.19 |
| C12      | 1.22±0.20 | 1.40±0.10 | 1.91±0.26 | 1.91±0.16 | 2.27±0.18 | 3.07±0.32 | 4.92±0.19 |
| H123     | 2.27±0.18 | 3.07±0.32 | 4.92±0.19 |

Significant differences (p<0.05, ANOVA) are indicated by black dots.

Comparisons of EC_{50} for peak amplitude.

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**Table 5.** Potency of serotonin for human, mouse and chimeric 5-HT3A receptors against charge.

| receptor | C123 | M123 | C23 | C123 | H123 | C123 | C123 |
|----------|------|------|-----|-----|------|------|------|
| C123     | 0.65±0.02 | 0.96±0.06 | 1.22±0.13 | 1.24±0.08 | 1.35±0.12 | 2.76±0.26 | 2.76±0.08 |
| M123     | 0.96±0.06 | 1.22±0.13 | 1.24±0.08 | 1.35±0.12 | 2.76±0.26 | 2.76±0.08 |
| C23      | 0.65±0.02 | 0.96±0.06 | 1.22±0.13 | 1.24±0.08 | 1.35±0.12 | 2.76±0.26 | 2.76±0.08 |
| C12      | 0.65±0.02 | 0.96±0.06 | 1.22±0.13 | 1.24±0.08 | 1.35±0.12 | 2.76±0.26 | 2.76±0.08 |
| H123     | 1.24±0.08 | 1.35±0.12 | 2.76±0.26 | 2.76±0.08 |

Significant differences (p<0.05, ANOVA) are indicated by black dots.

Comparisons of EC_{50} for charge (B).

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Figure 7. Antagonistic properties of clozapine against chimeric and mutant receptors. All 5-HT-induced currents through chimeric receptors and the P391R mutant were dose-dependently reduced by clozapine in a competitive manner, however, with different potencies. Representative traces for the control and the effects of different concentrations of clozapine on chimeric 5-HT₃ receptor-mediated currents. The application duration of 5-HT was 2 s.

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The functional characterization of these mutants revealed a significant increase in the EC50 for 5-HT of the P391R mutant [26]. Consequently, this mutation may thereby also affect clozapine pharmacology. In fact, the experiments with the P391R mutant revealed a considerable increase in the antagonistic potency of clozapine. Furthermore, the calculation of Kb for clozapine demonstrates that the intracellularly located point mutation affects the extracellular binding for clozapine via two mechanisms: either directly by increasing the binding affinity and/or indirectly by decreasing the EC50 for 5-HT.

In previous studies, two novel 5-HT3A polymorphisms, 178-T/C and 1596-A/G, have been reported [50]. These polymorphisms were not related to the therapeutic response to clozapine [50]. However, these polymorphisms are located outside the domain identified in our study. On the other hand, a polymorphism in the large intracellular domain region within the 5-HT3A receptor gene has recently been shown to affect the clinical response to risperidone treatment [51]. Although the genetic findings available so far suggest a putative role of the 5-HT3A receptor gene in the pathophysiology of schizophrenia and the response to antipsychotic treatment, no data are available on genetic variants within the extracellular domain of this receptor in schizophrenic patients. As such, based on our results further genetic studies should look more closely at the respective sequence responsible for clozapine affinity.

**Table 7. Potency of clozapine for human and murine 5-HT3A receptors and different 5-HT3A receptor chimeras against charge.**

| receptor | C123 | C123 | M123 | C123 | C123 | C123 | H123 |
|----------|------|------|------|------|------|------|------|
| C123     |      |      |      |      |      |      |      |
| C123     | 0.3  | 2.76±0.26 | 1.52±0.00 |
| C123     | 0.8  | 1.33±0.12 | 6.37±0.00 |
| M123     | 0.9  | 1.22±0.13 | 8.05±0.10 |
| C123     | 1.1  | 0.65±0.02 | 18.59±0.11 |
| P391R    | 1.3  | 2.73±0.06 | 5.92±0.1 |
| C123     | 5.8  | 0.96±0.06 | 65.85±0.01 |
| H123     | 9.2  | 1.24±0.08 | 83.32±0.02 |
| C123     | 13.4 | 2.76±0.08 | 62.17±0.01 |

Significant differences (p<0.05, ANOVA) are indicated by black dots. Comparisons of IC50 for peak charge.

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**Table 8. Calculation of the dissociation constant Kb of clozapine using the Cheng-Prusoff equation.**

| receptor | Kb (nM) | EC50 5-HT (µM) | IC50 clozapine (nM) |
|----------|---------|----------------|-------------------|
| C123     | 0.3     | 2.76±0.26      | 1.52±0.00         |
| C123     | 0.8     | 1.33±0.12      | 6.37±0.00         |
| M123     | 0.9     | 1.22±0.13      | 8.05±0.10         |
| C123     | 1.1     | 0.65±0.02      | 18.59±0.11        |
| P391R    | 1.3     | 2.73±0.06      | 5.92±0.1          |
| C123     | 5.8     | 0.96±0.06      | 65.85±0.01        |
| H123     | 9.2     | 1.24±0.08      | 83.32±0.02        |
| C123     | 13.4    | 2.76±0.08      | 62.17±0.01        |

The lowest Kb for clozapine were calculated for receptors containing the murine sequence 2 (0.3–1.1 nM). When 5-HT3 receptors contained the human sequence 2, clozapine affinity to the binding site was less potent and Kb ranged from 5.8–13.4 nM. These data demonstrate that the sequence between restriction site BstEII and SgrA1 (defined as sequence 2) of 5-HT3 receptors is important for the binding affinity of clozapine. The calculation of the dissociation constant of clozapine for the P391R mutant revealed a Kb of 1.3 nM. The EC50 for 5-HT (2.73±0.01 µM) has been taken from Thompson et al. (2006) [26].

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to the 5-HT<sub>3A</sub> receptor identified in the present study by detailed fine mapping strategies. The therapeutic relevance of the 5-HT<sub>3</sub> receptor in schizophrenia has recently been underlined by a placebo-controlled study with the 5-HT<sub>3</sub> receptor antagonist ondansetron as an add-on medication to a stable dose of risperidone [52], which showed a positive effect of ondansetron on negative symptoms and cognitive impairment.

Taken together, these studies suggest that the 5-HT<sub>3A</sub> receptor may contribute to the therapeutic efficacy of clozapine in schizophrenia and that the extracellular sequence close to the transmembrane domain TM1 within the 5-HT<sub>3A</sub> receptor identified in the present study may play a role for the unique pharmacological profile of clozapine.

References

1. Witschwendter CG, Fleischhacker WW (2005) Pathophysiology of schizophrenia and its impact on pharmacotherapy. Fortschr Neurol Psychiatr 73 Suppl 1:10–16.
2. Meisenzahl EM, Schmitt GJ, Schreuercker J, Moller HJ (2007) The role of dopamine for the pathophysiology of schizophrenia. Int Rev Psychiatry 19:337–345.
3. Brunello N, Masotto G, Stavola L, Markstein R, Riacagni G (1995) New insights into the biology of schizophrenia through the mechanism of action of clozapine. Neuropsychopharmacol 13:177–213.
4. Rammes G, Rupprecht R (2003) Modulation of ligand-gated ion channels by antipsychotics. Mol Neurobiol 35:160–174.
5. Mentes A, Moehl J, Takeda Y, Akazawa S (1995) Molecular cloning of human 5-hydroxytryptamine 3 receptor: heterogeneity in distribution and function among species. Mol Pharmacol 48:407–416.
6. Reynolds GP (1992) Developments in the drug treatment of schizophrenia. Trends Pharmacol Sci 13:116–121.
7. Abid-ArgEMA, Moore H (2003) Prefrontal DA transmission at D1 receptors and the pathology of schizophrenia. Neuroscientist 9:404–416.
8. Miyachi M (1996) Ventral tegmental area 5-HT<sub>3</sub> receptors: mesolimbic dopamine release and behavioral studies. Behav Brain Res 7:1–5.
9. Barnes NM, Sharp T (1999) A review of central 5-HT<sub>3</sub> receptors and their function. Neuropharmacology 38:1083–1132.
10. De Deurwaerdeer P, Stinus L, Spampinato U (1988) Opposite change of in vivo dopamine release in the rat nucleus accumbens and striatum that follows electrical stimulation of dorsal raphe nucleus: role of 5-HT<sub>3</sub> receptors. J Neurosci 18:6528–6538.
11. Blandina P, Goldfarb J, Green JP (1988) Activation of a 5-HT<sub>3</sub> receptor releases dopamine from rat striatalslice. Eur J Pharmacol 155:349–350.
12. Kriem B, Rostain JC, Abraini JH (1995) Involvement of 5-HT<sub>3</sub> receptor in the pressure-induced increase in striatal and accumbens dopamine release and the occurrence of behavioral disorders in free-moving rats. Neurosci Lett 197:37–40.
13. Liu W, Thielken RJ, Rodd MA, McBride VW (2006) Activation of serotonin-3 receptors increases dopamine release within the ventral tegmental area of Wistar and alcohol-prefering (P) rats. Alcohol 40:167–176.
14. Davies PA, Pinta M, Hamma MC, Peters JA, Lambert JJ, et al. (1999) The 5-HT<sub>3</sub>A subunit is a major determinant of serotonin-receptor function. Nature 397:359–363.
15. van Hoof JA, Vijnberg HP (2000) 5-HT<sub>3</sub>A receptors and neurotransmitter release in the CNS: a nerve ending story? Trends Neurosci 23:605–610.
16. Koyama S, Matsumoto N, Kubo C, Akaike N (2000) Presynaptic 5-HT<sub>3</sub>-receptor-mediated modulation of synaptic GABA release in the mechanically dissociated rat amygdala neurones. J Physiol-London 529(2):373–383.
17. Meneses A (1999) 5-HT<sub>3</sub>-system and cognition. Neuroscience & Biobehavioral Reviews 23:1111–1125.
18. Sugita S, Shen KZ, North RA (1992) 5-hydroxytryptamine is a fast excitatory neurotransmitter at 5-HT<sub>3</sub> receptors in rat amygdala. Neuron 8:199–203.
19. Roering B, Nelson DA, Katz LC (1997) Fast synaptic signaling by nicotinic acetylcholine and serotonin 5-HT<sub>3</sub> receptors in developing visual cortex. J Neurosci 17:8353–8362.
20. Hedges H, Sowinski P, Turner JJ, Fletcher AJ, Myers RM, Julius D (1999) Primary structure and functional expression of the 5-HT<sub>3</sub>A receptor, a serotonin-agonist ion channel. Science 254:432–437.
21. Peters JA, Hales TG, Lambert JJ (2005) Molecular determinants of single-channel conductance and ion selectivity in the Cyclo-loop family: insights from the 5-HT<sub>3</sub>-B receptor. Trends Pharmacol Sci 26:287–294.
22. Wetzel CH, Hermann B, Belc C, Pestel E, Rammes G, et al. (1998) Functional antagonism of gonadal steroids at the 5-hydroxytryptamine type 3 receptor. Mol Endocrin 12:1441–1451.
23. Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K<sub>i</sub>) and the concentration of inhibitor which causes 50% inhibition (IC<sub>50</sub>) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.
24. Eicke JL, Bertrand S, Galat JL, Devillers-Thiery A, Changeux JP, et al. (1993) Chaotropic nicotinic-serotoninergic receptor combines distinct ligand binding and channel specificities. Nature 366:479–483.
25. Hermann B, Wetzel CH, Pestel E, Zieglgansberger W, Eisensamer B, et al. (1996) Coexpression of the 5-HT<sub>3</sub>A serotonin receptor subunit alters the biophysics of the 5-HT<sub>3</sub>- receptor. Biophys J 74:1729–1733.
26. van Hoof JA, Vijnberg HP (1996) Selection of distinct conformational states of the 5-HT<sub>3</sub>A receptor by full and partial agonists. Br J Pharmacol 119:839–846.
27. Nylander B, Weiss B, Fischer C, Nothen MM, Popping P, et al. (2001) Serotonin receptor gene HTR3A variants in schizophrenic and bipolar affective patients. Pharmacogenetics 11:21–27.
28. Reeves DC, Lummis SC (2006) Detection of human and rodent 5-HT<sub>3</sub>-M receptor subunits by anti-peptide polyclonal antibodies. BMC Neurosci 7:28.
29. Hapfelmeier G, Tredt C, Haseneder R, Zieglgansberger W, Eisensamer B, et al. (2000) Cosynthesis of the 5-HT<sub>3</sub>A receptor subunit alters the biophysics of the 5-HT<sub>3</sub>- receptor. Biophys J 78:1547–1554.
30. van Hoof JA, Vijnberg HP (1996) Selection of distinct conformational states of the 5-HT<sub>3</sub>A receptor by full and partial agonists. Br J Pharmacol 119:839–846.
31. Nieder B, Weiss B, Fischer C, Nothen MM, Popping P, et al. (2001) Serotonin receptor gene HTR3A variants in schizophrenic and bipolar affective patients. Pharmacogenetics 11:21–27.
32. Hu XQ, Sun H, Peoples RW, Hong R, Zhang L (2006) An interaction involving 5-HT<sub>3</sub>B receptor subunit. Mol Pharmaco 10:1054–1062.
33. Thompson AJ, Lummis SC (2007) The 5-HT<sub>3</sub>- receptor as a therapeutic target. Expert Opin Ther Targets 11:527–540.
34. Kelley SP, Dunlop JJ, Kirkness EF, Lambert JJ, Peters JA (2003) A cytoplasmic and cell surface expression of homomeric and heteromeric 5-HT<sub>3</sub> receptors. Biophys J 84:1729–1733.
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Author Contributions

Conceived and designed the experiments: GR BE RR. Performed the experiments: CH BE ST CN. Analyzed the data: CH ST. Contributed reagents/materials/analysis tools: WZ RR. Wrote the paper: GR. Supervision: GR.
46. Yang J, Mathie A, Hille B (1992) 5-HT3 receptor channels in dissociated rat superior cervical ganglion neurons. J Physiol 448: 237–256.

47. Boddeke HW, Meigel I, Boeijinga P, Arbuckle J, Docherty RJ (1996) Modulation by calcineurin of 5-HT3 receptor function in NG108-15 neuroblastoma x glioma cells. Br J Pharmacol 118: 1036-1040.

48. van Hooft JA, Spier AD, Yakel JL, Lummis SC, Vijverberg HP (1998) Promiscuous coassembly of serotonin 5-HT3 and nicotinic alpha 4 receptor subunits into Ca2+-permeable ion channels. Proc Natl Acad Sci U S A 95: 11456–11461.

49. Iqbal MM, Rahman A, Husain Z, Mahmud SZ, Ryan WG, et al. (2003) Clozapine: a clinical review of adverse effects and management. Ann Clin Psychiatry 15: 33–48.

50. Gutierrez B, Arranz MJ, Huero-Díaz P, Dempster D, Matthiasson P, et al. (2002) Novel mutations in 5-HT3A and 5-HT3B receptor genes not associated with clozapine response. Schizophr Res 58: 95–97.

51. Gu B, Wang L, Zhang AP, Ma G, Zhao XZ, et al. (2008) Association between a polymorphism of the HTR3A gene and therapeutic response to risperidine treatment in drug-naive Chinese schizophrenia patients. Pharmacogenet Genomics 18: 721–727.

52. Akhondzadeh S, Mohammadi N, Noroozian M, Karamghadiri N, Ghoreishi A, et al. (2008) Added ondansetron for stable schizophrenia: A double blind, placebo controlled trial. Schizophr Res 107: 206–212.