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Intrinsic activity and comparative molecular dynamics of buspirone analogues at the 5-HT_{1A} receptors

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Abstract

In CNS, the 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptors exist in two different populations with different behavioural and physiological effects: (1) somatodendritic autoreceptors located pre-synaptically of 5-HT containing neurons and (2) receptors located post-synaptic to 5-HT containing neurons. Clinical studies have shown that 5-HT_{1A} partial agonists have anxiolytic properties, while antagonists of pre-synaptical autoreceptors shorten the onset time of selective serotonin reuptake inhibitors (SSRIs). In the present study, the pre- and post-synaptic activity of structural analogues of buspirone was evaluated in animal models. A three dimensional model of the 5-HT_{1A} receptor was used to study their interaction modes and helical displacements upon receptor binding. The predicted receptor–ligand interactions indicated similarities in the receptor binding modes for all buspirone analogues, and no clear relationship between receptor contact residues and activity at pre- and post-synaptic receptors. Comparative molecular dynamics (MD) simulations for 650 ps indicated that pre-synaptic antagonistic behaviour is connected to large displacements of transmembrane helix (TMH) 7 upon binding, while pre-synaptic agonistic behaviour is connected to large displacements of TMH2 and small displacements of TMH7. Post-synaptic partial agonist behaviour is connected to large displacements of TMH4 and TMH5 upon binding, while post-synaptic antagonists only slightly displace these helices.

Keywords: 5-HT_{1A} receptor; Buspirone analogues; Pre- and post-synaptic activity; Molecular mechanisms; Helical displacements

1. Introduction

G-protein coupled receptors (GPCRs) transduce signals across the cell membrane by receiving a ligand stimulus at the external side and activate a heterotrimeric G-protein bound in the interior of the cell. Due to technical difficulties in the handling and purification of integral membrane proteins, the structural information about GPCRs is progressing very slowly, and the X-ray structure of bovine rhodopsin is the only known structures at an atomic resolution [1,2].

Structural, functional and pharmacological characteristics have divided the 5-hydroxytryptamine (5-HT) receptors into seven major families (5-$HT_{1–7}$), with one or more family subtypes [3,4]. Except for 5-HT_3 receptors, the 5-HT receptors are GPCRs. One of the best characterised of the 5-HT receptors is the 5-HT_{1A} receptor subtype, which belongs to the G_i/G_o coupled receptors. The 5-HT_{1A} receptors inhibits adenylyl cyclase, increase the potassium conductances by regulating inward rectifying potassium channels, and decrease the opening of voltage gated calcium channels [5]. The 5-HT_{1A} receptors have been found to exist in two functionally distinct populations in the central nervous system with different pharmacology [3–6]: (1) somatodendritic autoreceptors, located pre-synaptically on cell bodies and dendrites of raphe nuclei regions and (2) post-synaptic to 5-HT containing neurons in forbrain regions.

Abbreviations: FBP, flat body posture; FT, forepaw treading; GPCRs, G-protein coupled receptors; LLR, lower lip retraction; MD, molecular dynamics; ps, picosecond; RESP, restrained electrostatic potentials; rms, root mean square; SSRI, selective serotonin reuptake inhibitors; TMH, transmembrane α-helix; 5-HT, 5-hydroxytryptamine

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The discovery of buspirone as a relatively potent but not selective partial agonist at 5-HT_{1A} receptors with anxiolytic properties [7] focused the attention onto the 5-HT_{1A} receptor as a potential target for anxiolytic drugs. However, the underlying mechanism of action for the anxiolytic effects of 5-HT_{1A} ligands is not fully understood. It is evident that also the 5-HT_{2A} receptor [8] and the 5-HT_{2C} receptor [9] are involved in the regulation of anxiety, and it has been suggested that ligands combining agonist properties at 5-HT_{1A} receptors with antagonist properties at 5-HT_{2A} and/or 5-HT_{2C} receptors may have greater efficacy than buspirone in producing anxiolytic effects [10]. SSRIs are currently the first line therapy for depression. Their antidepressant activity is believed to be exerted by increased 5-HT transmission. A major problem associated with SSRIs is their delayed onset of action, connected to increased activity at pre-synaptic 5-HT_{1A} autoreceptors. Upon chronic administration of SSRIs a desensitisation of the pre-synaptic 5-HT_{1A} autoreceptors occurs, and after 2–6 weeks the antidepressant effect is seen. Blockade of the 5-HT_{1A} autoreceptors should result in an advanced therapeutic action of the SSRIs, and administration of pindolol, a mixed β-adrenoreceptor/5-HT_{1A} receptor antagonists, has been shown to potentiate the antidepressant effects of SSRIs [6,11].

Activation of the 5-HT_{1A} receptors leads to a number of behavioural and physiological effects. Stimulation of the receptors cause, e.g. increased food intake in rats, LLR in rats, sex dependent modulation of sexual behaviour in rats, and hypothermia in mice [12]. The hypothermia produced by 5-HT_{1A} agonists in mice has been connected to the activation of pre-synaptic 5-HT_{1A} autoreceptors resulting in decreased 5-HT transmission [13–15], while the activation of post-synaptic 5-HT_{1A} receptors has been connected to induction of LLR or behavioural syndrome (FBP, FT) in reserpinised rats [4,16].

In previous modelling [17–19] and experimental studies [16,20] we have studied molecular determinants discriminating between the 5-HT_{1A} and the 5-HT_{2A} serotonin receptors. In the present study mice hyopthermia and LLR or a behavioural syndrome (FBP and FT) in reserpinised rats were used to evaluate the activity of buspirone analogues at pre- and post-synaptic 5-HT_{1A} receptors. Protein modelling was used for comparative MD simulations of the receptor complexed with different buspirone analogues having different activity at pre- and post-synaptic 5-HT_{1A} receptor.

2. Methods

2.1. In vivo experiments

The activity of the buspirone analogues was tested on male Wistar rats (250–280 g) or on male Albino-Swiss mice (26–29 g). The animals were kept at a room temperature (21 ± 1 °C) on a natural day–night cycle, and housed in plastic boxes (55 cm × 35 cm × 20 cm) in groups of 8 (rats) or 30 (mice) animals. The animals had free access to food (Bacutil pellets) and tap water throughout the experiments. Each experimental and control group consisted of six to eight animals. The compounds were administered as suspensions in a 1% aqueous solution of Tween 80, while 8-hydroxy-(di-*n*-propylamino)tetralin (8-OH-DPAT), reserpine and (*S*)-*N*-*tert*-butyl-3-[4-(2-methoxyphenyl)piperazin-1-yl]-2-phenylpropanamide ((*S*)-WAY 100135) were administrated as solutions in physiological saline. The buspirone analogues were administered intraperitoneally (i.p.), while 8-OH-DPAT, reserpine and (*S*)-WAY 100135 were administrated subcutaneously (s.c.) in a volume of 4 (rat) and 10 (mouse) ml/kg. The control groups received the same amounts of solvent. 8-OH-DPAT·HBr was purchased from RBI, (*S*)-WAY 100135 was synthesised by Dr. J. Boksa, Institute of Pharmacology, Polish Acadamy of Sciences, and reserpine in ampoules was purchased from Ciba-Geigy. Statistical significance of the results was evaluated by one-way analysis of variance followed by Newman–Keuels post-hoc test.

2.1.1. LLR in rats

The LLR studies were conducted according to the method described by Berendsen et al. [21]. The animals were individually placed in cages, and scored at 15, 30 and 45 min (observation time: 45 s) after administration of the buspirone analogues or 8-OH-DPAT. The scoring was as follows: 0, lower incisors invisible; 0.5, partly visible; 1, clearly visible; giving a maximum score of 3 for each rat. In another experiment, the effect of the buspirone analogues or (*S*)-WAY 100135 on 8-OH-DPAT (1 mg kg)-induced LLR was tested. The buspirone analogues were administered 45 min before the 8-OH-DPAT injection, and the animals were observed (scored) at 15, 30 and 45 min after the 8-OH-DPAT injections.

2.1.2. Behavioural syndrome in reserpinised rats

Reserpine (1 mg/kg) was administered 18 h before the tests. The animals were individually placed in cages 5 min before injection of the buspirone analogues or 8-OH-DPAT. The observation sessions lasted 45 s and started 3 min after drug administration, and were repeated every 3 min over a period of 15 min. FBP and reciprocal FT were scored using a ranked intensity scale: 0, absent; 1, equivocal; 2, present; 3, intense; according to Tricklebank et al. [22], giving a maximum score of 15 for each symptom over the time period of 15 min.

The effect of the buspirone analogues or (*S*)-WAY 100135 on the 8-OH-DPAT(5 mg/kg)-induced behavioural syndrome was also tested. The buspirone analogues or (*S*)-WAY 100135 were administered 45 min before 8-OH-DPAT, and animals were scored 3, 6, 9, 12, and 15 min after the injection of 8-OH-DPAT.

2.1.3. Body temperature in mice

The rectal body temperature of mice (2.5 cm deep, Ellab Thermometer, Denmark) was recorded at 30, 60, 90 and 120 min after the injection of buspirone analogues or 8-OH-DPAT. The effect of the buspirone analogues (**1**), (**4**) or (*S*)-WAY 100135 on 8-OH-DPAT (5 mg kg)-induced hypothermia was also tested. The compounds were administered 45 min before 8-OH-DPAT. The observation sessions started 15 min after the injection of 8-OH-DPAT and were repeated at 30, 45 and 60 min. In another experiment, the effect of (*S*)-WAY 100135 (10 mg/kg) on the hypothermia induced by compounds (**2**) and (**3**) was investigated. (*S*)-WAY 100135 was administered 30 min before the tested compounds. The rectal body temperature was measured 30 min after their injection. The results were expressed as a change in the body temperature (ΔT) with respect to the basal body temperature measured at the beginning of the experiments (the mean value out of two measurements).

2.2. Molecular modelling

Molecular mechanics energy mimisations and MD simulations were performed with the Amber 5.0 programs using the Cornell force field [23]. The Gaussian94 program was used for ab initio calculations of ligand molecules. Interactive molecular graphics was done with the Midas-Plus [24] and ICM [25] software. Energy minimisations of the receptor model and of receptor–ligand complexes were performed by 500 steps of steepest descent minimisation followed by 2000 steps of conjugate gradient minimisation until convergence. The convergence criteria was a 0.02 kcal/mol Å rms difference for the norm of the energy gradient between successive steps. During energy minimisations, the cut off radius for non-bonded interactions was 15 Å.

The local microscopic dielectricity in soluble proteins depends on the atom position [26], and for a GPCR the dielectricity of regions exposed to water will also differ from that of the regions buried inside the membrane. A distance-dependent dielectric function with a dielectric constant of 4 ($\varepsilon = 4r$, r is interactomic distance) was used in the calculations Explicit water molecules were not included. In order to preserve the helical conformation of the heptahelical bundle during MD, extra forces (corresponding to 5 kcal/mol) were applied between the backbone oxygen atom of residue n and the backbone nitrogen atom of residues $n + 4$, excluding prolines. Extra intrahelical forces have previously been found [27,28] to be important for reproducing the experimentally observed rigid body helical movements [29,30]. The helical constrains were applied during all calculations. MD was performed with a cut off radius for non-bonded interactions at 12 Å, and a secondary cut off radius of 15 Å. All bonds involving hydrogen atoms were constrained during MD using the SHAKE option.

2.2.1. Construction of the receptor model

The amino acid sequence of the human 5-HT_{1A} receptor [31] was obtained from the Swiss-Prot database (http://www.expasy.ch/sprot/). An initial model of the heptahelical bundle was constructed from the X-ray structure of bovine rhodopsin [1] (PDB-acquisition code: 1F88) using the traditional homology modelling approach. The backbone atoms of the TMHs of bovine rhodopsin were kept, while the side chains were replaced with the corresponding amino acids of the human 5-HT_{1A} receptor. The rhodopsin structure and information about familiar conservation among the GPCRs (http://tinyGRAP.uit.no/fam1asel.html) were used to define the start and end positions of the seven TMHs. The start and end positions of the TMHs in the model were: TMH1, Ser^{34}–Leu^{63}; TMH2, Val^{70}–Leu^{99}; TMH3, Gln^{106}–Ile^{138}; TMH4, Arg^{151}–Leu^{173}; TMH5, Tyr^{195}–Arg^{217}; TMH6, Glu^{340}–Pro^{369}; TMH7, Leu^{380}–Tyr^{400}.

Initial models of the loops and terminals were copied from our previous 5-HT_{1A} receptor model [18], and connected to the heptahelical bundle. The entire receptor model was refined in several subsequent steps:

1. 20 ps of MD of loops and terminals at 300 K, while the TMHs were kept at fixed positions.
2. 20 ps MD at 300 K of all side chains in the model.
3. Energy minimisation of the entire receptor model.

Site-directed mutagenesis studies of GPCRs have shown that a pair of cysteines, being highly conserved among GPCRs forms a disulphide bond, and a disulphide bond between Cys^{109} in TMH3 and Cys^{187} in EC2 was present during all calculations.

2.2.2. Modelling of ligands

The initials structures of the buspirone analogues were constructed from the crystal structure of buspirone [20]. The nitrogen atom attached to the butyl fragment was protonated for all molecules. An initial geometry optimisation was performed, and atom point charges were calculated with the Gaussian94 program using an RHF/6-31G* basis set and RESP fitting [32]. The major strength of the RESP charges is that they optimally reproduce the intermolecular interaction properties of molecules with a simple two-body additive potential. The final RESP charges were obtained after a two-stage fit, where the second stage was performed to fit charges to methyl group hydrogens which are not equivalent by molecular symmetry. The ligands were energy optimised by 200 cycles of steepest descent minimisation and 800 cycles of conjugate gradient minimisation with the generated RESP charges.

2.2.3. Docking of ligands into the receptor model

In a previous study we were able to reproduce the ligand binding rank order of buspirone analogues at the 5-HT_{1A} receptor by calculating the receptor–ligand interaction

energies [17]. A very similar binding mode was later confirmed as the most realistic for other structure analogues of buspirone [18,19]. The present buspirone analogues were therefore docked into the receptor in a similar position, using a manual docking procedure. The imide moieties were in the direction of amino acids in TMH2 and TMH7 with a hydrogen bonding distance between an imide carbonyl oxygen and Ser^{393} in TMH7, while the aromatic quinolinyl or pyrimidynyl group interacted with amino acids in TMH5, TMH6 and TMH7. To obtain optimum matching between each ligand and the receptor model, rotation of some ligand torsional angles was necessary. The receptor–ligand complexes were energy minimised and used as an initial structures for MD. The protocol for the simulation was:

1. 0–30 ps heating of the complexes. The temperature was gradually increased from 0 to 300 K (an increase of 50 K each 5 ps MD);
2. 30–150 ps, equilibration at 300 K;
3. 150–650 ps of MD at 300 K with sampling of coordinates every 5 ps;
4. calculation of an average structure from the MD sampling period, using the Carnal module of the Amber package;
5. energy minimisation of the average complex.

A similar approach was also used for a MD simulation of the receptor model in absence of a ligand at the binding site. The Carnal module of the Amber program was used to calculate the rms differences of receptor domains between the average receptor structures after MD.

3. Results

3.1. Intrinsic activity

In order to determine post-synaptic 5-HT_{1A} receptor agonistic effects of the tested compounds ((**1**), (**2**), (**3**), and (**4**)), their ability to induce LLR in rats and a behavioural syndrome (FBP and FT) in reserpinised rats were tested (Tables 1 and 2). The 8-OH-DPAT-induced LLR and behavioural syndrome in rats depend on the stimulation of post-synaptic 5-HT_{1A} receptors [22,33]. Further, these symptoms also seem to be sensitive to 5-HT_{1A} receptor antagonists [22,34–37]. Therefore, the ability of the tested compounds to inhibit 8-OH-DPAT-induced LLR, FBP and FT was considered as antagonistic activity at post-synaptic 5-HT_{1A} receptors (Tables 1 and 2). 8-OH-DPAT-induced hypothermia in mice is anticipated to be connected to the activation of pre-synaptic 5-HT_{1A} receptors [15,36], and found to be abolished by the known 5-HT_{1A} antagonists [15,34,36,37]. The hypothermia in mice produced by some of the buspirone analogues, and reduced by (*S*)-WAY 100135, a known 5-HT_{1A} receptor antagonist was regarded as pre-synaptic 5-HT_{1A} agonistic activity (Tables 3 and 4). Similarly, the ability of the compounds to reverse 8-OH-DPAT-induced hypothermia was connected to pre-synaptic antagonist activity (Table 5).

Table 1
The induction of LLR (A) by compounds (**1**), (**2**), (**3**), (**4**) and their influence on the 8-OH-DPAT (1 mg/kg)-induced LLR (B) in rats

Compound	Dose (mg/kg)	LLR score (mean ± S.E.M.)	
		A[a]	B[b]
8-OH-DPAT	0.125	0.1 ± 0.1	
	0.25	0.8 ± 0.2*	
	0.5	2.0 ± 0.4*	
	1	2.9 ± 0.1*	
(*S*)-WAY 100135	2.5	0	2.4 ± 0.4
	5	0	1.8 ± 0.2#
	10	0	0.6 ± 0.2#
(**1**)	5	0	1.9 ± 0.3#
	10	0	1.1 ± 0.3#
	20	0	0.7 ± 0.3#
(**2**)	5	0.0 ± 0.0	1.6 ± 0.3
	10	0.3 ± 0.1	1.3 ± 0.3#
	20	0.4 ± 0.2	0.5 ± 0.2#
(**3**)	10	0.3 ± 0.2	1.7 ± 0.3##
	20	0.2 ± 0.2	1.3 ± 0.4##
(**4**)	5	1.3 ± 0.3*	2.3 ± 0.3
	10	1.8 ± 0.4*	2.5 ± 0.1
	20	1.9 ± 0.2*	1.4 ± 0.2#

[a] The respective score for vehicle treated groups was 0.1 ± 0.1.
[b] The respective score for 8-OH-DPAT treated groups was 2.8 ± 0.
* $P < 0.01$ vs. vehicle.
# $P < 0.01$ vs. 8-OH-DPAT.
$P < 0.05$ vs. 8-OH-DPAT.

In behavioural experiments, compounds (**2**), (**3**), and (**4**) behaved like 5-HT_{1A} receptors partial agonists, although their functional profile was not identical. Compound (**4**) alone at doses of 5–20 mg/kg evoked LLR in rats. Further, (**4**) induced symptoms of behavioural syndrome in reserpine pretreated rats; doses of 20 mg/kg it induced FBP, but not at lower does, while doses of 10–20 mg/kg induced FT. The compound also inhibited 8-OH-DPAT-induced LLR and FT. In mice, (**4**) did not change the body temperature and did not abolish hypothermia induced by 8-OH-DPAT, so a pre-synaptic 5-HT_{1A} activity in this model is negligible. Compound (**4**) was therefore considered as a 5-HT_{1A} post-synaptic partial agonist.

Compound (**3**) did not induce LLR in rats, but induced weak FBP in reserpinised rats. When these effects were induced by 8-OH-DPAT, compound (**3**) was able to inhibited both LLR and FBP, but not FT. Compound (**3**) also reduced mouse body temperature. This effect was abolished by (*S*)-WAY 100135. Compound (**3**) was therefore considered as a pre-synaptic agonist with a weak post-synaptic agonistic/antagonistic activity.

Compound (**2**) did not give LLR but induced weak FT in reserpinised rats. On the other hand (**2**) inhibited 8-OH-DPAT induced LLR and the serotonergic syndrome.

Table 2
Induction of serotonin syndrome by compounds (**2**), (**3**) and (**4**) (A) and their effect on the 8-OH-DPAT (5 mg/kg)-induced syndrome (B) in reserpinised rats

Compound	Dose (mg/kg)	Mean ± S.E.M.			
		A		B	
		FBP	FT	FBP	FT
Vehicle		0	0		
8-OH-DPAT	2.5	8.1 ± 1.4*	7.2 ± 1.6*		
	5	14.5 ± 0.2*	13.8 ± 1.6*		
Vehicle		0	0	15.0 ± 0	13.2 ± 0.9
(*S*)-WAY 100135	2.5	0	0	12.5 ± 0.7	6.8 ± 0.7
	5	0	0	9.0 ± 1.0#	5.5 ± 0.6#
	10	0	0	9.7 ± 1.0#	1.0 ± 0.3#
Vehicle		0	0	14.2 ± 0.5	12.7 ± 0.6
(**2**)	5	0	0	13.1 ± 0.4	12.0 ± 0.3
	10	10	1.7 ± 0.5	9.9 ± 1.1#	10.0 ± 0.4#
	20	1.8 ± 0.9	4.3 ± 0.8*	7.8 ± 0.5#	8.7 ± 0.4#
Vehicle		0	0	13.3 ± 0.5	1103.0 ± 0.9
(**3**)	10	1.5 ± 0.6	0	11.7 ± 0.9	9.2 ± 0.9
	20	5.0 ± 1.3*	0.8 ± 0.3	9.0 ± 1.6#	9.3 ± 1.0
Vehicle		0	0	14.2 ± 0.4	13.6 ± 0.6
(**4**)	5	0	0	12.1 ± 0.6	12.2 ± 0.9
	10	5.7 ± 1.2*	4.7 ± 0.6*	10.0 ± 0.9##	9.8 ± 1.1##
	20	11.8 ± 0.6*	7.0 ± 0.7*	7.0 ± 0.7#	5.8 ± 0.4#

* $P < 0.01$ vs. vehicle.
\# $P < 0.01$ vs. 8-OH-DPAT.
\## $P < 0.05$ vs. 8-OH-DPAT.

At high dose (20 mg/kg) (**2**) induced a weak short lasting decrease of mice body temperature (reduced by (*S*)-WAY 100135) exhibiting properties of a weak pre-synaptic agonist. Under the present experimental conditions, (**2**) exhibited properties of a 5-HT_{1A} post-synaptic antagonist (with very weak agonistic activity) and a weak pre-synaptic agonist.[1]

Compound (**1**) did not induce LLR in rats but inhibited these symptoms induced by 8-OH-DPAT. Unfortunately (**1**) was not tested in reserpinised rats. It did not change mouse body temperature but completely abolished 8-OH-DPAT induced hypothermia. Therefore (**1**) was considered as a potential pre- and post-synaptic 5-HT_{1A} receptors antagonist.

Similar effects as in the present study were previously used to classify (**5**) as partial agonist at both pre- and post-synaptic receptors and (**6**) as pre-synaptic antagonist and post-synaptic partial agonist [16].

3.2. Molecular modelling

3.2.1. Receptor structure

The heptahelical bundle was constructed using the X-ray structure of rhodopsin [1] as a template. The helical packing and spatial position of highly conserved amino acids are therefore very similar to the X-ray structure of rhodopsin. However, some structural differences were seen after refinements of the receptor model. In TMH1, rhodopsin has a proline and a phenylalanine corresponding to Leu^{52} and Ala^{55} in the 5-HT_{1A} receptor. These differences results in a slightly more outward bending TMH1 in rhodopsin than in the 5-HT_{1A} model. Rhodopsin has a segment of three threonine residues at the EC-end of TMH2 corresponding to Pro^{91}–Met^{92}–Ala^{93} in the 5-HT_{1A} receptor. These threonine residues interact with a glutamic acid in TMH3, resulting in a small kink in TMH2. A kink is also seen in this region of the 5-HT_{1A} model, but mainly due to Pro^{91}. There are also some differences in the region of the highly conserved aspartic acid in TMH2 between rhodopsin and the 5-HT_{1A} receptor. In rhodopsin this aspartic acid forms a hydrogen bond with a highly conserved asparagines in TMH2 and is connected to a serine and an asparagine residues in TMH7 via a water molecule. Both the serine and the asparagine in TMH7 are highly conserved in family A of GPCRs. A corresponding hydrogen bonding network is also seen in the 5-HT_{1A} receptor model, where Asp^{82} (TMH2) forms hydrogen bonds with the corresponding asparagine (Asn^{54}) in TMH1, and serine (Ser^{393}) in TMH7. In the 5-HT_{1A} model Asn^{392} (TMH7) also forms a hydrogen bond with Asp^{82} and are involved in constraining TMH2 and TMH7 relative to each other. However, previous [18,19] and present ligand docking and MD simulations indicated that the hydrogen bonding interactions of Asn^{392} and Ser^{393} with Asp^{82} are broken

[1] It has been also shown that compound (**2**) at 10 mg/kg exhibited some anxiolytic-like activity (was twice less active than buspirone) at Vogel's conflict test (W. Kostowski at al., unpublished).

Table 3
The effect of compounds (**1**), (**2**), (**3**) and (**4**) on the body temperature in mice

Compound	Dose (mg/kg)	$\Delta T \pm$ S.E.M. (°C)[a]			
		30 min	60 min	90 min	120 min
Vehicle		−0.2 ± 0.1	−0.2 ± 0.1	−0.3 ± 0.1	−0.2 ± 0.1
(*S*)-WAY 100135	5	−0.1 ± 0.2	−0.3 ± 0.2	−0.2 ± 0.1	−0.1 ± 0.1
	10	−0.3 ± 0.2	−0.2 ± 0.1	−0.1 ± 0.1	−0.2 ± 0.1
Vehicle		−0.3 ± 0.1	−0.3 ± 0.1	−0.3 ± 0.1	−0.1 ± 0.1
(**1**)	2.5	−0.2 ± 0.2	−0.3 ± 0.2	−0.3 ± 0.2	−0.2 ± 0.2
	5	−0.2 ± 0.1	−0.4 ± 0.1	−0.4 ± 0.1	−0.4 ± 0.1
	10	−0.7 ± 0.1	−0.3 ± 0.2	−0.4 ± 0.1	−0.3 ± 0.1
	20	−0.3 ± 0.1	−0.3 ± 0.1	−0.2 ± 0.2	−0.1 ± 0.2
Vehicle		0.3 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
(**2**)	5	0.2 ± 0.1	−0.2 ± 0.2	0.4 ± 0.2	0.0 ± 0.2
	10	0.7 ± 0.2	0.3 ± 0.1	0.0 ± 0.2	0.0 ± 0.3
	20	−1.1 ± 0.2*	−0.3 ± 0.2	−0.1 ± 0.2	−0.3 ± 0.1
Vehicle		0.1 ± 0.1	−0.3 ± 0.2	−0.4 ± 0.1	−0.4 ± 0.1
(**3**)	10	−0.9 ± 0.2*	−0.3 ± 0.2	−0.1 ± 0.2	−0.1 ± 0.2
	20	−1.9 ± 0.3*	−1.1 ± 0.2**	−1.1 ± 0.1**	−0.9 ± 0.1**
Vehicle		−0.3 ± 0.1	−0.2 ± 0.1	−0.2 ± 0.2	−0.2 ± 0.2
(**4**)	10	−0.1 ± 0.1	−0.4 ± 0.1	−0.2 ± 0.1	−0.1 ± 0.2
	20	−0.2 ± 0.2	−0.3 ± 0.2	−0.1 ± 0.1	−0.2 ± 0.1

[a] Absolute mean initial body temperatures were within a range of 36.4 ± 0.3 °C.
* $P < 0.01$ vs. respective vehicle group.
** $P < 0.05$ vs. respective vehicle group.

Table 4
Influence of (*S*)-WAY 100135 (10 mg/kg) on the hypothermia induced by compounds (**2**) and (**3**)

Compound (dose, mg/kg)	$\Delta T \pm$ S.E.M. (°C)[a]
Vehicle + vehicle	0.1 ± 0.1
Vehicle + (**2**) (20)	−1.7 ± 0.1*
(*S*)-WAY 100135 + (**2**) (20)	−0.8 ± 0.2#
Vehicle + vehicle	0.1 ± 0.1
Vehicle + (**3**) (20)	−1.0 ± 0.2*
(*S*)-WAY 100135 + (**3**) (20)	−0.3 ± 0.1#

[a] Absolute mean initial body temperatures were within a range of 36.5 ± 0.4 °C.
* $P < 0.01$ vs. vehicle + vehicle.
# $P < 0.05$ vs. vehicle + compound tested.

upon binding of buspirone analogues. Strong ionic interaction between Arg^{134} in TMH3 and Glu^{340} in TMH6 also brings the cytoplasmic ends of TMH3 and 6 in close proximity.

3.2.2. Ligand binding sites

The average receptor–ligand complexes after MD (Fig. 1) indicated that the binding interactions of the buspirone analogues with the receptor are very similar. The binding modes after MD (Fig. 2) were also very similar to the positions before MD. The most important ligand anchoring residues in the receptor seemed to be Asp^{116} in TMH3 that interacted with the protonated nitrogen atom of

Table 5
The influence of compounds (**1**) and (**4**) on the 8-OH-DPAT (5 mg/kg)-induced hypothermia in mice

Compound (dose, mg/kg)	$\Delta T \pm$ S.E.M. (°C)[a]			
	15 min	30 min	45 min	60 min
Vehicle + vehicle	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
Vehicle + 8-OH-DPAT	−1.3 ± 0.3*	−1.6 ± 0.2*	−1.8 ± 0.4*	−1.4 ± 0.3*
(*S*)-WAY 100135 (**5**) + 8-OH-DPAT	−0.2 ± 0.2#	−0.6 ± 0.5	−0.7 ± 0.4	−0.9 ± 0.3
(*S*)-WAY 100135 (10) + 8-OH-DPAT	0.2 ± 0.2#	0.0 ± 0.2#	0.3 ± 0.2#	0.1 ± 0.2#
Vehicle + vehicle	0.3 ± 0.1	−0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
Vehicle + 8-OH-DPAT	−1.1 ± 0.2*	−1.4 ± 0.3*	−1.4 ± 0.3*	−0.9 ± 0.3*
(**1**) (10) + 8-OH-DPAT	−0.2 ± 0.3**	−0.0 ± 0.4**	−0.2 ± 0.3*	0.2 ± 0.3*
Vehicle + vehicle	0.2 ± 0.1	−0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Vehicle + 8-OH-DPAT	−1.2 ± 0.2*	−1.2 ± 0.3*	−0.9 ± 0.4**	−0.8 ± 0.3
(**4**) (20) + 8-OH-DPAT	−0.7 ± 0.3**	−0.8 ± 0.4**	−0.8 ± 0.4**	−0.4 ± 0.4

[a] Absolute mean initial body temperatures were within a range of 36.6 ± 0.4 °C.
* $P < 0.01$ vs. vehicle + vehicle.
** $P < 0.05$ vs. vehicle + vehicle.
# $P < 0.01$ vs. vehicle + 8-OH-DPAT.

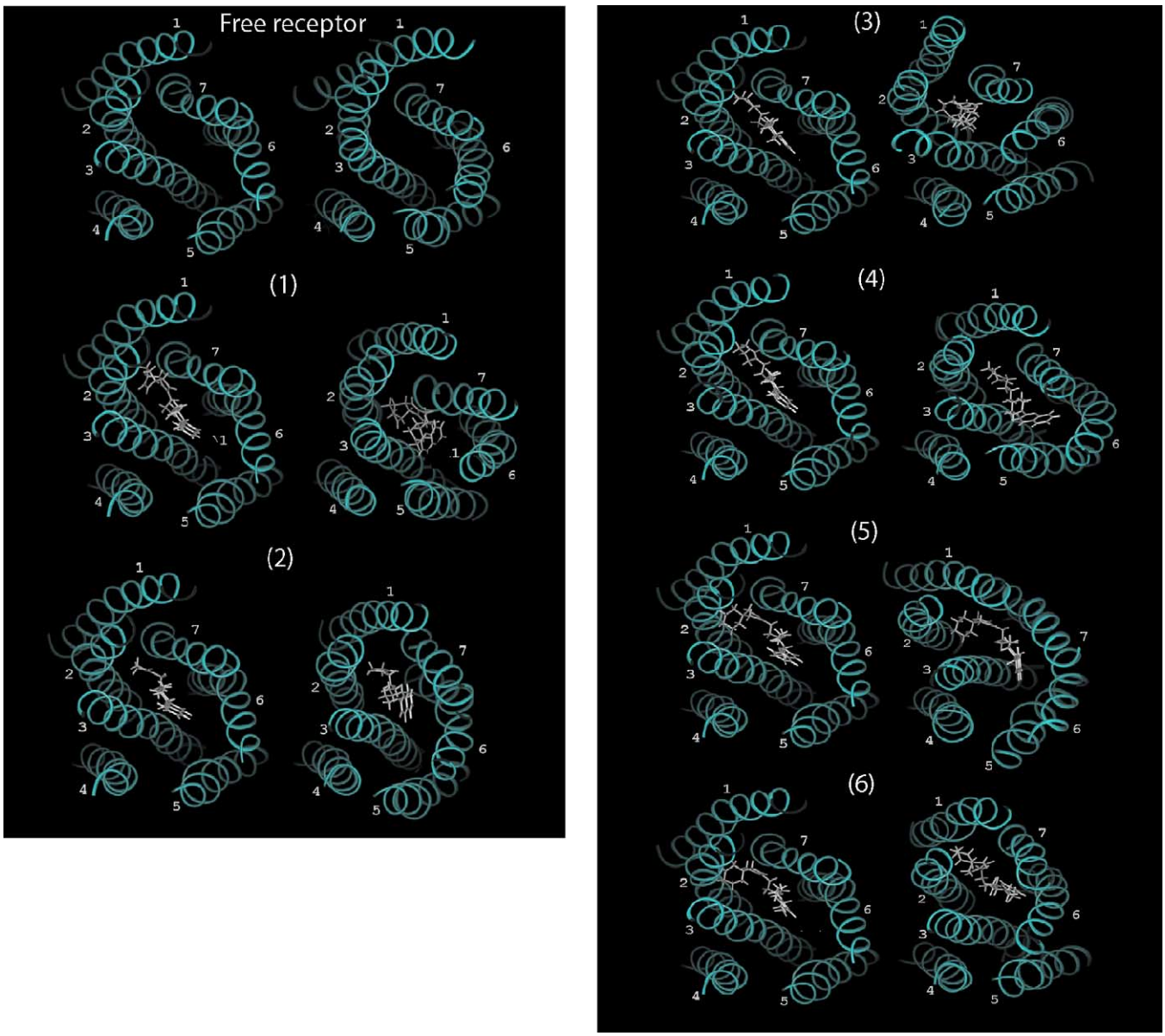


Fig. 1. The recepotor–ligand complexes after MD simulation, viewed from the extracellular side. The backbone Cα-carbon atoms of the TMHs and the ligands are shown in the figure.

the piperazine ring, and Ser^{393} in TMH7 that formed a hydrogen bond with a carbonyl group in the aromatic imide moiety (Table 7). TMH3 and TMH7 seemed to be the most important helixes for binding the buspirone analogues. Only (**5**) (Ile^{47}) and (**6**) (Leu^{46}) had van der Waals (vdw) contacts with TMH1. Both these ligands have a huge aromatic moiety that formed vdw contacts with TMH1 (Table 7). Table 7 indicates that (**2**) interacts with more amino acids in TMH2 than did the other ligands. Compounds (**1**), (**4**) and (**6**) had vdw contacts with Phe^{112} in TMH3 while the other ligands had not. Experimental studies indicated that (**1**) and (**6**) were antagonists at pre-synaptic 5-HT$_{1A}$ receptors, while (**4**) was inactive (Table 6). Compound (**2**), an agonist at pre-synaptic receptors, seemed to interact directly with fewer residues in TMH3 than did the other buspirone analogues, while TMH4 was not directly involved in binding to any of the ligands (Table 7). Only (**3**) interacted directly with amino acids in TMH5, while aromatic amino acids in TMH6 were important for binding to all ligands.

3.2.3. Ligand induced conformational states

The rms differences between specific receptor domains of the average receptor structures (150–650 ps) are shown in Table 8. Generally, the largest differences between the apo receptor and ligand bound receptor conformations after MD were in TMH2. The receptor structure after MD with (**2**), (**3**), (**5**) and (**6**) all had the largest deviation from the average free receptor in TMH2. The largest rms deviation between the average receptor structures after MD with the free receptor and the compound (**1**) bound recep-

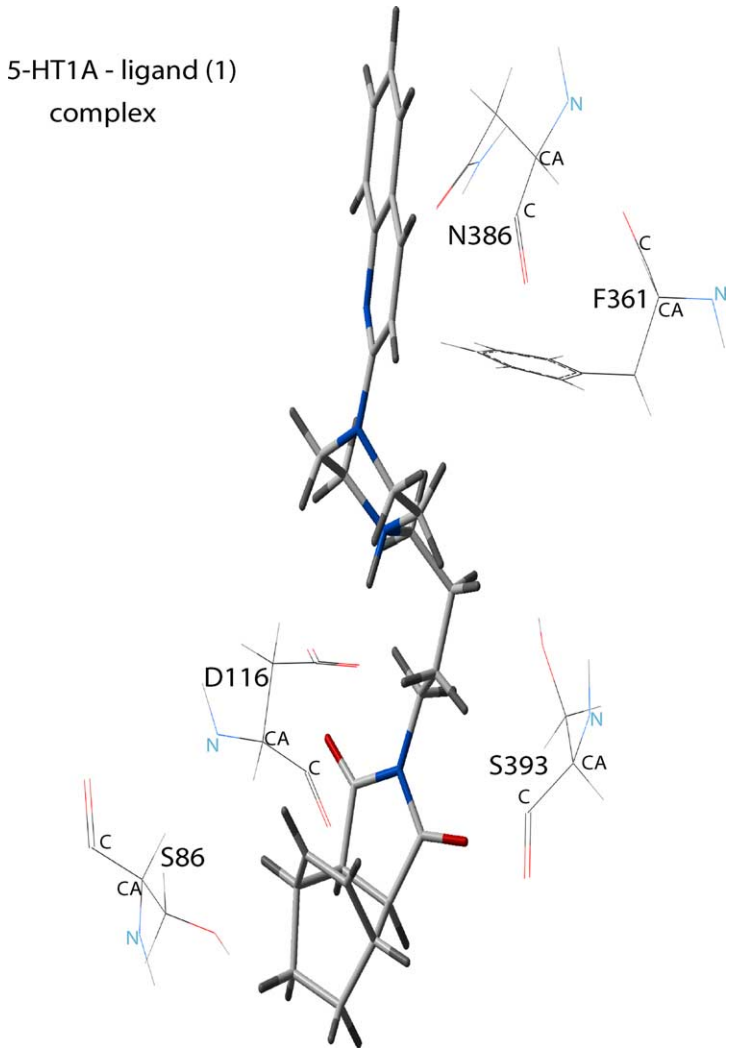


Fig. 2. The receptor binding sites of the buspirone analogues illustrated by the receptor–compund (**1**) complex after MD simulation. The most important amino acids for binding of the buspirone analogues are indicated in the figure. Colour coding of atoms: red, oxygen; blue, nitrogen.

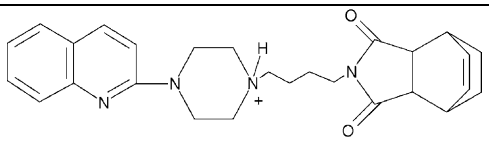
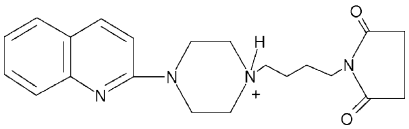
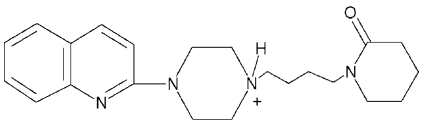
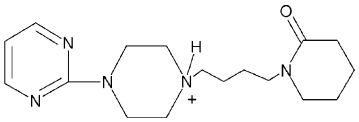
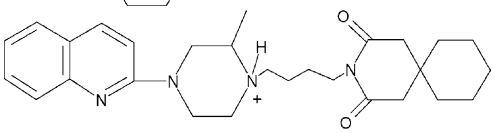
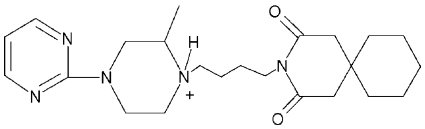
tor were in TMH7, while the corresponding differences to the compound (4) bound receptor were in TMH5. Both after MD with (**1**) and (**6**), TMH7 had very large rms deviation from the free receptor. Generally, the pre-synaptic partial agonists (**4**), (**5**) and (**6**) seemed to induce larger displacements into the TMHs than did the pre-synaptic antagonist (**1**), (**2**) and (**3**), and especially into TMH4 and TMH5 (Table 8). The displacements of TMH6 were also quite large for (**5**) and (**6**) compared with the other buspirone analogues.

4. Discussion

GPCRs constitute a huge family of membrane proteins that encompasses a wide range of functions, including various autocrine, paracrine and endocrine processes. The endogenous compounds activating GPCRs vary widely in structure and character, and their binding can be blocked or mimicked by synthetic analogues. GPCRs are the target for numerous drugs in today use, and ligand design targeting GPCRs is a primary task in numerous drug development projects. Information about GPCRs structure and mechanisms, and how ligands influence GPCR structure upon binding are therefore extremely important for developing new therapeutical agents.

The ligand binding modes suggested that the most important anchoring residues for binding of the buspirone analogues were Asp^{116} in TMH3 and Ser^{393} in TMH7. Asp^{116} interacted with the basic nitrogen atom of the ligands. Studies of many neurotransmitter receptors, including the 5-HT_{1A} receptor [38], have shown that interactions between the basic nitrogen atom and the carboxyl side chain of the aspartic acid in TMH3 is a major component for the binding affinity. Ser^{393} in TMH7 formed a hydrogen bond with a carbonyl group of the ligands. The importance of Ser^{393} was confirmed by the mutation of Ser^{393} into an alanine, which reduced the agonist binding with 86% [31]. Cys^{120} is located one helical turn closer to the cytoplasmic side in TMH3 than

Table 6
Molecular structure, affinities and behavioural effects of the buspirone analogues

Compound	Structure	K_i	Pre-5-HT$_{1A}$ hypothermia (mice)	Post-5-HT$_{1A}$ LLR (rats)
(**1**)		40	ANT	ANT
(**2**)		83	AGO	ANT
(**3**)		65	AGO	ANT
(**4**)		53	INACTIVE	PAG
(**5**)		13	PAG	PAG
(**6**)		29	ANT	PAG

The affinities (K_i) are given in nM. The effects of compounds (**5**) and (**6**) on pre- and post-synaptic 5-HT$_{1A}$ receptors are from a previous study [16]. Abbreviations: LLR, lower lip retraction; AGO, agonist; PAG, partial agonist; ANT, antagonist.

Asp^{116}, and corresponds to a serine in the 5-HT$_{2A}$ receptor. Mutation of the serine indicated that the residue is important for a proper orientation of 5-HT$_{2A}$ receptor agonists at the binding site [39]. Cys^{120} interacted with all the buspirone analogues (Table 7), suggesting that Cys^{120} has a similar role in 5-HT$_{1A}$ receptors. The buspirone analogues interacted with Asn^{386} in TMH7 (Table 7). A point mutation of Asn^{386} to valine increased the buspirone affinity four-folded [40]. In the receptor–ligand complexes Asn^{386} interacts with the aromatic ring system of the buspirone analogues. The complexes indicate that mutating Asn^{386} into a valine affects the ligand orientation at the binding site, and increases the number of stabilising hydrophobic contacts, and most probably increases the binding affinity.

Based on site directed mutageneis studies it is suggested that ionic interactions between the arginine (Arg^{134} in 5-HT$_{1A}$) of the highly conserved (D/E)RY motif at the cytoplasmic end of TMH3 and a highly conserved glutamic acid (Glu^{340} in 5-HT$_{1A}$) at the cytoplasmic end of TMH6 constrains the receptor in an inactive state, and that disrupting this interaction is a common switch in the activation of many GPCRs [41,42]. In the initial receptor model, the atomic distance between Arg^{134} and Glu^{340} was 2.6 Å. MD with a ligand increased the distance, but the cytoplasmic ends of TMH3 and TMH6 were still in close proximity. After MD with the post-synaptic antagonists (**1**), (**2**) and (**3**) the distance was 2.9, 3.0 and 3.1 Å, respectively, while the corresponding distance after MD with the post-synaptic partial agonists (**4**), (**5**) and (**6**) was 3.4, 4.2 and 3. 8 Å. Interestingly, the helical displacements induced by the post-synaptic partial agonists increased the atomic distance between Arg^{134} and Glu^{340} more than did the post-synaptic antagonists.

Due to the existence of two receptor populations in the CNS and the coupling to a variety of effectors, it is reasonable to believe that the 5-HT$_{1A}$ receptors exist in several active conformational states. Further, the receptor conformations giving full agonist activity at pre-synaptical and post-synaptical 5-HT$_{1A}$ receptors may be different. The molecular mechanisms and structural events that occur in the receptor leading to pre- and post-synaptic receptor activity are not understood. Additional information about these mechanisms is important for designing drugs that can act selectively at pre- and post-synaptic 5-HT$_{1A}$ receptors, and have a potential in the treatment of anxiety or depression.

Agonist induced hyptothermia in mice, LLR in rats and behavioural syndrome (FBP, FT) in reserpinised rats were

Table 7
Amino acids having van der Waals contact with the ligands in the energy minimised average complexes (150–650 ps of simulation)

Compound	Receptor domain	Amino acid residue
(**1**)	TMH2	Asp^{82}, Ser^{86}
	TMH3	Phe^{112}, Ile^{113}, Asp^{116}, Cys^{119}, Cys^{120}
	TMH6	Trp^{358}, Phe^{361}, Ala^{365}
	TMH7	Asn^{386}, Tyr^{390}, Ser^{393}, Leu^{394}
(**2**)	TMH2	Asp^{82}, Ser^{86}, Val^{89}, Leu^{90}
	TMH3	Asp^{116}
	TMH6	Trp^{358}, Phe^{361}
	TMH7	Asn^{386}, Tyr^{390}, Ser^{393}, Asn^{396}
(**3**)	TMH2	Asp^{82}, Ser^{86}
	TMH3	Asp^{116}, Cys^{119}, Cys^{120}
	TMH5	Thr^{196}, Ser^{199}
	TMH6	Trp^{358}, Ala^{365}
	TMH7	Ile^{385}, Asn^{386}, Tyr^{390}, Ser^{393}, Leu^{394}, Pro^{397}
(**4**)	TMH2	Asp^{82}, Ser^{86}
	TMH3	Phe^{112}, Asp^{116}, Cys^{119}, Cys^{120}
	TMH6	Phe^{361}
	TMH7	Gly^{389}, Tyr^{390}, Ser^{393}, Asn^{396}, Pro^{397}
(**5**)	TMH1	Ile^{47}
	TMH2	Ser^{86}
	TMH3	Ile^{113}, Asp^{116}, Cys^{119}, Cys^{120}
	TMH6	Trp^{358}, Phe^{361}
	TMH7	Gly^{382}, Ile^{385}, Asn^{386}, Gly^{389}, Ser^{393}, Asn^{396}
(**6**)	TMH1	Leu^{46}
	TMH2	Asp^{82}, Ser^{86}
	TMH3	Phe^{112}, Asp^{116}, Cys^{119}, Cys^{120}
	TMH6	Cys^{357}, Trp^{358}, Phe^{361}, Val^{364}
	TMH7	Asn^{386}, Gly^{389}, Tyr^{390}, Ser^{393}, Asn^{396}, Pro^{397}

previously used to classify (**5**) as partial agonists at both pre- and post-synaptic 5-HT_{1A} receptors, and (**6**) as a pre-synaptic antagonist and a post-synaptic partial agonist [16]. In the present study (**1**), (**2**) and (**3**) were classified as 5-HT_{1A} post-synaptic antagonists (although (**2**) and (**3**) at the highest dose induced weak symptoms of behavioural syndrome in rats), whereas (**4**) was classified as a partial agonist at post-synaptic 5-HT_{1A} receptors. Compound **1** was found to exhibit significant pre-synaptic antagonistic properties, while (**2**) and (**3**) behaved as pre-synaptic agonists. Compound (**4**) was inactive at pre-synaptic 5-HT_{1A} receptors in the hypothermia model.

Table 8
Rms difference of receptor domains (backbone atoms) between energy minimised average receptor structure

Receptor domain	Complex compared to apo receptor					
	(**1**)	(**2**)	(**3**)	(**4**)	(**5**)	(**6**)
TMH1	1.00	1.10	0.87	1.15	0.78	1.29
TMH2	1.26	1.43	1.63	1.61	2.06	2.00
TMH3	1.20	1.10	1.32	1.28	1.28	1.47
TMH4	0.67	0.99	0.69	1.02	1.55	1.14
TMH5	1.00	0.67	1.04	1.78	1.14	1.42
TMH6	0.92	1.35	0.98	1.21	1.41	1.61
TMH7	1.61	0.94	0.71	0.61	1.28	1.60

The complexes are averaged over 150–650 ps of simulation.

The average structures from the MD simulations provide information about the most populated receptor states explored by the simulation. Therefore, by comparing the receptor structure after MD of the free receptor with the receptor structures after MD of receptor–ligand complexes (Table 8), we may get insight into the structural displacements of receptor domains induced upon ligand binding.

Table 8 suggests that the post-synaptic partial agonists (**4**), (**5**) and (**6**) induce relatively larger displacements into the heptahelical bundle than did the post-synaptic antagonists (**1**), (**2**) and (**3**). The most striking difference between the post-synaptic agonist on one side and the post-synaptic antagonists at the other side is the larger displacements induced into TMH4 and TMH5 by the agonists (Table 8). Larger displacements upon binding of the partial agonist than upon binding of the antagonists were also observed for TMH2 and TMH6 (Table 8). TMH4 is connected to IC2, while TMH5 is connected to IC3. IC2 and IC3 are known as the main interaction sites for G-proteins [5,43]. The mutation of a highly conserved threonine (Thr^{149}) in IC2 of the 5-HT_{1A} receptor prevents the coupling to $G_{\alpha\beta}$ signalling, while $G_{\alpha i}$ induced signalling was preserved [5]. The larger helical displacements of TMH4 and TMH5 upon binding of the (**4**), (**5**) and (**6**) may contribute to a conformation giving a proper recognition of IC2 and IC3 by post-synaptically G-proteins, resulting in partial agonist pharmacology.

Compounds (**1**) and (**6**) induced larger displacements of TMH7 upon binding than the other ligands (Table 8). Both (**1**) and (**6**) are pre-synaptic antagonists (Table 6), indicating that pre-synaptic antagonists induce larger displacements into TMH7 than pre-synaptic agonist and partial agonists. Compound (**6**) induced much larger displacements of TMH4, TMH5 and TMH6 than did (**1**), which might explain that (**6**) is a post-synaptic partial agonist, while (**1**) is a post-synaptic antagonist.

The largest helical displacements into the heptahelical bundle upon binding of (**2**), (**3**), (**5**) and (**6**) were in TMH2. However, for compound (**2**) the deviation of TMH6 is almost similar to that of TMH2, and the ligand induced effects on TMH2 and TMH6 must be considered equal. The compounds (**2**), (**3**) and (**5**) all stimulate the pre-synaptic 5-HT_{1A} receptor, while (**6**) antagonise that receptor. However, a very important difference in helical displacements between (**6**) on one side and (**2**), (**3**) and (**5**) at the other side is that (**6**) also induced large displacements of TMH7 upon binding. This might suggest that stimulation of pre-synaptic receptor requires large helical displacements of TMH2 and relatively sparse displacements of TMH7 upon binding. Interestingly, (**5**) which is a partial agonist at pre-synaptical receptor induced larger displacement into TMH7 than did the full agonists (**2**) and (**3**), but less than did (**6**) which is a pre-synaptic antagonist. Compound (**4**) also induced large displacement of TMH2 and

relative sparse displacement of TMH7, suggesting that (**4**) should be a pre-synaptic agonist. However, the animal models indicated that compound (**4**) is inactive at pre-synaptic 5-HT_{1A} receptors (Table 6). This might be explained by the large displacement of TMH5 which was not seen for compounds (**2**), (**3**) and (**5**).

Based on analysis of sequence data and available structural information it is believed that the core function of signalling activation through a conformational change of the TMHs altering the cytoplasmic receptor surface and the G-protein interactions are shared by the entire rhodopsin family of GPCRs [44]. Therefore, the different helical displacements upon binding of the buspirone analogues may induce different conformations of the cytoplasmic receptor surfaces and affect the subsequent interactions with G-proteins, and explain their differences in intrinsic activity at pre- and post-synaptic 5-HT_{1A} receptors.

These results also support the assumption that full G-protein activation requires different receptor conformations at pre- and post-synaptic 5-HT_{1A} receptors. Based on the present results we might suggest that pre-synaptic antagonistic behaviour is connected to large displacements of TMH7 upon binding ((**1**) and (**6**)), while pre-synaptic agonistic behaviour might be connected to large displacements of TMH2 combined with sparse displacements of TMH7. Further, post-synaptic partial agonist behaviour might be connected to large displacements of TMH4 and TMH5 ((**4**), (**5**) and (**6**)), while pre-synaptical antagonist behaviour might be connected to smaller displacements of TMH4 and TMH5 upon binding.

Knowledge about the relationship between ligand structure, the ligand induced conformational changes of the receptor, and the activity at pre- and post-synaptic 5-HT_{1A} receptors may be important in order to enable design of new buspirone analogues with a therapeutical potential in anxiety or depression. The predicted 3D structures and ligand binding modes are also useful in designing mutagenesis experiments validating the structure of the binding sites.

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