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Histaminergic pharmacology of homo-oligomeric β3 γ-aminobutyric acid type A receptors characterized by surface plasmon resonance biosensor technology

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ABSTRACT

A surface plasmon resonance biosensor assay was established for studying the interactions of 51 histaminergic and 15 GABAergic ligands with homo-oligomeric β3 $GABA_A$ receptors. Detergent solubilized receptors were successfully immobilized via affinity-capture on biosensor surfaces. The interaction kinetics of both histaminergic and GABAergic ligands were very rapid but affinities could be determined by steady-state analysis. Binding of several GABAergic ligands was observed, in agreement with previous data. Histamine and 16 histaminergic ligands were detected to directly bind to β3 $GABA_A$ receptors with micromolar affinity ($K_D < 300$ μM), thus extending previous evidence that β3 $GABA_A$ receptors can interact with histaminergic ligands. Histamine exhibited an affinity for these receptors comparable to that for human histamine type 1 (H1) or type 2 (H2) receptors. Furthermore, 13 of these histaminergic ligands appeared to compete with histamine. The discovery that H2, H3 and H4 receptor ligands interact with β3 receptors indicates a unique histaminergic pharmacology of these receptors. Due to their low affinity for the homo-pentameric β3 receptors these histaminergic drugs are not expected to modulate these receptors at clinically relevant concentrations. The results support the use of the new biosensor assay for the identification of drugs interacting with full length receptors and for fragment-based drug discovery of high affinity ligands for β3 receptors. Drugs with high affinity and selectivity for these receptors can be used to clarify the question whether β3 receptors do exist in the brain, and provide new avenues for the development of therapeutically active compounds targeting this novel histamine binding site.

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and mediates most of its physiological action via γ-aminobutyric acid type A ($GABA_A$) receptors [1,2]. These receptors are known to be involved in a wide range of neurological functions, such as modulation of sleep, anxiety, depression, chronic pain, schizophrenia, epilepsy and recovery from stroke [2]. Recent studies also suggest that $GABA_A$ receptors might be involved in Fragile X Syndrome [3].

$GABA_A$ receptors belong to the superfamily of Cys-loop ligand-gated ion channels, further comprising nicotinic acetylcholine (nACh) receptors, serotonin type 3 ($5\text{-}HT_3$) receptors, glycine receptors and a Zn^{2+}-activated ion channel [1]. Each subunit of these pentameric receptors contains an extracellular domain harboring the ligand binding site and the characteristic disulfide bond (Cys-loop), a trans-membrane domain and an intracellular domain [4]. Genome sequencing has identified 19 genes for $GABA_A$ receptor subunits, contributing to the great heterogeneity of the receptor family [5]. Native receptors commonly consist of two α, two β and one γ subunit, forming a heteropentamer [6]. The specificity and function of ligand-gated ion channels in general, and $GABA_A$ receptors in particular, strongly depends on the subunit composition. In $GABA_A$ receptors, the GABA binding site is located at the interface of α and β subunits [7]. Benzodiazepines, clinically used allosteric modulators of $GABA_A$ receptors, primarily bind at the interface of α and γ subunits [8].

Homo-oligomeric $GABA_A$ receptors are useful model systems for exploration of receptor function and pharmacology [1]. They require only a single subunit for recombinant expression, are composed of five identical subunits, and do not have the problem of undefined structure and stoichiometry due to the simultaneous formation of

Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$ receptors, GABA type A receptors; SPR, surface plasmon resonance.

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hetero-oligomeric receptors with different stoichiometry and subunit arrangement [1]. From all 19 $GABA_A$ receptor subunits, only the 3ρ, the β3, and to a lesser degree the β1 subunits seem to readily form homo-oligomeric receptors [9]. Although the homo-oligomeric β3 receptors lack the ability of being activated by GABA and modulated by benzodiazepines, they carry allosteric binding sites for general anesthetics, like etomidate and propofol, and barbiturates, like pentobarbital [10,11]. In addition to the GABAergic pharmacology, β3 receptors have a histaminergic pharmacology since they are activated by histamine and can be blocked by histamine receptor antagonists such as thioperamide [12]. Whereas histamine is able to directly evoke currents in homo-oligomeric β2 or β3 receptors, it can only modulate GABA-evoked currents in α1β2 or α1β2γ2 receptors [12] or in a variety of other $GABA_A$ receptor subtypes [13]. Further studies with recombinant receptors of different subunit composition demonstrated that the histamine receptor antagonists tiotidine and famotidine have an inhibitory effect on GABA-evoked currents [14]. It is not yet clear what role these histaminergic ligands could play in GABAergic signal transmission but effects of histaminergic ligands on native $GABA_A$ receptors prepared from rat brains have been reported [15]. A possible cross-talk between GABAergic and histaminergic signal transmission has been proposed but evidence that such a phenomenon occurs in vivo is not yet available [13].

Homo-oligomeric β3 receptors have so far not been identified in mammalian brain but there is evidence for the existence of yet unidentified ionotropic histamine receptors [16]. However, the facile formation of such receptors in heterologous expression systems, makes it highly probable that such receptors are also formed in the brain and are located extrasynaptically. In the absence of a specific pharmacology, however, they cannot be identified. To possibly identify a specific histaminergic pharmacology of β3 receptors that could lead to the identification of such receptors being expressed in the brain, it was of interest to expand the known pharmacology of homo-oligomeric β3 receptors by identifying novel ligands.

To allow future fragment-based drug discovery with this receptor as a target, we investigated whether surface plasmon resonance (SPR) biosensor technology can be employed to detect direct interactions of rat homo-oligomeric β3 $GABA_A$ receptors with GABAergic and histaminergic ligands. The complexity of studying ion channels, particularly with SPR biosensor technology, arises mostly from the membrane spanning part. Reducing the complexity can be achieved by studying truncated ion channels that lack the trans-membrane domain. A prominent example is the acetylcholine-binding protein (AChBP), a soluble protein displaying structural similarity to nACh receptors and other members of the Cys-loop receptor superfamily [17]. An SPR biosensor assay has previously been developed in our group for the investigation of ligand interactions with AChBP [18]. A similar approach has now been taken to apply SPR biosensor technology for studies of full-length β3 $GABA_A$ receptors. It has enabled the study of interactions between small molecular ligands and β3 receptors. Previous reports on histamine and other histaminergic ligands acting on β3 receptors are supported and novel ligands have been identified, forming a distinct pharmacological fingerprint. The results support the feasibility of SPR for the identification of drugs interacting with full length receptors and are expected to stimulate the discussion about a potential cross-talk between GABAergic and histaminergic signal transmission.

2. Materials and methods

2.1. Production of homo-oligomeric β3 $GABA_A$ receptors

Generation of expression constructs and expression of rat homo-oligomeric β3 $GABA_A$ receptors were performed as described previously [19]. Briefly, a His_8-tag was introduced between amino acids 4 and 5 of the mature β3 protein using the *gene splicing by overlap extension* technique [20]. The mutated subunit was cloned into the pBacPAK8 transfer vector and used to generate recombinant baculoviruses in Sf9 cells (*Spodoptera frugiperda*). Single plaque forming units were picked and used to generate high titer working stocks. For expression, Sf9 cells at a concentration of 2.5×10^6 cells mL^{-1} and a multiplicity of infection of 1 was used. The infected cells were harvested 56–60 h post infection and subsequently stored at −80 °C.

The expression level of homo-oligomeric β3 $GABA_A$ receptors was determined by a radioligand binding assay using [^{3}H]EBOB [21]. Frozen pellets from infected cells were thawed, and cells were homogenized in 50 mM Tris/citrate buffer, pH 7.4, by using an Ultra-Turrax, followed by centrifugation (200,000 × *g*, 20 min, 4 °C). Cell pellets were resuspended in 50 mM Tris/citrate buffer, pH 7.4, at protein concentrations from 0.5 to 1.0 mg mL^{-1} as measured with the BCA protein assay kit (Thermo Fischer Scientific, Rockford, USA). For [^{3}H]EBOB binding assays, 100 μL of the membranes were incubated for 2 h at room temperature in a total volume of 500 μL containing 50 mM Tris/citrate buffer, pH 7.4, 200 mM NaCl and 50 nM [^{3}H]EBOB (26 Ci $mmol^{-1}$; NEN) in the presence or absence of 150 μM picrotoxin (Sigma–Aldrich, St. Louis, USA). Membranes were filtered through Whatman GF/B filters, and the filters were rinsed three times with 3.5 mL ice-cold 50 mM Tris/citrate buffer, pH 7.4. Radioactivity was measured by liquid scintillation counting (Packard). Specific binding was determined by subtracting non-specific binding in the presence of 150 μM picrotoxin from total [^{3}H]EBOB binding.

2.2. Membrane preparation and receptor solubilization

Two buffers were used for preparing membranes from Sf9-cells. Homogenization buffer (*buffer H*) consisted of 10 mM HEPES, 320 mM sucrose, pH 7.4 and one protease inhibitor tablet (Roche, Basel, Switzerland) per 50 mL buffer. Wash buffer (*buffer W*) was the same as buffer H, but did not contain sucrose. Buffers were chilled prior to usage and all steps were performed on ice or at 4 °C. Buffer H (10 mL per 100 mL culture volume) was added to Sf9 cell pellets expressing β3 $GABA_A$ receptors (*β3 sample*) and to Sf9 cell pellets not expressing the receptors (*control sample*). After thawing the pellets, the lysates were homogenized with a hand-homogenizer (Sartorius, Göttingen, Germany), 10 strokes were performed using a piston with a clearance of 0.06 mm (*L*), 10 with a clearance of 0.02 mm (*S*). The homogenate was centrifuged in a Beckman L7-55 ultracentrifuge (Beckman Coulter, Brea, USA) using a Ti60 rotor (60 min, 100,000 × *g*). The supernatant was discarded and buffer H (5 mL per 100 mL culture volume) was added to β3 and control samples. Homogenization (10× *L*) was followed by ultracentrifugation (60 min, 50,000 × *g*). The supernatant was discarded and buffer W (5 mL per 100 mL culture volume) was added. Samples were hand-homogenized (10× *L*), followed by ultracentrifugation (60 min, 50,000 × *g*). Buffer W (3 mL per 100 mL culture volume) was added, samples were hand-homogenized (10× *L*) and aliquots were stored at −80 °C.

Solubilization buffer (*buffer S*) consisted of 25 mM Tris–HCl, 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, pH 8.5, 0.5% deoxycholate (DOC) (Sigma–Aldrich), 0.15% n-dodecyl-β-D-maltopyranoside (DDM) (Affymetrix, Santa Clara, USA), 0.05% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 0.05% 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) (both from Avanti Polar Lipids, Alabaster, USA) and one protease inhibitor tablet per 50 mL buffer. Buffers were chilled prior to usage and solubilization was performed on ice or at 4 °C. Frozen membranes were thawed and centrifuged (60 min, 50,000 × *g*). The supernatant was discarded, buffer S (5 mL per

Table 1
GABAergic (italics) and histaminergic ligands used in the present study. The two starred (*) ligands R05-4864 and PK 11195 are also known as ligands of the peripheral benzodiazepine receptor.

Ligand	M [g mol^{-1}]	Ligand	M [g mol^{-1}]
4-Piperidine-sulfonic acid	*165.2*	Dimaprit	161.3
Alfaxalone	*332.5*	Diphenhydramine	255.4
Baclofen	*213.7*	Doxepin	279.4
Etazolate	*289.3*	Famotidine	337.4
Etomidate	*244.3*	Fexofenadine	501.7
Flumazenil	*303.3*	Histamine	111.1
Flurazepam	*387.9*	HTMT	382.4
GABA	*103.1*	ICI 162,846	306.3
Muscimol	*114.1*	Imetit	170.2
Pentobarbital	*225.3*	Immepip	165.2
*PK 11195**	*352.9*	Immethridine	159.2
Propofol	*178.3*	Impentamine	153.2
*Ro5-4864**	*319.2*	Iodophenpropit	414.3
SR 95531	*287.3*	JNJ 10181457	312.4
THIP	*140.1*	JNJ10191584	278.7
		JNJ7777120	277.7
(R)-α-Methylhistamine	125.2	Ketotifen	309.4
(S)-α-Methylhistamine	125.2	Loratidine	382.9
(S)-Dimethindene	292.4	Mepyramine	285.4
2-Pyridylethylamine	122.2	Methimepip	179.3
4-Methylhistamine	125.2	Mirtazepine	265.4
A-943931	295.4	N^{α}-Methylhistamine	125.2
A-987306	327.4	Proxyfan	216.3
Aminopotentidine	477.6	Ranitidine	314.4
Amthamine	157.2	ROS 234	241.3
Astemizole	458.6	Terfenadine	471.7
BF 2649	295.8	Thioperamide	292.4
Burimamide	212.3	Tiotidine	312.4
Carcinine	182.2	Triprolidine	278.4
Cetirizine	388.9	VUF 5681	193.3
Cimetidine	252.3	VUF 8430	161.2
Clemastine	343.9	Zolantidine	381.5
Clobenpropit	308.8	Zotepine	331.9
Conessine	356.6		

100 mL culture volume) was added and samples were hand-homogenized (10× *L*). Solubilization took place on an orbital shaker for 3 h, followed by ultracentrifugation (30 min, 100,000 × *g*). Aliquots of the supernatant were stored at −80 °C.

2.3. Ligands

A selection of 51 histaminergic and 15 GABAergic ligands were used (Table 1). Histaminergic ligands were purchased from Tocris (Bristol, UK), except histamine, thioperamide and famotidine that were purchased from Sigma Aldrich. The GABAergic ligands 4-piperidine-sulfonic acid, flurazepam, GABA, muscimol, pentobarbital, PK 11195, Ro5-4864 and THIP were purchased from Sigma Aldrich. Alfaxalone, baclofen, etazolate, etomidate, flumazenil, propofol and SR 95531 were purchased from Tocris.

Stock solutions of histamine (100 mM) and GABA (1 M) were prepared by dissolving the solid compounds in the respective running buffer prior to each interaction experiment. All other compounds were diluted from stock solutions (50 mM in dimethyl sulfoxide (DMSO)) to the highest concentration used in the interaction analyses. The pH of ligands that were analysed at concentrations greater than 2 mM was controlled and adjusted if necessary.

2.4. Biosensor surface preparation

SPR based biosensor studies were performed using Biacore T200 and S51 instruments and CM3 and CM5 sensor chips (GE Healthcare, Uppsala, Sweden). Sensor surfaces were prepared by antibody capture, as illustrated in Fig. 1. First, a polyhistidine antibody (R&D systems, Minneapolis, USA) was covalently immobilized on two spots (Biacore S51) or in two flow cells (Biacore T200) of the sensor surface by standard amine coupling chemistry [22] at a temperature of 25 °C. The antibody, in 10 mM Na-acetate, pH 5.5 (GE Healthcare), was injected for 7 min (5 μL min^{-1}) at a concentration of 45 μg L^{-1} for immobilization on CM5 sensor chips, and of 110 μg L^{-1} on CM3 sensor chips. After the covalent immobilization, the temperature was reduced to 10 °C. The running buffer (*buffer R*) consisted of 25 mM Tris–HCl, 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, 0.15% DDM and 0.2% DMSO, pH 7.4. Solubilized membranes containing the β3 receptors were injected for 30 min (2 μL min^{-1}), generating *β3 receptor surfaces*, whereas *control surfaces* were generated by using membranes without the over-expressed β3 receptors. During interaction analyses, the ligands were injected over the surfaces either in parallel (Biacore S51) or in series (Biacore T200).

2.5. Interaction analysis

Interaction analyses were performed at 10 °C. Ligands, diluted into buffer R, were injected in 2-fold dilution series in ascending order for 20–30 s (30 μL min^{-1}). Buffer injections served as blank samples. Minor variations in the DMSO concentration of samples were corrected by creating a standard curve in a range from 0.15 to 0.25% DMSO, a process referred to as *solvent correction*. Analyses were repeated with freshly prepared biosensor surfaces at least in triplicates.

2.6. Data analysis

Initial data analysis was performed with Biacore T200 Evaluation Software 1.0 (GE Healthcare). In addition to *solvent correction*,

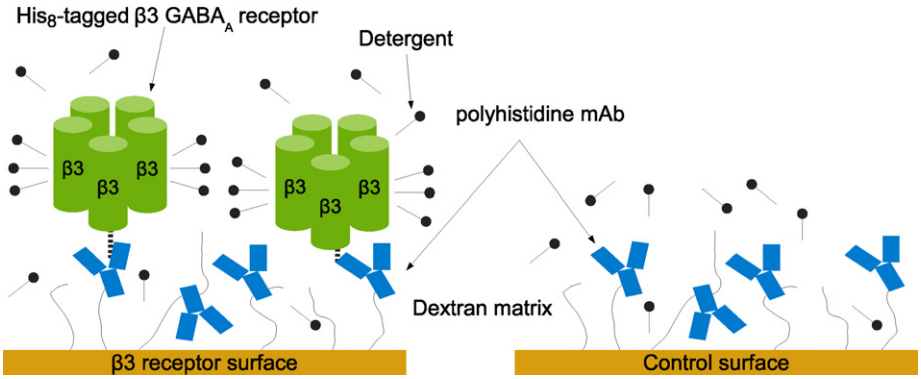

Fig. 1. Scheme of sensor surfaces for SPR based interaction studies. Left: detergent solubilized β3 $GABA_A$ receptors immobilized via affinity capture using a monoclonal polyhistidine antibody forming *β3 receptor surfaces*. Right: *Control surfaces* formed from solubilized membranes without overexpressed β3 receptors.

sensorgrams from control surfaces were subtracted from sensorgrams from β3 receptor surfaces (*reference subtraction*), and signals from blank injections were further subtracted (*blank subtraction*). The average signals within a 5 s interval were extracted 4 s before injection stop (report point *binding late*). Data were imported into the BeacticaSprint 1.02 software (Beactica AB, Uppsala, Sweden). Equilibrium dissociation constants (K_D) were determined by non-linear regression analysis of steady-state signals (R) as a function of ligand concentration ([L]) using a Langmuir isotherm equation (Eq. (1)). The equation contained a parameter for the offset (m) to correct for baseline level shifts. The parameter R_{max} represents the maximal signal under saturating conditions.

$$R = \frac{R_{max} \times [L]}{K_D + [L]} + m \quad (1)$$

2.7. Competition analysis

Competition analysis was performed under the same experimental conditions as for the interaction analysis (see Section 2.5). Each experimental series consisted of separate injections of 1 mM histamine, 50 μM ligand and a mixture of both ligands, also at 1 mM and 50 μM, respectively. Buffer injections served as blank samples. Biacore T200 Evaluation Software 1.0 was used for solvent correction, reference and blank subtraction and to extract the signals at the *binding late* report point. The report point data were further analysed using Microsoft Excel (Microsoft Corporation, Redmond, USA). Blank subtracted data from separate injections of histamine and the respective ligand were added and taken as the signal level under non-competitive conditions ($R_{no\text{-}comp}$). The standard deviation of the histamine signals within one independent experiment (σR) was used as the estimate of uncertainty. A ligand was regarded as a competitor, when the blank-subtracted signal of the mixture (R_{mix}) was significantly lower than the signal under non-competitive conditions in three independent experiments, expressed as $R_{mix} < R_{no\text{-}comp} \pm 3 \times \sigma R$.

3. Results

3.1. Production of β3 receptors

The expression level of His_8-tagged β3 $GABA_A$ receptors in Sf9 cells as determined by radioligand binding using [^{3}H]EBOB was 10 pmol receptors per mg protein.

3.2. Biosensor surface preparation

The β3 receptors were specifically and stably immobilized on SPR biosensor surfaces, using antibody capture. Immobilization of the polyhistidine antibody resulted in sensor surface densities of ≈4000 response units (RU) (1 RU ≡ 1 pg mm^{-2} [23]) on CM3 sensor chips and ≈11,000 RU on CM5 sensor chips. Capture of β3 samples resulted in sensor surface densities between 2000 and 4000 RU, irrespective of the type of sensor chip. Just a small portion of proteins bound non-specifically to the control surfaces (100–200 RU). The β3 receptor surface baseline was stable over time.

3.3. Biosensor based interaction analysis

3.3.1. Binding of histaminergic ligands to homo-oligomeric β3 $GABA_A$ receptors

Histamine is among the smallest molecules listed in Table 1 and detecting interactions of such a small molecule with SPR biosensor technology is a considerable challenge. Nevertheless, despite low signal intensities ($R_{max} < 3$ RU), a direct interaction between histamine and immobilized β3 receptors was clearly detected (Fig. 2). Interactions were too rapid to determine kinetic parameters but steady-state affinity analysis could reliably be performed and was not dependent on the injection times used. The interaction between histamine and β3 receptors was found to have an affinity (K_D) of 100 μM (Table 2, reference data for human histamine H1–4 receptors are from [24–27]).

The developed biosensor assay provided the required sensitivity to measure small molecular ligand interactions with immobilized β3 receptors during more than 200 injection cycles (15–20 h). The newer Biacore T200 instrument displayed a considerably reduced signal/noise ratio compared to the Biacore S51 instrument. However, both instruments were equally suited for the present study.

It was of interest to apply the assay for interaction analysis of a selection of commercially available histaminergic ligands. In total, 51 histaminergic ligands were analysed regarding their interactions with β3 $GABA_A$ receptors. Of these, 17 ligands were identified to interact with a K_D of less than 300 μM (Table 2).

Other histamine H1 receptor ligands, such as the agonist histamine trifluoromethyl toluidide (HTMT) and antagonist mepyramine, were not found to interact with the β3 receptors.

Binding of histamine H2 receptor agonists, except histamine, was not detected but three H2 receptor antagonists were identified to bind with higher affinity than histamine, tiotidine (K_D = 33 μM, Fig. 3), burimamide (K_D = 33 μM) and famotidine (K_D = 81 μM).

Several histamine H3/H4 receptor ligands were found to interact with the β3 receptors. Two histamine derivatives displayed both higher and lower affinities than histamine, these are the H3/H4 receptor agonists and stereoisomers (S)-α-methylhistamine (K_D = 51 μM) and (R)-α-methylhistamine (K_D = 180 μM). Further H3/H4 receptor agonists interacting with the β3 receptors were imetit (K_D = 51 μM, Fig. 3), immepip (K_D = 69 μM) and proxyfan (K_D = 110 μM). Three of the tested H3/H4 receptor antagonists bound to β3 receptors, namely

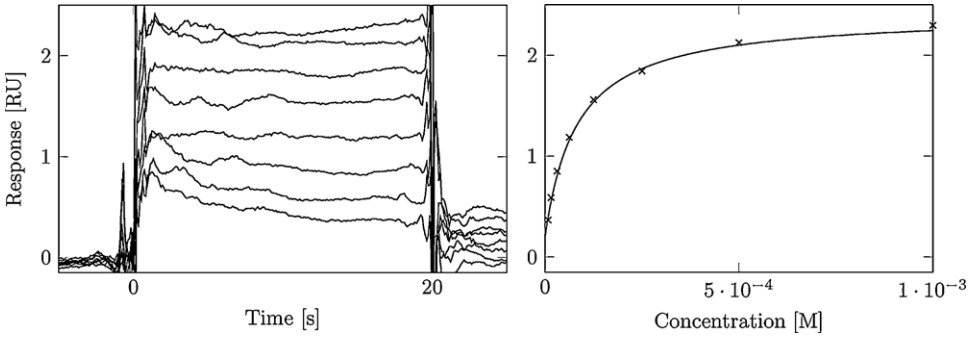

Fig. 2. Interaction between histamine and immobilized β3 $GABA_A$ receptors. Left: sensorgrams from the injection of histamine in 2-fold dilution series from 1000 to 8 μM. Right: steady state signals plotted as a function of concentration with a fitted Langmuir binding isotherm (solid line).

Table 2
Affinities (K_D, $pK_D \pm SD$) for 17 histaminergic ligands that interacted with homo-oligomeric β3 $GABA_A$ receptors. The number of independent replicates (n) is indicated. Activities and potencies of the histaminergic ligands at human histamine H1–4 receptors (hHR) are summarized. The data is taken from [24–27].

Ligand	K_D [μM]	pK_D	n	Histamine receptor activity	pK_i at hHR
Thioperamide	13	4.9 ± 0.1	5	H3/H4 antagonist	6.9 ± 0.1/7.3 ± 0.1
JNJ7777120	28	4.6 ± 0.3	5	H4 antagonist	7.8 ± 0.1
4-Methylhistamine	32	4.5 ± 0.3	6	H4 agonist	7.3 ± 0.1
Tiotidine	33	4.5 ± 0.1	7	H2 antagonist	7.8 ± 0.2
Burimamide	33	4.5 ± 0.2	4	H2/H3 antagonist, H4 agonist	5.4 ± 0.2/7.9 ± 0.1/7.4 ± 0.1
A-987306	46	4.3 ± 0.3	6	H4 antagonist	8.3 ± 0.2
Imetit	51	4.3 ± 0.3	6	H3/H4 agonist	8.8 ± 0.1/8.2 ± 0.1
(S)-α-Methylhistamine	51	4.3 ± 0.1	5	H3/H4 agonist	7.2 ± 0.1/5.4 ± 0.1
VUF 8430	54	4.3 ± 0.4	5	H4 agonist	7.5 ± 0.1
Clobenpropit	57	4.2 ± 0.1	5	H3 antagonist, H4 agonist	8.6 ± 0.1/8.1 ± 0.1
Immepip	69	4.2 ± 0.2	5	H3/H4 agonist	9.3 ± 0.1/7.7 ± 0.1
Famotidine	81	4.1 ± 0.2	8	H2 antagonist	7.8 ± 0.1
Histamine	98	4.0 ± 0.2	9	Endogenous H1/H2/H3/H4 agonist	4.2 ± 0.1/4.3 ± 0.1/8.0 ± 0.1/7.8 ± 0.1
Proxyfan	110	4.0 ± 0.3	6	H3/H4 agonist	7.9 ± 0.1/7.3 ± 0.1
A-943931	120	3.9 ± 0.2	6	H4 antagonist	8.3 ± 0.1
(R)-α-Methylhistamine	180	3.7 ± 0.3	7	H3/H4 agonist	8.2 ± 0.1/6.6 ± 0.1
Iodophenpropit	300	3.5 ± 0.1	3	H3/H4 antagonist	8.2 ± 0.1/7.9 ± 0.1

thioperamide (K_D = 13 μM, Fig. 3), clobenpropit (K_D = 57 μM) and iodophenpropit (K_D = 300 μM).

Also selective histamine H4 receptor ligands, like 4-methylhistamine (K_D = 32 μM, Fig. 3) were detected to bind to β3 receptors. Moreover, the four selective H4 receptor antagonists JNJ7777120 (K_D = 28 μM), A-987306 (K_D = 46 μM, Fig. 3), VUF 8430 (K_D = 54 μM) and A-943931 (K_D = 120 μM) complete the list of histaminergic ligands interacting with homo-oligomeric β3 $GABA_A$ receptors.

3.3.2. Binding of GABAergic ligands to homo-oligomeric β3 $GABA_A$ receptors

As a comparison, the interactions of GABAergic ligands with immobilized β3 $GABA_A$ receptors were also studied. The experiments demonstrated that known GABAergic ligands were confirmed to bind to this receptor subtype. Low micromolar affinities, slightly higher than of histamine (Table 3), were determined for etomidate, propofol (both shown in Fig. 4), PK-11195, Ro5-4864 and etazolate. GABA was included in the analyses as a negative control and no binding was detected up to concentrations of 100 mM. Similarly, no binding was obtained for the $GABA_A$ receptor agonists muscimol, 4-piperidine sulfonic acid, or THIP, the $GABA_A$ receptor antagonist SR 95531, the $GABA_B$ receptor agonist baclofen, the benzodiazepine flurazepam, or the benzodiazepine site antagonist flumazenil, all tested up to 100 μM.

Pentobarbital and alfaxalone did not show reproducible binding. Interaction data of alfaxalone repeatedly displayed secondary effects that did not allow quantification of the interaction (Fig. 5). Shortly after the ligand injection, signals increased with increasing ligand concentration, with signal levels approaching saturation. With increasing injection time, the signal levels progressed towards inverse concentration dependence where higher concentration resulted in lower negative signals.

3.3.3. Competition analysis

Histaminergic ligands (Table 2) and GABAergic ligands (Table 3) were subjected to competition analyses. From the 16 histaminergic ligands that interacted with β3 receptors (Table 2 and Fig. 6), 13 ligands appeared to compete with histamine. As exemplified in Fig. 7A for these 13 ligands, the signals of a mixture of 1 mM histamine and 50 μM ligand (R_{mix}) were significantly lower than the signal under non-competitive conditions ($R_{no\text{-}comp}$) in three independent experiments, expressed as $R_{mix} < R_{no\text{-}comp} \pm 3 \times \sigma R$. Representative sensorgrams from the competition analysis of A-987306 are shown in Fig. 7B. In contrast to the histaminergic ligands, the GABAergic ligands did not appear to compete with histamine.

A different experimental design was used to validate the applied assay for competition analysis using famotidine and tiotidine. Both ligands were injected in 2-fold concentration series, and then the concentration series were repeated in the presence of histamine (500 μM). The affinity for famotidine decreased from $pK_D = 4.1 \pm 0.2$ to an apparent affinity ($pK_{D'}$) in the presence of histamine of $pK_{D'} = 3.6$, for tiotidine from $pK_D = 4.5 \pm 0.1$ to $pK_{D'} = 4.0$. This is also demonstrated by a weaker curvature of the steady-state affinity plots, exemplified for famotidine in Fig. 7C. Repeated measurements of histamine (0.5–1000 μM) indicated stable conditions during the measurements, thereby validating the principal assay for competition analysis.

Table 3
Affinities (K_D, $pK_D \pm SD$) for 5 GABAergic ligands interacting with homo-oligomeric β3 $GABA_A$ receptors. The number of independent replicates (n) is indicated.

Ligand	K_D [μM]	pK_D	n
Etomidate	38	4.4 ± 0.3	7
Propofol	42	4.4 ± 0.3	6
PK-11195	61	4.2 ± 0.2	3
Ro5-4864	69	4.2 ± 0.2	3
Etazolate	79	4.1 ± 0.3	10

4. Discussion

In the present study, a new strategy for studying the interactions of ligands with ligand-gated ion channels has been pursued by developing an SPR biosensor assay. Such an assay allows the detection of direct interactions between ligands and receptors. The assay has successfully been applied to measure and characterize interactions with micromolar affinities of GABAergic and histaminergic ligands with homo-oligomeric β3 $GABA_A$ receptors.

4.1. Successful immobilization of β3 receptors on SPR biosensor surfaces

The SPR biosensor method was based on affinity capture of β3 receptors from solubilized membranes from Sf9 cells overexpressing the receptors. It even enabled the detection of small ligands, such as histamine (111 g mol^{-1}), demonstrating an exceptional sensitivity for studying interactions with trans-membrane receptors.

Interaction studies with membrane proteins using SPR biosensors have been performed in the past. A strategy involving immobilization of the target protein and subsequent reconstitution in a lipid membrane on the sensor surface has been pursued

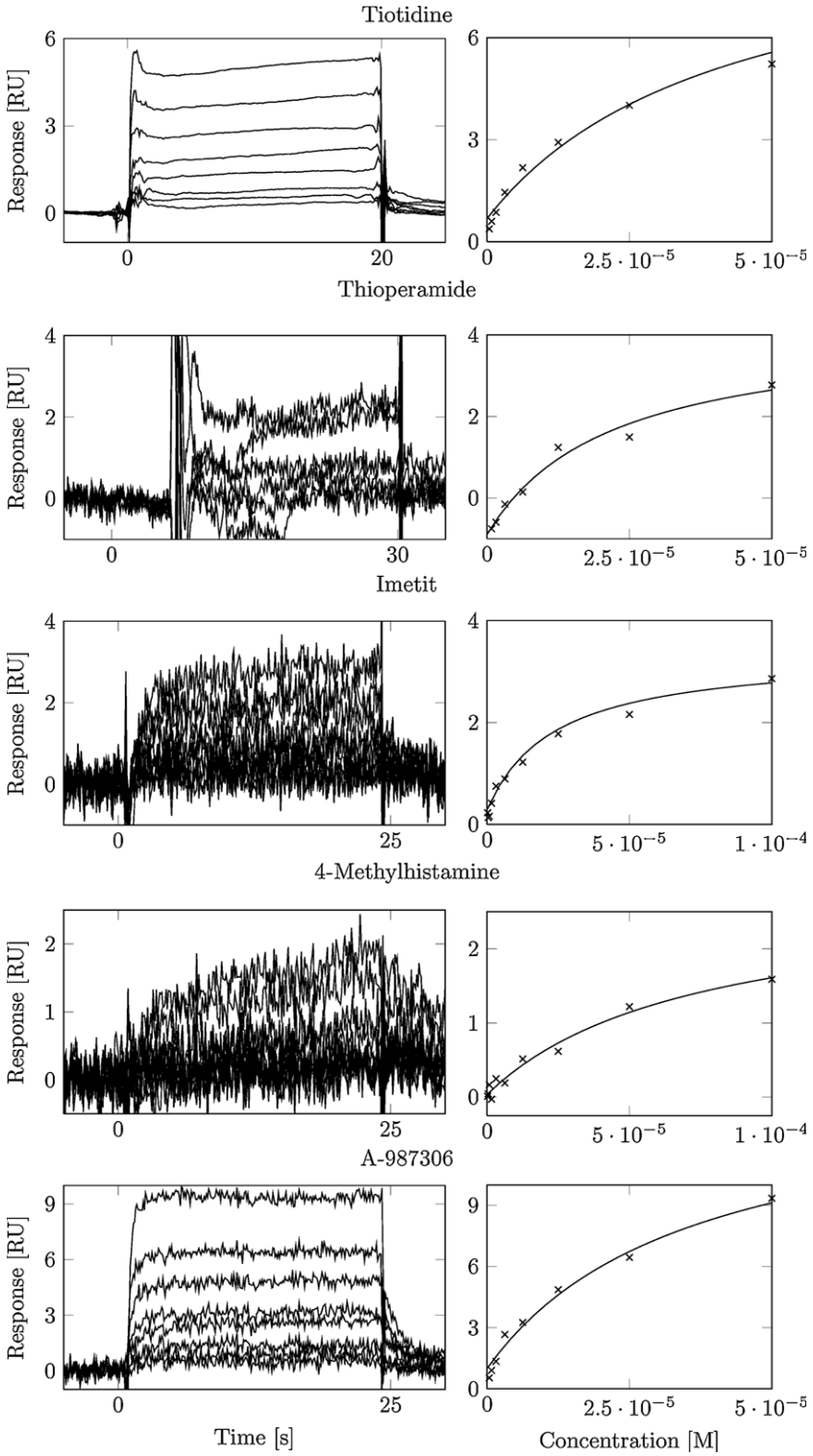

Fig. 3. Interactions of selected histaminergic ligands interacting with immobilized β3 $GABA_A$ receptors. Left: sensorgrams from 2-fold dilution series. Right: steady-state signals plotted as a function of ligand concentration with a fitted Langmuir binding isotherm (solid line).

for membrane proteins such as rhodopsin [28] and β-secretase-1 [29]. Immobilization and interaction studies of detergent-solubilized receptors have also been successfully performed for cytokine receptors [30] and β-adrenergic receptors [31]. In the present study, reconstitution of detergent solubilized β3 receptors on the sensor surface was not successful. However, ligand binding could be detected when immobilized β3 receptors were kept detergent-solubilized during interaction analyses.

In previous studies, different detergents have been successfully employed for functional solubilization of $GABA_A$ receptors, thereby preserving their pentameric assembly state. For instance, affinity-purified $GABA_A$ receptors from bovine brain remained functional after solubilization with 5 mM CHAPS and 4 mM $C_{12}E_9$ [32], demonstrated by modulation of [^{3}H]muscimol binding by etomidate. In a later study, hetero-oligomeric $GABA_A$-receptors were expressed in HEK293 cells and functionally

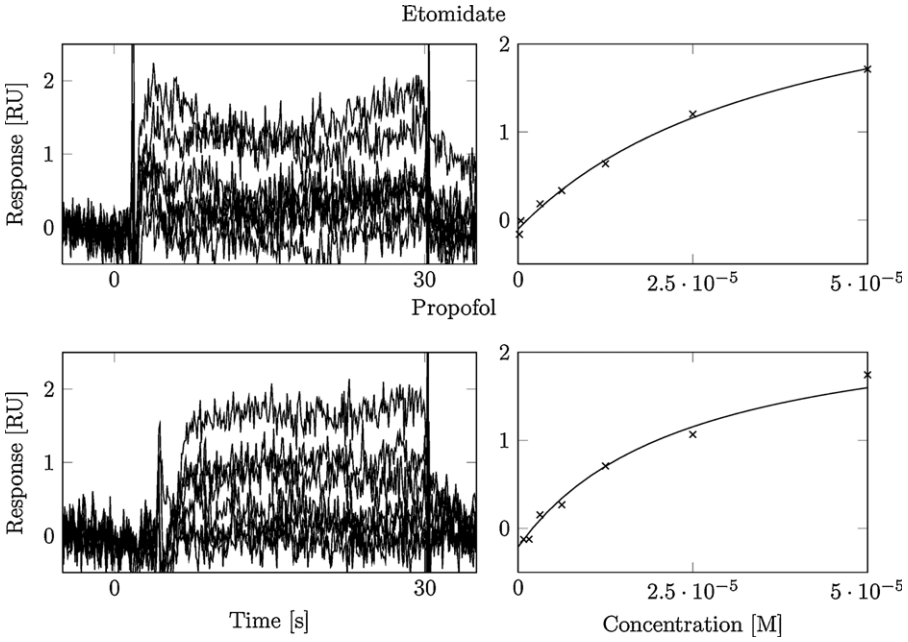

Fig. 4. Interaction of the general anesthetics etomidate and propofol with immobilized β3 $GABA_A$ receptors. Left: sensorgrams from injection of 2-fold dilution series from 0.8 to 50 μM. Right: steady-state signals plotted as a function of ligand concentration with a fitted Langmuir binding isotherm (solid line).

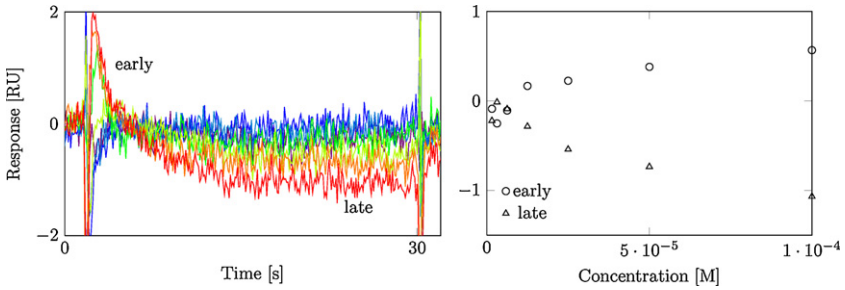

Fig. 5. Sensorgrams (left) of alfaxalone displaying secondary effects when injected over β3 biosensor surfaces. A concentration dependent increase shortly after the injection of alfaxalone (open circles) turns into a concentration dependent decrease of signal levels (open triangles).

solubilized by using 2.5 mM DDM [33]. A similar buffer was used for the present SPR-based interaction analysis. For receptor solubilization, DDM alone was not sufficient; instead the addition of lipids (POPC, DHPC) and another detergent (DOC) to the solubilization buffer was required. Generally, the immobilized β3 $GABA_A$ receptors were very sensitive towards different solubilization conditions, representing a great challenge during assay development. We were not able to obtain reliable data until we used the conditions for solubilization and interaction analysis described under Section 2.

It has recently been demonstrated that DOC, a bile acid, can block $GABA_A$ receptors with an IC_{50} of 0.17 mM, i.e. approximately 75-times lower than the detergent concentration used during solubilization [34]. In addition it has been demonstrated that detergent-solubilized $GABA_A$ receptors although retaining their ability to bind [^{3}H]muscimol or [^{3}H]flunitrazepam with high affinity, more or less rapidly lose their ability to exhibit pentobarbital modulation of [^{3}H]muscimol or [^{3}H]flunitrazepam binding [35]. Thus, a direct inhibition or loss of some drug binding sites by conformational changes induced in detergent-solubilized receptors cannot be excluded. Nevertheless, interaction with other drug binding sites can be reliably investigated as long as the data obtained are in agreement with those from more intact receptor preparations in the absence of detergents.

4.2. A direct binding assay for $GABA_A$ receptors

The pharmacological effect of a receptor agonist or antagonist is initiated by its binding to the receptor [36]. Today, several methods for measuring ligand-binding and its effects are available. For example, radioligand binding assays either directly measure binding of a high affinity radioligand, or indirectly measure the competitive displacement or allosteric inhibition of binding of this radioligand [10,21,36]. The effects elicited by receptor–ligand binding, for instance the chloride flux through $GABA_A$ receptors upon channel opening, can be measured by using electrophysiological techniques, like patch-clamp [12,37].

The developed SPR biosensor assay measures direct binding between receptor and ligand and the obtained binding signal is not dependent on induced effects, e.g. channel opening and ion flux. In contrast to a direct radioligand binding assay, SPR biosensor assays are able to measure weaker interactions with high micromolar affinities, and do not require labeling of any of the interacting partners. An additional advantage of the SPR based method is the high throughput that can be achieved. It was possible to perform roughly 200 ligand injections (≈20 h) on a biosensor surface until a new preparation was required. This would be sufficient for identification of novel ligands by fragment library screening (≈1 × 10^3 compounds) [38,39]. Once identified, receptor–ligand

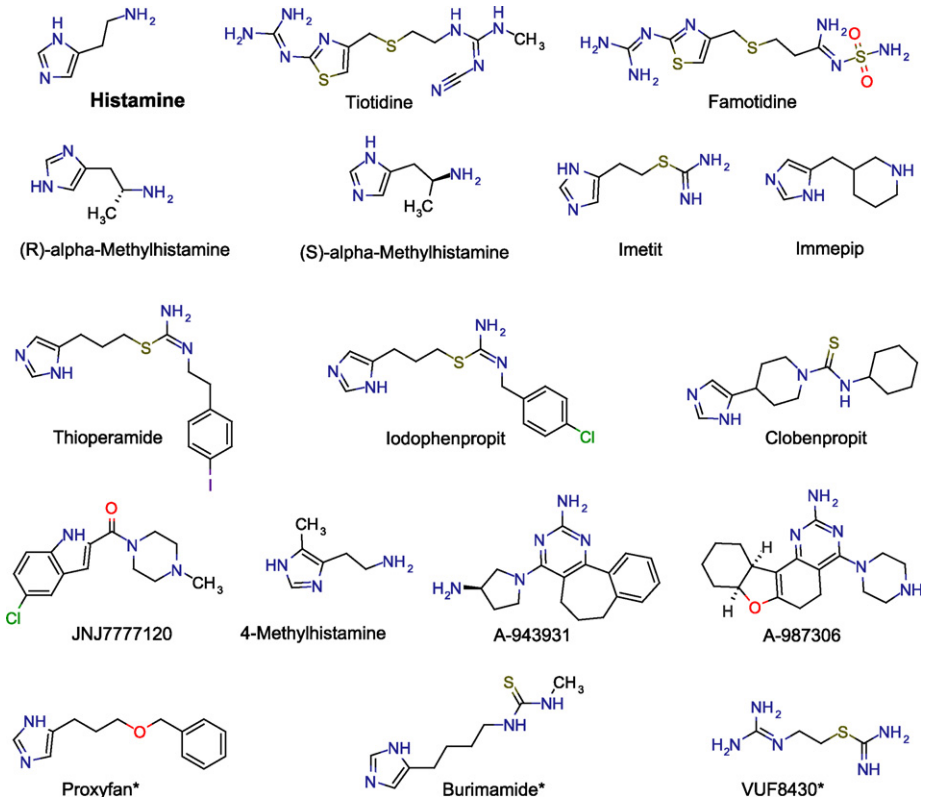

Fig. 6. Structures of histaminergic ligands interacting with homo-oligomeric β3 $GABA_A$ receptors and competing with histamine. Starred (*) ligands did not appear to compete with histamine.

interactions can be studied in more detail by the method described here and by established complementary methods with the ability to investigate the physiological effect of channel opening and potency of the respective compounds.

4.3. β3 $GABA_A$ receptors exhibit a distinct histaminergic pharmacology

It has been demonstrated previously that $GABA_A$ receptors display a distinct histaminergic pharmacology [12,13]. The results of the present study are in agreement with previous work and further expand the list of histaminergic ligands for the β3 receptor.

4.3.1. Interaction of β3 $GABA_A$ receptors with histamine H1 receptor ligands

The affinity of histamine was shown to be in the same order of magnitude as the median effective concentration (EC_{50}) on human homo-oligomeric β3 receptors, determined in electrophysiological studies of *Xenopus laevis* oocytes [12]. Furthermore, the affinity of histamine is similar to its pK_i at human H1 and H2 receptors ($K_D = 4.0 \pm 0.2$ (β3), $pK_i = 4.2 \pm 0.1$ (H1), $pK_i = 4.3 \pm 0.1$ (H2) [24]). Direct binding of HTMT, a H1/H2 receptor agonist, was not detected, despite previous data showing that HTMT blocks histamine-evoked currents with a median inhibitory concentration (IC_{50}) in the micromolar range [12]. The difference may be attributed to the different species of the recombinant β3 receptor subunits in the two studies, as has been described for β3 $GABA_A$ receptor subunits before [10,11], or to conformational changes induced in the detergent solubilized receptor. The lack of binding of mepyramine (pyrilamine) found in the present study is in agreement with previous results [12]. Other H1 receptor ligands tested, like astemizole and terfenadine, were not seen to bind in the present study.

4.3.2. Interaction of β3 $GABA_A$ receptors with histamine H2 receptor ligands

The determined K_D of famotidine, a H2 receptor antagonist, is similar to the IC_{50} for blocking histamine-evoked currents in *X. laevis* oocytes [12]. In studies with defined hetero-oligomeric $GABA_A$ receptors, expressed in human embryonic kidney 293 cells, both famotidine and tiotidine (H2 receptor antagonists) inhibited GABA-evoked currents with IC_{50} values close to the determined affinities of the present study [14]. However, homo-oligomeric β3 receptors were not included in this particular study. Competition between histamine and famotidine has been described previously [12] and was confirmed by the present SPR based interaction analysis. Similarly, it could be demonstrated that the combined signal of tiotidine and histamine in the SPR analysis was reduced compared to the sum of the individual signals of these compounds, indicating that these compounds compete for binding at β3 receptors as well. It has to be kept in mind, however, that the competition assay employed cannot distinguish between competition caused by the ligands binding to the same or different sites. In addition to famotidine and tiotidine, burimamide bound to β3 receptors but did not compete with histamine. No direct binding was detected for dimaprit (H2 receptor agonist) and cimetidine (H2 receptor antagonist), supporting previous results from electrophysiological studies [12].

4.3.3. Interaction of β3 $GABA_A$ receptors with histamine H3/H4 receptor ligands

Thioperamide, an H3/H4 receptor antagonist, was able to bind to homo-oligomeric β3 receptors and to compete with histamine binding. The K_D of thioperamide determined in this study was similar to the IC_{50} of this compound for the inhibition of histamine-evoked currents as demonstrated previously [12]. Both H3 and H4 receptors display stereoselectivity for (R)-α-methylhistamine over

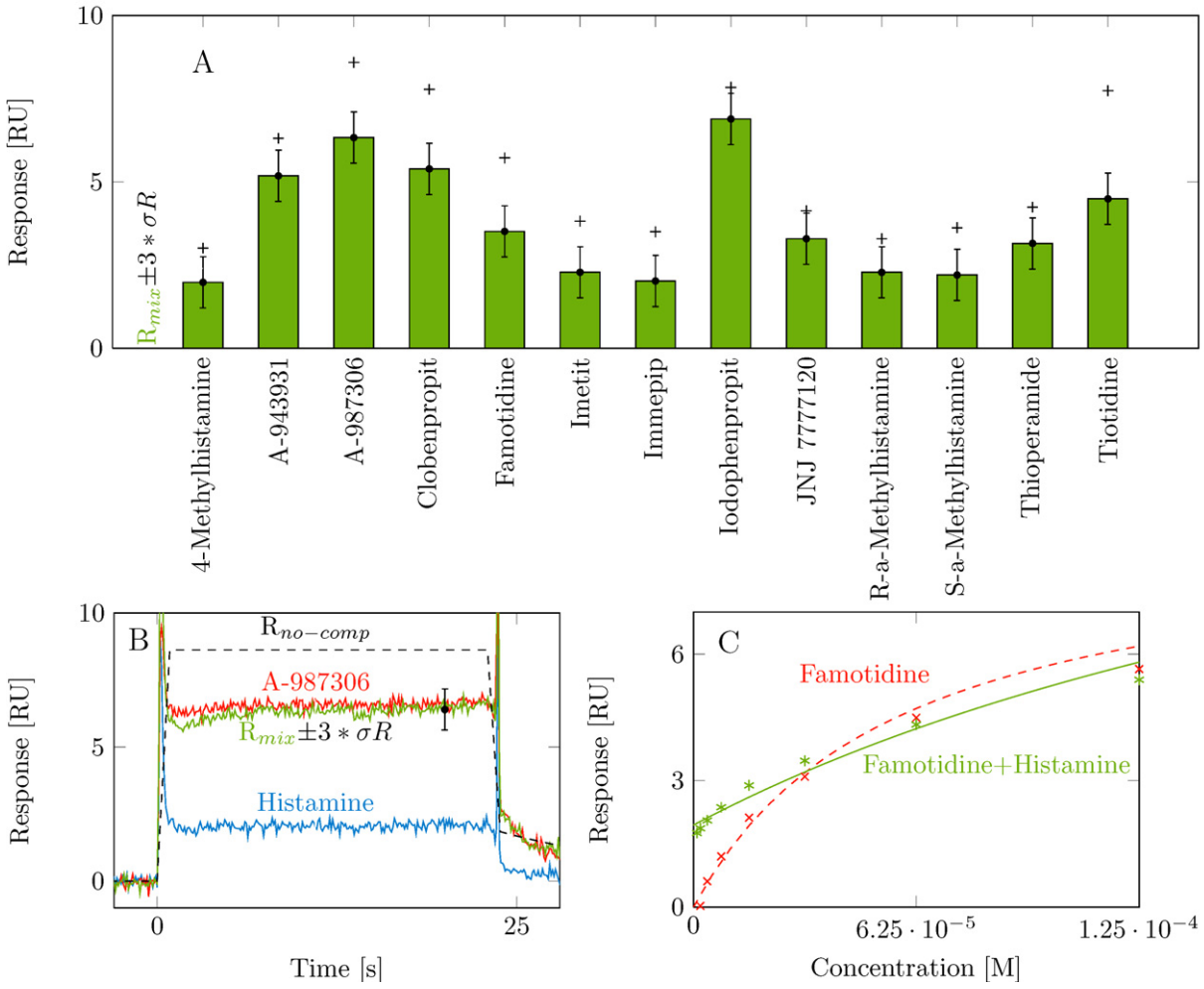

Fig. 7. Competition analysis of ligands competing with histamine. (A) Bars: signal levels from the injection of a mixture of 1 mM histamine and 50 μM of a ligand (R_{mix}). The standard deviation σR was taken from repeated histamine injections ($n > 15$). Points (+): sum of the individual signals of histamine and the ligand when injected separately, representing the theoretical signal under non-competitive conditions ($R_{no\text{-}comp}$). (B) Blank and reference-subtracted sensorgrams used for competition analysis, exemplified for A-987306. (C) Effect of 500 μM histamine on steady-state signals of famotidine injected in a 2-fold dilution series from 1 to 125 μM in the absence (dashed, x) and presence (solid, *) of histamine.

(S)-α-methylhistamine, and the level of stereoselectivity is higher at H3 receptors [24]. The stereoselectivity of β3 receptors favors the (S)- over the (R)-isomer, indicating distinct binding sites for these ligands at $GABA_A$ and histamine H3/H4 receptors.

4.3.4. Interaction of β3 $GABA_A$ receptors with histamine H4 receptor ligands

It is known that imidazole-derived H3 receptor ligands have considerable effects on H4 receptors [24]. Interestingly, binding to immobilized β3 receptors could also be detected for selective H4 receptor ligands. 4-Methylhistamine, the first selective and potent agonist to the H4 receptor [24], directly interacted with β3 receptors, as well as the H4 antagonists JNJ7777120, A-987306, A-943931 and VUF 8430. With the exception of VUF 8430, the other interacting selective H4 receptor ligands competed with histamine. Reference data for selective H4 receptor ligands acting at β3 receptors are not available.

4.4. Interaction analysis of GABAergic ligands

Although it has been shown that GABA does not act as an agonist on β3 receptors [10,11], the endogenous ligand was included in the present study since it was of interest to investigate whether GABA binds to homo-oligomeric β3 receptors but lacks the ability to open the channels. Binding of GABA was not detected up to a concentration of 100 mM, indicating that there is no detectable interaction between GABA and β3 receptors. This conclusion is supported by the absence of detectable interactions with other $GABA_A$ receptor agonists, such as muscimol, THIP, or 4-piperidine-sulfonic acid, of the $GABA_A$ receptor antagonist SR 95531, or of the $GABA_B$ receptor agonist baclofen. In addition, agonists (flurazepam) or antagonists (flumazenil) at the benzodiazepine binding site of hetero-oligomeric α1β3γ2 receptors did not bind to the homo-oligomeric β3 receptors. Both Ro5-4864 and PK-11195 are ligands at the peripheral benzodiazepine receptor, the mitochondrial 18 kDa translocator protein, where they bind with low nanomolar affinity. These compounds, however, also exhibit a micromolar potency for displacement of [^{35}S]TBPS from homo-oligomeric β3 receptors in receptor binding studies [10] and these results were confirmed in the present study by measuring direct interaction of these compounds with β3 receptors using the SPR technique.

In contrast to previous data [10], binding of alfaxalone and pentobarbital could not be reliably measured. Interaction data with pentobarbital were not reproducible. The signal levels seemed to be independent of the injected ligand concentration and the data could therefore not be rationally analysed. The difficulties in the detection of binding of pentobarbital or

alfaxalone might have resulted from non-accessible or conformationally distorted binding sites after solubilization of the receptor. Interaction data of alfaxalone displayed strong secondary effects that did not allow steady-state affinity or kinetic analysis (Fig. 5). Similar effects have been detected in SPR based interaction studies of AChBP and were mechanistically resolved as slow agonist-induced conformational changes [18]. However, there is insufficient information for a similar interpretation of the alfaxalone interaction.

Binding was detected for etomidate, propofol and etazolate with affinities similar to the potencies for displacing [^{35}S]TBPS from binding to homo-oligomeric β3 receptors determined in a previous study [10]. Propofol and etomidate seem to interact with binding pockets within the trans-membrane domain of $GABA_A$ receptors. Some of the β3 receptor binding sites at the trans-membrane domain are thus likely to be structurally intact after detergent solubilization. Propofol has recently been co-crystallized with the bacterial pentameric ligand-gated ion channel from *Gloeobacter violaceus* (GLIC) and it has been suggested that binding sites in the trans-membrane intra-subunit pockets of this receptor allow general anesthetics to modulate the function of pentameric ligand-gated ion channels [40]. Furthermore, the etomidate binding site, identified by photoaffinity labeling, is situated in the trans-membrane domain at the interface of α and β subunits of $GABA_A$ receptors [32]. A similar inter-subunit binding site might also be present in homo-oligomeric β3 receptors that do not contain α subunits.

None of the GABAergic ligands demonstrated to interact with β3 receptors in this study, including the two compounds known as mitochondrial translocator protein ligands, appeared to compete with histamine, possibly indicating that these ligands bind to different sites than histamine.

5. Conclusion

In the present study, a novel SPR biosensor assay was established that allowed the investigation of the direct interaction of histaminergic compounds with homo-oligomeric β3 $GABA_A$ receptors. Data obtained were in agreement with results of previous studies and extended the list of histaminergic compounds interacting with homo-oligomeric β3 $GABA_A$ receptors. Although the affinity of histamine for homo-oligomeric β3 receptors is in the same order of magnitude as the pK_i at human histamine H1 and H2 receptors, the K_D-values of specific histamine receptor ligands are roughly three orders of magnitude lower than the respective pK_i-values (Table 2) of native histamine H1–4 receptors. The histaminergic pharmacology of homo-oligomeric β3 $GABA_A$ receptors, thus, does not match with any of the four metabotropic histamine receptors, In addition, tiotidine and famotidine are derivatives of cimetidine, but neither activity [12] nor binding of cimetidine could be detected at β3 $GABA_A$ receptors. Together with the different stereoselectivity regarding (S)-α-methylhistamine and (R)-α-methylhistamine at β3 receptors, these findings further support a distinct structure of the histamine binding site of these ionotropic receptors.

So far, homo-oligomeric β3 receptors have not been identified in the mammalian brain due to a lack of suitable tools: a specific pharmacology for these receptors has not been identified and β3 subunit-specific antibodies cannot be used for detection, because they also identify hetero-oligomeric $GABA_A$ receptors containing β3 subunits. However, in heterologous expression systems homo-oligomeric β3 receptors are easily formed, making their existence in the brain very probable. In addition, there is pharmacological and physiological evidence for histamine receptors in mammalian brain that are yet unidentified [16]. In invertebrates, two histamine-gated chloride channels have been identified that belong to the Cys-loop receptor family and are closely related to $GABA_A$ and glycine receptors [16]. It is thus possible that homo-oligomeric β3 $GABA_A$ receptors formed in mammalian brain could be such histamine-gated ion channels. And histamine concentrations released at synapses [12] seem to be sufficient to activate these receptors, since their affinity for histamine is similar to that of human H1 or H2 receptors.

From the present study as well as from previous studies it is not expected that the currently available histaminergic drugs, due to their low affinity, are able to modulate homo-oligomeric β3 receptors at therapeutic concentrations [12,24]. Famotidine and tiotidine, two H2 receptor antagonists, have been shown to inhibit GABA-evoked currents, and it was speculated that these ligands can cause seizures or analgesia [14]. However, in the same study it was concluded that the IC_{50}-values required were too high to cause such effects. This does not exclude, however, that high affinity drugs could be developed that modulate these homo-oligomeric β3 receptors. SPR biosensor based assays could help in the development of such drugs because they have the ability to measure low affinity interactions of fragments with receptors and are ideal tools for fragment-based drug discovery. Drugs with high affinity and selectivity for homo-oligomeric β3 receptors could answer the question whether these receptors do exist in the brain and provide new avenues for the development of therapeutically active compounds targeting this novel histamine binding site.

Acknowledgments

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