Molecular analysis of the interaction of the four histamine receptor subtypes with antidepressant and antipsychotic drugs

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Fakultät für Chemie und Pharmazie der Universität Regensburg

vorgelegt von
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aus Regensburg

2010

Der experimentelle Teil dieser Arbeit entstand in der Zeit von Januar 2007 bis April 2010 unter Leitung von Herrn Prof. Dr. R. Seifert am Institut für Pharmakologie und Toxikologie der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie – der Universität Regensburg.

Das Promotionsgesuch wurde eingereicht im September 2010.

Tag der mündlichen Prüfung: 29. Oktober 2010

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Danksagungen

An dieser Stelle möchte ich ganz herzlich all den Menschen danken, die zum Gelingen dieser Arbeit beigetragen haben:

Herrn Prof. Dr. Roland Seifert für die Gelegenheit, an einem so interessanten und vielseitigen Projekt arbeiten zu dürfen, für seine kompetente fachliche Anleitung, die hilfreichen Ratschläge und Ideen, für seine ehrliche und freundliche Art und seine konstruktive Kritik beim Verfassen dieser Arbeit. Vielen Dank für die entgegengebrachte Geduld und das Vertrauen!

Herrn Prof. Dr. Dr. Ekkehard Haen für seine wissenschaftlichen Anregungen im Bereich des Therapeutischen Drug Monitorings und die Erstellung des Zweitgutachtens,

Herrn Prof. Dr. Jörg Heilmann für die Übernahme des Amtes des Drittprüfers und Herrn Prof. Dr. Dr. Wolfgang Wiegrebe für den Vorsitz in der Prüfungskommission,

Frau Dr. Andrea Straßer für die Bereitstellung ihrer Expertise und das Molecular Modelling zum H_1 und H_4 Rezeptor sowie für die wissenschaftlichen Ratschläge,

Herrn Tobias Holzammer für die Durchführung des Molecular Modellings der H_2 Rezeptor-modelle und für die Hilfestellung bei der Interpretation der Aufnahmen sowie Herrn Prof. Dr. Stefan Dove für seine fachliche Unterstützung,

Herrn Michael J. Keiser von der University of California, San Francisco, USA für die Durchführung des *Similarity Ensemble Approach* und für die Hilfestellung bei der Interpretation der Aufnahmen,

Herrn Dr. Erich Schneider für die kritische Betrachtung meiner Arbeit, seine stete Geduld bei der Beantwortung zahlreicher Fragen und seinen tiefgründigen Humor,

der Histamin-„Truppe“ mit Dr. David Schnell, Dr. Patrick Igel, Miroslaw Lopuch und Dr. Hendrik Preuss für manche Hilfestellung und Diskussion und vor allem Irena Brunskole für die gewissenhafte Korrektur meiner Arbeit,

der DAAD-Austauschstudentin Rince Wong (Vancouver, Kanada) sowie meinen Wahlpflicht-praktikantinnen Sissi Auburger und Marjeta Sema für ihre Beiträge und ihren Einsatz,

meiner Bürokollegin Dr. Miriam Erdorf für unzählige anregende Diskussionen, lange unter-haltsame Laborabende, ihren Enthusiasmus und für die schöne gemeinsame Zeit auch außerhalb der Universität,

meinen Bürokollegen Dr. Nina Lotter, Dr. Corina Matzdorf und Dr. Hesham Taha sowie meinen Laborkollegen Dr. Martin Göttle, Dr. Melanie Hübner, Dr. Kathrin Nickl und Sarah Sutor für zahlreiche wissenschaftliche und nicht-wissenschaftliche Diskussionen und das angenehme Klima,

Herrn Prof. Dr. Frieder Kees für seine wissenschaftlichen Ratschläge und die zeitweise Betreuung, für sein organisatorisches Geschick und die heiteren Unterhaltungen in den Teepausen,

Herrn Prof. Dr. Jens Schlossmann für die Möglichkeit, auch nach dem Weggang von Herrn Prof. Dr. Seifert alle praktischen und theoretischen Arbeiten am Lehrstuhl abschließen zu können,

Frau Dr. Katharina Wenzel-Seifert für ihre vielen konstruktiven Ratschläge beim Erstellen meiner Präsentationen und die hilfreichen Diskussionen,

Frau Gertraud Wilberg für ihre Unterstützung bei den Western-Blots, für die Sf9-Zellkultur und für ihre stete Hilfsbereitschaft sowie Frau Astrid Seefeld für die Hilfestellung bei den GTPase-Assays,

Frau Rita Prenzyna für ihr jederzeit offenes Ohr und die stets freundliche Unterstützung bei allen organisatorischen Angelegenheiten,

allen Kollegen des Lehrstuhls für ihre Kollegialität, Hilfsbereitschaft und das gute Arbeitsklima,

meinen Eltern, meinem Bruder und meiner Schwägerin für ihre stete Unterstützung und den Rückhalt,

vor allem aber meinem Mann Tobias, auf den ich mich immer verlassen kann.

Per aspera ad astra

Seneca

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT_xR	serotonin receptor subtypes
αAR	α-adrenoceptor
AC	adenylyl cyclase
Akt	protein family important in mammalian cellular signalling
AMI	amitriptyline
AMO	amoxapine
ATP	adenosine 5`-triphosphate
βAR	β-adrenoceptor
B_{max}	maximum specific binding of a ligand
$[Ca^{2+}]_i$	intracellular concentrations of free calcium
cAMP	cyclic 3`,5`-adenosine monophosphate
CBZ	carbamazepine
CLO	clozapine
CLD	*N*-desmethylclozapine
CLN	clozapine *N*-oxide
COMT	catechol-*O*-methyltransferase
CPM	clomipramine
cpm	counts per minute
CPX	chlorprothixene
CPZ	chlorpromazine
CNS	central nervous system
D_XR	dopamine receptors
DA	dopamine
DAG	1,2-diacylglycerol
DAT	dopamine active transporter
DBP	dibenzepin
DDD	daily defined doses
DOPA	3,4-dihydroxyphenylalanine
DPM	desipramine
DXP	doxepin
ECL1, ECL2, ECL3	1^{st}, 2^{nd} and 3^{rd} extracellular loops of a G protein-coupled receptor
EC_{50}	agonist concentration which induces 50% of the maximum effect
EDTA	ethylenediaminetetraacetic acid (Ca^{2+}-chelator)
E_{max}	maximum response relative to histamine (1.00)

EPS	extrapyramidal-motoric symptoms
FLAG	octapeptide epitope for the labeling of proteins
FPZ	fluphenazine
$G\alpha_{i2}$	α-subunit of a G protein that inhibits adenylyl cyclase
$G\alpha_{q/11}$	α-subunit of a G protein that stimulates phospholipase C
$G\alpha_s$	α-subunit of a G protein that stimulates adenylyl cyclase
$G_s\alpha_S$	short splice variant of the G protein $G\alpha_s$
GABA	γ-amino butyric acid
GAIP	Gα-interacting protein, also classified as RGS19
Gβγ	βγ-subunits of a heterotrimeric G protein
GDP	guanosine 5'-diphosphate
gp	guinea pig
GPCR	G protein-coupled receptor
G protein	guanine nucleotide-binding proteins
GSK3	glycogen synthase kinase 3
[γ-^{32}P]GTP	[γ-^{32}P]guanosine 5`-triphosphate
[γ-^{33}P]GTP	[γ-^{33}P]guanosine 5`-triphosphate
GTP	guanosine 5`-triphosphate
GTPase	large family of hydrolases that bind and hydrolyze GTP
GTPγS	guanosine 5`-[γ-thio]triphosphate
h	human
H_1R, H_2R, H_3R, H_4R	histamine receptor subtypes
hH_2R-$G_s\alpha_S$	fusion protein of hH_2R and short splice variant of $G\alpha_s$
HA	histamine
HAL	haloperidol
HDC	histidine decarboxylase
ICL1, ICL2, ICL3	1st, 2nd and 3rd intracellular loops of a G protein-coupled receptor
IC_{50}	functional assay: antagonist (inverse agonist) concentration suppressing 50% of an agonist induced effect radioligand binding assay: ligand concentration inhibiting the binding of a radioligand by 50%
IMI	imipramine
IP_3	inositol-1,4,5-trisphosphate
K_d	dissociation constant (saturation binding assay)
kDa	kiloDalton
K_i	dissociation constant (competition binding assay)
K_b	dissociation constant (functional GTPase assay)
LMZ	levomepromazine

LOX	loxapine
LPM	lofepramine
mAChR	muscarinic acetylcholine receptor
MAO	monoamine oxidase
MAPK	mitogen-acitivated protein kinase
MEP	mepyramine
min	minute(s)
MIR	mirtazapine
MPT	maprotiline
mRNA	messenger ribonucleic acid
MRZ	mesoridazine
MSN	mianserin
NAMH	N^{α}-methylhistamine
NaSSA	noradrenergic and specific serotonergic antidepressant
n.d.	not determined
NE	norepinephrine
NET	norepinephrine transporter
NTL	nortriptyline
OLA	olanzapine
OPI	opipramol
pA_2	negative decadic logarithm of the concentration of antagonist that causes a concentration ratio of agonist of r=2
PCP	prochlorperazine
P_i	inorganic phosphate
PKA, PKB, PKC	protein kinases of the types A, B or C
PLC	phospholipase C
PMZ	promethazine
PPZ	perphenazine
PRX	paroxetine
PTL	protriptyline
r^2	coefficient of determination
RGS	regulator of G protein signalling
RIS	risperidone
rpm	revolutions per minute
SARI	serotonin antagonist and reuptake inhibitor
S.D.	standard deviation
SERT	serotonin transporter
Sf9	insect cell line of *Spodoptera frugiperda*

SMILES	simplified molecular input line entry specification
SNDRI	selective norepinephrine-dopamine reuptake inhibitor
SNRI	selective norepinephrine reuptake inhibitor
SRZ	sulforidazine
SSNRI	selective serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressants
TIO	tiotidine
TM1-TM7	numbering of transmembrane domains of a G protein-coupled receptor
TMP	trimipramine
Tris	tris(hydroxymethyl)aminomethan
TRZ	thioridazine

The author declares that the following work presented in this volume was written by none other than herself. Unless indicated otherwise, all work was performed or supervised by the author.

The statistics-based chemoinformatic approach in Chapter C.3 was performed by M. J. Keiser (Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA, USA).

Molecular modelling at H_1 and H_4 receptor described in Chapter C.4.3 to C.4.6 was performed by Dr. A. Straßer (Department of Pharmaceutical/Medicinal Chemistry II, University of Regensburg, D-93040 Regensburg, Germany), while molecular modelling at H_2 receptor described in Chapter C.4.1 and Chapter C.4.2 was performed by T. Holzammer (Department of Pharmaceutical/Medicinal Chemistry II, University of Regensburg, D-93040 Regensburg, Germany).

A. Introduction

A.1 General introduction to different classes of psychiatric drugs

Psychiatric medications *sensu stricto* are drugs used to influence the mental state of patients and to eliminate or reduce mental disorders. Various compounds for treatment are nowadays available and according to their effects on psychopathological symptoms they are classified into six main groups:

- Stimulants mostly exert excitatory effects on the brain and can improve physical and mental abilities such as wakefulness, alertness and locomotion over a short time. Well-known stimulants are legal ones like caffeine and nicotine, norepinephrine-dopamine reuptake inhibitors such as methylphenidate and bupropion available on prescription and illicit performance enhancers like amphetamine and cocaine. Therapeutically, they are used for the treatment of disorders such as attention deficit hyperactivity disorder, narcolepsy and as anorexigenics.
- Anxiolytics are substances with calming effects onto the psyche and help to manage anxiety or sleep disorders. Benzodiazepines and azapirones are frequently applied prototypes.
- Depressants – also called “downers” – diminish mental or physical functions or activities and are used as hypnotics, sedatives and anesthetics. Frequently applied depressant substances are ethanol and benzodiazepines, as well as diverse other classes of drugs (*e.g.* antihistamines, anticholinergics, β-adrenoceptor (βAR) antagonists, dissociatives, muscle relaxants or non-benzodiazepines).
- Mood stabilizers help attenuate sustained and intense mood shifts that emerge with bipolar and schizoaffective disorder. Drugs with mood stabilizing effects are *e.g.* anticonvulsants like valproic acid, lamotrigine and carbamazepine (CBZ) as well as lithium.
- Antidepressants are the most important drugs for the therapy of affective disorders and help managing clinical depression, anxiety and dysthymia as well as eating disorders and borderline personality disorder. Often applied prototypes are amitriptyline (AMI), doxepin (DXP) and opipramol (OPI) and mirtazapine (MIR) (Schwabe and Paffrath, 2009).
- Antipsychotics or neuroleptics are used for the treatment of psychosis such as schizophrenia and mania and help to diminish symptoms like hallucination or delusion. Most

commonly used antipsychotic drugs are for example promethazine (PTZ), haloperidol (HAL), risperidone (RIS) and olanzapine (OLA) (Schwabe and Paffrath, 2009).

Prescriptions of psychiatric drugs have increased dramatically in the last decade, especially for antipsychotics and antidepressants. While in 2008 279 Million "defined daily doses" (DDD) of antipsychotics (1999: 224 Million DDD) were prescribed, the prescriptions of antidepressants even increased to 974 Million DDD (1999: 385 Million DDD) (Schwabe and Paffrath, 2009). Not only the striking number of applications of these two psychiatric drug classes, but also their quantity of interaction sites in the organism (receptors, reuptake transporter and other targets), resulting in a wide variety of desired and unwanted effects, make investigations on these substances so interesting and important.

A.1.1 Distinction between depression and schizophrenia

About 10-20% of the human population is affected by depression at least once in their life and 1% of all adults suffer from schizophrenia which, therefore, makes them the most prevalent mental disorders. They are classified by a standard diagnostic classification system, the *International Statistical Classification of Diseases and Related Health Problems* (ICD) by the *World Health Organization* (WHO), currently in its 10th revision.

The term depression is derived from the Latin word *deprimere* ("to press down") and is affiliated to mood disorders. These affective disorders are categorized in Chapter V of ICD into groups F30-F39 and subclassified *inter alia* in unipolar disorders, such as depressive episodes (ICD-10 F32) and recurrent depressive disorders (ICD-10 F33), and bipolar affective disorders (ICD-10 F31). Typical symptoms of depression are very low mood, anhedonia, anxiety, worthlessness, hopelessness up to thoughts of death or suicide. Sometimes also physical complaints, such as insomnia as well as fatigue, headaches or weight gain as well as loss, or other psychopathological conditions like delusions and hallucinations occur. The bipolar disorder or manic-depressive disorder, besides the depressive episodes, also displays manic episodes, in which people experience an elevation of mood and increased activity, inadequate to the circumstances. Abnormal behavior like aggression and intolerance as well as impaired judgment are the consequence.

Often used expressions related to depression are:

- Dysthymia: a form of chronic, long-lasting and less severe major depressive disorder
- Atypical depression: characterized by mood reactivity, *i.e.* improved mood in response to positive events, excessive sleep and increased appetite
- Seasonal affective disorder: occurs in the less light-intense seasons autumn or winter and resolves with the beginning of spring
- Postpartum depression: an intense depression experienced by women after giving birth and can last up to three months

Although many efforts were made to elucidate the nature and causes of depression the understanding is still incomplete. Triggered by life events, also a hereditary component plays a prominent role. Some drugs for long-term use are known to cause and impair depressive symptoms. The treatment with antidepressants addresses the monoamine hypothesis and adjusts an imbalance of the neurotransmitters serotonin (5-HT), norepinephrine (NE) and dopamine (DA) (Chapter A.1.3.). Due to the diversity of effects of some therapeutically used antidepressants on the neurotransmitter systems, the sole cause of an imbalance seems unlikely and calls for other models. The common time of depression onset is between the age of 20 and 30 years. Its diagnosis is accomplished by reports of the patient or relatives and friends and a mental status exam. Possible treatment options are antidepressant medication and psychotherapy or counseling, less commonly deep brain stimulation, sleep deprivation and electroconvulsive therapy. Phototherapy and physical exercise can positively influence mood disorders, too.

The term schizophrenia, coined by the Swiss psychiatrist Eugen Bleuler in 1908 (Fusar-Poli and Politi, 2008), originates etymologically from the ancient Greek *σχίζειν* and *φρήν* and means “split mind” describing the distinct disorders of thinking, affect, perception, lethargy and personality. ICD defines schizophrenia in groups F20-29 together with several subtypes which distinguish in the specificity of symptoms. This disorder is often classified by positive symptoms referring to symptoms that are not experienced in normal life circumstances and include delusions, hallucinations and thought disorder. Negative symptoms are more unspecific and are normally also found in non-schizophrenic individuals affected by other diseases and requires differential diagnosis: poverty of speech (alogia),

blunted affect, lack of motivation (avolition), inability to experience pleasure (anhedonia) and lack of desire to form relationships (asociality). These symptoms mainly contribute to a poor quality of life.

Alike depression many efforts were made to clarify the nature and causes of schizophrenia, but the understanding is nevertheless still incomplete. Several investigations suggest a hereditary component, but also neurobiology, early environment, psychological and social processes may contribute. Further, some therapeutically used drugs may cause or worsen schizophrenic symptoms. The common time of onset is between the age of 20 and 30 years. It is affecting both sexes equally but in males often earlier in age. Like for depression, no laboratory test exists and the diagnosis is made by the patient's self-reported experiences and behavior observed by relatives and friends and a mental status exam. However, Carlsson coined the DA hypothesis in 1978 when he postulated a dopaminergic hyperactivity in schizophrenic subjects (Carlsson, 1978). Therefore, the main constituent of treatment is antipsychotic medication with the various types of available drugs primarily suppressing dopamine activity at DA receptors D_2R and D_4R (Chapter A.1.2.3). Additional psychotherapy and social intervention are also important instruments while electroconvulsive therapy may be indicated for treatment-resistant individuals. In more severe cases and episodes involuntary hospitalization is needed to reduce the risk for themselves and others. Due to the number of diversified symptoms and various affected drug targets, depression as well as schizophrenia may not only be the consequence of a single disorder but rather a combination of syndromes.

A.1.2 Examined antidepressant and antipsychotic drugs

In our investigations we examined 34 different drugs and metabolites employed in the treatment of depression and schizophrenia and listed them below by drug groups. We focused on an examination of previous developments. The more selective advancements such as selective serotonin-norepinephrine reuptake inhibitors (SSNRIs), selective norepinephrine reuptake inhibitors (SNRIs), selective norepinephrine-dopamine reuptake inhibitors (SNDRIs) and serotonin antagonist and reuptake inhibitors (SARIs) display fewer side effects.

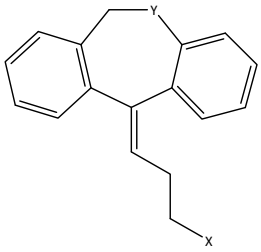
A.1.2.1 Antidepressants

For the treatment of mood disorders commonly drugs including tricyclic antidepressants (TCAs), tetracyclic antidepressants, monoamine oxidase (MAO) inhibitors, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors are used. The first antidepressant was imipramine (IMI), which was one of several iminodibenzyl compounds developed as antihistamine by Geigy Pharmaceuticals in the 1940s and structurally similar to the first true antipsychotic chlorpromazine (CPZ) synthesized in 1950. The antidepressant properties of IMI were discovered in 1957 by Roland Kuhn more or less by chance (Kuhn, 1957). Already in 1951, the antituberculosis medication isoniazide was found to enhance the well-being of moribund patients then dancing in the hallway (Robitzek *et al*., 1952). Numerous variants of TCAs with small variations in the structure were introduced in the following years. In the early 1970s the SSRI fluoxetine was developed, becoming one of the first blockbusters (Wong *et al*., 1975).

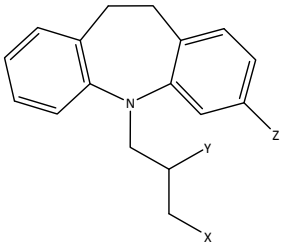
A.1.2.1.1 Non-selective antidepressants

A.1.2.1.1.1 Tricyclic antidepressants

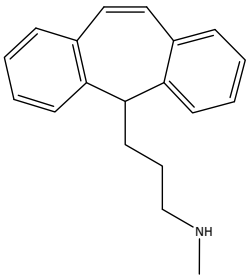
Tricyclic antidepressants block the reuptake of the neurotransmitters norepinephrine (NE) and serotonin (5-HT) and, thus, increase their concentrations in the synaptic cleft (Fig. A.1). They are named after their chemical structure (three aromatic rings) and are used for the treatment of major depressive disorder, dysthymia, bipolar disorder and a number of other medical disorders. Despite their consistent structural appearance the pharmacological effects of TCAs are widely varying and were, therefore, in the past categorized by Kielholz (1971) in groups of psychomotoric inhibiting, psychomotoric neutral and psychomotoric stimulatory antidepressants. This categorization is, however, strongly simplified and is not used anymore.



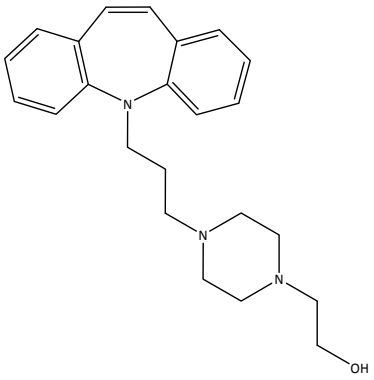
amitriptyline, AMI, X = $N(CH_3)_2$, Y = C
nortriptyline, NTL, X = $NH(CH_3)$, Y = C
doxepin, DXP, X = $N(CH_3)_2$, Y = O



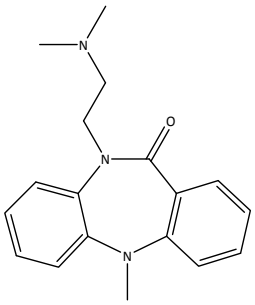
desipramine, DPM, X = $NH(CH_3)$, Y = H, Z= H
imipramine, IMI, X = $N(CH_3)_2$, Y = H, Z = H
clomipramine, CPM, X = $N(CH_3)_2$, Z = Cl
trimipramine, TPM, X = $N(CH_3)_2$, Y = CH_3, Z = H
lofepramine, LPM, X = $N(CH_3)CH_2COC_6H_4Cl$,
Y = H, Z = H



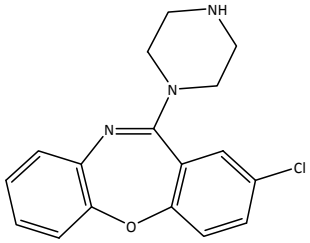
protriptyline, PTL



opipramol, OPI



dibenzepin, DBP



amoxapine, AMO

Fig. A.1. Structures of tricyclic antidepressants.

Besides the blockade of the monoamine reuptake, TCAs also modulate other receptors and produce many side effects like antimuscarinic effects such as dry mouth, constipation, blurry vision and cognitive disorders. Also, sedation as well as the modulation of food intake and weight gain may occur by histamine H_1R blockade, while reflex tachycardia and hypotension are α_1-adrenergic receptors (α_1AR) related, the latter increasing the risk of tumbling of older patients.

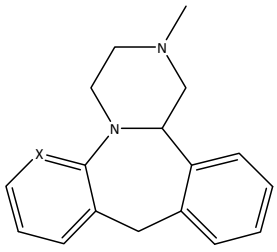
In contrast, trimipramine (TMP) is only a weak reuptake inhibitor of monoamines and is, therefore, often considered as atypically. Its main effects are exerted by a potent antagonism at serotonin 5-HT_2 receptors (5-HT_2Rs), α_1AR, muscarinic acetylcholine receptors (mAChRs) and histamine H_1 receptor (H_1R), less potent at 5-HT_1R, D_2R and α_2AR. The therapeutic effects like potent antidepressant activity, sedation and anxiolysis are accompanied by potent anticholinergic and antiadrenergic side effects. Due to its antagonism at D_2R, also

antipsychotic activity was observed with low incidence of extrapyramidal-motoric symptoms (EPS) (Eikmeier *et al*., 1991). OPI shows a high affinity to σ_1 receptor (Müller *et al*., 2004), but also acts as antagonist at 5-HT_2R, D_2R, mAChR and H_1R with a low to moderate affinity. In contrast to other TCAs, OPI does not inhibit the reuptake of 5-HT and NE.

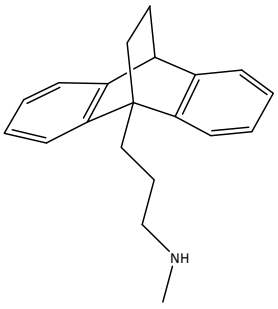
Bioavailability ranges between 50–80% and elimination half-life is varying substantially. The TCAs are effectively metabolized by cytochrome P450 2D6 hepatic enzymes, which implicates possible interactions with cytochrome P450-inhibiting substances leading to increased or even toxic plasma concentrations of TCAs. Due to a low lethal dose, the risk of abusive application of an overdose for the realization of suicidal thoughts is relatively high. However, TCAs are still used because of their effectiveness, especially in treatment-resistant variants. Despite the development of more selective drugs like selective serotonin reuptake inhibitors (SSRIs) with less frequent and intense side effects TCAs are – although prescribed less commonly – an important and effective medication, specifically in severe cases of major depression.

A.1.2.1.1.2 Tetracyclic antidepressants

Like the TCAs also tetracyclic antidepressants are non-selective monoamine reuptake inhibitors (Fig. A.2). They contain four heterocyclic rings of atoms, but apart from that share most of the properties with TCAs. Chemically, also mianserin (MSN) and MIR belong to this group but display also antagonistic α_2-adrenoceptor (α_2AR) properties which increase noradrenergic and serotonergic tonus. (Chapter A.1.2.1.2.2).



mianserin, MSN, X = CH
mirtazapine, MIR, X = N



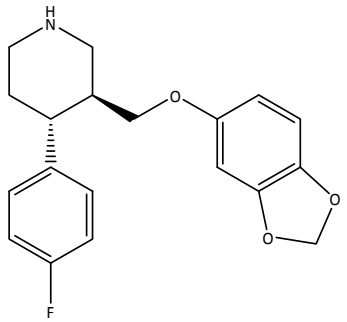
maprotiline, MPT

Fig. A.2. Structures of tetracyclic antidepressants.

A.1.2.1.2 Selective antidepressants

A.1.2.1.2.1 Selective serotonin reuptake inhibitors

Current standard in treatment of depressive disorders are SSRIs like fluoxetine, citalopram or paroxetine (PRX) (Fig. A.3). Their selective inhibition of 5-HT reuptake compensates the imbalance of serotonergic neurotransmission. Postsynaptic 5-HT_{1A}R and 5-HT_{2A}R and presynaptic autoreceptors may also be modulated by the increased neurotransmitter concentration in the synaptic cleft. Adverse effects such as nausea, diarrhea and changes in appetite are observed less frequently than with the more unselective TCAs or MAO inhibitors as affinity to αAR, muscarinic and histamine receptors (H_xR) are much lower, although effects as drowsiness, anxiety, insomnia, dry mouth, nervousness, decreased appetite, weight gain and several types of sexual dysfunction may still occur. Due to the serotonergic modulation a prevalent side effect is nausea or rather vomiting. A low affinity to H_1R prevents sedative effects. Thus, an intermittent benzodiazepine medication is indicated until onset of the antidepressive effects. The serotonin syndrome is an idiosyncratic adverse drug reaction occurring during therapeutic drug use of antidepressants. It is a potentially life-threatening consequence of exceeding serotonergic activity in central nervous system (CNS) and periphery and causes cognitive, autonomic and somatic effects. In children and adolescents administration of most SSRIs is contraindicated because of juvenile suicide (attempts).



paroxetine, PRX

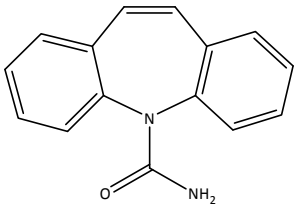
Fig. A.3. Structure of the selective serotonin reuptake inhibitor PRX.

A.1.2.1.2.2 Noradrenergic and specific serotonergic antidepressants

Although they chemically belong to tetracyclic antidepressants, MSN does not inhibit the reuptake of neurotransmitters and MIR does only marginally. Both substances rather act by antagonizing various receptors such as 5-HT_{2A}R, 5-HT_{2C}R and 5-HT_3R and H_1R. By blockade of presynaptic α_2AR at serotonergic and noradrenergic synapses the concentration of both neurotransmitters is increased so that they are designated as noradrenergic and specific serotonergic antidepressants (NaSSAs).

A.1.2.2 Mood stabilizers

Mood stabilizers are often also anticonvulsant substances like CBZ (Fig. A.4), lithium or valproic acid, which are administered for bipolar disorders alone or in combination with other medication. They are indicated for acute treatment and long-term relapse prophylaxis, while they are ineffective for a depressive period. CBZ decreases excitability of the brain cells by stabilizing the inactivated state of voltage-gated sodium channels. Due to an induction of CYP450 enzymes in the liver CBZ displays a very high potential for drug interactions, like decreasing the blood concentrations of valproic acid, warfarin, phenytoin and theophylline.



carbamazepine, CBZ

Fig. A.4. Structure of the anticonvulsant and mood stabilizing drug CBZ.

A.1.2.3 Antipsychotics

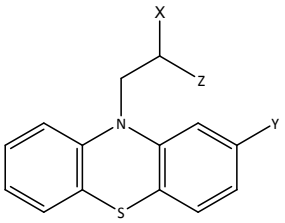
Rational psychopharmacology was developed in the early 1950s, when Paul Charpentier developed CPZ, a phenothiazine derivative which improved thinking and emotional behavior in psychotic patients and (Healy, 2004). CPZ was chosen as reference substance and its neuroleptic potency, *i.e.* the antipsychotic effect of a drug in comparison to its dose, was set to a value of 1. The earlier developed promethazine (PMZ) exhibits only a fraction of CPZ`s potency and is, therefore, mainly used as H_1R antihistamine. In the course

of time several advancements were made, differing in structure, neuroleptic potency, as well as efficacy and mechanism of action, respectively. Thus, a differentiation into typical and atypical antipsychotics – also known as first and second generation antipsychotics – was made.

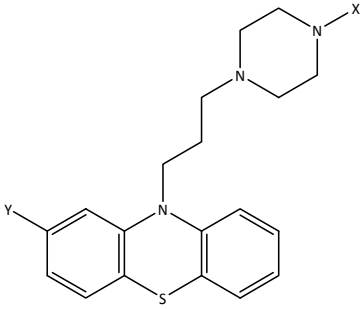
A.1.2.3.1 Typical antipsychotics

A.1.2.3.1.1 Phenothiazines

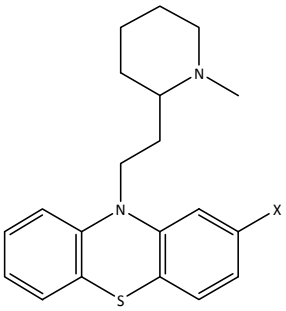
Based on the phenothiazine structure of CPZ (Fig. A.5) various analogues were developed with considerable differences in their neuroleptic potencies, *i.e.* their relative effectiveness at a certain given dose. The low-potency antetype CPZ exhibits only few EPS like akathisia, akinesia or pseudoparkinsonism but shows more effects by blocking H_1R (*e.g.* sedation), α_1AR (*e.g.* orthostasis) and muscarinic targets (*e.g.* dry mouth). By contrast, the highly potent fluphenazine (FPZ) shows also effects with low doses and produces less antihistaminic, α-adrenergic and anticholinergic effects but has a high incidence for EPS. Perphenazine (PPZ) is an antipsychotic with a medium potency, *i.e.* a CPZ-equivalency of fifteen. Although rare, reported side effects are the potentially lethal neuroleptic malignant syndrome and agranulocytosis manifested in a reduction of white blood cells.



promethazine, PTZ,
$X = N(CH_3)_2$, $Y = H$, $Z = CH_3$
chlorpromazine, CPZ,
$X = CH_2N(CH_3)_2$, $Y = Cl$, $Z = H$
levomepromazine, LMZ,
$X = CH_2N(CH_3)_2$, $Y = OCH_3$, $Z = (R)\text{-}CH_3$



prochlorperazine, PCP, $X = CH_3$, $Y = Cl$
perphenazine, PPZ, $X = CH_2\ CH_2OH$, $Y = Cl$
fluphenazine, FPZ, $X = CH_2\ CH_2OH$, $Y = CF_3$

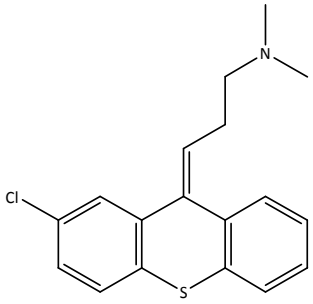


thioridazine, TRZ, $X = SCH_3$
mesoridazine, MRZ, $X = SOCH_3$
sulforidazine, SRZ, $X = SO_2CH_3$

Fig. A.5. Structures of first generation antipsychotics of the phenothiazine type.

A.1.2.3.1.2 Thioxanthenes

The chemically closely related thioxanthenes differ from the phenothiazines mainly by a carbon atom with a double bond to the side chain at position 10. The prototype chlorprothixene (CPX) (Fig. A.6), therefore, exhibits similar properties to the phenothiazines, the blockade of D_2R, 5-HT_2R, α_1AR, mAChR and H_1R contributing to the side effects. In contrast to other antipsychotics, EPS are observed rarely.

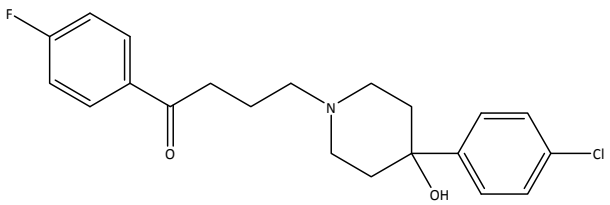


chlorprothixene, CPX

Fig. A.6. Structure of a first generation antipsychotic of the thioxanthene type.

A.1.2.3.1.3 Butyrophenones

The most widely used classical antipsychotic drug of the butyrophenone derivatives is haloperidol (HAL) (Fig. A.7), specifically acting against delusions and hallucinations. Due to its strong blockade of central antidopaminergic receptors in the mesocortex and the limbic system, it is classified as a highly potent neuroleptic. D_2R antagonism in the nigrostriatal pathways is liable for the high frequency of EPS and for the release of prolactin in anterior pituitary, the latter resulting in galactorrhea. The blockade of D_2R in the periphery accounts for its strong antiemetic activity, while the antihistaminic and anticholinergic properties often cause hypotension, dry mouth and constipation.



haloperidol, HAL

Fig. A.7. Structure of a first generation antipsychotic of the butyrophenone type.

A.1.2.3.2 Atypical antipsychotics

Atypical or second generation antipsychotics affect the brain's dopamine pathways preferentially *via* D_3R and D_4R, interact more with the limbic- than with the striatal system, and combine D_2R and 5-HT_2R antagonism, whereby they cause less EPS than typical antipsychotics. However, their mechanism of action is not fully understood and rather differs from drug to drug. A higher rate of responders, efficiency also in subjects with treatment-resistant schizophrenia, lower risk of suicides and an improved quality of life are distinguishing properties, especially for clozapine (CLO) (Fig. A.8). This dibenzodiazepine shows high affinity for the D_4R and interferes with other dopaminergic receptors only to a low extent. Its (side) effect profile is predominantly influenced by a strong antagonism at H_1R (causing sedation and weight gain), 5-$HT_{2A}R$ (antipsychotic action) and 5-$HT_{2C}R$ (weight gain), α_1AR (orthostatic hypotension) and mAChR (reduced EPS). A similar binding profile is exhibited by *N*-desmethylclozapine (CLD), which most likely contributes to CLO`s atypical effects. By contrast, clozapine *N*-oxide (CLN) shows little or no affinity to most targets. However, the beneficial therapeutic effects of CLO are contrasted by undesirable side effects. Besides a distinct weight-gain and cardiac toxicity CLO fell in disgrace because 1% of patients develop drug-induced agranulocytosis, an acute and severe suppression of the immune system with absolute neutrophil counts of less than 100 cells/µl blood. Closely related to CLO is loxapine (LOX) which is sometimes also classified as typical antipsychotic. Like its structure analogue it may cause hypersalivation by agonistic activity at M_4 in the salivary glands. Metabolization by *N*-demethylation generates amoxapine (AMO) which is classified as antidepressant but exhibits also antipsychotic properties (Apiquian *et al*., 2003). With olanzapine (OLA) and its higher affinity for 5-HT_2R compared to D_2R, as well as risperidone (RIS) and its high affinity for D_2R and several serotonin receptor subtypes, atypical antipsychotics with a more favorable side effect profile and less requirement for monitoring were developed, but the efficacy of CLO is still unrivaled.

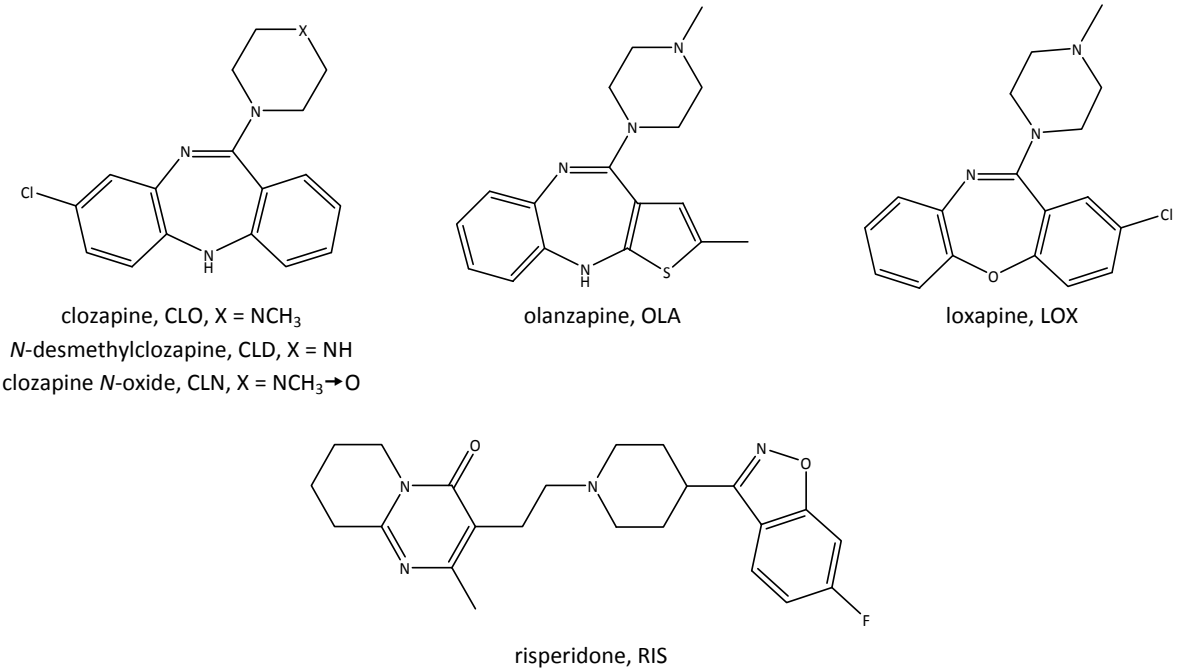


Fig. A.8. Structures of second generation antipsychotics.

A.1.3 Mechanisms of drug action

As already pointed out in the Chapters A.1.2.1 and A.1.2.3, psychiatric medications show very complex receptor profiles. The priority targets used in the treatment of depression and schizophrenia are presented in the following.

The majority of the TCAs act primarily as monoamine reuptake inhibitors by blocking the norepinephrine transporter (NET) and the serotonin transporter (SERT). Thereby, the extracellular concentrations of these neurotransmitters are elevated and further neurotransmission is enhanced. The affinity of TCAs for the dopamine transporter (DAT) is negligible and dopamine levels are, therefore, not influenced. The more selective SNRIs, SSRIs and SSNRIs act similarly on the specific transporters. MAO inhibitors block the enzymatical degradation of neurotransmitters by the monoamino oxidase and lead to increased neurotransmitters concentrations, too.

The source of the neurotransmitter 5-HT in brain are mainly neurons of the raphe nuclei, with a projection into the entire brain (Fig. A.9). Its prevalently inhibitory effects on the post-synaptic membrane influence emotion (particularly mood), appetite, sleep, sensoric perception, including pain, and higher cognitive functions like memory and learning. 5-HT is generated from tryptophan (Trp) *via* 5-hydroxytryptophan, which is catalyzed by the

enzymes tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase. It is transported and stored in vesicles which are released by exocytosis. Postsynaptically, 5-HT binds to 5-HT_1R coupled to G_i proteins or to 5-HT_2 receptors. Binding of 5-HT to presynaptic 5-HT_1R inhibits its own release. The action of the neurotransmitter is terminated by reuptake of 5-HT which can be blocked by tricyclic antidepressant drugs. Finally, 5-HT is degraded to 5-hydroxyindoleacetic acid by MAO and aldehyde dehydrogenase.

Additionally to their reuptake inhibition, many TCAs act as high-affinity antagonists at the 5-HT_{2A}R, 5-HT_{2C}R, 5-HT_6R, 5-HT_7R, α_1AR and H_1R as well as mAChR contributing to their therapeutic efficacy as well as their unwanted side effects.

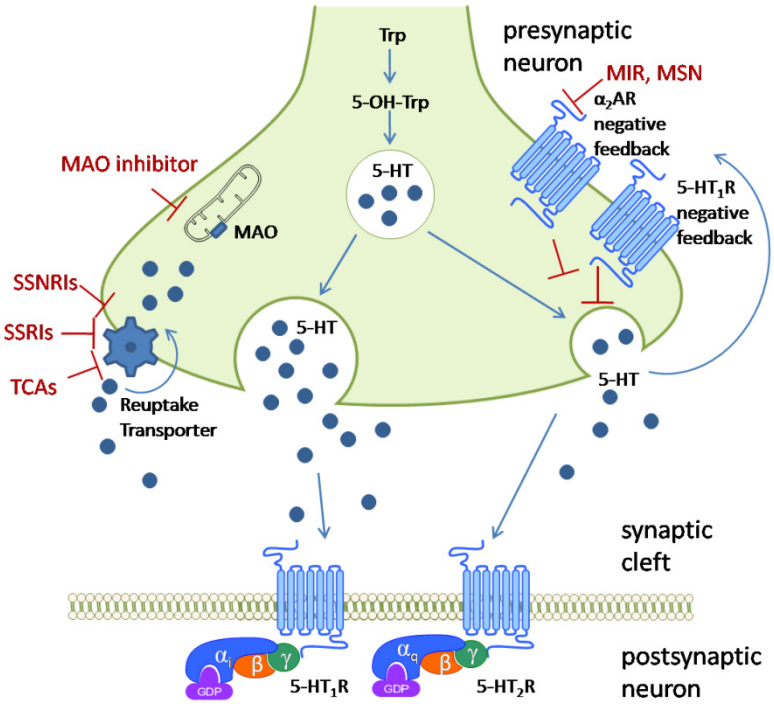


Fig. A.9. Serotonergic neurotransmission in the CNS and the priority targets used in the treatment of depression.

NE is synthesized largely in the locus coeruleus with projections to almost every other region of the nervous system and also in the lateral tegmental area with projections targeting the hypothalamus (Fig. A.10). It is involved in many general functions like emotion, sleep and wakefulness, neuroendocrine function, temperature regulation. Tyrosine (Tyr) is metabolized to DOPA, dopamine (DA) and finally NE, catalyzed by the enzymes tyrosine hydroxylase, DOPA decarboxylase and finally dopamine-β-hydroxylase. Vesicles transport and store NE, which is released by exocytosis. NE binds postsynaptically to α_1AR, which leads to the modulation of Ca^{2+} channels, as well as to mostly presynaptic α_2AR, which is linked to

adenylyl cyclase and modulates K^+ channels. Direct actions of the βγ-subunits of G proteins on K^+ channels are mediated by NE-activated α_2AR which leads to phosphorylation of ion channels. Binding of the neurotransmitter or MIR and MSN to presynaptic α_2AR inhibits further NE and 5-HT release by a negative feedback mechanism. Inhibition of presynaptic reuptake by antidepressants leads to a neurotransmitter surplus in the cleft and the following down-regulation of βAR and presynaptic α_2AR as well as up-regulation of α_1AR. Both antidepressants and antipsychotics display affinities to αAR mediating unwanted side effects like orthostatic hypotension and reflex tachycardia. NE action is terminated by its reuptake, blocked by antidepressant drugs. The degradation of NE is carried out by MAO or catechol *O*-methyl transferase (COMT).

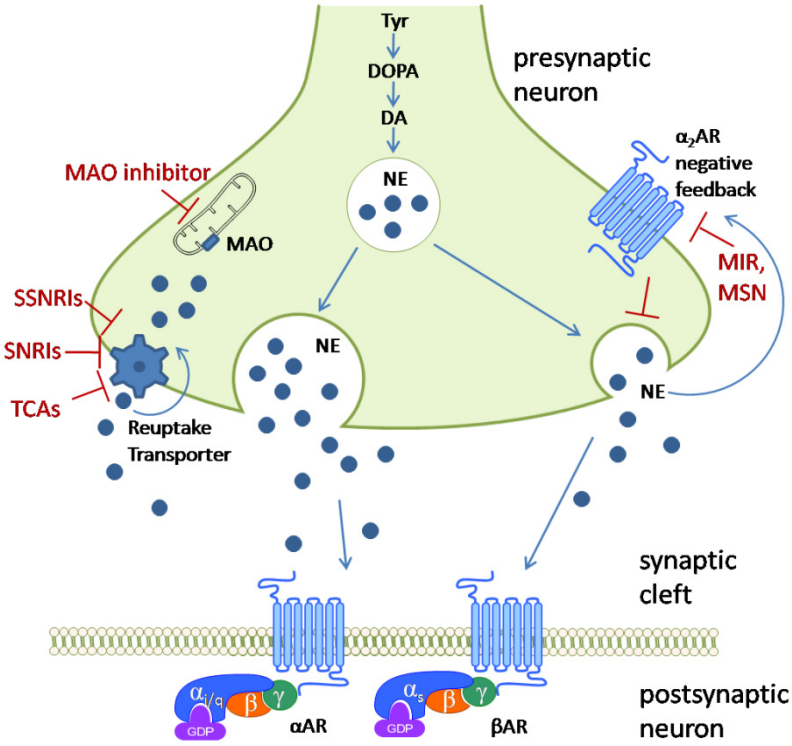


Fig. A.10. Noradrenergic neurotransmission in the CNS and the priority targets used in the treatment of depression.

Supplementary, the surplus of neurotransmitters through antidepressant therapy is also suggested to increase brain derived neutrophic factor, associated with neurogenesis and improvement of neuronal plasticity (Shirayama *et al.*, 2002; Eisch *et al.*, 2003). The adaptive changes by up- and down-regulation of receptors and neuronal plasticity may explain the delayed onset of two to six weeks of clinical effects of antidepressants.

DA is involved in both intellectual and motoric functions. It is synthesized enzymatically from Tyr *via* DOPA, primarily in the substantia nigra and ventral tegmental area (Fig. A.11). Projections reach to the basal ganglia (forming the nigrostriatal pathway) and the prefrontal cortex and amygdala (forming the mesolimbic pathway). Vesicles transport, store and release DA by exocytosis (inhibited by reserpine). Levels of the second messenger cyclic 3´,5´-adenosine monophosphate (cAMP) increase by the binding of DA to receptors of the D_1R family, coupled to the stimulatory G protein (G_s), or decrease by activation of inhibitory G proteins (G_i) by members of the D_2R family, prevalent in nigrostriatal and mesolimbic areas. Binding of the neurotransmitter to presynaptic D_2R inhibits its own release *via* an inhibitory feedback. Typical antipsychotic drugs such as butyrophenones inhibit D_2R-transmission, whereas agonists such as apomorphine stimulate D_2R and can produce schizophrenic-like behavior. DA action is terminated by reuptake into the synapse and inactivation by MAO and COMT.

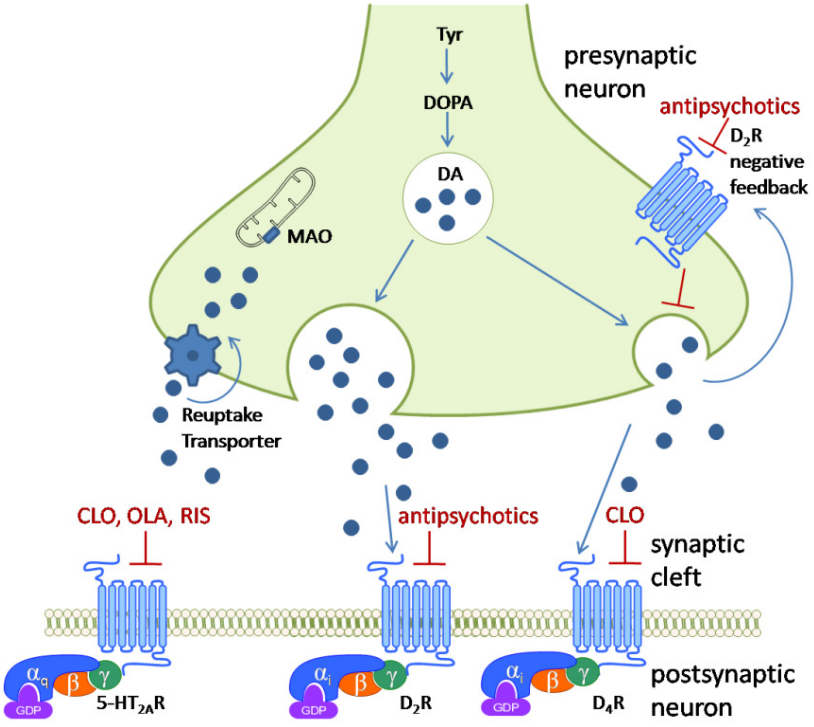


Fig. A.11. Neurotransmission in the CNS and the priority targets used in the treatment of schizophrenia.

The stimulation of the mesolimbic projection in prefrontal cortex and amygdala causes pleasure and, therefore, DA antagonists applied to the nucleus accumbens suppress reward systems. Parkinson's disease is caused by degeneration of nigrostriatal neurons and characterized by tremor, muscle rigidity, poor balance and difficulty in initiating movement

or even loss of the same (akinesia). Therapeutic occupancy at D_2R ranges hereby between 60 to 80%. EPS are mainly caused by exceeding this threshold. Atypical antipsychotics show a reduced occupancy here, including a fast dissociation rate of CLO and a higher affinity for serotonin 5-$HT_{2A}R$ over dopamine D_2R (Meltzer, 1999) or even partial D_2R agonism in the case of aripiprazole (Miyamoto *et al*., 2005). Furthermore, atypical antipsychotics differ in their ability to modulate dopaminergic D_1R, D_3R and D_4R and serotonergic 5-$HT_{1A}R$, 5-$HT_{2C}R$, 5-HT_3R, 5-HT_6R, 5-HT_7R (Lieberman *et al*., 2008; Miyamoto *et al*., 2005).

A.1.4 Methods for therapeutic drug monitoring

The term therapeutic drug monitoring (TDM) describes a field of clinical pharmacology focused on the determination of blood plasma or serum concentrations of diverse drugs for an optimized therapeutic dosage. Prerequisite herefore is a correlation of the measured plasma or serum drug concentration and the yielded pharmacological effect, in case of antipsychotics for example the improvement of schizophrenic symptoms as well as minimal side effects, like extrapyramidal reactions (Hiemke *et al*., 2004). Due to this given relationship TDM is deployed in many indication fields: antidepressants (TCAs, SSNRIs, SSRIs), antipsychotics (such as CLO, OLA, RIS), antiepileptics and mood stabilizers like CBZ and lithium, sedatives such as midazolam, the anesthetic thiopental and the anticonvulsant pentobarbital, aminoglycoside antibiotics like gentamicin, antimycotics such as imidazoles or echinocandins, or antiarrhythmic agents like amiodarone. Commonly, these drugs display a narrow “therapeutic index” so that insufficient levels result in undertreatment and extreme concentrations easily lead to tissue damage or toxicity, specifically for therapy of children or elderly. Particularly in the treatment with psychiatric drugs the problem of patient compliance is substantial and TDM helps to control a reliable intake. Also with regard to the pharmacoeconomical aspects monitoring the blood concentration can be valuable. The primary methods utilized for TDM are immunoassays like the heterogeneous radioimmunoassay or the homogeneous fluorescence immunoassay and enzyme immunoassay, and gas liquid chromatography (GLC) or the widely used high-performance liquid chromatography (HPLC) with various detection systems. Combined with mass spectrometry (MS or MS/MS) the limit of detection is reduced. With the help of chromatographic assays parent drugs and metabolites may be measured simultaneously, whereas immunoassay response is limited by antibody specificity or antibody cross-reactivity with a

view to the often applied polypharmacology, but their simple performance with automated instruments distributed them widely (Greiner, 2008). For optimization, the measured plasma concentrations are evaluated in comparison to literature-reported therapeutic reference ranges. As investigations for some drugs are incomplete, lacking target ranges may be compensated by plasma concentrations observed at therapeutic drug doses (Baumann *et al*., 2004).

Unfortunately, the brain as main target of antidepressant and antipsychotic therapeutics is not accessible for direct drug monitoring. The blood-brain barrier causes a variable penetration of the mostly lipophilic therapeutic agents to the brain. This may lead to imbalanced concentrations of drug in circulating blood and cerebrospinal fluid. Nevertheless, investigations in animals have shown that brain concentrations of atypical antipsychotics correlate with blood levels (Aravagiri *et al*., 1999). An investigation of the DA receptor occupancy in patients is achieved by positron emission tomography (PET) (Farde *et al*., 1988). Radioactive PET ligands like [^{11}C]raclopride and [^{18}F]fallypride are hereby displaced from D_2R binding by antipsychotics, amongst others *e.g.* by HAL (Fitzgerald *et al*., 2000), OLA (Kapur *et al*., 1998; De Haan *et al*., 2003) and RIS (Nyberg *et al*., 1995). The measured plasma concentrations of the drugs correlate well with the receptor occupancy. Therefore, plasma concentrations may be used as a valid measure of brain concentrations at its primary target structure (Hiemke *et al*., 2004).

A.2 General introduction to the family of histamine receptors

G protein-coupled receptors (GPCRs) are the largest and most multifaceted family of cell-surface receptors. Nearly 2% of the human genome is made up by this superfamily. Seven transmembrane (TM) characteristics are shown by about eight hundred genes assessed by hydrophobicity plots of amino acid sequences (Vassilatis *et al*., 2003). From a therapeutic point of view GPCRs have a very high impact, as drugs binding to them are beneficial for a variety of human diseases, including psychiatric disorders, pain, inflammation, asthma, obesity, cancer as well as cardiovascular, metabolic and gastrointestinal diseases. Approximately 50% of all modern drugs act on GPCR targets. However, only 40 GPCRs are affected by these drugs (Wise *et al*., 2004; Jacoby *et al*., 2006; Lagerström and Schiöth, 2008) which leaves an enormous potential for further research.

Upon binding a ligand on the extracellular side or in the transmembrane binding pocket the signal is transduced *via* a G protein into the cell (Fredriksson *et al.*, 2003). The seven transmembrane receptors are built up by an extracellular amino terminus, seven α-helical TM domains connected by three extracellular (ECL1, ECL2 and ECL3) and three intracellular (ICL1, ICL2 and ICL3) loops and an intracellular carboxyl terminus (Fig. A.12).

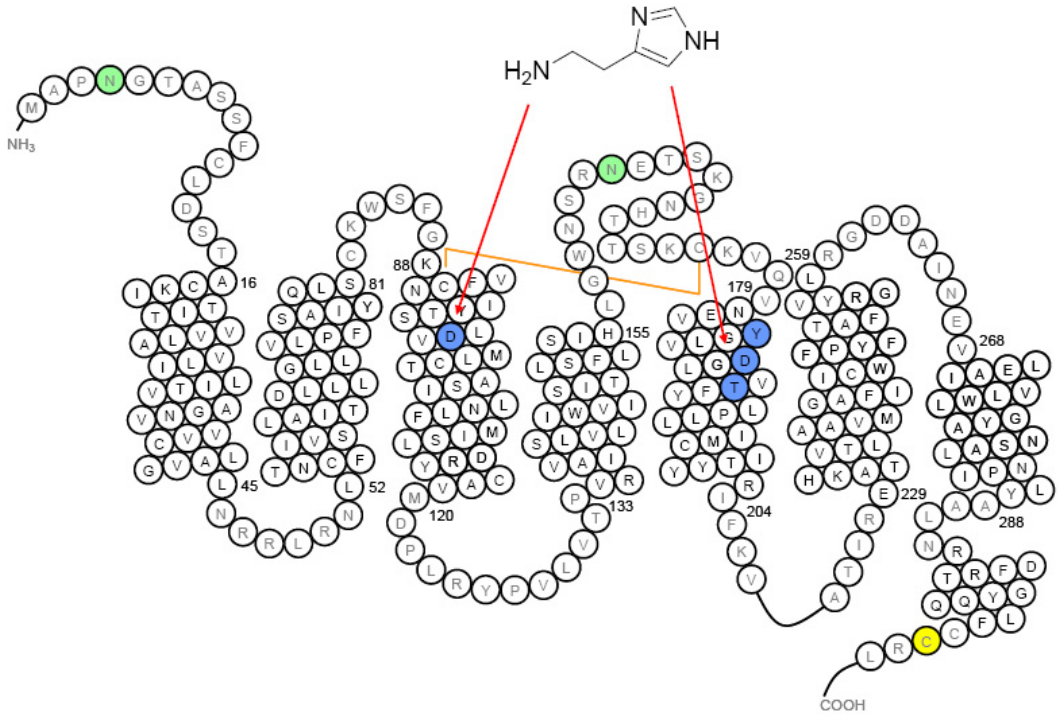


Fig. A.12. Snake representation of the human H_2R. N4 and N162 (green) are *N*-glycosylated and C305 (yellow) is palmitoylated. A disulfide bond between C91 and C174 is represented by an orange line. D98, Y182, D186 and T190 that probably interact with HA are colored in blue. Sixteen amino acids of ICL3 and 51 amino acids of the C-terminus are omitted for reasons of clarity. Adapted from Preuss *et al.*, 2007b.

Six families of GPCRs are to be distinguished phylogenetically. The HA receptors belong to family A (also family I or the rhodopsin-like family). This class contains receptors for odorants, small molecules such as biogenic amines, peptides and glycoprotein hormones. For their function, 20 highly conserved amino acids and a disulfide bridge between the first and second extracellular loop (ECL1 and ECL2) are crucial structural features. Most of the conserved amino acids are located in the cytoplasmic half of the protein. The seven α-helices are collocated counter-clockwise in the cell membrane when viewed from the extracellular side.

The generation of a high-resolution crystal structure of bovine rhodopsin was a breakthrough in GPCR research giving insight into the three-dimensional architecture of a

mammalian family A receptor (Palczewski *et al*., 2000). Recently, the first crystal structure of a human GPCR, the β_2-adrenoceptor ($h\beta_2AR$), was presented by Kobilka and co-workers (Rasmussen *et al*., 2007). By construction of a receptor/T4-lysozyme fusion protein an alternative high-resolution structure of $h\beta_2AR$ was yielded (Rosenbaum *et al*., 2007). However, all these GPCR structures display the receptors in their inactive state. Therefore, the determination of high-resolution receptor structures in the active-state will be the next challenge in crystallography of GPCRs. Nevertheless, GPCRs are dynamic in nature and crystal structures represent only snapshots of specific states. To learn more about the activation process of GPCRs on an atomic level, biophysical studies like nuclear magnetic resonance will have to supplement X-ray crystallography, (Ratnala, 2006; Kofuku *et al*., 2009). In the meantime, several different classic experimental approaches and molecular modelling techniques need to be combined to investigate the field of ligand binding, receptor activation and G protein/effector coupling for GPCRs.

A.3 The endogenous neurotransmitter and local mediator histamine

The first report of histamine (HA, 2-(1*H*-imidazol-4-yl)ethanamine) was its synthesis by Windaus and Vogt (1908). Two years later, Sir Henry Dale and colleagues isolated this amine from ergot (Barger and Dale, 1910). The pharmacological characterization was conducted in the following years (Dale and Laidlaw, 1910; Dale and Laidlaw, 1911; Dale and Laidlaw, 1919). These early investigations characterized the fundamental effects of HA, like the stimulation of cardiac contractility, stimulation of smooth muscles from the gut and respiratory tract and induction of shock-like syndrome when injected into animals. Isolated from liver and lung HA was first verified as an endogenous substance (Best *et al*., 1927). However, certain HA-effects such as the stimulation of gastric acid secretion were not inhibited by the prototypical “antihistamines”. Therefore, the existence of two distinct HA receptor subtypes was postulated (Ash and Schild, 1966). This was confirmed by synthesis of burimamide, a compound that competitively antagonized HA-induced gastric acid secretion (Black *et al*., 1972). In the early 1980s, a third histamine receptor subtype was predicted when studies on rat cerebral cortex showed that HA inhibited its own release not antagonizable by known antihistamines (Arrang *et al*., 1983). In the 1990s, advancements of molecular biology enabled cloning of the H_1R (Yamashita *et al*., 1991), the H_2R (Gantz *et al*., 1991b) and later on also of the H_3R (Lovenberg *et al*., 1999). At the turn of the millennium,

Oda and co-workers identified and cloned the sequence of an additional HA receptor and termed it H_4R (Oda *et al*., 2000) which was confirmed independently by other groups almost simultaneously (Nakamura *et al*., 2000; Liu *et al*., 2001; Morse *et al*., 2001; Nguyen *et al*., 2001; Zhu *et al*., 2001; O'Reilly *et al*., 2002).

HA receptors belong to family A of GPCRs and are classified in four subtypes: H_1R, H_2R, H_3R and H_4R. The average sequence homology between the subtypes is around 20%. H_3R and H_4R share the highest overall sequence homology of about 40%. An overview of the most important properties of histamine receptors is given in Table A.1.

As a ubiquitous messenger molecule HA is one of the most important local mediators and neurotransmitters. High concentrations of HA are found in the skin, the gastrointestinal tract and the lung. Mast cells and basophils store HA in specific granules. In response to various immunological or non-immunological stimuli these can release the neurotransmitter rapidly in large amounts by degranulation. For the regulation of gastric acid secretion HA is also produced in enterochromaffin-like cells. Within the CNS, HA is stored in vesicles of histaminergic neurons, located exclusively in the tuberomamillary nucleus of the posterior hypothalamus (Haas and Panula, 2003). These neurons are involved in the regulation of fundamental brain functions such as sleep/wakefulness, energy homeostasis and cognition (Haas and Panula, 2003). The “neo-synthesized HA” is released without prior storage. The production modulated by cytokines was found in hematopoietic cells, dendritic cells, macrophages, platelets and T cells.

H_1R	
Gene localization	3p25
Amino acids	487
Agonists	2-methylhistamine, 2-(3-trifluoromethylphenyl)histamine, histaprodifen(s)
Antagonists/inv. agonists	chlorpromazine, chlorpheniramine, mepyramine, cetirizine, astemizole, clemastine, terfenadine, loratadine, triprolidine
Expression pattern	airway and vascular smooth muscles, nerve cells, hepatocytes, endothelial and epithelial cells, neutrophils, hematopoietic cells
Signal transduction	coupling to $G\alpha_{q/11}$, PLC↑, production of IP_3 and DAG, $[Ca^{2+}]_i$↑, PKC↑
(Patho)physiological functions	rhinitis, conjunctivitis, urticaria, asthma, anaphylaxis, bronchoconstriction and vascular permeability in the lung, immune response
H_2R	
Gene localization	5q35.2
Amino acids	359
Agonists	dimaprit, amthamine, impromidine, arpromidine
Antagonists/inv. agonists	cimetidine, raniditine, tiotidine, famotidine, aminopotentidine
Expression pattern	gastric parietal cells, right atrial and ventricular muscle, airway and vascular smooth muscles, nerve cells, promyelocytic leukemic cells, hematopoietic cells
Signal transduction	coupling to $G\alpha_s$, AC↑, [cAMP]↑, protein kinases↑, $[Ca^{2+}]_i$↑; alternative coupling to $G\alpha_{q/11}$ in some systems
(Patho)physiological functions	gastric acid secretion, chronotropic and inotropic activity, vascular permeability, bronchodilation, hypotension, cell proliferation, differentiation, immune response
H_3R	
Gene localization	20q13.33
Amino acids	445
Agonists	(R)-α-methylhistamine, imetit, immepip
Antagonists/inv. agonists	thioperamide, ciproxyfan, clobenpropit, iodoproxyfan, JNJ-5207852
Expression pattern	histaminergic neurons
Signal transduction	coupling to $G\alpha_{i/o}$, AC↓, [cAMP]↓
(Patho)physiological functions	pre-synaptic autoreceptor (controlling HA release) and heteroreceptor (controlling release of dopamine, serotonin, norepinephrine, GABA, acetylcholine), obesity, attention deficit hyperactivity disorder, epileptic seizures
H_4R	
Gene localization	18q11.2
Amino acids	390
Agonists	OUP-16, iodophenpropit, imetit
Antagonists/inv. agonists	JNJ-7777120, thioperamide
Expression pattern	hematopoietic and immunocompetent cells, low expression in brain, liver and lung
Signal transduction	coupling to $G\alpha_{i/o}$, AC↓, [cAMP]↓, $[Ca^{2+}]_i$↑, MAPK↑
(Patho)physiological functions	chemotaxis in mast cells and eosinophils, control of IL-16 production by CD^{8+} lymphocytes, bronchial asthma, conjunctivitis, atopic dermatitis

Table A.1. Overview on human histamine receptors.

HA is synthesized by the enzyme L-histidine decarboxylase (HDC) by decarboxylation of the amino acid L-histidine in the cytosol. The vesicular monoamine transporter VMAT2 transports HA from the cytosol into the secretory granules (Kazumori *et al.*, 2004). Inactivation occurs by an oxidative deamination or methylation to imidazole-4-acetaldehyde and N^{τ}-methylhistamine. The histaminergic neurotransmission is illustrated in Fig. A.13.

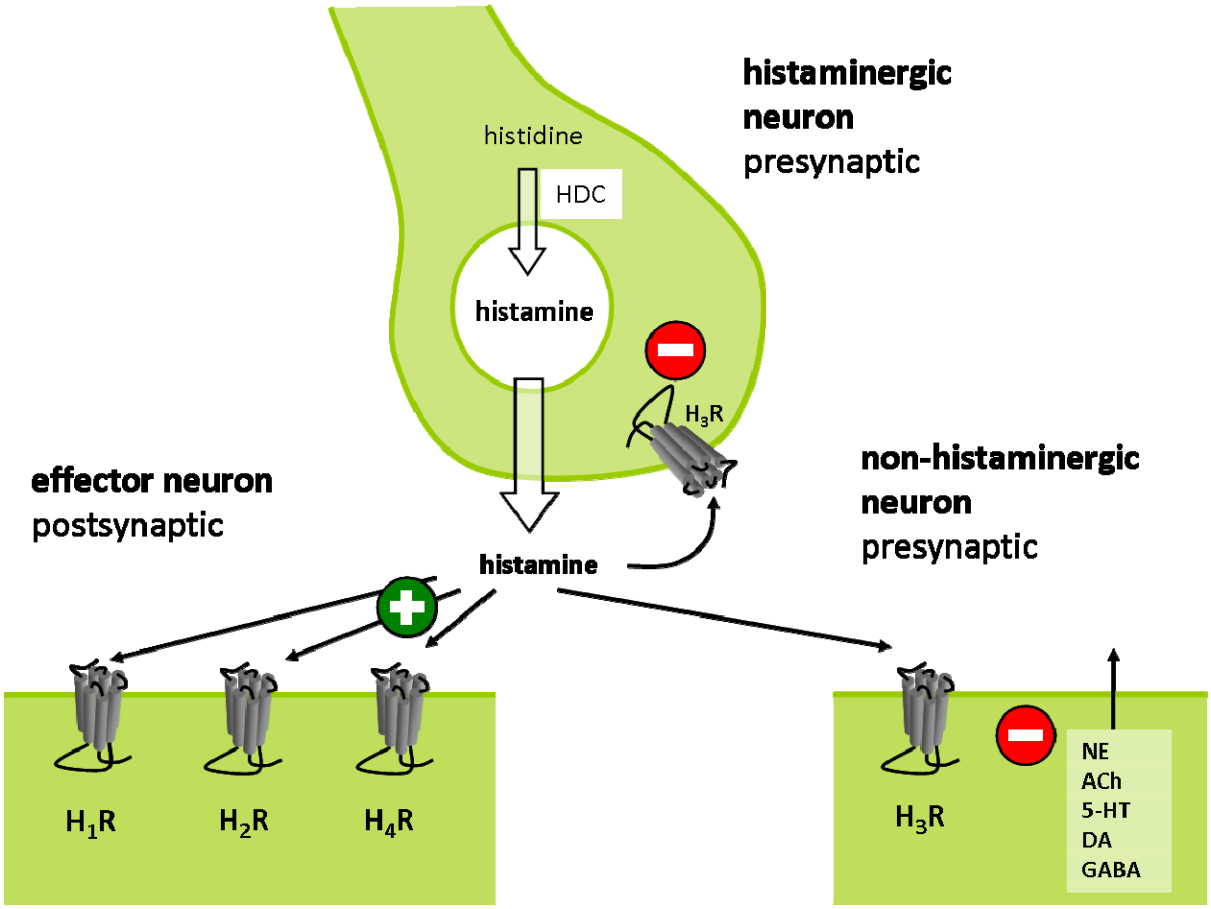


Fig. A.13. Histaminergic neurotransmission of H_xR in the nervous system. Modified from Schnell, 2010.

A.4 G protein-cycle and examination methods

When a ligand binds to a GPCR embedded in the cell membrane, the conformation of the GPCR changes and a G protein (inactive state) couples to the receptor. The thereby attained active state of the receptor protein then specifically interacts with a precoupled or free heterotrimeric G protein, consisting of a Gα-subunit and a Gβγ-heterodimer, located at the cytosolic side of the membrane. Guanosine 5'-diphosphate (GDP) is then released from the Gα-protein and a ternary complex between the agonist-bound active receptor and nucleotide-free G protein is formed. This complex is characterized by a high affinity for agonists. Subsequently, the binding of GTP to Gα activates the G protein complex, which

leads to a further conformational change and then dissociates into GTP-bound Gα-subunit and Gβγ-dimer, which can influence effector proteins and continue the signal cascade. Due to the intrinsic GTPase activity of Gα, the induced effector modulation is terminated after a certain period of time and GTP is hydrolyzed to GDP and P_i. After the cleavage of phosphate, the Gα- and Gβγ-subunit reassociate and the heterotrimer is ready to interact with another activated receptor. The G protein-cycle is illustrated in Fig. A.14.

The approach of radioligand binding assays takes advantage of low dissociation rate constants of high-affinity ligands, specifically for agonists at the ternary complex. This complex between the membrane-associated active receptor bound to an agonist and nucleotide-free G protein can be separated from free ligand by filtration through glass-fiber filters and determined by liquid scintillation counting. In the steady-state GTPase assay, a radioactively labeled GTP derivative is used. After binding to the Gα-subunit, [γ-^{32}P]GTP is hydrolyzed to GDP and radioactive $^{32}P_i$ by the intrinsic GTPase activity of Gα. The released amount of $^{32}P_i$ under steady-state conditions can be determined by liquid scintillation counting. In the GTPγS binding assay the GDP/GTP exchange at the Gα-subunit is determined kinetically. In contrast to [γ-^{32}P]GTP, [^{35}S]GTPγS cannot be hydrolyzed by the Gα-subunit and subsequently, the [^{35}S]GTPγS-labeled Gα subunit accumulates. The complex of Gα-subunit/[^{35}S]GTPγS remains membrane-associated and cannot be filtrated through glass-fiber filters. The [^{35}S]GTPγS remaining on the filters can be determined by liquid scintillation counting (Harrison and Traynor, 2003).

Activity of G proteins is also receptor independently modulated by a family of proteins named regulators of G protein-signalling (RGS). These proteins may accelerate the rate-determining hydrolysis of Gα-bound GTP to GDP and P_i and the following reassociation of Gα/GDP- and Gβγ-subunits (Neitzel and Hepler, 2006; Willars, 2006; Wieland *et al*., 2007).

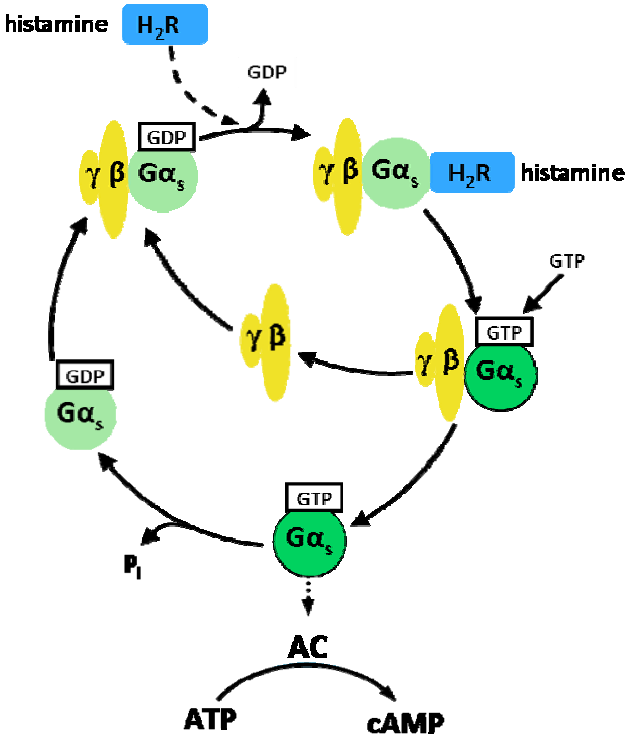


Fig. A.14. Gα protein activation/deactivation-cycle after GPCR stimulation of the H_2R by an agonist. Adapted from Seifert, 2005.

Continuous or repeated stimulation of a GPCR by an agonist often results in desensitization and, a loss of cellular sensitivity by phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs). This is followed by β-arrestin binding and uncoupling of the G protein, and GPCR internalization *via* clathrin-coated pits. The receptor then can return to the plasma membrane (receptor recycling) or is degraded in lysosomes (Hanyaloglu and von Zastrow, 2008).

Heterotrimeric G proteins may be divided into four classes: $G_{i/o}$, G_s, $G_{q/11}$ and $G_{12/13}$. The different subtypes of activated Gα-subunits can selectively stimulate ($G\alpha_s$) or inhibit ($G\alpha_{i/o}$) adenylyl cyclase, activate phospholipase Cβ ($G\alpha_{q/11}$) or interact with guanine nucleotide exchange factors ($G\alpha_{12/13}$). Thereby, the production of second messengers such as cAMP, inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) is modulated. In the consequence, a fast cellular response is induced, such as the regulation of enzyme activity or change in intracellular ion concentrations. The second messenger cAMP can activate protein kinase A (PKA) or the mitogen-activated protein kinase (MAPK) pathway both modulating gene expression. Moreover, by interacting directly with phospholipase Cβ, AC or certain ion channels activated Gβγ-dimers can also trigger cellular effects (Birnbaumer, 2007).

A.5 Two-state model and constitutive activity

To describe the interaction between a GPCR, the G protein and a ligand mathematically, different models have been developed based on the law of mass action. In the ternary complex model, the activation of the G protein requires the binding of an agonist to the receptor. However, it was found that GPCRs can be spontaneously active, a phenomenon referred to as constitutive activity (Seifert and Wenzel-Seifert, 2002). Constitutive activity is observed in many wild-type GPCRs, *e.g.* β_2AR, 5-$HT_{2A/C}$R, H_4R and the formyl peptide receptor (Gether *et al*., 1995; Seifert and Wenzel-Seifert, 2003; Berg *et al*., 2008; Schneider *et al*., 2009). GPCR mutations with increased constitutive activity might be a source of some diseases (Seifert and Wenzel-Seifert, 2002). The existence of constitutive receptor activity was integrated in the extended ternary complex model (ETC model) (Lefkowitz *et al*., 1993; Samama *et al*., 1993) which is also referred to as the two-state model of receptor activation (Leff, 1995). This model claims that GPCRs can isomerize from an inactive state (R) to an active state (R*) independently of agonist binding (Fig. A.15 A). A receptor in the R* state binds and activates G proteins, resulting in a cellular response. According to the two-state model, ligands can be classified as agonists, neutral antagonists and inverse agonists (Fig. A.15 B). Agonists stabilize the active R* state, inverse agonists the inactive R state of a GPCR. Partial agonists or inverse agonists possess a lower efficacy towards G protein activation or inhibition, relative to the endogenous (full) agonist which produces a maximum biological response (efficacy). Neutral antagonists do not possess any intrinsic activity but antagonize the effects of agonists and inverse agonists competitively.

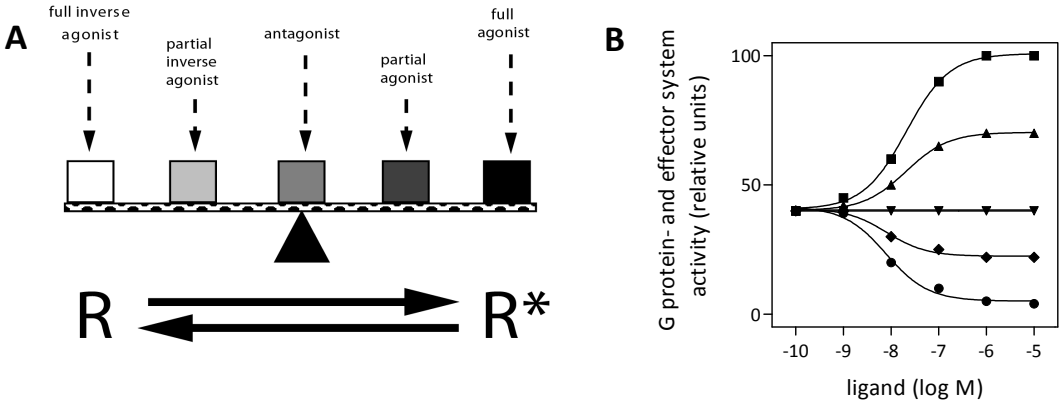


Fig. A.15. The two-state model of GPCR activation. A, GPCRs are able to isomerize from an inactive state (R) to an active state (R*). Ligands are classified according to their capability of shifting the equilibrium to either side of both states. Adapted from Seifert, 2005. **B**, Differential responses in an effector system upon binding of full agonists (■), partial agonists (▲), antagonists (▼), partial inverse agonists (♦) and full inverse agonists (●). Adapted from Seifert, 2005.

A.6 Sf9 cells and various other histamine receptor model systems

Numerous methods are available to investigate ligand binding, receptor activation and G protein/effector coupling. Specific applications, advantages and disadvantages, are referred to each method. Various basic steps in signal transduction of a GPCR can be investigated with a baculovirus/Sf9 cell expression system (Fig. A.16) (Seifert, 2005). Derived from *Spodoptera frugiperda* pupal ovarian tissue, Sf9 cells are very suitable for protein expression, especially GPCRs (Aloia *et al*., 2009).

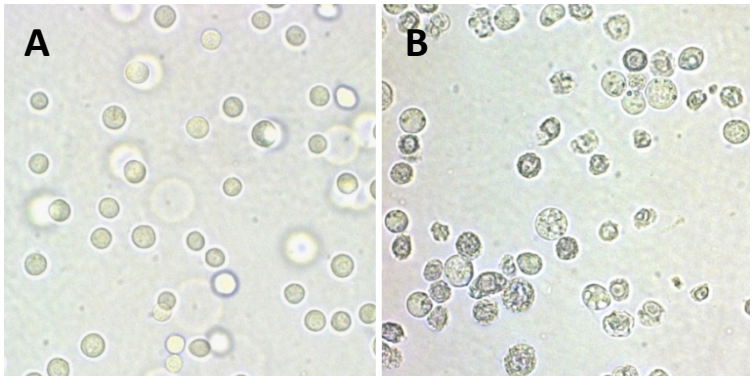


Fig. A.16. Uninfected Sf9 cells (A) and Sf9 cells after transfection with recombinant baculoviruses (B). Adapted from J. von der Ohe, Institute of Pharmacology, Medical School of Hannover.

Recombinant baculoviruses, double-strained DNA-viruses which infect only non-vertebrate hosts, are used as expression vectors (Preuss *et al*., 2007a; Schneider *et al*., 2009). The preferred system for large-scale recombinant protein expression is *Autographa*

lines. The BD BaculoGold™ linearized baculovirus DNA from BD Biosciences contains the DNA for a non-viable virus. A viable virus is reconstituted only by co-transfection of insect cells with the viral DNA and the construct included in the complementing transfer vector. The foreign cDNA to be expressed has to be cloned into the transfer vector (Fig. A.17). High expression levels can be achieved for a GPCR or G protein (Seifert *et al*., 1998; Ratnala *et al*., 2004; Schneider *et al*., 2009). A correct folding of the recombinant protein as well as disulfide bond formation are provided by this expression system. Endogenous constitutively active GPCRs or relevant amounts of other receptors are not expressed by Sf9 cells. Advantageous is also the excellent signal to noise ratio, which is caused by limited endogenous G protein signalling (Quehenberger *et al*., 1992; Wenzel-Seifert *et al*., 1998; Brys *et al*., 2000; Seifert and Wenzel-Seifert, 2003).

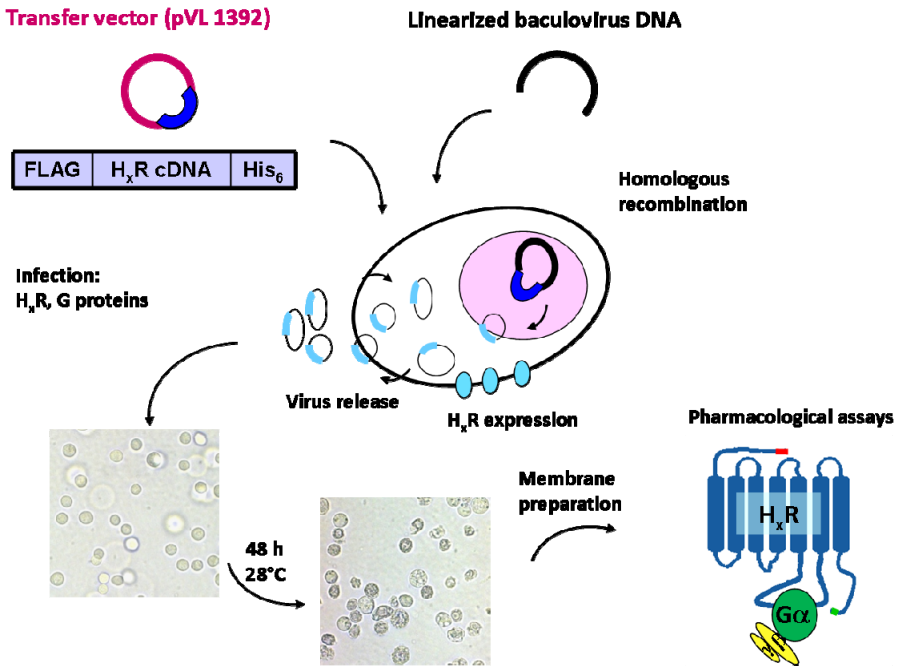


Fig. A.17. Generation of recombinant H_xR baculoviruses, protein expression and membrane preparation.

In this work, studies were exclusively performed with broken-cell preparations (membranes) and not whole cells. Thus, contaminations with agonists can be eliminated through centrifugation and resuspension of the membrane. The elimination of endogenous HA in whole cells or native brain tissue can be very difficult if not impossible. Otherwise, tissues derived from sterile–kept $HDC^{-/-}$ mice fed with HA-free food would be required.

The Chinese hamster ovary cell line (CHO) is a commonly used system for long-term, stable gene expression. The cells grow rapidly and yield high amounts of protein. For

investigating the H_2R, CHO cells deficient in dihydrofolate reductase were transfected with $pSVH_2$ as vector (Traiffort *et al*., 1992b). Upon exposure to the H_2R antagonists cimetidine and ranitidine the receptor was up-regulated time- and dose-dependently (Smit *et al*., 1996). In contrary, the human HL-60 promyelocytes constitutively express H_2R. Hence, investigations for this AC activating receptor are feasible at a non-artifical human model system. Furthermore, by differentiation with dibutyryl-cAMP, HL-60 leukemia cells additionally express H_1R (Seifert *et al*., 1992).

The COS cell line was generated by immortalizing kidney CV-1 cells of the African green monkey cell line with monkey virus SV40 (Jensen *et al*., 1964). Transiently transfected COS-7 cells produce recombinant proteins, for example tagged H_2R (Shayo *et al*., 2001). Also, a stable transfection of human H_3R or H_4R cDNA in human SK-N-MC neuroblastoma cells is possible (Lovenberg *et al*., 1999; Liu *et al*., 2001). For functional analysis of human H_4R (hH_4R) the cell line was additionally containing a cAMP-responsive element (CRE)-driven β-galactosidase reporter gene and cAMP accumulation was measured indirectly by absorbance readout of β-galactosidase activity (Liu *et al*., 2001). However, measurements in reporter gene assays may be susceptible for interference of other processes in signal transduction due to its distance to the actual receptor activation event. Human embryonic kidney cells (HEK 293) are cultured easily, transfected very readily and, therefore, widely used. Although derived of human origin, the transformation with DNA of adenovirus 5 made the HEK cells to a rather artificial model (Graham *et al*., 1977). Nevertheless, for observing single transfected genes and their expressed proteins, HEK cells are a feasible model system for various GPCRs, *e.g.* H_xRs or cannabinoid receptors (Morse *et al*., 2001; Hann *et al*., 2004; Geiger *et al*., 2010).

A.7 The histamine H_2 receptor in the brain

Histaminergic neurons arise from the tuberomamillary nucleus in the posterior hypothalamus and spread their axons all over the mammalian brain (Fig. A.18). All of the four known H_xRs are expressed in the CNS. They mostly control excitability and plasticity and serve for several functions like maintaining wakefulness and attention. By forming a network with other transmitter systems, also higher brain functions are controlled such as emotion, aggression, learning and memory, arousal, sleep/wake cycle, appetite and immunity (Watanabe and Yanai, 2001; Haas *et al*., 2008).

The expression of H_4R in distinct deep laminae and cortex in humans, mouse thalamus, hippocampal stratum lucidum and cerebral cortex was reported only recently and its function is still unclear (Connelly *et al*., 2009). The H_1R-mediated actions in brain were revealed early by the use of the classical antihistamines and characterization of the $H_1R^{-/-}$ mouse. But H_3R was associated with the brain from the very beginning and soon correlated with the release of other monoamines. The impact of H_2R on neurotransmission is still poorly understood. A reason for this may be that the only available selective H_2R antagonist zolantidine, which sufficiently penetrates the blood-brain barrier, was never introduced for a therapeutic use (Ganellin, 1992). Autoradiographic localization in guinea pig found the H_2R to be distributed heterogeneously in brain with high densities in basal ganglia, amygdala, hippocampus and cortex (Haas *et al*., 2008). The large association of H_2R with neurons (Pollard and Bouthenet, 1992) suggests that many postsynaptic actions of HA are mediated by this receptor (Ruat *et al*., 1990; Vizuete *et al*., 1997). Colocalizations of H_1R and H_2R in some regions indicate synergistic interactions of these two receptor subtypes. This was supported by the suppression of locomotor hyperreactivity induced by methamphetamine in $H_{1/2}$R-deficient mice (Ogawa *et al*., 2009). The H_2R antagonist cimetidine was also accounted for an anti-tumor activity against glioblastomas (Lefranc *et al*., 2006). Further, H_2R-deficient mice show selective cognitive disorders along with an interference of long-term potentiation in hippocampus (Dai *et al*., 2007; Haas *et al*., 2008) and an inhibition of the enhanced thalamic firing of nociceptive neurons (Mobarakeh *et al*., 2005; 2006).

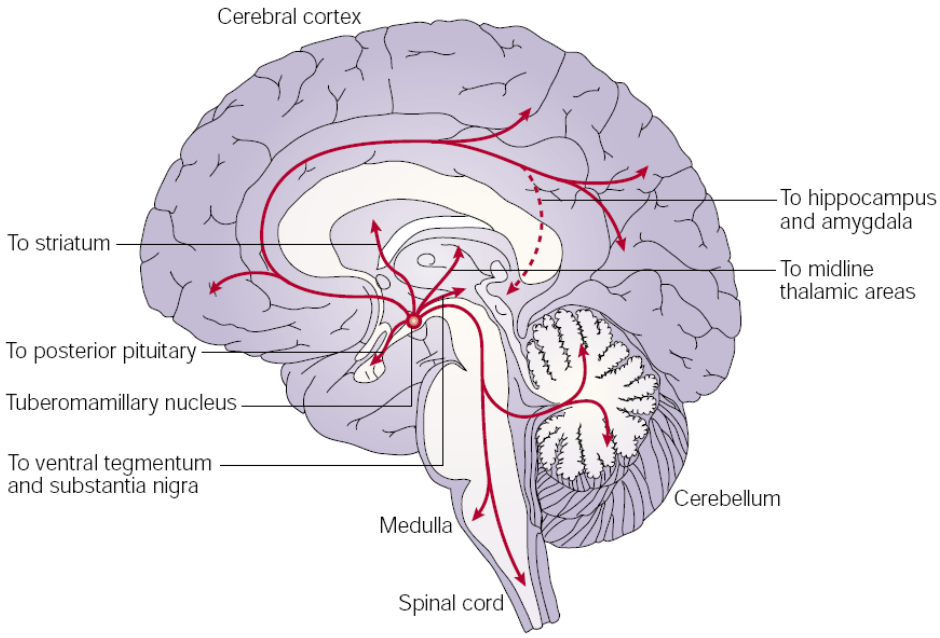


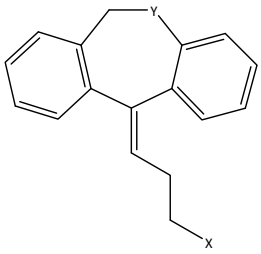
Fig. A.18. The histaminergic system in the human brain. The histaminergic fibers emanating from the tuberomamillary nucleus project to and arborize in the whole central nervous system. Adapted from Haas and Panula, 2003.

A.8 Scope and objectives

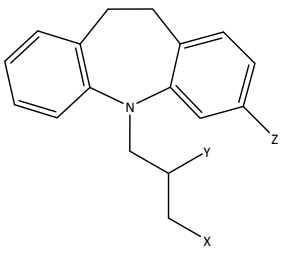
The local mediator and neurotransmitter histamine plays an important (patho)physiological role in a number of processes by activating four specific histamine receptors, *i.e.* H_1, H_2, H_3 and H_4 receptors (H_xRs) which all belong to the large family of GPCRs and are very important drug targets. $H_{1\text{-}3}$Rs are already well examined with potent and selective agonists and antagonists being available. While the H_1R is located in CNS as well as endothelium and regulates physiological functions like alertness and vasodilatation, H_2R can be found in parietal cells (H^+ secretion), cardiomyocytes (positive inotropy) and also in different brain regions like basal ganglia and the limbic system (Traiffort *et al.*, 1992a). Zolantidine is the only existing H_2R antagonist sufficiently penetrating the blood-brain barrier, but was never introduced for therapeutical use. Therefore, the precise function of the cerebral H_2R is still poorly defined (Ganellin, 1992). The H_3R is localized presynaptically at neurons regulating neurotransmitter release. In contrast, the function and pharmacological properties of the H_4R are still incompletely understood. It is primarily expressed in hematopoietic cells, specifically T-lymphocytes, mast cells and eosinophils (Oda *et al.*, 2000), but also in brain (Connelly *et al.*, 2009) suggesting an involvement of the H_4R mainly in immune reactions and inflammatory processes.

Antipsychotic and antidepressant drugs show affinity to H_xRs, mostly to the H_1R, which is known to cause the sedative (side) effects of these compounds (Richelson, 1979). Hence, we asked the question whether antipsychotic drugs also interact with other H_xRs, thereby contributing to potentially desired or unwanted effects. In order to better understand the interactions between these compounds and H_xRs, we expressed the different histamine receptor subtypes in Sf9 insect cells. We determined the affinities (K_i-values) of 34 antipsychotics and antidepressants (Fig. A.19 to A.23) by performing radioligand binding studies using [^{3}H]mepyramine (H_1R), [^{3}H]tiotidine (H_2R), [^{3}H]N^{α}-methylhistamine (H_3R) and [^{3}H]histamine (H_4R) as radioligands. The functional data (potencies (EC_{50} and K_b, respectively) and efficacies (E_{max})) were assessed in steady-state GTPase assays. Hence, examination of all tested substances could be performed in a single expression system. The obtained data was then compared with the corresponding therapeutic reference ranges to reveal the possible interactions and specify those by molecular modelling. Clinicians may use these receptor binding data to reduce or avoid drug interactions and adverse effects (Richelson and Souder, 2000).

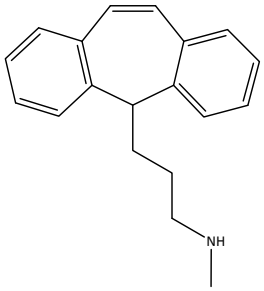
The lipophilicity of antipsychotics and antidepressants facilitates penetration of the blood-brain barrier. Accordingly, the affinity of the psychiatric medication to H_xRs, especially H_2Rs, in the CNS may contribute to their antidepressant and antipsychotic effects as well as to unwanted side effects, as the role of the H_2R in the regulation of brain function is still not understood.



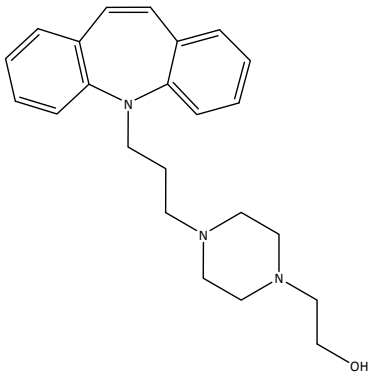
amitriptyline, AMI, X = $N(CH_3)_2$, Y = C
nortriptyline, NTL, X = $NH(CH_3)$, Y = C
doxepin, DXP, X = $N(CH_3)_2$, Y = O



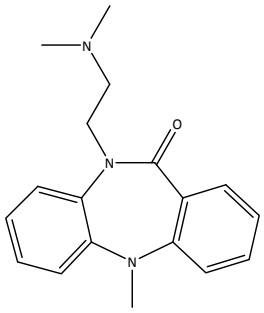
desipramine, DPM, X = $NH(CH_3)$, Y = H, Z= H
imipramine, IMI, X = $N(CH_3)_2$, Y = H, Z = H
clomipramine, CPM, X = $N(CH_3)_2$, Z = Cl
trimipramine, TPM, X = $N(CH_3)_2$, Y = CH_3, Z = H
lofepramine, LPM, X = $N(CH_3)CH_2COC_6H_4Cl$,
Y = H, Z = H



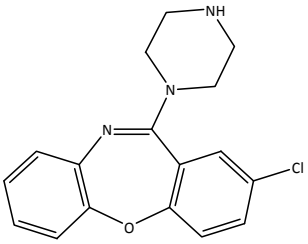
protriptyline, PTL



opipramol, OPI

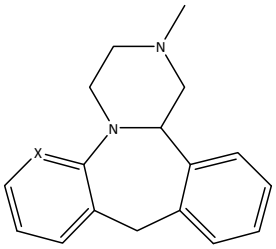


dibenzepin, DBP

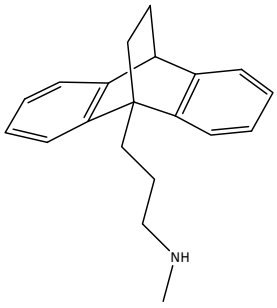


amoxapine, AMO

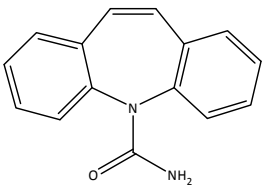
Fig. A.19. Structures of tricyclic antidepressants.



mianserin, MSN, X = CH
mirtazapine, MIR, X = N

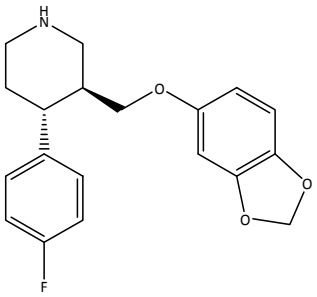


maprotiline, MPT

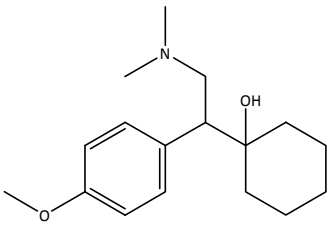


carbamazepine, CBZ

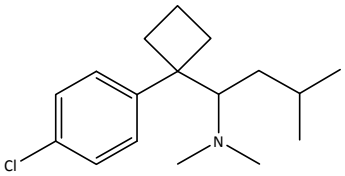
Fig. A.20. Structures of tetracyclic antidepressants (MSN, MIR and MPT) and a mood stabilizer (CBZ).



paroxetine, PRX

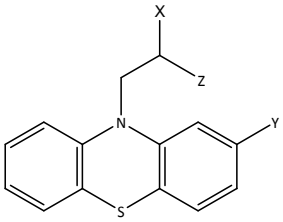


venlafaxine, VFX

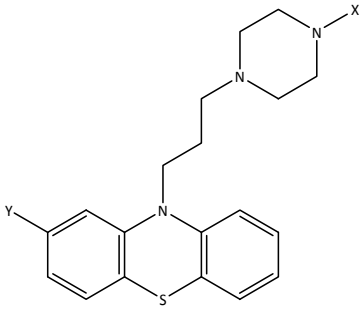


sibutramine, SBT

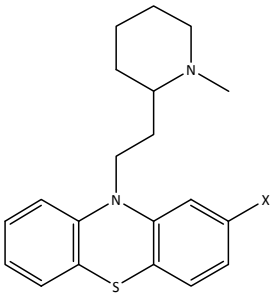
Fig. A.21. Structures of a selective serotonin reuptake inhibitor (PRX) and serotonin-norepinephrine reuptake inhibitors (VFX and SBT).



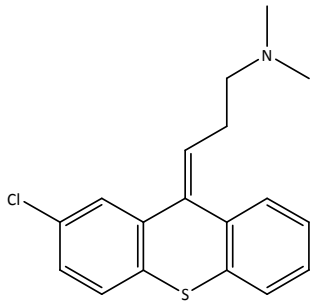
promethazine, PTZ,
X = $N(CH_3)_2$, Y = H, Z = CH_3
chlorpromazine, CPZ,
X = $CH_2N(CH_3)_2$, Y = Cl, Z = H
levomepromazine, LMZ,
X = $CH_2N(CH_3)_2$, Y = OCH_3, Z = (*R*)-CH_3



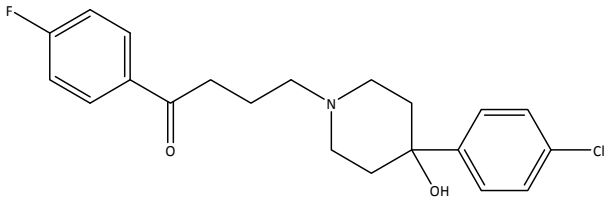
prochlorperazine, PCP, X = CH_3, Y = Cl
perphenazine, PPZ, X = CH_2 CH_2OH, Y = Cl
fluphenazine, FPZ, X = CH_2 CH_2OH, Y = CF_3



thioridazine, TRZ, X = SCH_3
mesoridazine, MRZ, X = $SOCH_3$
sulforidazine, SRZ, X = SO_2CH_3



chlorprothixene, CPX



haloperidol, HAL

Fig. A.22. Structures of first generation antipsychotics.

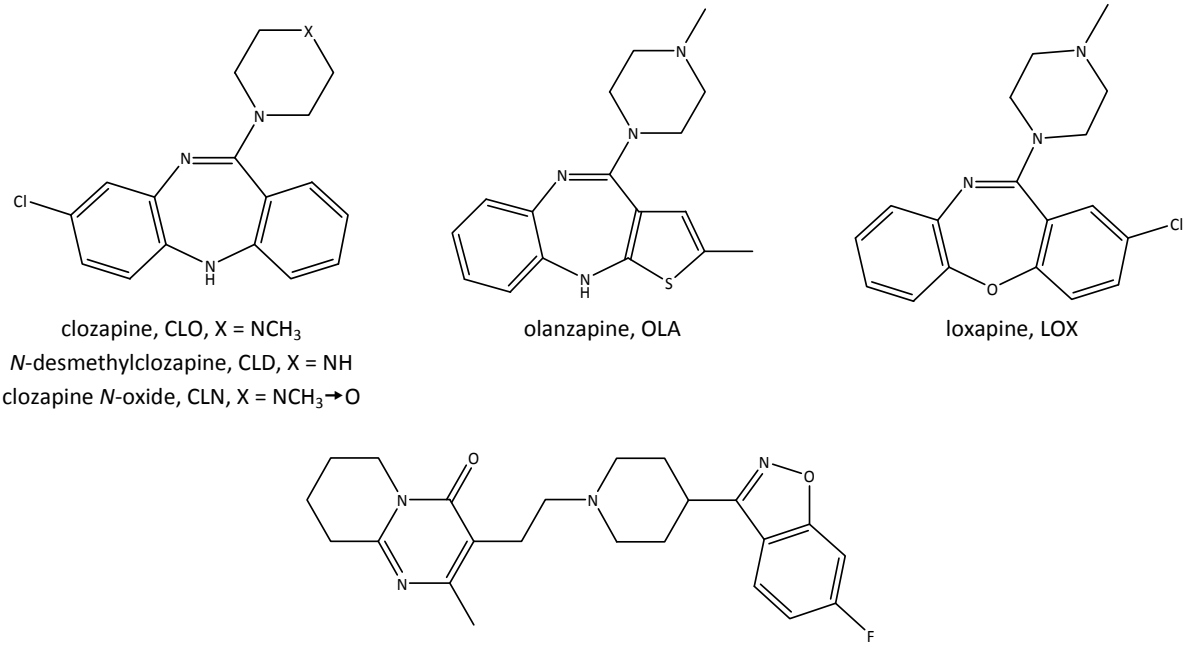


Fig. A.23. Structures of second generation antipsychotics.

B. Materials and Methods

B.1 Materials

B.1.1 Equipment

Analytical balance	BP 211D	Sartorius, Göttingen
	Extend	Sartorius, Göttingen
Autoclave (steam sterilizer)	Varioklav 135S	Thermo Electron, Oberschleißheim
Cell incubator	C24KC Refrigerated Incubator Shaker	New Brunswick Scientific, Edison, NJ, USA
Centrifuge	Sorvall Super T21	Thermo Scientific, Langenselbold
	Eppendorf 5417R	Eppendorf, Hamburg
	Multifuge 3L-R	Heraeus, Hanau
	GR4i Jouan	Thermo Electron, Waltham, MA, USA
Freezer	Arctis	AEG, Frankfurt am Main
Glass ware	diverse shapes and sizes	Schott, Mainz
Harvester	M-48	Brandel, Gaithersburgh, MD, USA
Heat block	Digital Heatblock	VWR, West Chester, PA, USA
Heating plate and stirrer	MR3001	Heidolph Instruments, Schwabach
Hemocytometer		Marienfeld, Lauda-Königshofen
Homogenizer	Dounce homogenizer	B. Braun, Melsungen
Microscope	Olympus CK2	Olympus, Tokyo, Japan
Millipore water Purification system	Milli-Q Water	Millipore, Schwalbach
pH-Meter	pH526	WTW, Weilheim
Photometer	Bio-Photometer	Eppendorf, Hamburg
Pipette	diverse volumes	Abimed, Langenfeld
Pipette controller	Accujet	Brand Tech, Wertheim
Platform shaker	Innova 2000	New Brunswick Scientific, Edison, NJ, USA

Power supply for SDS-Page/Western blot	Power Pac 200 Basic Power Supply	Bio-Rad, München
Scintillation-counter	Liquid Scintillation Analyzer Tri-Carb 2800 TR	PerkinElmer, Waltham, USA
Tissue culture hood	S@feflow 1.2	Nunc, Wiesbaden-Biebrich
Ultra low temperature freezer	U 570 Premium	New Brunswick Scientific, Edison, NJ, USA
Vertical elelctrophoresis system	Mini-PROTEAN TetraCell	Bio-Rad, München
Vortex shaker	Reax top	Heidolph, Schwalbach
Water bath	28L	VWR, Darmstadt
Western blot	Mini Trans-Blot Cell	Bio-Rad, München
X-ray film processor	Cawomat 2000 IR	CAWO, Schrobenhausen

B.1.2 Chemicals, enzymes and antibodies

[^{3}H]histamine	histamine dihydrochloride, [Ring, Methylenes-^{3}H(N)]-, specific activity: 10-40 Ci/mmol	Perkin Elmer, Boston, MA, USA
[^{3}H]mepyramine	[pyridinyl 5-^{3}H]-, specific activity: 20-30 Ci/mmol	Perkin Elmer, Boston, MA, USA
[^{3}H]N^{α}-methylhistamine	methylhistamine dihydrochloride, N^{α}-[methyl-^{3}H]-, specific activity: 74-85 Ci/mmol	Perkin Elmer, Boston, MA, USA
[^{3}H]tiotidine	tiotidine (ICI 125, 211), [Methyl-^{3}H]-, specific activity: 70-90 Ci/mmol	Perkin Elmer, Boston, MA, USA
[^{3}H]trimipramine	trimipramine hydrochloride, [N-methyl-^{3}H], specific activity: 80 Ci/mmol	American Radiolabeled Chemicals, Saint Louis, MO, USA
[^{32}P]H_3PO_4 and [^{33}P]H_3PO_4	phosphorus$^{32/33}$ radionuclide orthophosphoric acid, specific activity: 8,500-9,120 Ci/mmol	Perkin Elmer, Boston, MA, USA
Acrylamide 30% (m/V)	acrylamide/bis-acrylamide	Sigma-Aldrich, Taufkirchen
Activated charchoal		Sigma-Aldrich, Taufkirchen

Amitriptyline HCl	3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*] cycloheptene-5-ylidene)-*N*,*N*-dimethyl-1-propanamine hydrochloride	Sigma-Aldrich, Taufkirchen
$(NH_4)_2S_2O_8$	ammonium persulfate	Sigma-Aldrich, Taufkirchen
Amoxapine	2-chloro-11-(piperazin-1-yl) dibenzo[*b,f*] [1,4]oxazepine	Sigma-Aldrich, Taufkirchen
Antibiotic	gentamicin sulfate	Cambrex Bio Science, Walkersville, MD, USA
Antibodies	**primary**: anti-FLAG M1 antibody, anti-G$\alpha_{i1/2}$ antibody, anti-RGS4 antibody	Sigma-Aldrich, Taufkirchen Calbiochem, La Jolla,CA, USA
	secondary: anti-mouse (goat), anti-rabbit, anti-goat (donkey)	Santa Cruz, Santa Cruz, CA, USA
AppNHp	adenosine 5′-[β,γ-imido] triphosphate	Roche, Mannheim
ATP	adenosine 5′-triphosphate	Roche, Mannheim
BD BaculoGold	Transfection kit	BD PharMingen, San Diego, CA, USA
Benzamidine	benzenecarboximidamide	Acros Organics, Geel, Belgium
H_3BO_3	boric acid	Merck, Darmstadt
Bromphenol blue	3',3",5',5"-tetrabromophenolsulfonphthalein	Sigma-Aldrich, Taufkirchen
BSA	bovine serum albumine	Sigma-Aldrich, Taufkirchen
Carbamazepine	5*H*-dibenzo[*b,f*]azepine-5-carboxamide	Sigma-Aldrich, Taufkirchen
$CaCl_2$	calcium chloride	Merck, Darmstadt
cAMP	cyclic adenosine monophosphate	Sigma-Aldrich, Taufkirchen
Chlorpromazine HCl	3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethyl-propan-1-amine hydrochloride	Sigma-Aldrich, Taufkirchen
Chlorprothixene HCl	2-chloro-9-(3-dimethylaminopro-pylidene)thioxanthene hydrochloride	Sigma-Aldrich, Taufkirchen
CK	creatine kinase	Roche, Mannheim

Clomipramine HCl	3-(3-chloro-10,11-dihydro-5*H*-dibenzo [*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine hydrochloride	Biotrend, Köln
Clozapine	8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b,e*][1,4]-diazepine	RBI, Natick, MA, USA
Clozapine *N*-oxide	8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b,*e](1,4)diazepine *N*-oxide	Sigma-Aldrich, Taufkirchen
CP	phosphocreatine disodiumsalt	Sigma-Aldrich, Taufkirchen
DC Protein Assay Reagent A, B and S	colorimetric assay kit	Bio-Rad, München
Desipramine HCl	3-(10,11-dihydro-5*H*-dibenzo[*b,f*] azepin-5-yl)-*N*-methylpropan-1-amine hydrochloride	Biotrend, Köln
Dibenzepin HCl	10-(2-(dimethylamino)ethyl)-5-methyl-5*H*-dibenzo[*b,e*][1,4]diazepin-11(10*H*)-one hydrochloride	Novartis, Basel, Switzerland
Diphenhydramine HCl	2-(diphenylmethoxy)-*N,N*-dimethylethanamine hydrochloride	Biotrend, Köln
DMSO	dimethyl sulfoxide	Merck, Darmstadt
Doxepin HCl	(*E*,*Z*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine hydrochloride	Biotrend, Köln
DTT	dithiothreitol	Sigma-Aldrich, Taufkirchen
ECL Western Blotting	Detection reagent peroxide and Luminol	Pierce Biotechnology, Rockford, IL, USA
EDTA	ethylenediaminetetraacetic acid	Merck, Darmstadt
Ethylenimine	oligomer mixture	Sigma-Aldrich, Taufkirchen
Famotidine	3-([2-(diaminomethyleneamino) thiazol-4-yl]methylthio)-*N'*-sulfamoylpropanimidamide	Sigma-Aldrich, Taufkirchen
FCS	fetal calf serum	Biochrom, Berlin, Germany

Fluphenazine · 2HCl	2-[4-[3-[2-(trifluoromethyl)-10*H*-phenothiazin-10-yl]propyl]-piperazin-1-yl]ethanol dihydrochloride	Sigma-Aldrich, Taufkirchen
GDP	guanosine 5'-diphosphate	Roche, Mannheim
Glycerol	87% solution	AppliChem, Darmstadt
GTP	guanosine 5'-triphosphate	Roche, Mannheim
Haloperidol	4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidyl]-1-(4-fluorophenyl)-butan-1-one	Sigma-Aldrich, Taufkirchen
HCl	hydrochloric acid	Merck, Darmstadt
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Sigma-Aldrich, Taufkirchen
Histamine	2-(1*H*-imidazol-4-yl)ethanamine	Sigma-Aldrich, Taufkirchen
Imipramine HCl	3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride	Novartis, Basel, Schweiz
Insect-Xpress	culture medium for Sf9 cells	Lonza, Walkersville, MD, USA
KCl	potassium chloride	Merck, Darmstadt
KH_2PO_4	potassium dihydrogen phosphate	Merck, Darmstadt
Leupeptin	*N*-acetyl-L-leucyl-L-leucyl-L-argininal	Merck, Darmstadt
Levomepromazine HCl	(2*R*)-3-(2-methoxyphenothiazine-10-yl-)-*N*,*N*,2-trimethylpropanamine hydrochloride	Bayer Vital, Leverkusen
Lofepramine	*N*-(4-chlorophenethyl)-3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*-methylpropan-1-amine	Tocris Bioscience, Bristol, UK
Loxapine succinate	2-chloro-11-(4-methylpiperazin-1-yl)dibenzo[*b,f*][1,4]oxazepine succinate	Sigma-Aldrich, Taufkirchen
Maprotiline HCl	*N*-methyl- 9,10-ethanoanthracene-9(10*H*)- propanamine hydrochloride	Sigma-Aldrich, Taufkirchen
MeOH	methanol	Merck, Darmstadt
Mesoridazine besylate	10-{2-[(*RS*)1-methylpiperidin-2-yl]ethyl}-2-methylsulfinyl-10*H*-phenothiazine besylate	Sigma-Aldrich, Taufkirchen

$MgCl_2 \cdot 6H_2O$	magnesium chloride hexahydrate	Merck, Darmstadt
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate	Merck, Darmstadt
Mianserin HCl	(±)-2-methyl-1,2,3,4,10,14b-hexahydrodibenzo[*c,f*]pyrazino[1,2-*a*]azepine hydrochloride	Sigma-Aldrich, Taufkirchen
Mirtazapine	(±)-1,2,3,4,10,14*b*-hexahydro-2-[11*C*]methylpyrazino(2,1-*a*)pyrido (2,3-*c*)(2)benzazepine	Biotrend, Köln
Na_2HPO_4	disodium hydrogen phosphate	Merck, Darmstadt
NaCl	sodium chloride	Merck, Darmstadt
$NaH_2PO_4 \cdot H_2O$	sodium dihydrogen phosphate	Merck, Darmstadt
NaOH	sodium hydroxide	Merck, Darmstadt
N-desmethylclozapine	8-chloro-11-piperazinyl-5*H*-dibenzo[*b,e*][1,4]diazepine	Tocris Bioscience, Bristol, UK
NH_4SO_4	ammonium sulfate	Merck, Darmstadt
Nortriptyline HCl	3-(10,11-dihydro-5*H*-dibenzo[a,d] cyclohepten-5-ylidene)-*N*-methyl-1-propanamine hydrochloride	Biotrend, Köln
Olanzapine	2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzo-diazepine	Biotrend, Köln
Opipramol HCl	4-[3-(5*H*-dibenz[*b,f*]azepin-5-yl)propyl]- 1-piperazinethanol hydrochloride	Novartis, Basel, Switzerland
Paroxetine maleate	(3*S*,4*R*)-3-[(2H-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl) piperidine maleate	Sigma-Aldrich, Taufkirchen
Perphenazine	2-[4-[3-(2-chloro-10*H*-phenothiazin-10-yl) propyl]piperazin-1-yl]ethanol	Sigma-Aldrich, Taufkirchen
PMSF	phenylmethanesulfonyl fluoride	Sigma-Aldrich, Taufkirchen
Ponceau S	Acid Red 112, diazo dye	Sigma-Aldrich, Taufkirchen
Prestained protein molecular weight marker		Fermentas, St. Leon-Rot
Prochlorperazine dimaleate	2-chloro-10-[3-(4-methyl-1-piperazinyl)propyl]-10*H*-phenothiazine dimaleate	Sigma-Aldrich, Taufkirchen

Promethazine HCl	(*RS*)-*N,N*-dimethyl-1-(10*H*-phenothiazin-10-yl)propan-2-amine hydrochloride	Sigma-Aldrich, Taufkirchen
Protriptyline HCl	3-(5*H*-dibenzo[*a,d*][7]annulen-5-yl)-*N*-methylpropan-1-amine hydrochloride	Sigma-Aldrich, Taufkirchen
Risperidone	4-[2-[4-(6-fluorobenzo[*d*]isoxazol-3-yl)-1-piperidyl]ethyl]-3-methyl-2,6-diazabicyclo[4.4.0]deca-1,3-dien-5-one	Biotrend, Köln
Rotiszint eco plus	scintillation cocktail	Carl Roth, Karlsruhe
SDS	sodium dodecyl sulfate	Merck, Darmstadt
Sulforidazine	10-[2-(1-methylpiperidin-2-yl)ethyl]-2-(methylsulfonyl)-10*H*-phenothiazine	Novartis, Basel, Switzerland
TEMED	tetramethylethylenediamine	Sigma-Aldrich, Taufkirchen
Thioperamide maleate	*N*-cyclohexyl-4-(1*H*-imidazol-4-yl)piperidine-1-carbothioamide maleate	Tocris Bioscience, Bristol, UK
Thioridazine HCl	10-{2-[(*RS*)-1-Methylpiperidin-2-yl]ethyl}-2-methylsulfanyl-phenothiazine hydrochloride	Sigma-Aldrich, Taufkirchen
Trimipramine maleate	(±)-3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*,2-trimethylpropan-1-amine maleate	Sigma-Aldrich, Taufkirchen
Tris	tris(hydroxymethyl)aminomethane	USB Corporation, Cleveland, OH, USA
Tween 20	polysorbate 20	Merck, Darmstadt
Zolantidine dimaleate	*N*-[3-[3-(1-piperidinylmethyl) phenoxy]propyl]-2-benzothiazolamine dimaleate	Sigma-Aldrich, Taufkirchen

B.1.3 Consumables

Cuvettes	diverse sizes	Eppendorf, Hamburg
Glass microfiber filters	Whatman GF/C	Schleicher+Schuell, Maidstone, Kent, UK
Injection needles	20G, 21G, 27G BD Microlance	Becton Dickinson, Heidelberg
Insulin syringe	1 ml, sterile	B. Braun, Melsungen
Mini vials	6 ml	Sarstedt, Nümbrecht
Multipet tips	diverse volumes	Brand, Wertheim
Nitrocellulose membrane	0.45 µm Trans-Blot Transfer medium	Bio-Rad, München
Photo film	Amersham hyperfilm ECL	GE Healthcare, Little Chalfont, Buckinghamshire, UK
Pipette tips	10 µl, 100 µl, 1,000 µl,	Sarstedt, Nümbrecht
Serological pipettes	1 ml, 5 ml, 10 ml, 25 ml, sterile	Sarstedt, Nümbrecht
Single–use syringe	2 ml, 5 ml, 10 ml, 20 ml, sterile	B. Braun, Melsungen
Test tubes	1.5 ml micro tubes	Sarstedt, Nümbrecht
	2 ml micro tubes	Eppendorf, Hamburg
	15 ml and 50 ml Falcon tubes	Sarstedt, Nümbrecht
	4 ml	KABE, Nümbrecht
Transfer pipette	2 ml, disposable	Sarstedt, Nümbrecht

B.1.4 Buffers

PBS buffer (pH = 7.4)	**Lysis buffer (pH = 7.4)**	**Binding buffer (pH = 7.4)**
137 mM NaCl	10 mM Tris/HCl	75 mM Tris/HCl
2.6 mM KCl	1 mM EDTA	1 mM EDTA
0.5 mM $MgCl_2$	0.2 mM phenylmethane-sulfonyl fluoride	12.5 mM $MgCl_2$
0.9 mM $CaCl_2$	10 µg/ml benzamidine	
1.5 mM KH_2PO_4	10 µg/ml leupeptin	
0.8 mM Na_2HPO_4		

B.2 Methods

B.2.1 Solution protocols: handling of the antipsychotic and antidepressant ligands

Most of the commercially available ligands are highly lipophilic. Their solubility in water is very low, which implies dissolving them in aqueous solutions such as binding buffer is not possible at all. However, solubility in dimethyl sulfoxide (DMSO) or ethanol is sufficient to prepare at least 10 mM stock solutions. Ethanol has several disadvantages compared to DMSO. Firstly, ethanol evaporates easily, making it difficult to ensure accurate concentrations present in either stored stock solutions or dilutions. Secondly, ethanol is toxic for cell proteins, whereas DMSO can be used in concentrations of up to 5% (v/v) in Sf9 cell membranes without affecting receptor protein function, except membrane preparations expressing H_2R. Therefore, stock solutions of the ligands (10 mM each) were prepared in and stored at -20°C for up to 3 months without loss of pharmacological activity. Dilutions of ligands were prepared in such a way that the DMSO concentration was 50% (v/v) and that the final DMSO concentration in all assay tubes was 5% (v/v). A final volume percentage of up to 5% DMSO assured accurate and stable solutions without affecting receptor protein, except for H_2R, where concentrations of 5% (v/v) decreased the measured affinities and potencies by up to factor 5. Lowering the DMSO concentration to 1% (v/v) resulted in cloudy suspensions when diluting ligands.

The more hydrophilic ligands (TCAs except lofepramine (LPM)) could be solved in 10% (v/v) DMSO (10 mM), so that the final concentration of DMSO in the tubes was 1% (v/v). While affinities at H_3R and H_4R are very low and, therefore, high concentrations of the ligands were needed, examinations at H_1R and H_2R require only lower concentrated ligand solutions so that the DMSO content could be reduced to 1% or even 0.1% (v/v) final.

B.2.2 Generation of recombinant baculoviruses, cell culture and membrane preparation

Baculoviruses encoding recombinant proteins were generated in Sf9 cells using the BaculoGOLD™ transfection kit according to the manufacturer's instructions. Sf9 cells were cultured in 250 or 500 ml disposable Erlenmeyer flasks at 28°C and shaking at 125 rpm in an incubation shaker in SF 900 II medium supplemented with fetal calf serum to 5% (v/v) and

gentamicin sulfate to 0.1 mg/ml. Supplementation of fetal calf serum is not absolutely necessary, but cells grow better and show higher GPCR expression levels if serum is added. Sf9 cells were maintained at a density of 0.5 to 6.0 x 10^6 cells/ml.

After initial transfection, high-titer virus stocks were generated by two sequential virus amplifications. In the first amplification, cells were seeded at 2.0 x 10^6 cells/ml and infected with a 1:100 dilution of the supernatant from the initial transfection. Cells were cultured for 7 days, resulting in the death of virtually the entire cell population. The supernatant fluid of this infection was harvested and stored under light protection at 4°C. In a second amplification, cells were seeded at 3.0 x 10^6 cells/ml and infected with a 1:20 dilution of the supernatant fluid from the first amplification. Cells were cultured for 48 hr and the supernatant fluid was harvested. After the 48 hr culture period, the majority of cells showed signs of infections (*e.g.* altered morphology, viral inclusion bodies), but most of the cells were still intact. The supernatant fluid from the second amplification was stored under light protection at 4°C and used as routine virus stock for membrane preparations. To ensure the purity and identity of the viruses, the total ribonucleic acid of infected Sf9 cells was isolated, the cDNA was derived *via* reverse transcription and fragments representative for the constructs were PCR-amplified and analyzed by restriction digestion.

For transfection, cells were seeded at 3.0 x 10^6 cells/ml and infected with a 1:100 dilution of high-titer baculovirus stocks encoding either hH_1R, hH_2R-$G_s\alpha_S$, hH_3R or hH_4R as well as $G\alpha_{i2}$- and $G\beta_1\gamma_2$-protein. In some transfections, RGS4 or GAIP were additionally coexpressed. Cells were cultured for 48 hr and checked for signs of infection before membrane preparation.

Sf9 membranes were prepared as described previously (Seifert *et al*., 1998; Wenzel-Seifert and Seifert, 2000). All membrane preparation steps were conducted at 4°C in 50 ml Falcon tubes. Briefly, cells were washed once by centrifuging for 10 min at 1,000 rpm, discarding the supernatant and resuspending the cell pellet in 50 ml of PBS-buffer. After repeating the centrifugation step, the supernatant was discarded and the pellet was suspended in 15 ml of lysis buffer using EDTA (1 mM) and phenylmethylsulfonyl fluoride (0.2 mM), benzamidine (10 µg/ml) and leupeptine (10 µg/ml) as protease inhibitors and homogenized in a 15 ml Dounce homogenizer with 25 strokes. After centrifugation at 500 rpm for 5 min, the pellet contained the nuclei and unbroken cells and the supernatant contained the membranes. Therefore, the supernatant was carefully transferred to a plastic

Sorvall tube and spun down at 18,000 rpm for 20 min in a Sorvall centrifuge. The pellet containing the membranes was resuspended in 20 ml of lysis buffer and again centrifuged as described above. The resulting membrane pellet was suspended in 25 ml of binding buffer and homogenized by a syringe with 20 strokes. Protein concentrations were determined using the DC protein assay kit according to the instructions of the manufacturer. This assay allows the protein determination in the presence of reducing agents or detergents and is based on a colorimetric reaction according to the Lowry method.

The membrane suspension was aliquoted into 25 tubes at 1 ml each for storage at -80°C until use. At this temperature, GPCRs and G proteins are functionally and structurally stable for up to 4 years. By SDS-PAGE and immunoblotting with specific antibodies the expression of FLAG-tagged histamine receptors, G protein subunits and RGS proteins was confirmed (see Chapter B.2.3).

B.2.3 SDS-PAGE and immunoblot analysis

Membrane proteins were diluted in Laemmli-buffer and separated on SDS polyacrylamide gels containing 12% (w/v) acrylamide at 110 V for 120 min. Proteins were then transferred with 250 mA for 120 min onto 0.45 µm nitrocellulose membranes and then reacted with M1 antibody (1:1,000), anti-$G\alpha_{i1/2}$ (1:1,000) and RGS4 antibody (1:500) immunoglobulins G (IgGs). Immunoreactive bands were visualized by enhanced chemo-luminescence, using goat anti-mouse IgG (M1 Ig), anti-rabbit IgG (anti-$G\alpha_{i1/2}$ Ig) and donkey anti-goat IgG (RGS4 Ig) respectively, coupled to peroxidase. Electrochemoluminescence-stained blots were exposed to X-ray films.

Membranes of Sf9 cells expressing H_xR plus mammalian G proteins were prepared and analyzed *via* immunoblot. As shown in Fig. B.1 A, the M1 anti-FLAG antibody stained the hH_xR proteins as well as the fusion proteins. Due to a higher degree of *N*-glycosylation, the bands of hH_1R appeared at ~85 kDa (kDa) (Straßer *et al.*, 2008a). Some weak bands were visible in a range from 25 to 30 kDa. The expected molecular mass of the hH_2R is ~33 kDa (Gantz *et al.*, 1991a; 1991b; Fukushima *et al.*, 1997). The fusion protein hH_2R-$G_s\alpha_S$ was detected as strong band at ~80 kDa. The predicted molecular mass of the hH_3R is ~47 kDa and was detected as a strong band. The hH_4R signal consisted of more bands in the range between 37 and 44 kDa which are due to receptor glycosylation in the receptor N-terminus (Asn-5 and Asn-9) (van Rijn *et al.*, 2006; Schneider *et al.*, 2009). As shown in

Fig. B.1 B, we coexpressed hH_3R and hH_4R with $G\alpha_{i2}$, $G\beta_1\gamma_2$ and non-fused RGS4. In these membranes, RGS4 was detected with specific anti-RGS4 Igs. Likely due to differently glycosylated species, H_4R-GAIP shows very broad and diffuse bands. To visualize the coexpressed Gα-subunits, a $G\alpha_{i1/2}$ antibody was used. The proteins were detected at the expected molecular mass (~40 kDa) (Schnell *et al*., 2010).

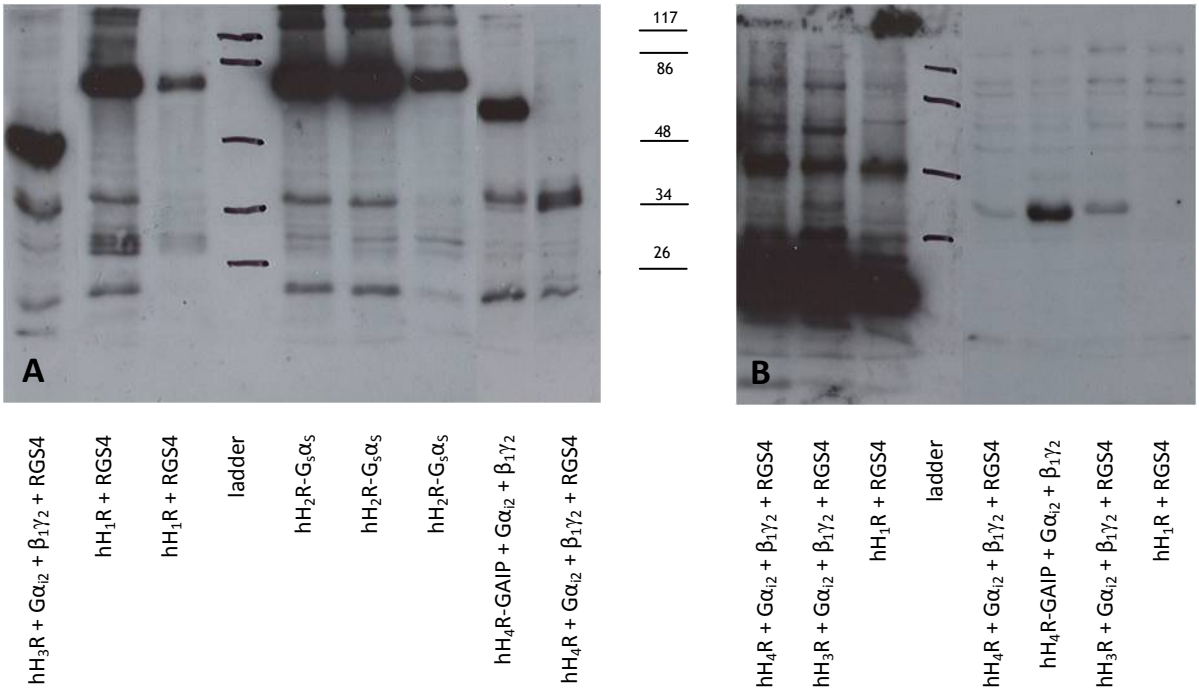


Fig. B.1. Immunological detection of the expression of recombinant proteins in Sf9 cells. In each lane, 10 µg of membrane protein was loaded onto the gel. Numbers in the middle of both gels designate masses of marker proteins in kDa. In **A**, the FLAG-tags of the four different types of histamine receptors were detected by the M1 monoclonal antibody (anti-FLAG Ig). In **B**, on the right side, membranes expressing a $G\alpha_{i2}$ subunit were detected by the anti-$G\alpha_{i2}$ IgG. On the left hand side of **B**, RGS4 proteins are displayed.

B.2.4 Radioligand binding assay

Radioligand binding assays were performed as previously described for the H_1R (Seifert *et al*., 2003), H_2R (Kelley *et al*., 2001), H_3R (Schnell *et al*., 2010) and H_4R (Schneider *et al*., 2009). H_1R assays: Sf9 insect cell membranes coexpressing the hH_1R and RGS4 were employed, H_2R assays: Sf9 insect cell membranes expressing the hH_2R-$G_s\alpha_S$ fusion protein were employed, H_3R assays: Sf9 insect cell membranes coexpressing the hH_3R, mammalian $G\alpha_{i2}$ and $G\beta_1\gamma_2$ were employed, H_4R assays: Sf9 insect cell membranes coexpressing the hH_4R, mammalian $G\alpha_{i2}$ and $G\beta_1\gamma_2$ were employed.

The membranes were thawed and sedimented by a 10-min centrifugation at 4°C and 15,000*g* and resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Each tube (total volume 250 µL) contained 25 µg (hH_1R), 45 µg (hH_3R), 75 µg (hH_4R) or 100 µg (hH_2R) of membrane protein. Competition binding experiments were performed in the presence of 5 nM of [^{3}H]mepyramine (hH_1R), 20 nM of [^{3}H]tiotidine (hH_2R), 3 nM of [^{3}H]N^{α}-methylhistamine (hH_3R) or 10 nM of [^{3}H]histamine (hH_4R) and increasing concentrations of unlabeled ligands. Non-specific binding was determined in the presence of tritiated radioligand plus 10 µM antagonist (diphenhydramine for H_1R, famotidine for H_2R or thioperamide for H_3R and H_4R). Incubations were conducted for 60 min at 25°C and shaking at 250 rpm. Bound radioligand was separated from free radioligand by filtration through GF/C filters pretreated with 0.3% (m/v) polyethyleneimine, followed by three washes with 2 ml of ice-cold binding buffer using a Brandel Harvester. After an equilibration phase of at least 12 hr, filter-bound radioactivity was determined by liquid scintillation counting. The experimental conditions chosen ensured that not more than 10% of the total amount of radioactivity added to binding tubes was bound to filters.

Absolute binding (pmoles of radioligand bound per mg of membrane protein) was calculated as follows:

$$\frac{pmol}{mg} = \frac{(cpm\ total\ -\ cpm\ non\text{–}specific) \times pmol\ radioligand}{cpm\ total\ added \times mg\ protein}$$

Equation 1

Explanations:

cpm total:	filter-bound radioactivity of radioligand from assay tubes, except from those tubes containing an antagonist for determination of non-specific binding
cpm non-specific:	filter-bound radioactivity of radioligand from assay tubes containing the antagonists diphenhydramine (H_1R), famotidine (H_2R) or thioperamide (H_3R and H_4R)
pmol radioligand:	absolute amount of radioligand present in the assay tubes
cpm total added:	the radioactivity of the radioligand added to each tube (no filtration)
mg protein:	absolute amount of membrane protein added per tube (0.025–0.100 mg)

B.2.5 Steady-state GTPase assay

GTPase activity assays were performed as previously described for the H_1R (Seifert *et al*., 2003), H_2R (Kelley *et al*., 2001), H_3R (Schnell *et al*., 2010) and H_4R (Schneider *et al*., 2009). H_1R assays: Sf9 insect cell membranes coexpressing the hH_1R and RGS4 were employed, H_2R assays: Sf9 insect cell membranes expressing the hH_2R-$G_s\alpha_S$ fusion protein were employed, H_3R assays: Sf9 insect cell membranes coexpressing the hH_3R, mammalian $G\alpha_{i2}$, $G\beta_1\gamma_2$ and RGS4 were employed, H_4R assays: Sf9 insect cell membranes coexpressing the hH_4R-GAIP fusion protein, mammalian $G\alpha_{i2}$ and $G\beta_1\gamma_2$ were employed.

The membranes were thawed, sedimented by centrifugation at 4°C and 13,000 rpm for 10 min. Membranes were resuspended in 10 mM Tris/HCl, pH 7.4. Each assay tube contained Sf9 membranes expressing the respective H_xR subtype (10 µg protein/tube), $MgCl_2$ ($H_{1/2}R$ assays: 1.0 mM, $H_{3/4}R$ assays: 5.0 mM), 100 µM EDTA, 100 µM adenosine 5`-triphosphate (ATP), 100 nM guanosine 5`-triphosphate (GTP), 100 µM adenosine 5`-[β,γ-imido]triphosphate, 1.2 mM creatine phosphate, 20 µg creatine kinase and 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4 and the investigated ligands at various concentrations. All H_4R assays additionally contained 100 mM NaCl. Histamine was added to the reaction mixtures (final concentrations: H_1R: 1 µM; H_2R: 1 µM) for the determination of K_b-values (antagonist mode of the GTPase activity assay).

Reaction mixtures (80 µl) were incubated for 2 min at 25°C before the addition of 20 µl of [γ-^{32}P]GTP (0.1 µCi/tube) or [γ-^{33}P]GTP (0.05 µCi/tube). All stock and work dilutions of [γ-^{32}P]GTP and [γ-^{33}P]GTP were prepared in 20 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min at 25°C. The addition of 900 µl slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0 terminated the reactions. The charcoal absorbs nucleotides but not P_i. Charcoal-quenched reaction mixtures were centrifuged for 7 min at room temperature at 13,000*g*. Six hundred microliters of the supernatant were removed and $^{32}P_i$/$^{33}P_i$ was determined by liquid scintillation counting. Enzyme activities were corrected for spontaneous degradation of radiolabeled GTP. Spontaneous degradation of radiolabeled GTP was determined in tubes containing all components described above, plus a very high concentration of unlabeled GTP (1 mM) which competes with [γ-^{32}P]GTP/ [γ-^{33}P]GTP and prevents hydrolysis of radiolabeled GTP by enzymatic activities present in Sf9 membranes. Spontaneous [γ-^{32}P]GTP/[γ-^{33}P]GTP degradation was <1% of the total amount

of radioactivity added. The experimental conditions chosen ensured that not more than 20% of the total amount of radiolabeled GTP added was converted to $^{32}P_i/^{33}P_i$.

GTPase activity (pmoles of P_i released per mg of membrane protein per min) was calculated as follows:

$$\frac{pmol}{mg \times min} = \frac{(cpm\ total - cpm\ GTP) \times pmol\ GTP\ unlabeled \times 1.67}{cpm\ total\ added \times min\ incubation \times mg\ protein}$$

Equation 2

Explanations:

cpm total:	radioactivity of [γ-^{32}P]GTP/[γ-^{33}P]GTP counted in the 600 µl aliquot taken from all assay tubes except those containing 1 mM GTP
cpm GTP:	radioactivity of [γ-^{32}P]GTP/[γ-^{33}P]GTP counted in the 600 µl aliquot taken from the assay tubes containing 1 mM GTP
pmol GTP unlabeled:	absolute amount of substrate present in the assay tubes; *i.e.* with 100 nM GTP, 10 pmoles of GTP were present in the 100 µl reaction mixture; the small amount of radiolabeled GTP may be neglected
1.67:	factor correcting the fact that only 600 µl out of 1,000 µl in the assay tubes were counted
cpm total added:	the radioactivity of [γ-^{32}P]GTP/[γ-^{33}P]GTP added to each tube (no charcoal addition)
min incubation:	assays were routinely conducted for 20 min
mg protein:	absolute amount of membrane protein added per tube (0.01 mg)

B.2.6 Prediction of off-targets by *Similarity Ensemble Approach*

For the prediction of new off-targets we used a statistics-based chemoinformatics approach called *Similarity Ensemble Approach* (SEA) (Keiser *et al*., 2007). By comparison of targets by the similarity of the ligands that bind to them, so-called expectation values are obtained which quantify a statistical significance of the observed similarity between drug and ligand. The simplified molecular input line entry specification (SMILES) formulas of the 34 drugs were compared against ligands drawn from the MDL Drug Data Report. The maintained predictions were analyzed retrospectively against known associations drawn from ChEMBL 02 database.

B.2.7 Construction of active and inactive state hH_xR models with different compounds in the binding pocket

For generation of the inactive and active hH_1R and hH_4R models, the sequence of the hH_xR was aligned to $h\beta_2AR$ (Ballesteros *et al*., 2001). Based on this alignment, the homology models of hH_1R and hH_4R were generated using the crystal structure of the $h\beta_2AR$ (Protein Data Bank code 2rh1) (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Rosenbaum *et al*., 2007). The software package SYBYL 7.3 (Tripos, St. Louis, MO) was used as described (Straßer *et al*., 2008b; Deml *et al*., 2009; Igel *et al*., 2009). Loops with different length, compared with the $h\beta_2AR$, were modeled using the Loop Search module of SYBYL 7.3. Because of the lack of sufficient experimental data concerning the structure of the ICL3-loop and parts of the C-terminus, both were included only partially in the modelling studies. This approximation should not have much influence on the modelling of the ligand binding-mode. Subsequently, the receptor was minimized carefully.

The modelling studies for the H_2R were performed using a homology model of the $h\beta_2AR$ (Protein Data Bank code 2rh1) (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Rosenbaum *et al*., 2007) published elsewhere (Ghorai *et al*., 2008). The docking of both TMP enantiomers and TIO was first performed manually in different positions in consideration of the probable binding site of the endogenous ligand HA (Del Valle *et al*., 1995). For TIO, mutational data of the H_2R (Gantz *et al*., 1992) and a pharmacophoric model derived from rigid TIO analogues (Haaksma *et al*., 1992) were considered. Next, several runs with the automated docking program FlexiDock of the SYBYL software package were performed. By a genetic algorithm the program is able to translate and rotate the ligand as well as to vary the torsion angles of both ligand and receptor amino acid side chains. The retrieved results were energy minimized with the force field MMFF94s (Halgren, 1999).

B.2.8 Miscellaneous

Ligand structures were illustrated using ChemDraw Ultra 11.0 (CambridgeSoft, Cambridge, MA, USA). Protein was determined using the DC protein assay kit. All analyses of experimental data were performed with the Prism 5 program (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using Student`s *t* test. K_b-values were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

C. Results

C.1 Analysis of antidepressants and antipsychotics at hH_1R and hH_2R

Cpd.	hH_1R			hH_2R			Therapeutic reference ranges [nM]
	K_i [nM] ± S.D.	K_b [nM] ± S.D.	Inv. Eff. ± S.D.	K_i [nM] ± S.D.	K_b [nM] ± S.D.	Inv. Eff. ± S.D.	
AMI	1.3 ± 0.7	1.8 ± 0.1	-0.07 ± 0.01	67 ± 14	112 ± 45	-0.12 ± 0.01	255-637[2]
AMO	8.0 ± 1.4	9.4 ± 3.9	-0.05 ± 0.01	n.d.	1,297 ± 195	-0.11 ± 0.01	637-1,594[1]
CBZ	> 100 µM	> 100 µM	ineffective	n.d.	> 100 µM	ineffective	25.3-50.8 µM[2]
CPZ	3.1 ± 1.7	3.4 ± 0.7	-0.28 ± 0.02	n.d.	1,534 ± 720	-0.22 ± 0.02	84-844[2]
CPX	1.1 ± 0.06	2.1 ± 0.1	-0.12 ± 0.03	n.d.	592 ± 56	-0.15 ± 0.01	57-568[2]
CPM	13 ± 2.0	9.0 ± 1.8	-0.10 ± 0.004	209 ± 12	168 ± 66	-0.14 ± 0.01	500-1,281[2]
CLO	2.6 ± 0.8	4.3 ± 1.1	-0.04 ± 0.01	n.d.	528 ± 78	-0.09 ± 0.01	1,071-1,836[2]
CLD	4.5 ± 0.9	4.9 ± 0.8	-0.11 ± 0.01	n.d.	1,624 ± 224	-0.14 ± 0.02	151-4,297[4]
CLN	2,919 ± 860	3,388 ±1,085	-0.05 ± 0.01	n.d.	> 100 µM	ineffective	75[5]
DPM	68 ± 11	21.2 ± 4.2	-0.08 ± 0.01	1,548 ± 243	1,439 ± 702	-0.12 ± 0.01	330-991[2]
DBP	24 ± 2.7	14 ± 3.4	-0.05 ± 0.01	1,966 ± 136	3,174 ± 154	-0.09 ± 0.01	85-850[3]
DXP	1.2 ± 0.3	1.2 ± 0.3	-0.07 ± 0.01	198 ± 137	344 ± 79	-0.12 ± 0.02	158-475[2]
FPZ	2.2 ± 0.2	65 ± 20	-0.16 ± 0.02	n.d.	16,702 ± 1,729	-0.14 ± 0.01	1.0-4.0[2]
HAL	1,947 ± 644	2,056 ± 43	-0.03 ± 0.02	n.d.	1,161 ± 289	-0.07 ± 0.01	13-45[2]
IMI	7.6 ± 2.0	5.7 ± 1.3	-0.10 ± 0.03	613 ± 394	791 ± 92	-0.13 ± 0.01	552-947[2]
LMZ	1.7 ± 0.7	3.2 ± 0.2	-0.20 ± 0.03	n.d.	596 ± 130	-0.17 ± 0.01	41-164[2]
LPM	243 ± 94	203 ± 79	-0.23 ± 0.02	4,415 ± 1,354	5,684 ± 1,243	-0.18 ± 0.01	12-42[3]
LOX	2.3 ± 0.4	7.3 ± 2.5	-0.07 ± 0.02	n.d.	1,221 ± 204	-0.10 ± 0.03	30-91[6]
MPT	1.4 ± 0.2	2.9 ± 1.0	-0.11 ± 0.03	782 ± 42	857 ± 118	-0.13 ± 0.01	398-637[2]
MSN	1.2 ± 0.3	3.3 ± 0.4	-0.10 ± 0.03	445 ± 95	450 ± 16	-0.12 ± 0.004	50-233[2]
MIR	1.3 ± 0.3	2.6 ± 0.2	-0.05 ± 0.01	n.d.	1,676 ± 206	-0.08 ± 0.01	151-301[2]
NTL	3.0 ± 1.0	5.0 ± 1.6	-0.09 ± 0.02	648 ± 73	877 ± 289	-0.14 ± 0.01	233-567[2]
OLA	3.1 ± 0.5	5.2 ± 1.1	-0.05 ± 0.005	n.d.	951 ± 87	-0.07 ± 0.01	64-256[2]
OPI	6.0 ± 1.1	8.5 ± 1.1	-0.08 ± 0.01	4,498 ± 749	6,261 ± 617	-0.12 ± 0.01	140-550[3]
PRX	12,703 ± 2,190	23,870 ± 15,967	-0.05 ± 0.04	n.d.	8,960 ± 1,088	-0.15 ± 0.04	157-269[2]
PPZ	2.6 ± 1.2	3.6 ± 0.7	-0.13 ± 0.03	n.d.	2,817 ± 15	-0.15 ± 0.01	1.5-6.0[2]
PCP	6.0 ± 2.6	4.5 ± 1.8	-0.17 ± 0.002	n.d.	2,412 ± 135	-0.16 ± 0.01	27-134[6]
PMZ	1.0 ± 0.2	1.7 ± 0.3	-0.14 ± 0.01	n.d.	197 ± 65	-0.15 ± 0.01	176-703[6]
PTL	7.2 ± 1.0	13 ± 1.4	-0.11 ± 0.01	399 ± 32	688 ± 188	-0.14 ± 0.01	266-950[1]
RIS	54 ± 11	22 ± 11	-0.07 ± 0.03	n.d.	202 ± 42	-0.07 ± 0.02	49-146[2]
TRZ	2.4 ± 0.2	8.6 ± 0.7	-0.14 ± 0.04	n.d.	454 ± 112	-0.17 ± 0.02	491-4,914[2]
MRZ	2.4 ± 0.5	3.5 ± 0.04	-0.08 ± 0.02	n.d.	1,228 ± 248	-0.12 ± 0.01	388-2,587[6]
SRZ	3.4 ± 1.0	6.6 ± 3.0	-0.08 ± 0.01	n.d.	1,205 ± 283	-0.07 ± 0.01	159-601[5]
TMP	1.5 ± 0.5	4.7 ± 1.3	-0.08 ± 0.01	41 ± 5.3	44 ± 12	-0.13 ± 0.01	365-853[2]

Table C.1. Affinities (K_i), inhibiting potencies (K_B) and inverse agonist efficacies (Inv. Eff.) of antidepressant and antipsychotic drugs at hH_1R + RGS4 and hH_2R-$G_s\alpha_S$. Radioligand binding assay and GTPase assay were

performed with Sf9 membranes as described in Chapters B.2.4 and B.2.5. Reaction mixtures contained Sf9 membranes expressing receptor and G proteins and antagonists at concentrations from 1 nM to 100 µM as appropriate to generate saturated competition curves. To determine the inverse agonist efficacies (Inv. Eff.), the effects of antagonists at a fixed concentration (10 µM to 100 µM) on basal GTPase activity were assessed and referred to the stimulatory effect of 100 µM HA (= 1.00). Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Values are given in nanomolar and are the means ± S.D. of two to six experiments performed in duplicate and triplicate. n.d. = not determined

[1] Lexi-comp, 2010; *s.v.* "therapeutic reference range"

[2] Baumann *et al*., 2004; values are designated as "therapeutic reference ranges"

[3] Gutteck and Rentsch, 2003; values are designated as "therapeutic ranges"

[4] Olesen *et al*., 1995; values are designated as "serum ranges"

[5] Baumann *et al*., 2004; values are designated as "dose related plasma concentrations"

[6] Schulz and Schmoldt, 2003; values are designated as "therapeutic blood-plasma/blood-serum concentrations"

For the examination of potential side effects of antidepressant and antipsychotic drugs related to H_xRs we determined their affinities (K_i), potencies (K_B) and inverse agonist efficacies (Inv. Eff.) and compared them with the particular therapeutic reference range, when available, or the therapeutic plasma concentration. Data for hH_1R and hH_2R are summarized in Table C.1 and Fig. C.1.

Nearly all examined compounds acted as weak partial inverse agonists with affinities/potencies in the low nanomolar range at H_1R. The tricyclic antidepressants desipramine (DPM) and LPM exhibited affinities/potencies in the higher nanomolar range, while the antipsychotics CLN, HAL, dibenzepin (DBP) and RIS as well as the mood stabilizer CBZ and the selective 5-HT reuptake inhibitor PRX showed no relevant affinities and, therefore, are not likely to cause any side effects *via* H_1R in comparison to all other investigated drugs. All inverse agonist efficacies were in the range between -0.04 and -0.28, relative to HA.

All compounds also decreased GTPase activities below basal values and, thus, showed partial inverse agonistic behavior at hH_2R, but most affinities and potencies varied between the low nanomolar and micromolar range. As the most outstanding structures we identified the TCAs TMP, AMI, clomipramine (CPM), DXP, IMI and protriptyline (PTL), while LPM showed only moderate potency. For hH_2R, also antipsychotics with phenothiazine structures like PMZ and thioridazine (TRZ) and its metabolite mesoridazine MRZ, the thioxanthene CPX as well as the atypical antipsychotic CLO and its metabolite CLD displayed a reasonable potency. Again, CBZ, CLN, HAL and PRX showed no relevant potencies there. In summary, we

determined for 12 of 34 compounds (*i.e.* 35%) affinities/potencies below the concentrations that are likely to be reached *in vivo* under therapy.

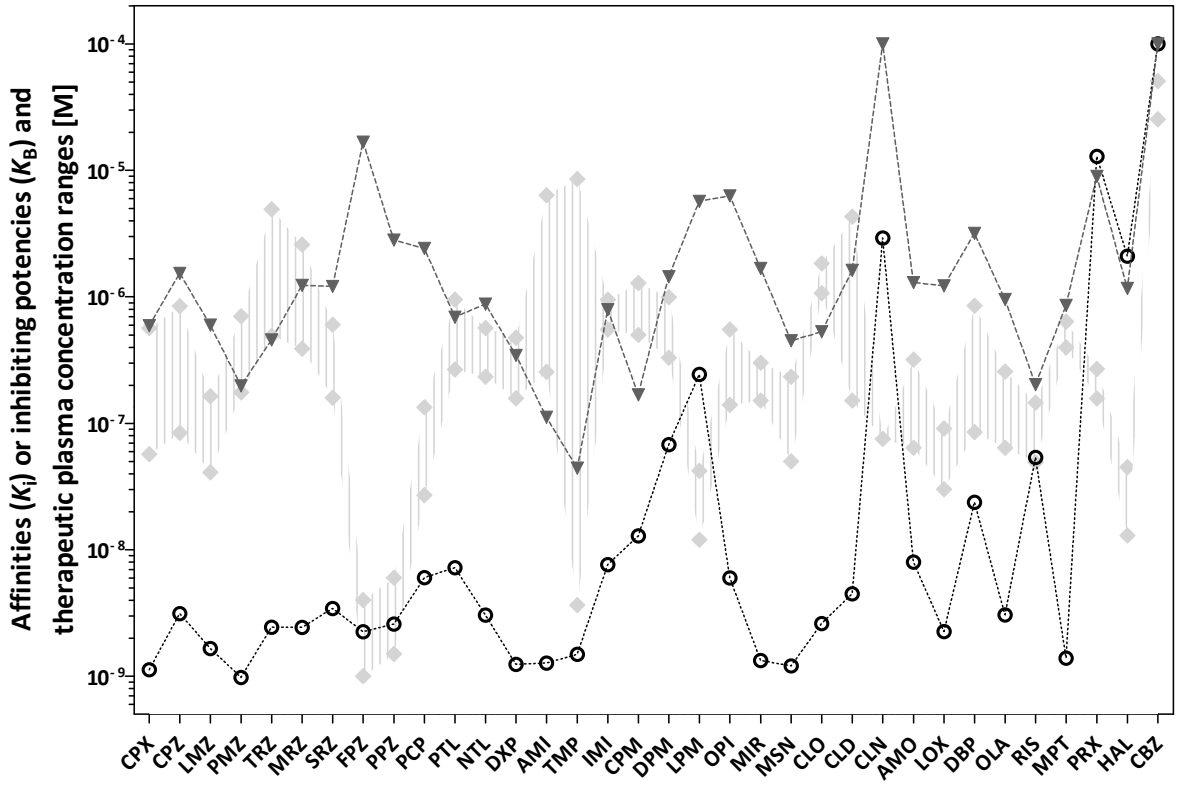


Fig. C.1. Affinities (K_i) or inhibiting potencies (K_B) of antidepressant and antipsychotic drugs to hH_1R + RGS4 (○) and hH_2R-$G_s\alpha_S$ (▼) in comparison to their therapeutic reference ranges (◆). Drugs were ordered according to structural similarities to visualize structure–activity relationship. Plasma/serum concentration was used if a therapeutic reference range was not available. Data points shown are the means of two to six independent experiments performed in duplicates or triplicates. A summary of all results is shown in Table C.1.

C.2 Analysis of antidepressants and antipsychotics at hH_3R and hH_4R

Also for hH_3R and hH_4R, affinities (K_i), potencies (EC_{50}/IC_{50}) and efficacies (E_{max}/Inv. Eff.) of all compounds were examined and compared with the particular therapeutic reference range (when available) or the therapeutic plasma concentration, both summarized in Table C.2 and Fig. C.2. Nearly all examined compounds showed strong partial inverse agonistic behavior in the high micromolar range at H_3R. No effects at all were determined for the mood stabilizer CBZ and the antipsychotic metabolite CLN. All inverse agonist efficacies ranged between -0.18 and -0.95, relative to HA. Clinically relevant interactions of the ex-

amined compounds can be excluded as the necessary concentrations are not reached under therapy.

Cpd.	hH_3R			hH_4R			Therapeutic reference ranges [nM]
	K_i [µM] ± S.D.	IC_{50} [µM] ± S.D.	Inv. Eff. ± S.D.	K_i [µM] ± S.D.	EC_{50}/IC_{50} [µM] ± S.D.	E_{max} / Inv. Eff. ± S.D.	
AMI	76 ± 31	> 100	-0.59 ± 0.04	27 ± 8.9	> 100	-0.68 ± 0.12*	255-637[2]
AMO	> 100	99 ± 59	-0.37 ± 0.11*	6.4 ± 1.3	8.0 ± 0.4	-0.36 ± 0.07	637-1,594[1]
CBZ	> 100	> 100	ineffective	> 100	> 100	ineffective	25.3-50.8 µM[2]
CPZ	41 ± 8	8.1 ± 2.2	-0.71 ± 0.10$^{\diamond}$	15 ± 5.4	6.9 ± 1.7	-0.96 ± 0.12$^{\diamond}$	84-844[2]
CPX	27 ± 6	62 ± 31	-0.71 ± 0.13$^{\diamond}$*	3.2 ± 0.9	2.9 ± 1.3	0.39 ± 0.13$^{\diamond}$	57-568[2]
CPM	9.7 ± 0.2	51 ± 22	-0.39 ± 0.08$^{\diamond}$	5.8 ± 0.5	22 ± 2.1	-0.71 ± 0.17$^{\diamond}$	500-1,281[2]
CLO	> 100	> 100	-0.49 ± 0.12*	1.2 ± 0.3	1.7 ± 0.2	0.66 ± 0.09	1,071-1,836[2]
CLD	53 ± 19	> 100	-0.61 ± 0.02*	1.8 ± 0.5	2.6 ± 1.1	0.36 ± 0.20	151-4,297[4]
CLN	> 100	> 100	ineffective	> 100	> 100	0.28 ± 0.08	75[5]
DPM	> 100	> 100	-0.51 ± 0.12*	9.6 ± 3.5	63 ± 15	-0.61 ± 0.08	330-991[2]
DBP	> 100	> 100	-0.44 ± 0.14*	> 100	> 100	-0.21 ± 0.10*	85-850[3]
DXP	39 ± 16	77 ± 27	-0.66 ± 0.05	15 ± 1.7	17 ± 4.1	-0.20 ± 0.05	158-475[2]
FPZ	24 ± 6	50 ± 22	-0.69 ± 0.11$^{\diamond}$*	24 ± 7.9	> 100	-0.67 ± 0.22$^{\diamond}$	1.0-4.0[2]
HAL	> 100	> 100	-0.18 ± 0.04*	> 100	> 100	ineffective	13-45[2]
IMI	> 100	> 100	-0.54 ± 0.12*	24 ± 9.7	59 ± 19	-0.78 ± 0.13*	552-947[2]
LMZ	> 100	> 100	-0.84 ± 0.07*	75 ± 22	> 100	-1.17 ± 0.11*	41-164[2]
LPM	79 ± 4.1	3.4 ± 1.1	-0.42 ± 0.08	36 ± 12	9.2 ± 2.7	-0.55 ± 0.06	12-42[3]
LOX	55 ± 15	> 100	-0.71 ± 0.12*	8.6 ± 1.2	> 100	ineffective	30-91[6]
MPT	67 ± 30	> 100	-0.71 ± 0.12*	84 ± 37	> 100	-1.06 ± 0.11*	398-637[2]
MSN	96 ± 19	> 100	-0.95 ± 0.13*	> 100	> 100	-1.17 ± 0.21*	50-233[2]
MIR	83 ± 22	> 100	-0.56 ± 0.08*	> 100	37 ± 4.0	-0.22 ± 0.08*	151-301[2]
NTL	46 ± 18	> 100	-0.63 ± 0.10*	6.9 ± 1.4	> 100	-0.82 ± 0.04*	233-567[2]
OLA	> 100	17 ± 10	-0.33 ± 0.13	17 ± 8.3	38 ± 8.3	0.57 ± 0.08	64-256[2]
OPI	62 ± 23	61 ± 8.1	-0.74 ± 0.11	> 100	> 100	-0.49 ± 0.16*	140-550[3]
PRX	95 ± 17	> 100	-0.56 ± 0.12$^{\diamond}$	44 ± 4.5	> 100	-0.34 ± 0.10$^{\diamond}$*	157-269[2]
PPZ	46 ± 12	16 ± 3.3	-0.78 ± 0.12$^{\diamond}$	> 100	15 ± 4.5	-0.54 ± 0.15$^{\diamond}$*	1.5-6.0[2]
PCP	17 ± 4.0	22 ± 6.2	-0.82 ± 0.12$^{\diamond}$	18 ± 3.9	27 ± 9.2	-0.51 ± 0.14$^{\diamond}$*	27-134[6]
PMZ	> 100	> 100	-0.76 ± 0.10	77 ± 8.9	> 100	-1.10 ± 0.15*	176-703[6]
PTL	> 100	> 100	-0.54 ± 0.10*	15 ± 8.2	> 100	-0.43 ± 0.05*	266-950[1]
RIS	> 100	90 ± 38	-0.27 ± 0.10	> 100	> 100	ineffective	49-146[2]
TRZ	12 ± 0.7	27 ± 9.4	-0.87 ± 0.07$^{\diamond}$	14 ± 4.5	> 100	-0.93 ± 0.17$^{\diamond}$*	491-4,914[2]
MRZ	40 ± 8.8	36 ± 3.9	-0.83 ± 0.13	> 100	74 ± 16	-1.21 ± 0.11	388-2,587[6]
SRZ	33 ± 22	95 ± 9.1	-0.71 ± 0.11	> 100	86 ± 12	-0.66 ± 0.14	159-601[5]
TMP	> 100	> 100	-0.68 ± 0.12	44 ± 5.9	> 100	-0.93 ± 0.13*	365-853[2]

Table C.2. Affinities (K_i), potencies (EC_{50} or IC_{50}) and efficacies (E_{max}) or inverse agonist efficacies (Inv. Eff.) of antidepressant and antipsychotic drugs at $hH_3R + G\alpha_{i2} + \beta_1\gamma_2$ and $hH_4R + G\alpha_{i2} + \beta_1\gamma_2$ (respectively hH_4R-GAIP + $G\alpha_{i2} + \beta_1\gamma_2$). Radioligand binding assay and GTPase assay were performed with Sf9 membranes as described in Chapters B.2.4 and B.2.5. Reaction mixtures contained Sf9 membranes expressing receptor and G proteins and antagonists at concentrations from 1 nM to 500 µM as appropriate to generate saturated competition curves.

To determine the inverse agonist efficacies (Inv. Eff.), the effects of antagonists at fixed concentrations (100 µM to 500 µM) on basal GTPase activity were assessed and referred to the stimulatory effect of 100 µM HA (= 1.00). In case of unspecific effects by ligands at higher concentrations, E_{max}/inverse efficacy was measured at 100 µM, as indicated by $^{\Diamond}$. If saturation was not achieved within these concentration ranges, the inverse agonist efficacies were determined at 100 or 500 µM and are indicated by $^{\Diamond}$* and *, respectively. Data were analyzed by non-linear regression and were best fit to sigmoidal concentration/response curves. Values are given in micromolar and are the means ± S.D. of two to six experiments performed in duplicate and triplicate.

[1] Lexi-comp, 2010; *s.v.* "therapeutic reference range"

[2] Baumann *et al*., 2004; values are designated as "therapeutic reference ranges"

[3] Gutteck and Rentsch, 2003; values are designated as "therapeutic ranges"

[4] Olesen *et al*., 1995; values are designated as "serum ranges"

[5] Baumann *et al*., 2004; values are designated as "dose related plasma concentrations"

[6] Schulz and Schmoldt, 2003; values are designated as "therapeutic blood-plasma/blood-serum concentrations"

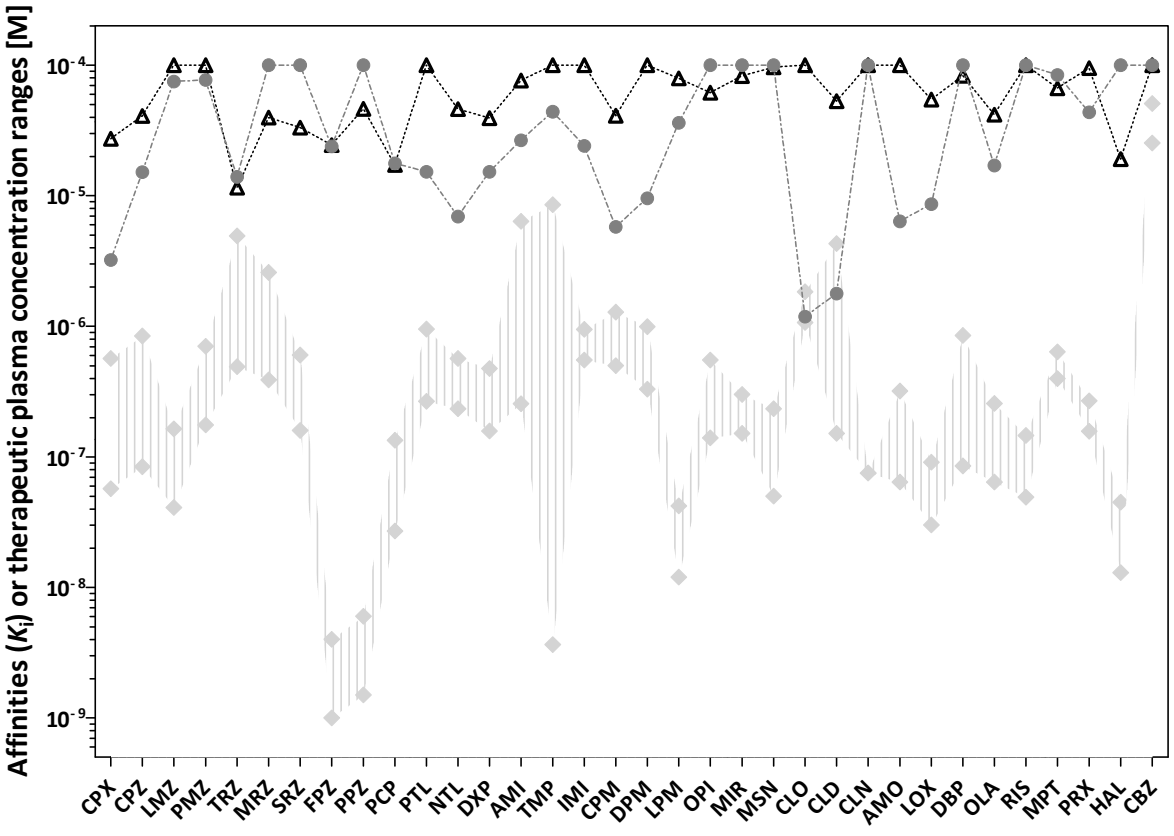


Fig. C.2. Affinities (K_i) of antidepressant and antipsychotic drugs to $hH_3R + G\alpha_{i2} + \beta_1\gamma_2$ (△) and $hH_4R + G\alpha_{i2} + \beta_1\gamma_2$ (●) in comparison to their therapeutic reference ranges (◆). Drugs were ordered according to structural similarities to visualize structure–activity relationship. Plasma/serum concentration was used if no therapeutic reference range was applicable. Data points shown are the means of two to six independent experiments performed in triplicates. A summary of all results is shown in Table C.2.

A different picture reveals the hH_4R: most of the compounds also displayed partial inverse agonistic behavior at hH_4R (E_{max} -0.20 to -1.21, relative to HA), but the atypical antipsychotics CLO, CLD, CLN and OLA as well as the typical antipsychotic CPX with its thioxanthene structure acted as partial agonists with efficacies from 0.28 to 0.66, relative to HA. Affinities and inhibiting potencies varied in the micromolar range. Compared with the therapeutic reference ranges or plasma concentrations the only relevant interaction is possibly given for CLO and CLD, while all other affinities/potencies are beyond the reference ranges.

C.2.1 Representative competition binding curves for hH_xR

Some representative data sets summarized in Table C.1 and Table C.2 are depicted in the following as competition binding curves of all four H_xRs (Fig. C.3). All binding isotherms were monophasic with a Hill slope close to unity, indicative for a single ligand binding site. Apparent is the wide range of affinities at H_xRs which is obtained by the different compounds except of hH_3R where affinities were all very low. The competition of the SSRI PRX could not be saturated at hH_1R which coincides with the fact that PRX is a more selective drug and, therefore, less sedating than the other examined compounds, *e.g.* TCAs (Hassan *et al.*, 1985).

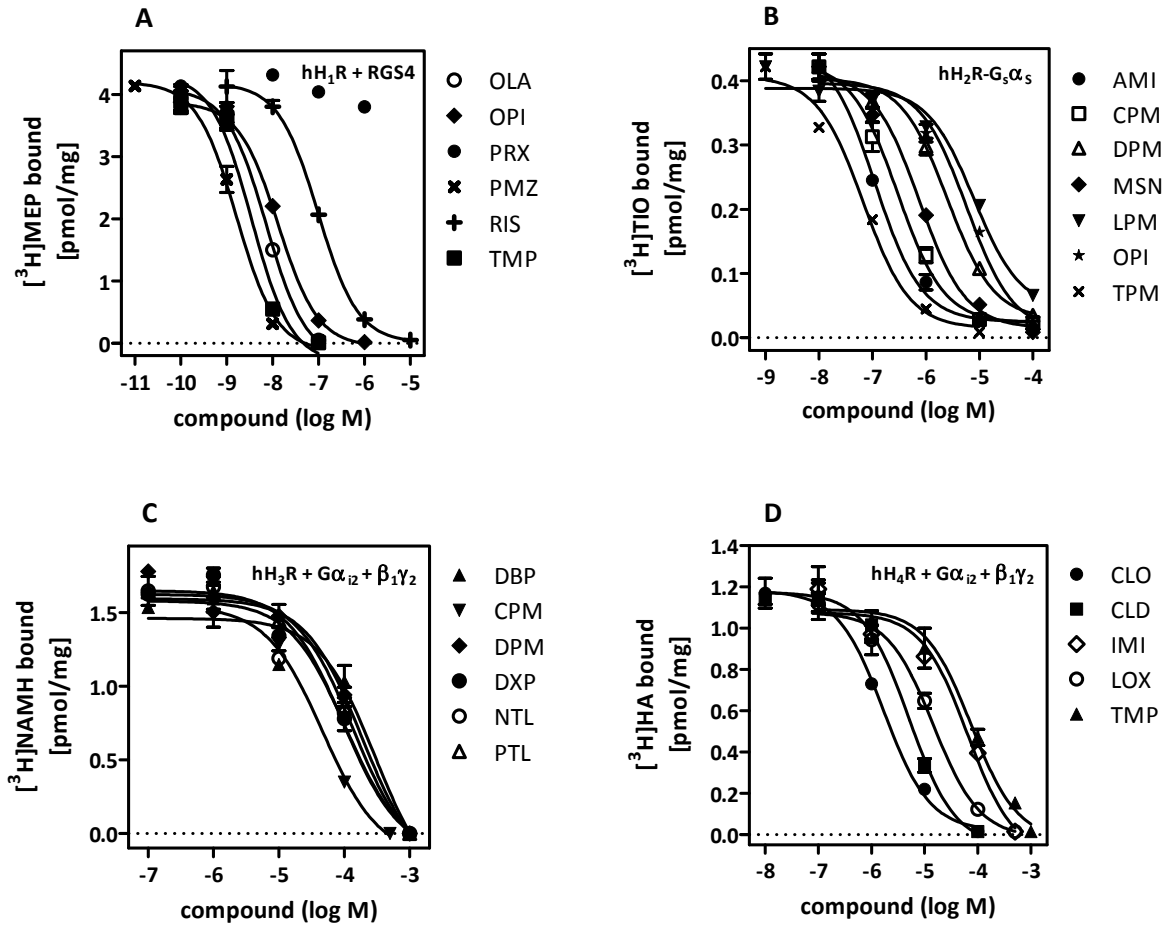


Fig. C.3. Competition bindings of H_xR [^{3}H]radioligands and selected drugs in Sf9 membranes expressing hH_xR. **A**, competition binding with [^{3}H]MEP at hH_1R + RGS4 as described in Chapter B.2.4. Reaction mixtures contained Sf9 membranes (25 µg of protein per tube) expressing the recombinant proteins, 5 nM [^{3}H]MEP and ligands at the concentrations indicated on the abscissa. **B**, competition binding with [^{3}H]TIO at hH_2R-$G_s\alpha_S$ as described in Chapter B.2.4. Reaction mixtures contained Sf9 membranes (100 µg of protein per tube) expressing the recombinant proteins, 20 nM [^{3}H]TIO and ligands at the concentrations indicated on the abscissa. **C**, competition binding with [^{3}H]NAMH at hH_3R + $G\alpha_{i2}$ + $\beta_1\gamma_2$ as described in Chapter B.2.4. Reaction mixtures contained Sf9 membranes (45 µg of protein per tube) expressing the recombinant proteins, 3 nM [^{3}H]NAMH and ligands at the concentrations indicated on the abscissa. **D**, competition binding with [^{3}H]HA at hH_4R + $G\alpha_{i2}$ + $\beta_1\gamma_2$ as described in Chapter B.2.4. Reaction mixtures contained Sf9 membranes (75 µg of protein per tube) expressing the recombinant proteins, 10 nM [^{3}H]HA and ligands at the concentrations indicated on the abscissa. Data points shown are the means ± S.D. Three to five independent experiments were performed in triplicates. A summary of all results is shown in Table C.1 and Table C.2.

C.2.2 Representative concentration/response curves for drugs at hH_xR in the GTPase assay

Some representative data sets summarized in Table C.1 and Table C.2 are depicted in the following as concentration/response curves of all four H_xRs (Fig. C.4). While all substances with relevant potencies acted as inverse agonists displaying a very narrow range of efficacies at hH_1R and hH_2R, potencies at hH_3R and hH_4R were much lower and also the efficacies relative to HA varied over a wider span, even up to full inverse agonists. At the hH_4R receptor four of the tested compounds even acted as partial agonists, although there is no noticeable similarity of structures between CLO, its metabolites and OLA on the one hand and CPX on the other.

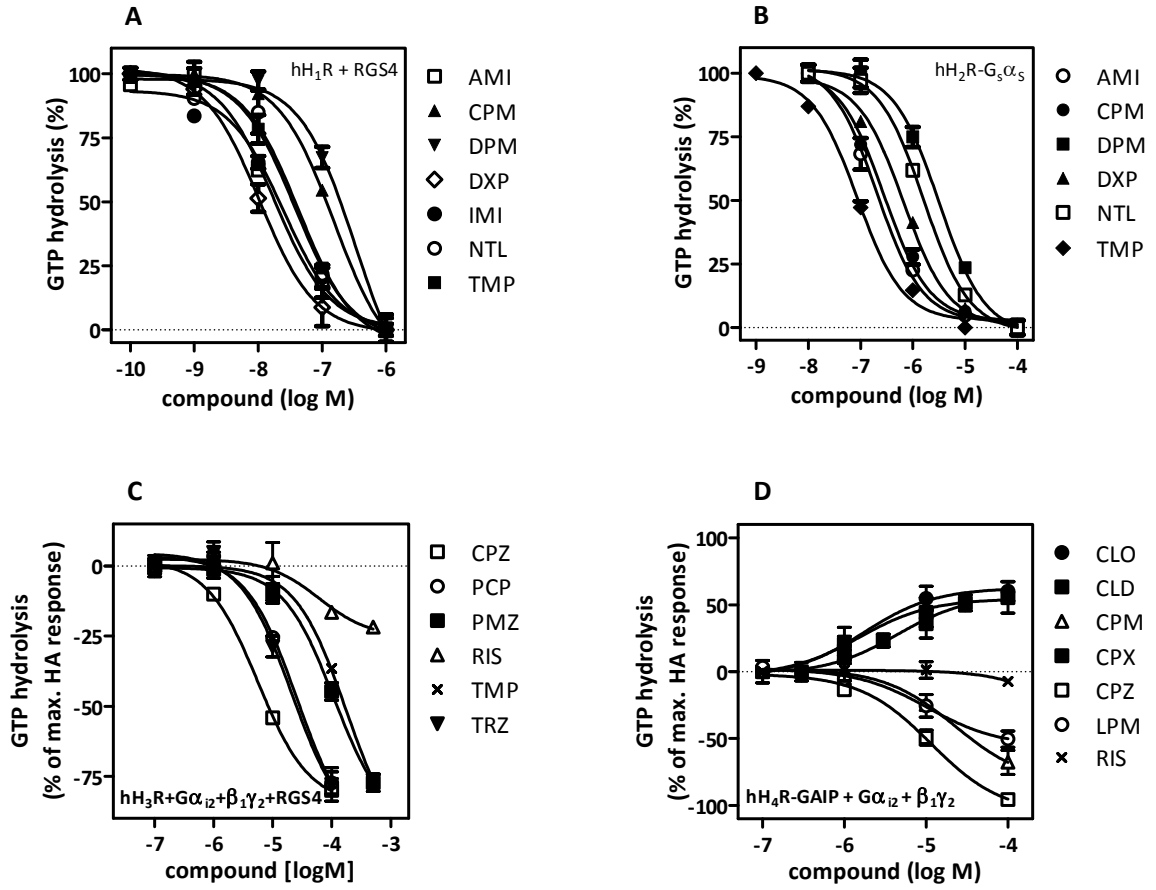


Fig. C.4. Concentration-dependent alteration of GTP hydrolysis by antidepressants and antipsychotics in membranes expressing hH_1R + RGS4, hH_2R-$G_s\alpha_s$, hH_3R + $G\alpha_{i2}$ + $\beta_1\gamma_2$ + RGS4 or hH_4R-GAIP + $G\alpha_{i2}$ + $\beta_1\gamma_2$. GTPase activity in Sf9 membranes was determined as described in Chapter B.2.5. Reaction mixtures contained membranes (10 µg of protein/tube) expressing receptor, proteins and drugs at concentrations indicated on the abscissa. In **A** and **B**, HA was added to the reaction mixtures (1 µM) for the determination of K_b-values, while in **C** and **D**, the agonist/inverse agonist mode was performed in absence of HA. Data shown are the means ± S.D. Two to six independent experiments were performed in duplicates. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves.

C.3 Prediction of ligand binding by *Similarity Ensemble Approach*

A very promising approach in the search for new off-targets emerged from statistics-based chemoinformatics. The previously published *Similarity Ensemble Approach* (SEA) explores possible interactions computationally by comparing targets – not like bioinformatic methods by sequence or structural similarity among the targets – but rather by the similarity of the ligands that bind to them, expressed as expectation values (E-values) (Keiser *et al.*, 2007). The generated E-values (Table C.3) hereby do not equate the experimentally

determined affinities or potencies, they rather quantify a statistical significance of the observed similarity between drug and ligand.

Cpd.	hH_1R		hH_2R		hH_3R		hH_4R	
	E-value	K_i-value [M]	E-value	K_b-value [M]	E-value	K_i-value [M]	E-value	K_i-value [M]
AMI	1.23×10^{-12}	1.27×10^{-9}	4.59×10^{-11}	1.12×10^{-7}	---	---	---	---
AMO	3.36×10^{-9}	7.97×10^{-9}	---	---	---	---	5.70×10^{-14}	6.37×10^{-6}
CPZ	1.22×10^{-9}	3.13×10^{-9}	7.49×10^{-2}	1.53×10^{-6}	---	---	---	---
CPX	7.65×10^{-12}	1.13×10^{-9}	5.39×10^{-9}	5.92×10^{-7}	---	---	---	---
CPM	2.04×10^{-9}	1.29×10^{-8}	2.21×10^{-6}	1.68×10^{-7}	---	---	---	---
CLO	3.77×10^{-4}	2.60×10^{-9}	---	---	---	---	1.47×10^{-15}	1.19×10^{-6}
CLN	---	---	---	---	---	---	1.75×10^{-14}	$>1.00 \times 10^{-4}$
DPM	4.18×10^{-1}	6.77×10^{-8}	5.12×10^{-5}	1.44×10^{-6}	---	---	---	---
DBP	---	---	7.02×10^{-2}	3.17×10^{-6}	---	---	---	---
DXP	8.78×10^{-14}	1.24×10^{-9}	4.38×10^{-6}	3.44×10^{-7}	---	---	---	---
FPZ	2.38×10^{-6}	2.25×10^{-9}	2.33×10^{-3}	1.67×10^{-5}	---	---	---	---
HAL	7.61×10^{-35}	1.95×10^{-6}	8.24×10^{-16}	1.16×10^{-6}	1.34×10^{-7}	$>1.00 \times 10^{-4}$	---	---
IMI	2.23×10^{-13}	7.61×10^{-9}	2.44×10^{-14}	7.91×10^{-7}	---	---	---	---
LMZ	1.23×10^{-1}	1.66×10^{-9}	---	---	---	---	---	---
LPM	1.50×10^{-8}	2.43×10^{-7}	3.19×10^{-5}	5.68×10^{-6}	1.12×10^{-1}	7.90×10^{-5}	---	---
LOX	2.30×10^{-11}	2.25×10^{-9}	---	---	---	---	3.61×10^{-25}	8.63×10^{-6}
MPT	4.82×10^{-10}	1.39×10^{-9}	1.01×10^{-36}	8.57×10^{-7}	---	---	---	---
MSN	3.60×10^{-12}	1.20×10^{-9}	7.65×10^{-12}	4.50×10^{-7}	---	---	---	---
MIR	6.32×10^{-1}	1.33×10^{-9}	4.18×10^{-1}	1.68×10^{-6}	---	---	---	---
NTL	3.21×10^{-8}	3.04×10^{-9}	1.24×10^{-7}	8.77×10^{-7}	---	---	---	---
OLA	5.15×10^{-6}	3.05×10^{-9}	---	---	---	---	6.50×10^{-7}	1.70×10^{-5}
OPI	2.16×10^{-5}	5.99×10^{-9}	1.05×10^{-2}	6.26×10^{-6}	9.63×10^{-1}	6.15×10^{-5}	---	---
PRX	---	---	---	---	9.58×10^{-1}	9.51×10^{-5}	---	---
PPZ	7.65×10^{-12}	2.57×10^{-9}	---	---	---	---	---	---
PCP	1.18×10^{-12}	6.01×10^{-9}	2.01×10^{-2}	2.41×10^{-6}	1.12×10^{-2}	1.71×10^{-5}	3.67×10^{-24}	1.77×10^{-5}
RIS	6.36×10^{-6}	5.37×10^{-8}	---	---	---	---	---	---
TMP	---	---	1.08×10^{-4}	4.41×10^{-8}	---	---	---	---

Table C.3. Comparison of E-values and affinities (K_i) or inhibiting potencies (K_b) at hH_xR. Known-true-predictions are indicated in blue, under-predictions with a weak SEA score but a good K_i or K_b in grey and over-predictions with a good SEA score but a weak K_i or K_b in black. Data sets with no available SEA-predictions are indicated by ---. Data were analyzed by non-linear regression and were best fit to sigmoidal concentration/response curves. K_i-/K_b-values are the means of two to six experiments performed in duplicate and triplicate. K_i-/K_b-data are summarized completely in Table C.1 and Table C.2.

We determined predictions for one or more H_xR for 27 of the 34 compounds tested. The majority of them were obtained for H_1R, thereof eleven known-true-predictions and ten over-predictions with a good SEA score but a weak K_i- or K_b-value. For LMZ and CLO, in contrast, under-predictions resulted at H_1R with a good K_i- or K_b-value but a weak SEA score. These ligands or structurally similar compounds were obviously not registered in the used database yet, although at least for CLO hH_1R affinity was described before (Richelson and Nelson, 1984). A similar pattern was obtained for hH_2R. Here, we found six known-true-predictions, five over-predictions and several potential new interactions, preeminently TMP. Its high affinity to hH_2R was not reproduced adequately by the SEA. This finding is particularly surprising as the affinity of at least some TCAs for H_2R was reported before (Green and Maayani, 1977) and several of these compounds were subject of trials for a therapy of duodenal and gastric peptic ulcer disease (Ries *et al*., 1984; Wilson *et al*., 1985). Only few new targets were predicted for hH_3R: of five interactions four were under-predictions. However, since all those affinities are still exceeding therapeutic reference ranges, no clinically relevant interactions are to be expected. All ligands found for hH_4R are over-predictions: the achieved SEA score was much better than the corresponding dissociation constant K_i or K_b, *e.g.* CLN, OLA or prochlorperazine (PCP). Interestingly, all of the six substances are antipsychotics containing a piperazine-moiety in the side chain, but differ in the tricyclic ring system.

C.4 Molecular modelling

C.4.1 Binding of trimipramine to hH_2R

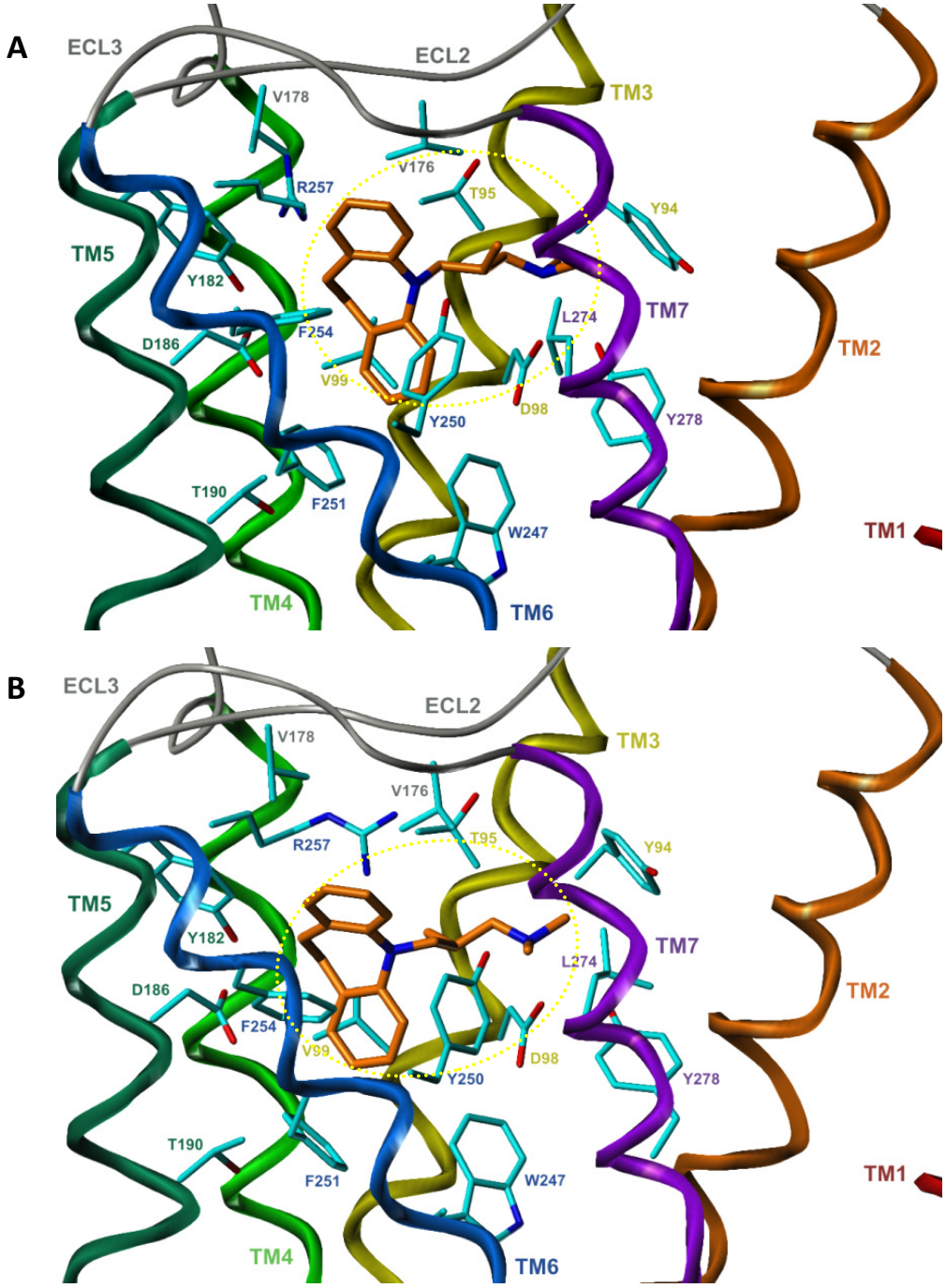


Fig. C.5. Side view of the hH_2R model in complex with (*R*)-TMP in A and (*S*)-TMP in B. The putative inverse agonist binding site and the extracellular components of the hH_2R are shown. TMP (carbon atoms in orange) was manually docked into the putative binding pocket. Colored ribbons represent the transmembrane domains TM1-TM7; thin grey lines represent extracellular loops ECL1, ECL2 and ECL3. The model – based on the crystal structure of the $h\beta_2AR$ – was generated as described under Materials and Methods in Chapter B.2.7.

While we used the racemate to generate the experimental results, we were able to differ the two enantiomers of TMP for the modelling studies. Two positions are possible binding to hH_2R. At physiological pH the protonated nitrogen of the side chain interacts with the negatively charged aspartic acid in TM3 (Asp-98) or TM5 (Asp-186). The docking mode with the charged nitrogen near Asp-98 as displayed in Fig. C.5 was preferred by the docking results. The amino acids contributing to binding belong to TM3, TM5, TM6 and TM7. The heterocycle of (*R*)- and (*S*)-TMP is positioned similarly and closely interacts with the hydrophobic amino acids Trp-247, Tyr-250, Phe-251, Phe-254 and a possible π-cation interaction with Arg-257 of TM6. This part of the binding pocket (Fig. C.6) is furthermore formed by Tyr-182, Asp-186 and Thr-190 of TM5 (Gantz *et al.*, 1992; Nederkoorn *et al.*, 1996) and amino acids Val-176 and Val-178 of ECL2, as well as Thr-95 and Val-99 of TM3. The 2-methyl group of (*R*)-TMP interacts with Leu-274 of TM7 and Val-176 of ECL2. The corresponding methyl group of the (*S*)-enantiomer points towards Val-176, Thr-95, Val-99 and Asp-98. Further contacts to the side chain of TMP occur with Tyr-250, Leu-274 and Tyr-278 of TM7, Tyr-94 and Asp-98 of TM3.

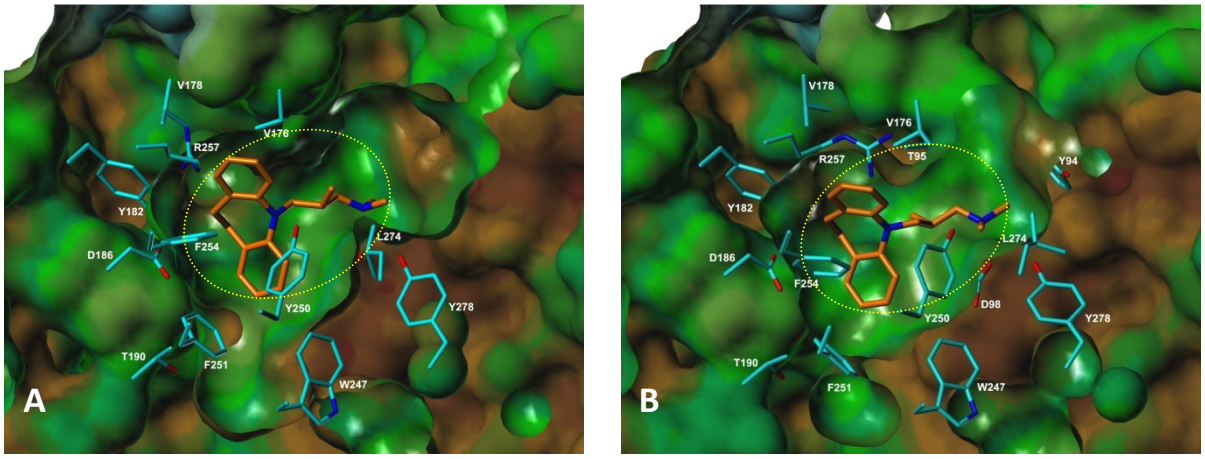


Fig. C.6. Electrostatic potential surface in the binding pocket of inactive hH_2R with (*R*)-TMP and (*S*)-TMP in its binding conformation. The electrostatic potential of the binding pocket surface is rather lipophilic (brown surface) and can, therefore, interact with the hydrophobic ring system of TMP (carbon atoms in orange, yellow dotted line). **A**, open binding pocket with inlaying (*R*)-TMP. **B**, open binding pocket with inlaying (*S*)-TMP. The model was generated as described in Chapter B.2.7.

C.4.2 Binding of tiotidine to hH_2R

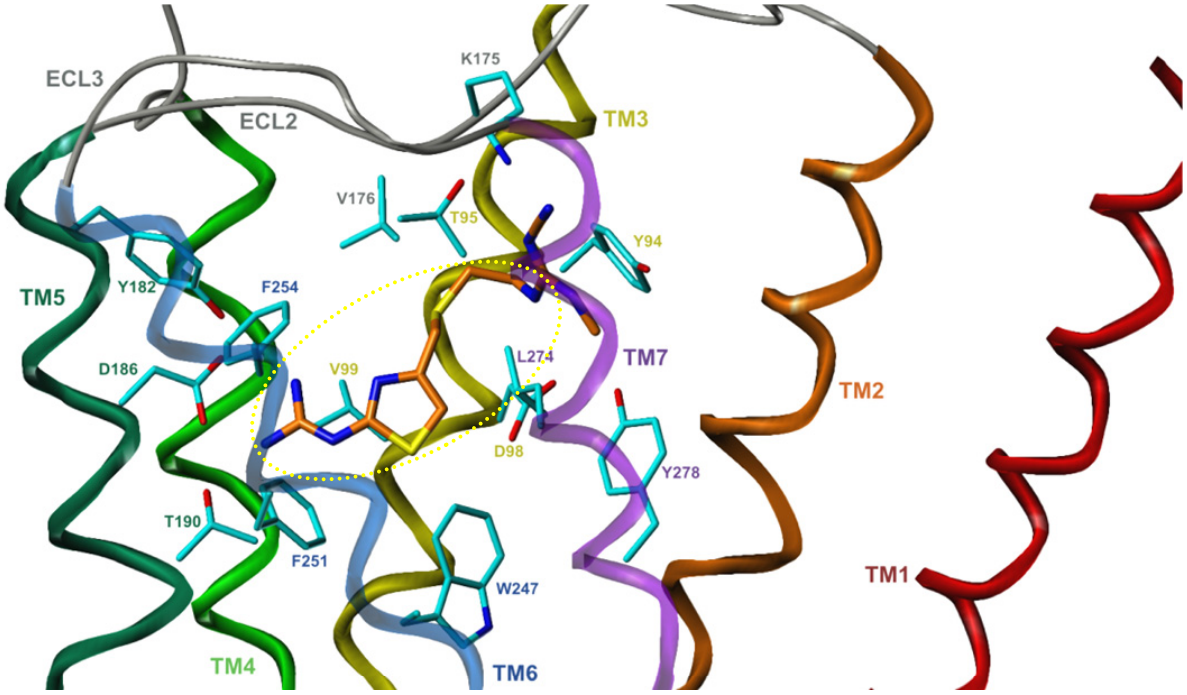


Fig. C.7. Side view of the hH_2R model in complex with TIO. The putative inverse agonist binding site and the extracellular components of the hH_2R are shown. TIO (carbon atoms in orange, yellow dotted line) was manually docked into the putative binding pocket. Colored ribbons represent transmembrane domains TM1-TM7; thin grey lines represent extracellular loops ECL1, ECL2 and ECL3. The model – based on the crystal structure of the $h\beta_2AR$ – was generated as described under Materials and Methods in Chapter B.2.7.

Interacting amino acids for TIO at hH_2R are similar to those which contribute to the TMP-binding. According to the docking results, most important are amino acids Asp-186 and Thr-190 of TM5 and Asp-98 of TM3 (Fig. C.7). They enable hydrogen bonds to the guanidine moiety and the cyanoguanidine moiety of TIO. Moreover, these amino acids were shown to be important for the binding of TIO (Gantz *et al.*, 1992). Lys-175 of ECL2 seems to be essential, too. In the docking result a further hydrogen bond between Lys-175 and the cyano group of TIO exists.

C.4.3 Binding of trimipramine to hH_1R

The data obtained in the steady-state GTPase assay show that TMP acts as an inverse agonist at hH_1R, quite similarly as at hH_2R. According to the docking results, the highly conserved Asp-107 in TM3, which interacts with TMP by electrostatic interactions, as well as the hydrophobic amino acids Trp-428, Tyr-431 and Phe-435 in TM6, which interact with the tricyclic ring system of TMP, are the most important are amino acids (Fig. C.8).

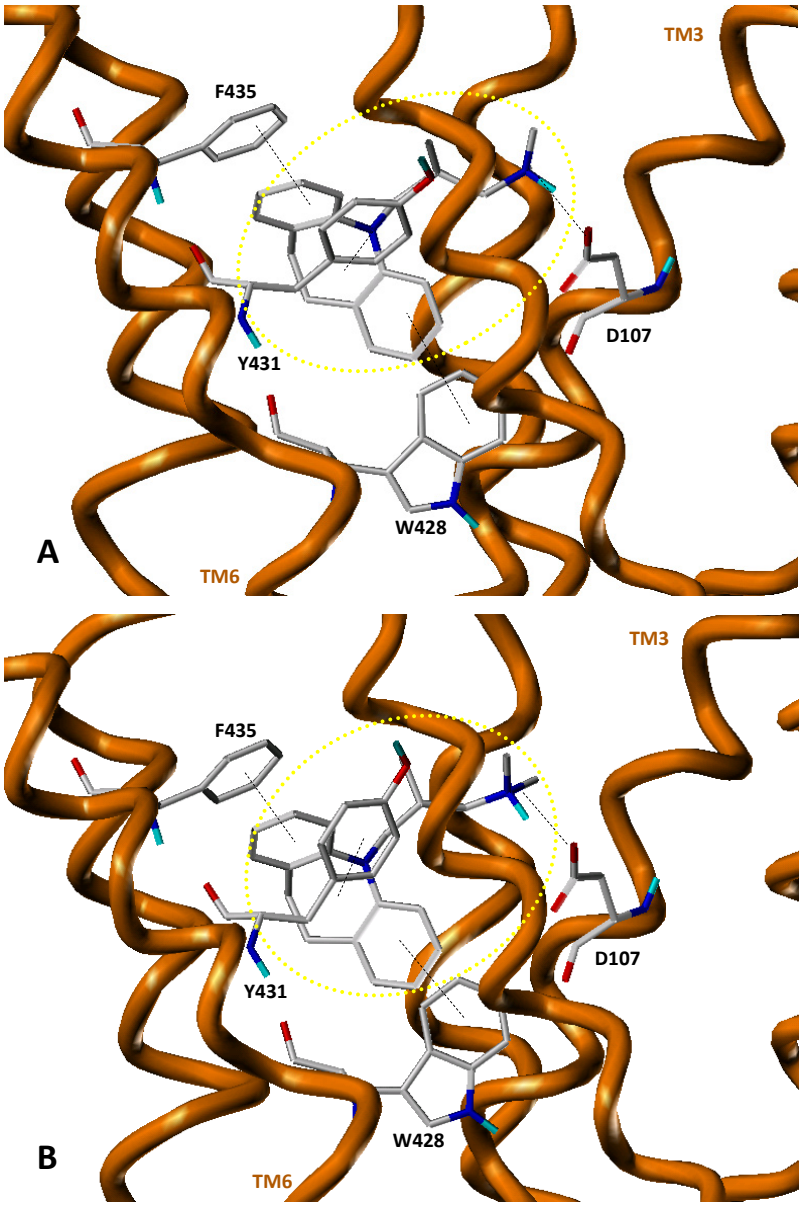


Fig. C.8. Side view of the hH_1R model in complex with (*R*)-TMP in A and (*S*)-TMP in B. The putative inverse agonist binding site of the hH_1R is shown. TMP (carbon atoms in grey, yellow dotted line) was manually docked into the putative binding pocket. Colored ribbons represent transmembrane domains TM1-TM7. The model was generated as described in Chapter B.2.7.

C.4.4 Binding of clozapine to hH_1R

The SEA predictions were not sufficient for the binding of CLO to the hH_1R. We, therefore, performed the docking of this atypical antipsychotic with this receptor. The interacting amino acids are similar to those which contribute to the binding of TMP. According to the docking results, the highly conserved Asp-107 in TM3, which stabilizes the position by electrostatic interactions, as well as the hydrophobic amino acids Trp-428, Tyr-431 and Phe-435 in TM6, which interact with the aromatic ring system, are the most important are amino acids (Fig. C.9).

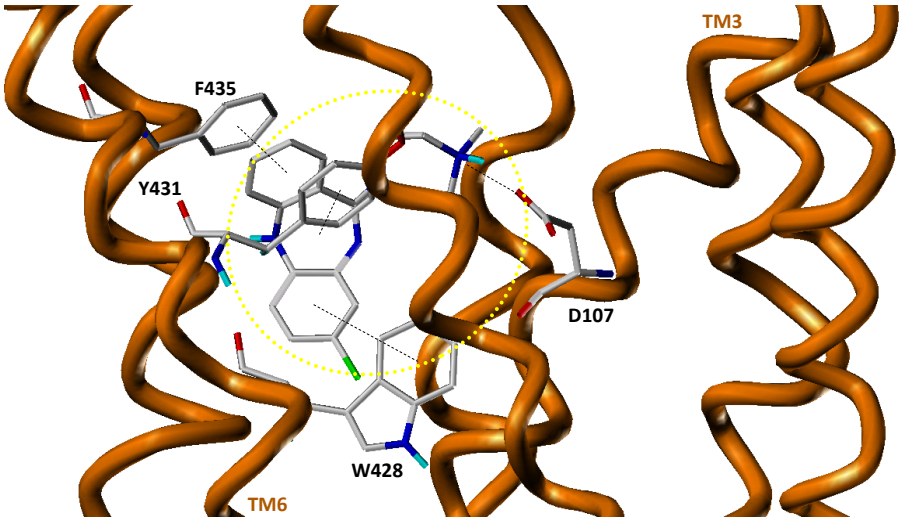


Fig. C.9. Side view of the hH_1R model in complex with CLO. The putative inverse agonist binding site of the hH_1R is shown. CLO (carbon atoms in grey, yellow dotted line) was manually docked into the putative binding pocket. Colored ribbons represent transmembrane domains TM1-TM7. The model was generated as described in Chapter B.2.7.

C.4.5 Binding of trimipramine to hH_4R

The affinity of TMP to the hH_4R is only marginal in comparison to the hH_1R and hH_2R. Nevertheless, we conducted docking studies for the TCA at this receptor, too. According to these studies, the most important interacting amino acids for TMP at hH_4R are electrostatic interactions with the highly conserved Asp-94 in TM3 and aromatic ring interactions with the hydrophobic amino acids Tyr-95 in TM3, Trp-316 and Tyr-319 in TM6 (Fig. C.10). However, TMP does not fit optimally into the binding pocket and the hydrophobic interactions are not established well, compared to the docking at hH_1R. In comparison to CLO, TMP contains no

structure-stabilizing piperazine ring and is more flexible. This property might be responsible for a diminished fitting into the binding pocket and, therefore, reduced affinity.

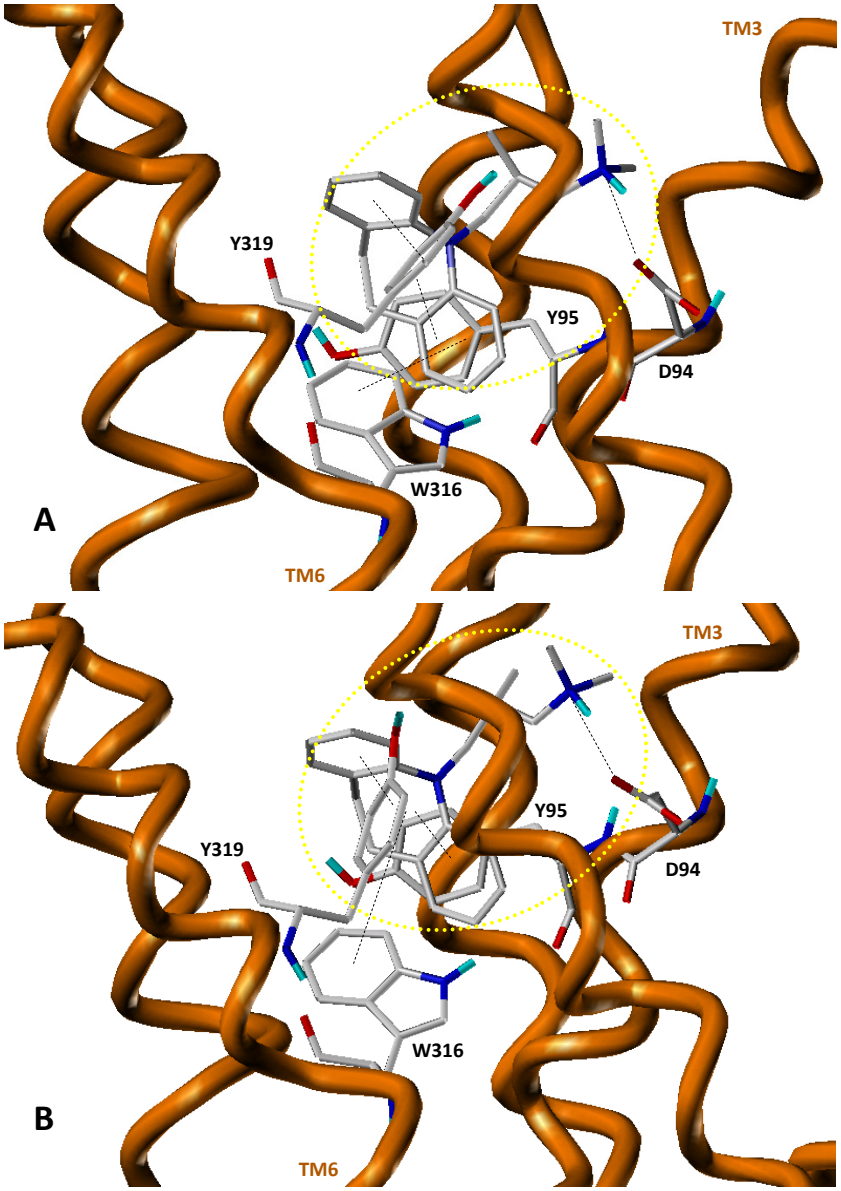


Fig. C.10. Side view of the hH_4R model in complex with (*R*)-TMP in A and (*S*)-TMP in B. The putative inverse agonist binding site of the hH_4R is shown. TMP (carbon atoms in grey, yellow dotted line) was manually docked into the putative binding pocket. Colored ribbons represent transmembrane domains TM1-TM7. The model was generated as described in Chapter B.2.7.

C.4.6 Binding of clozapine to hH_4R

The data obtained in the steady-state GTPase assays show that CLO acts as a partial agonist at hH_4R and, therefore, CLO was docked into the active state model of hH_4R (Fig. C.11). This activation is based on a change of the highly conserved Trp-316 in TM6 into a horizontal conformation, described as toggle switch (Crocker *et al.*, 2006). Interacting amino acids for CLO at hH_4R are similar to those which contribute to the TMP binding. According to the docking results, most important are amino acids Asp-94 in TM3 with its electrostatic interactions and the hydrophobic amino acids Tyr-95, Trp-316, Tyr-319 in TM6 and Tyr-340 in TM7 with the aromatic ring system. In contrary to the binding to hH_1R, CLO exhibits a different orientation at hH_4R which may explain the reduced affinity.

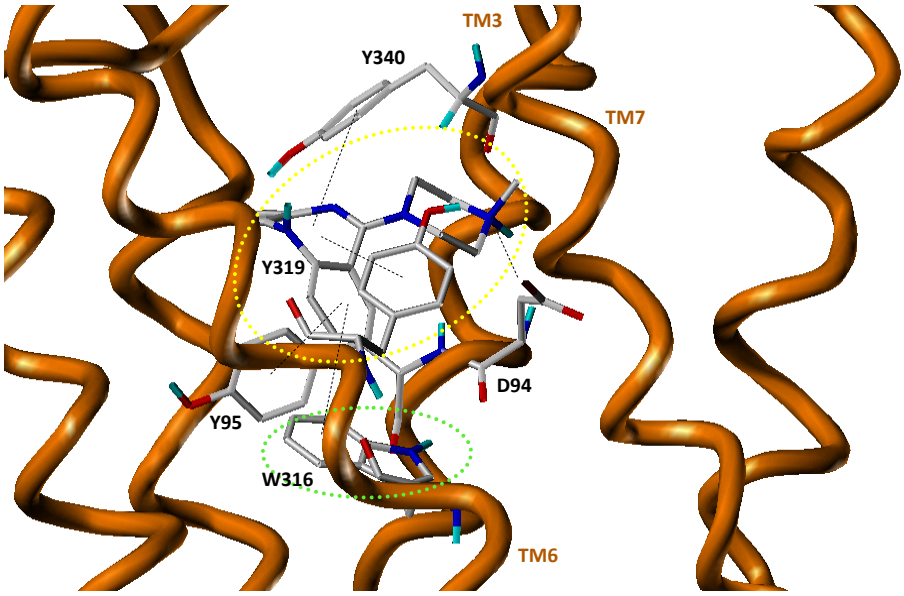


Fig. C.11. Side view of the hH_4R model in complex with CLO. The putative agonist binding site of the hH_4R is shown. CLO (carbon atoms in grey, yellow dotted line) was manually docked into the putative binding pocket. The green circle indicates the highly conserved Trp-316 in TM6, which is important element in the rotamer toggle switch during receptor activation. Colored ribbons represent transmembrane domains TM1-TM7. The model was generated as described in Chapter B.2.7.

In comparison to a previously described model by (Jongejan *et al.*, 2008) this model displays some differences: the nitrogen atom in position 1 of the piperazine system needs to adopt a planar conformation. Further, more likely than the boat conformation for the tri-cyclic component is that of a capsized boat. Finally, due to our calculations any interactions of the glutamate in TM5 with CLO seem to be improbable.

C.5 Schild analysis of trimipramine and tiotidine at hH_2R

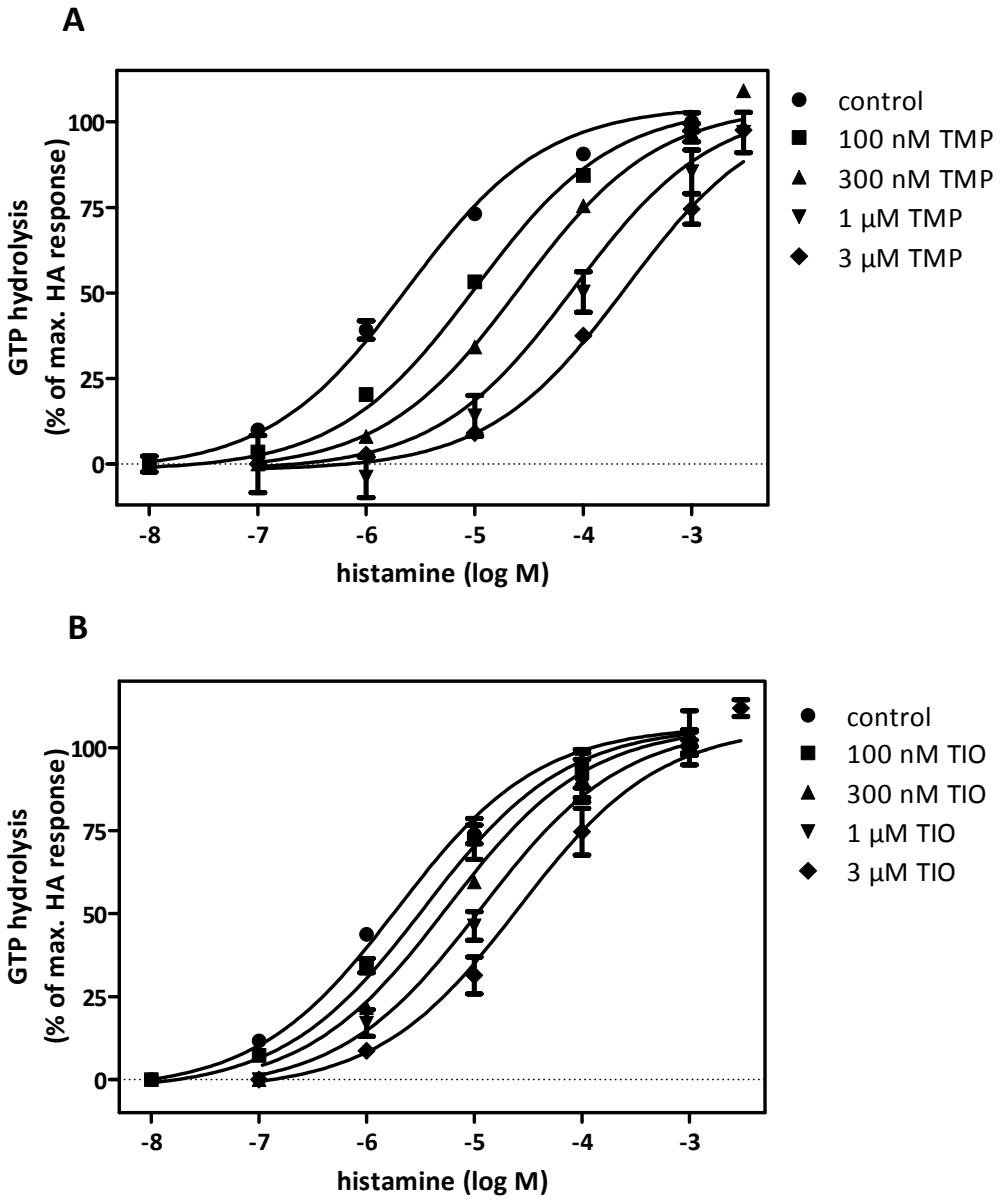


Fig. C.12. Concentration-dependent increase of GTPase activity by HA and competition with TMP (A) and TIO (B) in membranes expressing hH_2R-$G_s\alpha_S$. GTPase activity in Sf9 membranes was determined as described in Chapter B.2.5. Reaction mixtures contained membranes (10 µg of protein/tube) expressing receptor and proteins and HA at concentrations indicated on the abscissa. Through addition of various concentrations of TMP or TIO right shifts with consistent maximum stimulatory effect were yielded (TMP: pA_2 = 7.50 ± 0.07; Schild Slope = 1.02; r^2 = 0.99; TIO: pA_2 = 6.84; Schild Slope = 0.82; r^2 = 0.99). Data shown were performed in duplicates and analyzed by Gaddum/Schild EC_{50} shift.

A Schild analysis was conducted in order to illuminate the quality of TMP antagonism and to determine its pA_2 at the H_2R. To increasing concentrations of HA constant concentrations of TMP were added. Different concentrations of TMP caused a right-shift of these concentration/response curves due to a competition of HA with TMP for the binding pocket. As the E_{max} of the various concentration/response curves were constant and did not decline, TMP is a competitive antagonist of HA. According to the Law of mass action both ligands competed for the same binding site. The displacement of the agonist HA by TMP led to the right shift. As TMP is a competitive antagonist, it can also be redisplaced by increasing concentrations of HA so that the maximum efficacy of the agonist is still achieved. Quality of TMP antagonism was also validated by a Schild Slope ≈ 1 and a comparable pA_2-value in the low nanomolar range. The same behavior was found for TIO so that HA, TMP and TIO are likely to compete for the same binding site.

C.6 Comparison of various antidepressants and antipsychotics at hH_2R and gpH_2R

The analysis of a series of agonists and antagonists at human, canine, guinea pig and rat H_2R revealed that within the species isoforms most discrepancies were determined for hH_2R and gpH_2R. Data are summarized in Table C.4. All drugs acted consistently as weak inverse agonists (E_{max} -0.05 to -0.22). The inhibitory potencies were comparable at both receptor isoforms with significantly decreased K_b-values at gpH_2R for some structurally unrelated compounds. Inversely, only the potency for RIS was significantly lower at hH_2R than at gpH_2R. The correlation of pK_b-values of seven structurally similar TCAs for hH_2R and gpH_2R displayed no significant difference between these two isoforms, only a slight right-shift due to the higher pK_b-values at hH_2R (Fig. C.13).

Cpd.	hH_2R		gpH_2R	
	K_b [nM] ± S.D.	Inv. Eff. ± S.D.	K_b [nM] ± S.D.	Inv. Eff. ± S.D.
AMI	118 ± 45	-0.12 ± 0.01	174 ± 83	-0.12 ± 0.01
CPZ	1,534 ± 720	-0.22 ± 0.02	833 ± 70	-0.19 ± 0.02
CPX	592 ± 56***	-0.15 ± 0.01***	192 ± 33	-0.14 ± 0.01
CPM	168 ± 66	-0.14 ± 0.01	158 ± 46	-0.13 ± 0.01
DPM	1,439 ± 702	-0.12 ± 0.01	1,244 ± 251	-0.11 ± 0.004
DBP	3,174 ± 154	-0.09 ± 0.01	2,860 ± 635	-0.09 ± 0.003
DXP	344 ± 79	-0.12 ± 0.02	461 ± 22	-0.12 ± 0.01
FPZ	16,702 ± 1,729***	-0.14 ± 0.01	8,188 ± 1,075	-0.16 ± 0.01
IMI	791 ± 92**	-0.13 ± 0.01	539 ± 39	-0.12 ± 0.01
LMZ	596 ± 130	-0.17 ± 0.01	469 ± 39	-0.16 ± 0.01
LPM	5,684 ± 1,243**	-0.18 ± 0.01*	1,818 ± 151	-0.16 ± 0.01
LOX	1,221 ± 204**	-0.10 ± 0.03	726 ± 100	-0.10 ± 0.01
MPT	857 ± 118	-0.13 ± 0.01	720 ± 55	-0.12 ± 0.01
MSN	450 ± 16***	-0.12 ± 0.004	240 ± 38	-0.12 ± 0.01
NTL	877 ± 289	-0.14 ± 0.01	684 ± 33	-0.11 ± 0.03
OPI	6,261 ± 617**	-0.12 ± 0.01	3,801 ± 611	-0.11 ± 0.01
PRX	8,960 ± 1,088**	-0.15 ± 0.04	5,148 ± 271	-0.12 ± 0.003
PMZ	197 ± 65	-0.15 ± 0.01	133 ± 33	-0.14 ± 0.01
PTL	688 ± 188	-0.14 ± 0.01	754 ± 109	-0.13 ± 0.003
RIS	202 ± 42***	-0.07 ± 0.02	525 ± 68	-0.05 ± 0.03
TRZ	454 ± 112	-0.17 ± 0.02	357 ± 29	-0.16 ± 0.01
MRZ	1,228 ± 248*	-0.12 ± 0.01	807 ± 147	-0.12 ± 0.02
TMP	44 ± 12	-0.13 ± 0.01	56 ± 7	-0.10 ± 0.02

Table C.4. Inhibiting potencies (K_b) and inverse agonist efficacies (Inv. Eff.) of antidepressant and antipsychotic drugs at human and guinea pig (gp) H_1R + RGS4 and human and guinea pig H_2R-$G_s\alpha_S$. GTPase assay was performed with Sf9 membranes as described in Chapter B.2.5. Reaction mixtures contained Sf9 membranes expressing receptor and G proteins and antagonists at concentrations from 1 nM to 100 µM as appropriate to generate saturated competition curves. To determine the inverse agonist efficacies (Inv. Eff.), the effects of antagonists at fixed concentrations (10 µM to 100 µM) on basal GTPase activity were assessed and referred to the stimulatory effect of 100 µM HA (= 1.00). Data were analyzed by non-linear regression and were best fitted to sigmoid concentration/response curves. Shown are the means ± S.D. Two to five experiments were performed in duplicates and triplicates. The potencies of compounds at hH_2R-$G_s\alpha_S$ were compared with the corresponding potencies of compounds at gpH_2R- $G_s\alpha_S$ using the t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

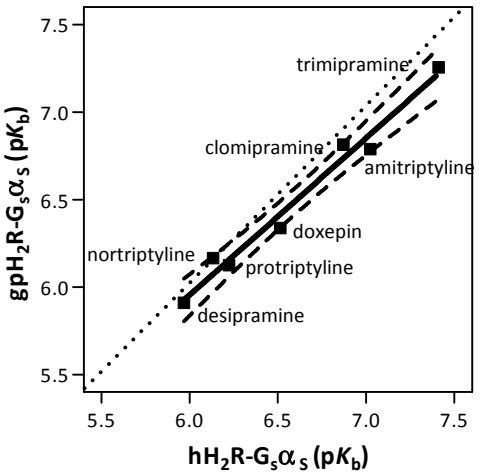


Fig. C.13. Correlation of potencies (pK_b) of various TCAs at hH_2R-$G_s\alpha_s$ and gpH_2R-$G_s\alpha_s$. Data shown were analyzed by linear regression: slope = 0.90 ± 0.06; r^2 = 0.98. The dashed lines indicate the 95% confidence intervals of the regression lines. The diagonal dotted line has a slope of 1 and represents a theoretical curve for identical values.

C.7 Agonism of antipsychotic drugs at hH_4R

Most of the 34 examined antidepressants and antipsychotics acted as inverse agonists at H_4R. Only four of them displayed partial agonistic activity (Fig. C.14). For the atypical antipsychotic CLO a slightly higher potency and efficacy (EC_{50} = 1.7 µM; E_{max} = 0.66) were determined than for its demethyled metabolite CLD (EC_{50} = 2.6 µM; E_{max} = 0.36). The other tested metabolite CLN acted also partial agonistic (E_{max} = 0.28), but due to the *N*-oxide with much lower potency of > 100 µM. OLA, which differs from CLO only by substitution of 2-methylthiophene, was as efficacious but much less potent (EC_{50} = 38 µM; E_{max} = 0.57). Although structurally dissimilar, the thioxanthene CPX exhibited similar pharmacological properties (EC_{50} = 2.9 µM; E_{max} = 0.39) as the atypical antipsychotics CLO and CLD, but only the two latter are likely to interact with hH_4R at therapeutic relevant concentrations.

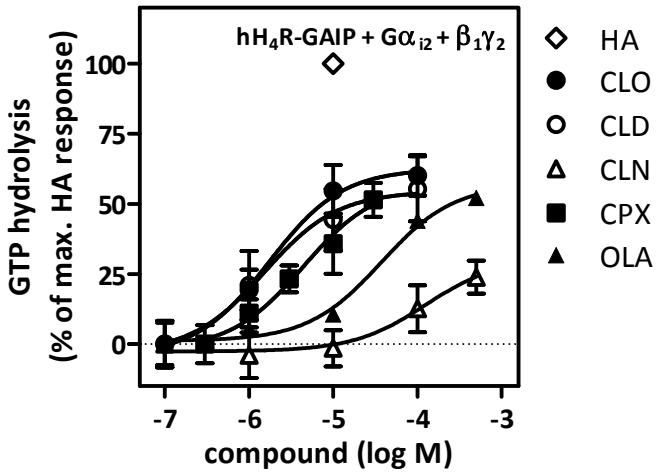


Fig. C.14. Concentration-dependent increase of GTP hydrolysis by various antipsychotics in membranes expressing hH_4R-GAIP + $G\alpha_{i2}$ + $\beta_1\gamma_2$. GTPase activity in Sf9 membranes was determined as described in Chapter B.2.5. Reaction mixtures contained membranes (10 µg of protein/tube) expressing receptor, proteins and ligands at concentrations indicated on the abscissa. Data shown are the means ± S.D. Three independent experiments were performed in duplicates. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves.

C.8 Inverse agonism of psychiatric drugs at hH_3R

All of the examined antidepressant and antipsychotic drugs except CBZ and CLN exhibited inverse agonism at hH_3R but to a varying extent (Fig. C.15). The TCAs CPM and LPM and the antipsychotics AMO, CLO, DBP, HAL, OLA and RIS acted as weak inverse partial agonists with E_{max} < -0.5. For the remaining compounds inverse agonist efficacies with more than half-maximal stimulation were determined. MSN revealed nearby fully inverse agonism relative to HA (E_{max} = -0.95). Due to the low potencies in comparison to the therapeutic plasma concentrations none of the drugs is likely to cause any effects related to H_3R under clinical conditions.

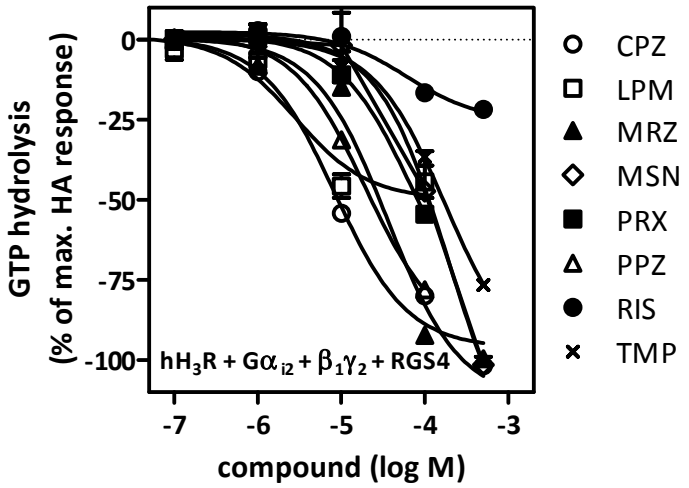


Fig. C.15. Concentration-dependent decrease of GTP hydrolysis by antidepressants and antipsychotics in membranes expressing $hH_3R + G\alpha_{i2} + \beta_1\gamma_2$ + RGS4. GTPase activity in Sf9 membranes was determined as described in Chapter B.2.5. Reaction mixtures contained membranes (10 µg of protein/tube) expressing receptor, proteins and ligands at concentrations indicated on the abscissa. Data shown are the means ± S.D. Three independent experiments were performed in duplicates. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves.

C.9 Direct G protein-stimulatory effects of ligands at higher concentrations

The main effects during a therapy with antidepressant and antipsychotic drugs are known to be the result of interaction with receptors like D_2R, 5-HT_xR, H_1R and α_1AR. Nevertheless, we found that some compounds of both medication groups activate G proteins at higher concentrations also in a receptor-independent manner because some drugs displayed varying agonistic or antagonistic properties depending on their concentrations. Therefore, assays were conducted with Sf9 membranes expressing only $G\alpha_{i2} + \beta_1\gamma_2$ but no H_xR. We found that the typical antipsychotics CPZ, CPX, FPZ, PPZ, PCP and TRZ, the SSRI PRX and the TCA CPM increased the hydrolysis of GTP to GDP and P_i at concentrations higher than 100 µM although no histamine receptor was present (Fig. C.16). The remaining substances showed no such effects within concentrations up to 500 µM and, thus, an involvement in the therapeutic effects of psychiatric medication can be excluded. The identified unspecific effects were not relevant for determination of affinities, potencies and efficacies of compounds since we did not consider ligand concentrations > 100 µM.

Such direct G protein-stimulatory effects of cationic-amphiphilic compounds were already described for mast cell-activating substances, like mastoparan, substance P and com-

pound 48/80 (Higashijima *et al*., 1988; Mousli *et al*., 1990a; 1990b) and H_1R agonists and antagonists, such as 2-(3-chlorophenyl)histamine (Seifert *et al*., 1994), diphenhydramine and chlorpheniramine, which stimulate HL-60 cells, basophils and mast cells without receptor activation (Burde *et al*., 1996).

All these substances share a physicochemical property: they are cationic amphiphiles. At physiological pH the nitrogen of the side chain is protonated to a quaternary ammonium cation. Furthermore, the structures can be divided into a lipophilic and a polar or cationic domain. The G protein-activating properties seem to depend on the arrangement of these domains and, thus, its basicity (Detert *et al*., 1996). Accountable for this alternative mode of G protein-regulation is possibly a novel group of proteins that act as receptor-independent activators of G protein signalling (AGS). Three different groups of AGS (AGS1-3) were identified in a pheromone response pathway in *Saccharomyces cerevisiae* and alter G protein-signalling by influencing nucleotide exchange or G protein-subunit interactions unrelated to a cell surface receptor (Cismowski *et al*., 2001; Blumer *et al*., 2005).

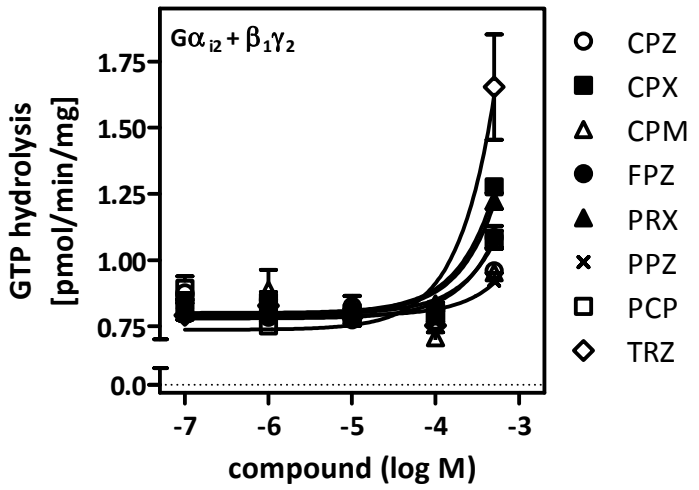


Fig. C.16. Direct G protein-stimulatory effects of ligands at higher concentrations. GTPase activity in Sf9 membranes expressing $G\alpha_{i2}$ + $\beta_1\gamma_2$ was determined as described in Chapter B.2.5. Reaction mixtures contained membranes (10 µg of protein/tube) expressing G proteins and ligands at concentrations indicated on the abscissa. Data shown are the means ± S.D. performed in duplicates.

C.10 [^{3}H]tiotidine saturation binding at hH_2R

[^{3}H]TIO is a commonly used radioligand for binding studies at H_2R with a dissociation constant (K_d) of 42.3 nM. Despite the use of 0.3% (m/V) polyethyleneimine a non-specific binding of more than 60% still occurred in Sf9 membranes with saturating radioligand concentrations (Fig. C.17). Obviously, the majority of ligand-free hH_2R-$G_s\alpha_S$ protein exists in a conformation that is not capable of binding [^{3}H]TIO (Kelley *et al*., 2001).

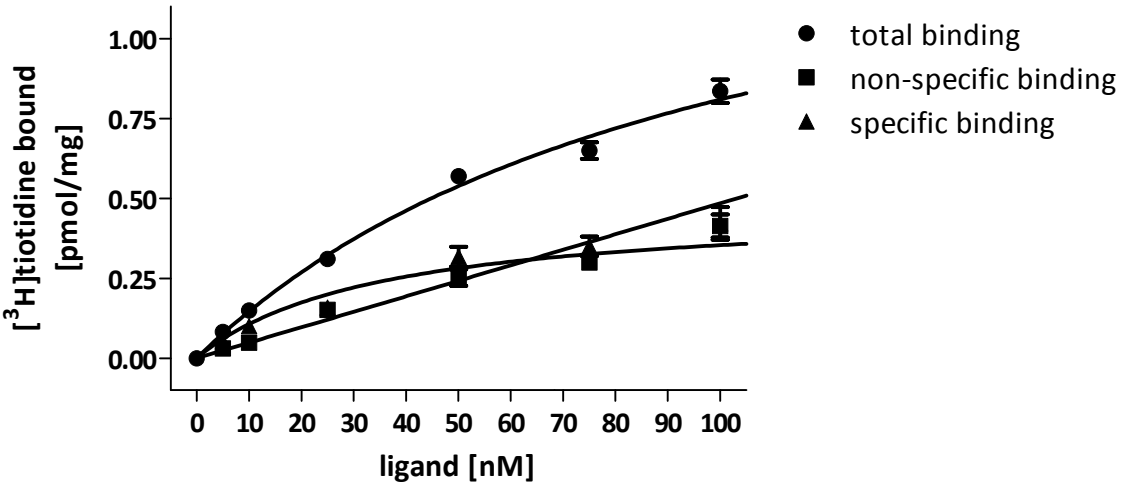


Fig. C.17. [^{3}H]tiotidine saturation binding in Sf9 cell membranes expressing hH_2R-$G_s\alpha_S$. Experiments were performed as described in Chapter B.2.4. Data were analyzed by non-linear regression and were best fitted to hyperbolic one-site saturation isotherms. The closed triangles (▲) show the data for the specific [^{3}H]TIO binding (K_d = 42.3 nM; B_{max} = 0.48 pmol/mg). Data points shown are the means ± S. D. performed in triplicates.

C.11 [^{3}H]trimipramine saturation binding at hH_2R

Due to the high affinity of TMP to hH_2R (K_i = 41 nM; K_b = 44 nM), the tritiated TCA is possibly an applicable radioligand for the use in Sf9 membranes. We determined a K_d of 45.2 nM for [^{3}H]TMP which is also in a comparable high range than that of [^{3}H]TIO. Non-specific binding of [^{3}H]TMP was even exceeding 80% (Fig. C.18). The non-selectivity and binding properties to several other targets such as 5-HT_xR, mAChR, H_1R, D_xR and αAR also limit the use of TMP as radioligand to recombinant systems expressing H_2R, so that [^{3}H]TIO is still superior to [^{3}H]TMP as radioligand.

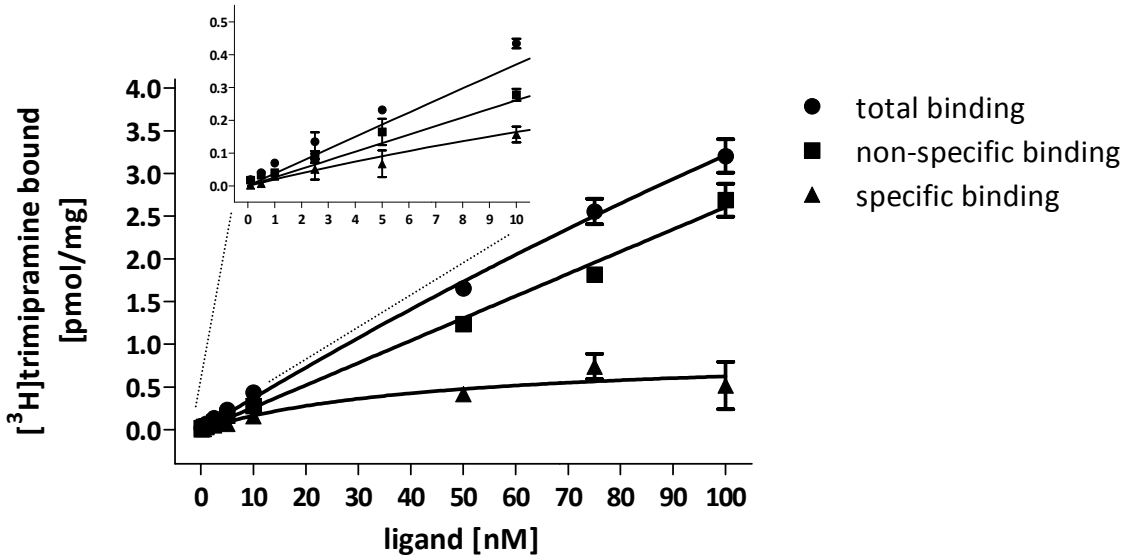
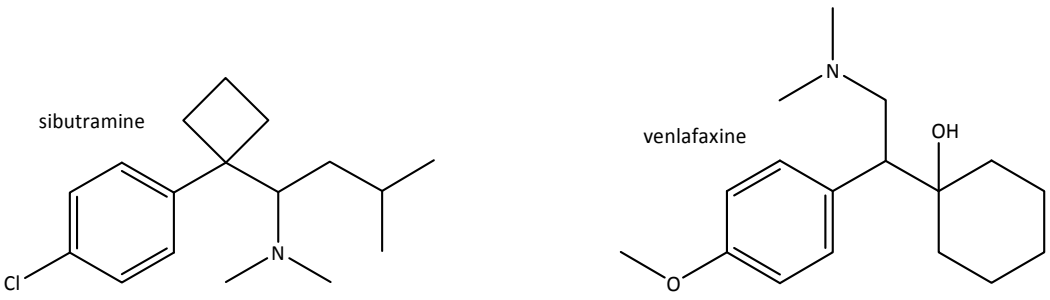


Fig. C.18. [^{3}H]trimipramine saturation binding in Sf9 cell membranes expressing hH_2R-$G_s\alpha_S$. Experiments were performed as described in Chapter B.2.4. Data were analyzed by non-linear regression and were best fitted to hyperbolic one-site saturation isotherms. The closed triangles (▲) show the data for the specific [^{3}H]TMP binding (K_d = 45.2 nM; B_{max} = 0.91 pmol/mg). The inset shows the first six data sets at a larger scale. Data points shown are the means ± S. D. performed in duplicates.

C.12 Functional analysis of sibutramine at hH_1R and hH_2R

Sibutramine is an appetite suppressant for the treatment of obesity and acts as SNRI. Its structure resembles that of amphetamine, which enhances a reverse function of DAT and SERT, and the SNRI venlafaxine. All compounds increase the concentrations of monoamines in the synaptic cleft and, thereby, induce satiety.



The mechanism of action is similar to that of TCAs and the pharmacological effects are mainly caused by its two active metabolites (Ryan *et al*., 1995). Although the drug was examined in animals and humans for its antidepressant properties 20 years ago (Buckett *et al*., 1988; Luscombe *et al*., 1989), it was never used for this indication. Because the

Sibutramine Cardiovascular Outcome Trial observed an increased risk of serious, non-fatal cardiovascular events, such as stroke or heart attack, the *European Medicines Agency* (EMA) suspended sibutramine from the European market (Williams, 2010). The increase of appetite and a consecutive weight gain is a common side effect for most psychiatric drugs and is often also associated to antagonism at H_1R (Kim *et al*., 2007). Many anti-histaminic drugs acting at H_1R also display these orexigenic properties (Sakata *et al*., 1988). Hypothalamic neuronal HA signalling *via* H_1R contributes to appetite modulation. Food intake was suppressed by injection of HA into rat ventromedial and paraventricular nuclei (Ookuma *et al*., 1993). The same effect was mediated by the appetite suppressor leptin which increased secretion of HA in the hypothalamus (Masaki and Yoshimatsu, 2006). In patients with anorexia nervosa the density of H_1R was increased (Yoshizawa *et al*., 2009). Thus, it is likely that the appetite-suppressing properties of endogenous HA are blocked by psychiatric drugs with H_1R antagonism such as CLO or TMP, whereas the lack of affinity to this receptor for sibutramine maintains the activating properties of HA in hypothalamic cells and suppresses appetite.

As sibutramine as well as venlafaxine (Westenberg and Sandner, 2006) and PRX are rather selective and have no affinity to H_1R within the therapeutic plasma concentrations (Table C.5), no weight gain is expected here which facilitates therapy of obese patients with depression. Studies of other orexigenic or anorexigenic receptors did not identify a novel binding site contributing to the induced weight gain (Theisen *et al*., 2007). Nevertheless, as psychiatric drugs show a broad receptor binding profile that is not fully understood yet, also other effects may still account for the modulation of food intake. The missing effect of sibutramine on hH_2R in contrary to many other psychiatric medications is likely to be associated with the potential lack of antidepressant quality – the indication originally developed.

Cpd.	hH_1R		hH_2R		Plasma concentration of main metabolites [µM]
	K_b [µM] ± S.D.	Inv. Eff. ± S.D.	K_b [µM] ± S.D.	Inv. Eff.	
sibutramine	20 ± 1.5	-0.10 ± 0.08	32 ± 3.5	-0.15	0.015 to 0.032†

Table C.5. Inhibiting potencies (K_b) and inverse agonist efficacies (Inv. Eff.) of sibutramine at hH_1R + RGS4 and hH_2R-$G_s\alpha_s$. GTPase assays were performed with Sf9 membranes as described in Chapter B.2.5. Reaction mixtures contained Sf9 membranes expressing receptor and G proteins, HA (1 µM) and sibutramine at concentrations from 100 nM to 500 µM as appropriate to generate saturated competition curves. To determine the inverse agonist efficacies (Inv. Eff.), the effects of antagonists at a fixed concentration (500 µM) on basal GTPase activity were assessed and referred to the stimulatory effect of 100 µM HA (= 1.00). Data were analyzed by non-linear regression and were best fit to sigmoidal concentration/response curves. Values are given in micromolar and are the means ± S.D. of two to five experiments performed in duplicate.

† plasma concentrations of the main metabolites were calculated according to Talbot *et al*., 2010

D. Discussion

As shown in Chapter C, interactions between the examined psychiatric drugs and the four HA receptor subtypes are complex. Because of similarities between affinities, potencies and therapeutic reference ranges it is likely that some of these interactions are also therapeutically relevant. Thus, the number of interaction sites of the single drug has to be extended. This “polypharmacology” through interaction with multiple molecular targets may be crucial for its therapeutic efficacy, but also pivotal for its side effect profile (Roth *et al*., 2004).

D.1 Structure-activity relationships for hH_xR

The key ligand-receptor interaction sites of the substances that were explored by molecular modelling are localized in TM3, most notably the highly conserved aspartic acid (hH_1R: Asp-107; hH_2R: Asp-98; hH_4R: Asp-94) and in TM6, most notably the highly conserved amino acids tryptophan (hH_1R: Trp-428; hH_2R: Trp-247; hH_4R: Trp-316) and tyrosine (hH_1R: Tyr-431; hH_2R: Tyr-250; hH_4R: Tyr-319), while Phe-435 and Phe-251 interact solely with ligands binding to hH_1R and hH_2R, respectively (Fig. D.1). Due to the low affinities for H_3R no interaction sites could be identified. The endogenous ligand HA interacts not only with amino acids in TM5, but also with the highly conserved aspartic acid in TM3. Besides, interaction of ligands with hH_1R, hH_2R and hH_4R is also mediated by other non-conserved amino acids in ECL2, TM5 and TM7. The highly conserved aspartic acid in TM3 is present in all receptors for biogenic amines and serves as a counter anion to the cationic amino group of the amines, which occurs under physiological conditions (Gantz *et al*., 1992) (*q.v.* the binding mode of HA in Fig. A.12). In contrast, the orientation of the highly conserved tryptophan in TM6 plays an integral part in stabilizing active or inactive conformation of the receptor described as toggle switch (Crocker *et al*., 2006). Therefore, interaction with these two amino acids is crucial for binding and functional effect of ligands at H_xR.

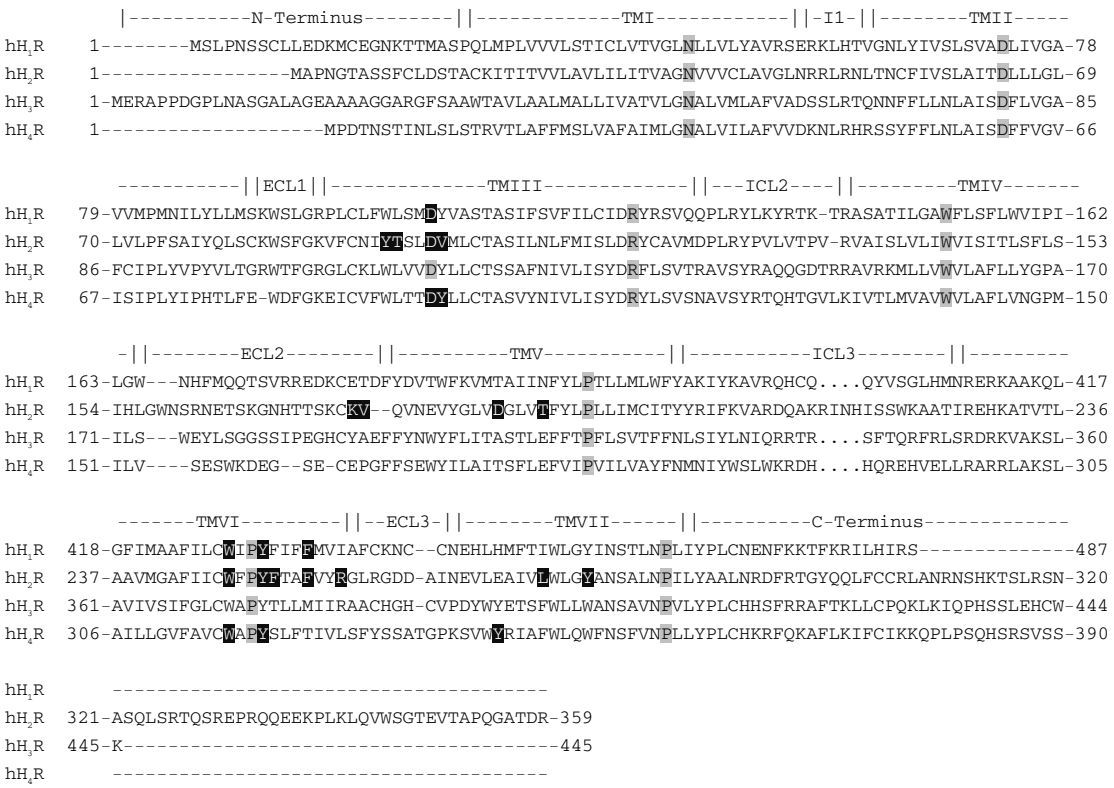


Fig. D.1. Alignment of the amino acid sequences of hH_1R, hH_2R, hH_3R and hH_4R. Dots in the sequences of hH_1R, hH_3R and hH_4R indicate incomplete amino acid sequence of the long ICL3. Hyphens indicate missing amino acids. Amino acids with gray shading are the most conserved amino acids, according to the numbering scheme used by Ballesteros *et al*. (2001). Amino acids in white with black shading indicate the amino acids that are proposed to interact in the binding pocket of the hH_xR models as described in Chapter C.4. The amino acid sequences are given in the one-letter code. The sequence alignment was performed as multi-sequence alignment using ClustalW 2.0 (Larkin *et al*., 2007) and subsequently edited manually.

Some minor structural changes of the examined ligands appear to be pivotal for affinity and potency to hH_xRs. Contrary to the observation previously made for other receptors (Richelson, 1982) that tertiary amine tricyclic antidepressants (AMI, CPM, DXP, IMI and TPM) are more potent ligands than their secondary amine counterparts (DPM, nortriptyline NTL and PTL), this is not true for hH_xR. The experimental conditions at pH 7.4 ensured that both types of TCAs were protonated according to their pK_a-values between 8.0 and 10.2. At hH_1R the secondary amine antidepressant PTL (K_b = 13 nM) was as potent as the tertiary amines CPM and IMI (K_b = 9.0 nM and 5.7 nM, respectively), while the highly lipophilic LPM (K_b = 203 nM) appeared to unveil its full effect not until it was metabolized to the secondary amine DPM (K_b = 21.2 nM). Also for hH_2R, a sole classification of TCAs in tertiary and

secondary amines as described by (Richelson, 1982; Kanba and Richelson, 1983) is not sufficient. While the tertiary amine compounds TMP, AMI and CPM (K_b = 44 nM, 112 nM and 344 nM, respectively) displayed the highest potencies measured for hH_2R, IMI (K_b = 791 nM) with its tertiary amine function was as potent as the secondary amines PTL and NTL (K_b = 688 nM and 877 nM, respectively). A closer examination of TMP, IMI and DPM (K_b = 1.4 µM) verifies that solely the difference of two methyl groups decreased potency by a factor of 20 and 30, respectively, suggesting that the furcation of the side chain in case of TMP is crucial and even more decisive for potency at hH_2R than the tertiary amine function. An elongation of the side chain like for LPM (K_b = 5.7 µM) and OPI (K_b = 6.2 µM) reduced potency of TCAs to the hH_2R even more. But obviously, also the heterocycle is important for the potency of TMP that shares the side chain with levomepromazine LMZ (K_b = 596 nM). For hH_4R, correlations between structure and affinity of TCAs yield a heterogeneous picture. Arborization of the side chain may also account for the high potency of the phenothiazine PMZ (K_b = 197 nM) to hH_2R. While insertion of a methoxy group into the heterocycle in case of the (*R*)-enantiomer LMZ (K_b = 596 nM) decreased potency by a factor of 3, an elongation of the side chain by integration of the branched methyl group and an additional chlorine substituent into the tricycle reduced potency in case of CPZ (K_b = 1.5 µM) by a factor of 8. A further elongation of the side chain like by an insertion of piperazine or a replacement of the chlorine substituent by a strongly electronegative trifluoromethyl group diminished potency even more (PPZ K_b = 2.8 µM; PCP K_b = 2.4 µM; FPZ K_b = 16 µM). The absence of the chlorine substituent combined with an exchange of the benzene ring for a thiophene ring in the heterocycle in case of OLA (K_b = 5.2 nM) yielded no changes in potency at hH_1R in comparison to CLO. At hH_2R, metabolization of CLO (K_b = 528 nM) to CLD (K_b = 1.6 µM) by demethylation reduced potency, while replacement of the diazepine structure by oxazepine did not affect the properties (AMO K_b = 1.3 µM; LOX K_b = 1.2 µM). The affinity of CLO (K_i = 1.2 µM) to hH_4R, in contrary, remained unchanged by demethylation (CLD K_i = 1.8 µM), whereas it is diminished for the dibenzoxazepine analogues AMO (K_i = 6.4 µM) and LOX (K_i = 8.6 µM). Metabolization of the atypical antipsychotic CLO to the *N*-oxide is accompanied by a loss of potency at all receptor subtypes. In conclusion, small changes in a drug may modify the pharmacological properties remarkably but not consistently for all receptor subtypes.

The application of the *Similarity Ensemble Approach* was largely insufficient for the predictions of interactions of antidepressants and antipsychotics with hH_xR. Interactions of the compounds with hH_1R are well-known, which is reflected in the numerous SEA scores for this receptor, although only 41% of these are known-true-predictions. For the hH_2R the approach yielded 22% of known-true-predictions, however, TMP was underpredicted. This is all the more astonishing as known-true-predictions were made for the structural similar TCAs AMI, DPM, DXP and NTL and their potency at hH_2R was described already in the late 1970s. Some over-predictions were yielded for the hH_4R. As all of these compounds contain a piperazine moiety and except for PCP are all analogues of dibenzodiazepines, these predictions are presumably based on the affinity of CLO to this receptor. This provided us with similar SEA scores for CLO and CLN, although their affinities diverge extremely. These deficiencies may be due to incomplete databases. Also, the tricyclic ring system and its kekulization may lead to a different encoding of the structures in SMILES formulas to that of the reference ligands in the databases so that congruence of the compared ligands often remained undetected. Moreover, small changes in the molecules like for CLO and CLN with high impact on their pharmacology are only inadequately taken into account by this approach. In conclusion, SEA may be a helpful tool for the additional screening of numerous ligands and to suggest new targets but may not replace the experimentally examination in the laboratory.

D.2 Relationship between histamine receptor function and psychiatric diseases

Several observations suggest a correlation between HA receptors and psychiatric diseases, above all depression and schizophrenia. In animals, a reduction of HA receptor function induced symptoms similar to depression in man (Nath *et al*., 1988; Ito *et al*., 1999). Further, histaminergic neurons are modulated also through $5\text{-}HT_{2C}R$ which influence higher brain functions and pathological states such as epilepsy and depression by pre-messenger RNA (mRNA) editing (Sergeeva *et al*., 2007) correlating with suicide (Schmauss, 2003; Haas *et al*., 2008). Also in schizophrenia, brain histamine seems to play a role. In various animal models of schizophrenia histamine turnover was enhanced (Browman *et al*., 2004; Dai *et al*., 2004; Fox *et al*., 2005; Faucard *et al*., 2006; Day *et al*., 2007). Moreover, increased levels of

the major HA-metabolite N^{τ}-methylhistamine were found in the cerebrospinal fluid of schizophrenic patients, particularly in those with pronounced negative symptoms and significantly related to the severity, indicating elevated histaminergic activity in brain (Prell *et al*., 1995; 1996). These observations suggest an involvement of histaminergic neurotransmission in the pathophysiology of depression and schizophrenia (Haas *et al*., 2008).

D.2.1 Histamine H_1 receptor

The first TCAs were synthesized as potential antihistamines, so that their antagonism at H_1R is not surprising. After discovering the presence of H_1R in brain it was discussed that some of their remarkable properties may be the consequence of a blockade of H_1R. Due to the fact that nearly all examined antidepressant and antipsychotic drugs display high affinity to hH_1R, the comparison with their therapeutic reference ranges renders this assumption plausible and explains their sedative effects (Richelson, 1979). LPM and its metabolite DPM showed the lowest affinities of the TCAs. Thus, together with CLN, PRX, HAL and CBZ they are not likely to interact with hH_1R and exhibit less sedating properties, clinically (Laux *et al*., 2001). As all of these substances are inverse agonists at H_1R, the observation of an antidepressant-like effect of H_1R agonists like 2-(3-trifluoromethylphenyl)histamine (Lamberti *et al*., 1998) is not consistent. Otherwise, antidepressants with inverse agonistic properties in the nanomolar range would thwart any antidepressant-like effect by H_1R agonism. Additionally, some of the first generation antihistamines act as 5-HT reuptake inhibitors in both animals and humans (Kanof and Greengard, 1978). Positron emission tomography studies using [^{11}C]DXP revealed a correlation of severity of clinical depression and decreased binding to H_1R in cortex and the cingulate gyrus (Kano *et al*., 2004; Haas *et al*., 2008). This may be explained by a reduced density of H_1R as well as an increased release of endogenous HA.

Also antipsychotic drugs exhibited high H_1R inverse agonistic properties. Analogous to depression, the number of H_1R in the frontal cortex of schizophrenics was reduced in postmortem binding studies using [^{3}H]mepyramine as a ligand (Nakai *et al*., 1991). Positron emission tomography studies in frontal and prefrontal cortices and in the cingulate gyrus of schizophrenic patients using [^{11}C]DXP produced the same output (Iwabuchi *et al*., 2005; Haas *et al*., 2008). Therefore, the reduced density of H_1R may be involved in the pathophysiology of schizophrenia. The H_1R antagonist mepyramine was also shown to impair working

memory in the prepulse inhibition test but improved reference memory on the radial-arm maze test in rats. So the blockade of H_1R may be a beneficial action of antipsychotics (Roegge *et al*., 2007).

The properties of antidepressant and antipsychotic drugs at H_1R are not consistent and, therefore, it remains uncertain if they account largely for their therapeutic efficacy or rather for their unwanted side effects, such as weight gain. However, also if sometimes displeasing from the patient`s view, sedative effects of many compounds may exhibit auxiliary value for the therapy of psychiatric diseases.

D.2.2 Histamine H_2 receptor – with focus on polymorphisms and schizophrenia

For twelve out of 34 examined antidepressants and antipsychotics we determined affinities and potencies for H_2R that lie below the reference ranges during therapy and render interaction of the ligand with the receptor likely. The detection of H_2R in brain and the fact that TCAs block the histamine induced cAMP production in mammalian brain (Green and Maayani, 1977; Kanof and Greengard, 1978; Kanof and Greengard, 1979) raised the question, if antidepressant activity of these compounds is associated with blockade of cerebral H_2R, similar to the sedative properties being caused by H_1R antagonism (Schwartz *et al*., 1981; Timmerman, 1989). AMI showed a biphasic inhibition of histamine-stimulated cAMP synthesis but a monophasic effect on dimaprit-stimulation and makes plausible that histamine stimulates cAMP synthesis in these cells by activating both H_1R and H_2R (Kanba and Richelson, 1983). The selective H_2R antagonist famotidine which may penetrate the blood-brain barrier to a low extent (Kagevi *et al*., 1987) was able to reduce positive and particularly negative symptoms in schizophrenic patients when given as a sole medication for schizophrenia or augmentarily (Kaminsky *et al*., 1990; Oyewumi *et al*., 1994; Martínez, 1999). Postmortem brains of schizophrenic patients showed selective alterations of H_xRs indicating the possible existence of pathologically altered histaminergic neurotransmission (Deutsch *et al*., 1997). Furthermore, various efforts were made to link schizophrenia to several polymorphisms of H_2R. Orange *et al*. (1996) reported an about 1.8 times increased incidence of the H_2R649G allele for the H_2R gene in subjects with schizophrenia, compared to the control population, and an elevation of even 2.8 times for the homozygous variant. These findings could not be verified by Ito and co-workers (2000), using the genetic material of individuals of different geographical areas. They allocated three additional H_2R gene

polymorphisms, but their incidence was, however, not significantly different from control (Ito *et al*., 2000). Additional four H_2R promoter polymorphisms were identified, although the differences were not significant. Due to a missing influence on receptor expression and an apparent lack of function, the participation of these variants in pathophysiology of schizophrenia is unlikely (Mancama *et al*., 2002). Although none of the reported H_2R variants is clearly related to this disease, based on the number of H_2R polymorphisms found recently, it is likely that more of these alternate variants will be identified, which may be associated with schizophrenia by causing altered coupling of the receptor (Deutsch *et al*., 1997).

Atypical antidepressants like TMP, MSN, tianeptine or iprindole do not or only weakly inhibit the reuptake of 5-HT or NE which is the commonly suggested mechanism of action for antidepressant drugs. However, they exhibit a similar therapeutic efficacy as “typical” antidepressants for which reason another mechanism of action may be mainly responsible for their antidepressive effects. But like for schizophrenia, the investigations of the cerebral H_2R in the pathophysiology of depression are inhomogeneous. Using the swimming despair test as a behavioral model of depression the H_1R antagonist mepyramine did not affect immobility induced by HA or the H_2R agonist impromidine while the H_2R antagonist cimetidine, IMI and DPM decreased it significantly. In conclusion, antidepressant drugs may block central H_2R and, thus, depression is ameliorated (Nath *et al*., 1988). In contrast, several cases of depression induced by cimetidine were reported (Johnson and Bailey, 1979; Crowder and Pate, 1980; Billings *et al*., 1981; Pierce, 1983). Billings *et al*. suggested an imbalance between H_1R and H_2R signalling by inhibition of the latter receptor, but disregards that antidepressants are also effective inhibitors of H_2R. Antell *et al*. (1989), however, negated any association of depression and cimetidine. Several H_2R antagonists were reported to penetrate the blood-brain barrier to a low extent (Jönsson *et al*., 1984; Kagevi and Wahlby, 1985) but it remains unclear if they may reach adequate cerebral concentrations to affect not only peripheral H_2R.

D.2.3 Histamine H_3 receptor

The H_3R plays an important role in modulating a variety of neuropharmacological effects including cognition, locomotion, sleep-wake status and epilepsy. Although some indirect hints exist, there is no evidence for a direct correlation between H_3R and depression yet. Studies in rat brain cortex showed that AMI counteracted a chronic stress-induced

decrease of the H_3R density, while it increased the density of the receptor when chronically administered in the non-stressed control group (Ghi *et al*., 1995). Investigations of mice in the forced swim test, a model for depression in animals, displayed a significant anti-depressant-like effect of the H_3R/H_4R antagonist thioperamide, which was prevented by the H_3R agonist (*R*)-α-methylhistamine (Lamberti *et al*., 1998; Peréz-García *et al*., 1999). In a modified study the antidepressive effect of thioperamide was examined together with its serotonergic and/or antioxidant mechanisms and indicated its antioxidant potential (Akhtar *et al*., 2005).

After the detection of an intermediate affinity of the atypical antipsychotic CLO for the H_3R in rat brain cortex (Kathmann *et al*., 1994; Rodrigues *et al*., 1995), the antipsychotic effects have been associated with this HA receptor subtype as well. The localization of this receptor and its function not only as autoreceptor but also as heteroreceptor influencing also monoamine concentrations are reasons in favor of this hypothesis (Ito, 2009). Moreover, the elevated hH_3R expression in the prefrontal cortex of schizophrenic post-mortem brain samples suggests a connection between hippocampus and cortical regions and a regulation *via* hH_3R (Jin *et al*., 2009).

The H_3R/H_4R antagonist thioperamide was shown to exert not only antidepressive effects but also antipsychotic-like properties by potentiating HAL-induced catalepsy, reducing amphetamine-induced hyperactivity and reducing apomorphine-induced climbing in mice. These effects were reversed by (*R*)-α-methylhistamine, indicating the involvement of H_3R, and suggest a potential for improving the refractory cases of schizophrenia (Akhtar *et al*., 2006). Also ciproxifan, a H_3R antagonist/inverse agonist, potentiates neurochemical and behavioral effects of HAL in the rat (Pillot *et al*., 2002) and modulates the effects of methamphetamine on neuropeptide mRNA expression in rat striatum (Pillot *et al*., 2003).

Due to the low affinity of CLO for the human H_3R isoform and the missing affinities of all other tested antipsychotic drugs for the H_3R, an antischizophrenic effect of this receptor is not likely at least for the substances studied herein. However, histamine neuron activity may also be modulated by a crosstalk of other co-localized receptors like a stimulation *via* blockade of the 5-$HT_{2A}R$ by several atypical antipsychotics (Morisset *et al*., 1999). Therefore, H_3R antagonists or inverse agonists are not useful for a stand-alone therapy of schizophrenic symptoms but might constitute a valuable add-on medication for the treatment of cognitive deficits in schizophrenic subjects (Tiligada *et al*., 2009). A currently ongoing study with the

H_3R inverse agonist tiprolisant may confirm the pro-cognitive properties (ClinicalTrials, 2010). Further, the combination of D_2-like receptor, 5-HT_2R and H_3R inverse agonism and decreased H_1R affinity in one compound may be a promising approach in the treatment of schizophrenic subjects (von Coburg *et al*., 2009; Tiligada *et al*., 2009).

Although there is no correlation of the tested substances and a modulation of their antidepressant and antipsychotic effects *via* H_3R it is possible that more potent ligands are able to alter the concentrations of diverse neurotransmitters in brain by H_3 auto- and heteroreceptor modulation.

D.2.4 Histamine H_4 receptor

In contrast to H_1R, H_2R and H_3R, the functional presence of the H_4R on neurons in the CNS has been revealed just recently. The involvement of the H_4R in brain diseases such as depression and schizophrenia is, therefore, still poorly understood and its potential as a target for new drugs, particularly in neurological diseases, needs to be elucidated. This finding will allow a further characterization of histaminergic neurotransmission in the mammalian brain in general (Connelly *et al*., 2009).

The only clinical relevant interaction for the H_4R we found was for CLO and its metabolite CLD. Our findings are in agreement with Nguyen *et al*. (2001), Smits *et al*., (2006) and Jongejan *et al*., (2008). However, we were not able to verify affinities for this GPCR in the nanomolar range for AMI, CPZ, DXP, PMZ and MSN, as measured by Nguyen and co-workers. Also, Lim *et al*. (2005), Venable and Thurmond (2006) and Deml *et al*. (2009) could not confirm the high-affinity binding observed by Nguyen *et al*. (2001) either. These discrepancies may be explained by differences in the expression systems (mammalian *vs.* Sf9 insect cells) that could affect receptor glycosylation as well as oligomerization and, therefore, the pharmacological properties. As the interactions of a number of prototypical hH_4R ligands with hH_4R expressed in Sf9 insect cells and mammalian cells were verified to be very similar (Lim *et al*., 2005; Schneider *et al*., 2009; Schneider and Seifert, 2009), also an inadvertent or endogenous expression of H_1R in the HEK293 cells used by Nguyen *et al*. (2001) is possible (Venable and Thurmond, 2006).

The atypical antipsychotic CLO together with its metabolite CLD exhibits unique properties in comparison to other drugs in the therapy of schizophrenia. Both are further the only substances of the examined ones that allow interaction with the H_4R at therapeutic

plasma concentrations. Whether and to what extent the agonistic behavior of CLO and CLD at H_4R contributes to atypicality of antipsychotics remains subject of further investigation.

D.3 Trimipramine at histamine H_2 receptor

The interaction of the antidepressants AMI, IMI, DBP and iprindole with H_2R linked to adenylyl cyclase in homogenates of guinea pig hippocampus was first reported by Green and Maayani (1977). This finding was independently confirmed for more compounds shortly afterwards by Kanof and Greengard (1978). However, the obtained potencies were questioned by impromidine-stimulated cAMP accumulation experiments in guinea pig hippocampal slices (Dam Trung Tuong *et al*., 1980). Also, several tricyclic and non-tricyclic antidepressants were shown to inhibit the effect of HA on the H_2R using rat isolated uterus. TMP and MSN displayed the highest potency, even superior to that of cimetidine whereas maprotiline (MPT) inhibited H_2R activity with the lowest potency (Alvarez *et al*., 1986). A comparison of the different preparations yielded a K_d of 2.4 µM for TMP in dissociated tissue and 0.003 µM in homogenates of guinea pig hippocampus. However, also DXP (K_d of 1.4 µM in dissociated tissue and 0.17 µM for homogenates) and AMI (K_d of 1.9 µM in dissociated tissue, 3.5 µM in slices and 0.034 µM for homogenates) showed very heterogeneous results (Kanba and Richelson, 1983). In contrast to the studies with homogenized guinea pig hippocampus (Kanba and Richelson, 1983), we were able to perform a saturable binding of [^{3}H]TMP to recombinant H_2R fusion protein (Fig. C.18), although non-specific binding was exceeding 80%. As our results (TMP: K_i = 41 nM; DXP: K_i = 198 nM; AMI: K_i = 67 nM) have been obtained with membrane fractions of infected Sf9 cells they correspond very closely with those yielded with the cell-free homogenates. Small differences may be explained by the different test systems and also by using native tissue with many other interaction sites for the multiple-target ligands. However, the discrepancy between data of homogenates and dissociated hippocampal tissue for all examined antidepressants and antipsychotics but not H_2R antagonists is striking. The potency of TMP is reduced 800-fold in the dissociated cell preparation than compared with the homogenates (Kanba and Richelson, 1983). Therefore, the therapeutic reference range of TMP (365-853 nM) and the yielded concentrations in plasma fit to data obtained at H_2R in

homogenates but do not fit to cerebral H_2R, in case they possess comparable properties as the examined brain slices or the dissociated tissue.

It is possible that the molecules have only remote access to receptors in intact tissue or dissociated cells which consist of large clumps of cells of about 100 µm (Schwartz *et al*., 1981; Kanba and Richelson, 1983). In this case, the data for dissociated cells with its augmented surface should be more varying in comparison to the brain slices (Dam Trung Tuong *et al*., 1980; Kanba and Richelson, 1983). Schwartz and co-workers suggested not only a different receptor conformation in the presence of high concentrations of ATP, Mg^{2+}, GTP and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, which are required for an optimal adenylyl cyclase activity in homogenates, but also the modifications of drug discriminatory characteristics of H_2R by cell disruption as possible sources for the discrepancies. For the investigations in native tissue also high concentrations of the ions Na^+, K^+, Ca^{2+} and PO_4^{3-} were used. A damage of the receptor by the homogenization step as ruled out by Kanba and Richelson can also be excluded by the similar findings with the recombinant H_2R fusion protein. Angus and Black (1980) suggested that also a secondary intracellular action of the drug like inhibition of the phosphodiesterase activity in the intact cell preparations may explain the discrepancies. Possibly, antidepressant and antipsychotic drugs interact with the H_2R differently in brain than with single cells and membrane fractions as well as in a diverse mode as mere H_2R antagonist do. As shown in Chapters C.4.1 and C.4.2 the binding mode of TMP and the H_2R antagonist TIO is, however, quite similar. TMP may displace HA by docking into the binding pocket. Like the endogenous ligand the charged quaternary ammonium of TMP interacts with Asp-98 as counterion. The binding pocket is formed by several amino acids in TM5 (Gantz *et al*., 1992; Nederkoorn *et al*., 1996) and two amino acids each in ECL2 and TM3. The chiral side chain is preeminent for high affinity binding of TMP because its absence reduced affinity by a factor of 20. This moiety interacts with amino acids in ECL2 and TM7 or TM3, respectively, depending on the orientation of the 2-methyl group of the (*R*)- and (*S*)-enantiomers. However, a significant preference for one of the two TMP enantiomers was not determinable in the modelling approach. Due to its heterocycle, TMP exhibits multiple interactions with the hydrophobic amino acids Trp-247, Tyr-250, Phe-251 and Phe-254 in TM6 while the diaminomethylidene amino moiety of TIO at this position interacts with Asp-186 and Thr-190 in TM5. The latter interactions are, however, not plausible for other H_2R antagonists except famotidine because they are lacking this partial

structure. Therefore, it is likely that there are indeed differences in the interaction of H_2R antagonists and TCAs with H_2R (Tsai and Yellin, 1984). Moreover, Beil *et al*. (1988) demonstrated that TMP, DXP and HAL interfere with H_2R in parietal cell in a non-competitive mode. The Schild plots of TMP and TIO performed in the Sf9 cell system, however, refer to a competitive binding at H_2R. To ascertain the role of H_2R to the mechanism of action of antidepressants and also antipsychotic drugs further experiments are indispensable to clarify why data in cell assemblies are varying to that in cell fractions and if interference of antidepressants to H_2R differs to that of H_2R antagonists.

D.3.1 Trimipramine for ulcer therapy

Simultaneously to the finding that TCAs inhibit H_2R linked to adenylyl cyclase in homogenates of guinea pig hippocampus (Green and Maayani, 1977) several studies investigated the use of these compounds for ulcer therapy, above all TMP. Therefore, low doses of the antidepressant were used, mainly 25-50 mg/day, while for the therapy of depression 200 mg/day on average are recommended (Baumann *et al*., 2004). TMP was effective in treatment of both duodenal and gastric ulcer (Myren *et al*., 1979). Additional advantages to the inhibiting gastric secretion were benefits by the antipain/depression effect of TCAs, their long half-lives, low cost and readily available serum monitoring (Ries *et al*., 1984). Berstad *et al*. (1980) showed that in combination with antacids TMP was nearly as effective as cimetidine. In another clinical trial for treatment of peptic ulcer disease, TMP was superior to placebo and as effective as cimetidine. Due to the fact, that this effect may be mediated by anticholinergic receptor modulation and because of the strong sedating properties of TCAs, the usefulness as first-line anti-ulcer agents was doubted and a possible usage was suggested only for a short-term treatment of duodenal ulcers and for patients unresponsive to conventional anti-ulcer therapy (Berardi and Caplan, 1983).

Other studies, however, reported that although TMP accelerated healing of duodenal ulcer it was inferior to cimetidine with respect to the rate of healing and the reduction of symptoms (Becker *et al*., 1983) and evoked frequently complaints of fatigue (Blum, 1985). Wilson *et al*. (1985) proved that TMP inhibited pentagastrin-stimulated secretion of acid by 13% and MSN by 38%. MSN inhibited overnight gastric secretion by 37%, while TMP increased it by 16%. Further, for TMP the cumulative percentages of patients with relapse of ulcers within twelve months was as high as no treatment while cimetidine, antacids,

ranitidine and sucralfate were significantly better (Hui *et al*., 1992). In summary, the evaluation of TMP in the treatment of ulcer is not significant and gives only inconsistent information relating to H_2R, as also its anticholinergic effects may play a role. Since more effective treatment options have been introduced the use of TMP for this indication is not reasonable anymore.

D.3.2 Connection between clinical profile and molecular affinities at H_xR

The sedative properties of antidepressants and antipsychotics seem to be correlated with the occupation of H_1R at clinical dosage. Drowsiness and sedation are often observed with TCAs like AMI (K_i = 1.3 nM), DXP (K_i = 1.2 nM) and TMP (K_i = 1.5 nM). In contrast, LPM and its metabolite DPM, the TCAs with the lowest affinity to hH_1R (K_i = 243 nM and 68 nM, respectively), are known to cause more agitation than sedation. Although their affinities are within the threshold of therapeutic reference ranges, the occupation of H_1R seems to be insufficient for sedation. This observation is also made for antipsychotics. While sedation is a determining aspect in therapy with CLO (K_i = 2.6 nM) and PMZ (K_i = 1.0 nM), for RIS (K_i = 54 nM) only moderate sedating properties were reported. FLU shows also a high affinity to H_1R. Due to its very low reference range, an adequate occupation of this receptor to mediate clinical effects is not secured. With affinities beyond the therapeutic reference ranges PRX (K_i = 13 µM) and HAL (K_i = 1.9 µM) display only low sedating properties. CBZ showed no affinity to this receptor at all which is in agreement with a lack of sedation (Laux *et al*., 2001; Lexi-comp, 2010). The association with weight gain is not consistent. Within the group of TCAs this side effect is most likely for AMI, although other TCAs had affinities in the same range. For DPM less or even no weight gain was reported (Stern *et al*., 1987; Fernstrom and Kupfer, 1988). Despite the low affinity of PRX to H_1R, weight gain may be experienced during therapy. This fact may account for a contribution of other orexigenic substances like $5\text{-}HT_{2C}R$ antagonists (Reynolds *et al*., 2006). The antipsychotics CLO, OLA, TRZ, CPZ, RIS and HAL also were shown to enhance weight gain. Its extent correlates with the measured affinities. Again, therapy with FLU has only small impact on weight gain due to its low blood concentration during therapy (Gitlin, 2007).

A correlation of clinical antidepressant dosage and affinity to H_2R as found for anti-psychotics and D_2R has not been observed while a correlation of clinical antidepressive effect and affinity to H_2R is difficult to assess due to the variety of potential therapeutic

effects like mood brightening, anxiolysis, agitation. Interestingly, also the antipsychotics TRZ, PPZ and CPZ were reported to exhibit antidepressive effects (Hollister *et al*., 1967; Raskin *et al*., 1970; Becker, 1983). But only the affinity of TRZ is within the therapeutic reference ranges and sufficiently high to mediate this effect *via* H_2R. For H_3R, the measured affinities are not sufficiently high in comparison to the therapeutic reference ranges to cause any clinical effect. This is also the fact for H_4R with exception of CLO and CLD. The atypical antipsychotic and its metabolite are, therefore, associated with the incidence of agranulocytosis.

D.4 Clozapine and histamine H_4 receptor – a possible cause for agranulocytosis

CLO showed the highest number of possible interactions with hH_xRs among the examined substances. The blood concentrations under therapy are sufficiently high to modulate hH_1R and hH_2R as well as hH_4R. Thereby, its properties change from an inverse agonist at hH_1R and hH_2R to a partial agonist at hH_4R and in the same order potencies increase from 4.3 nM, 528 nM to 1,700 nM. This also applies to the main metabolite CLD. The examined substances are known to decrease the number of circulating white blood cells, prevalently neutrophils. This leukopenia may impair to a severe and potentially lethal condition referred as agranulocytosis with less than 1,000 white blood cells/mm^3 or 500 granulocytes/mm^3 of blood (Ryabik *et al*., 1993). Clinical signs of this agranulocytosis are sudden fever, sore throat and quickly progressing infections like pneumonia up to sepsis. CLO is one of the numerous drugs causing agranulocytosis and a prevalence of 1%. The highest risk appears in the first eighteen weeks of treatment (Ryabik *et al*., 1993). Therefore, the white blood cell counts are weekly monitored during the first few months. A secondary infection may rise the mortality rate from 38% to 50% if the patient is not taken off from CLO (Claas, 1989; Krupp and Barnes, 1989). A treatment is possible with granulocyte colony-stimulating factor (G-CSF) (filgrastim) and granulocyte macrophage colony-stimulating factor (GM-CSF) (sargramostim), two human colony-stimulating cytokines (Delannoy and Géhenot, 1989; Palmblad *et al*., 1990). As the H_4R is mainly expressed on hematopoietic and immunocompetent cells and CLO is a potent partial agonist at H_4R it has been discussed if agonist activity at this receptor may be related to agranulocytosis (Ito, 2009). The modifica-

tion of differentiation through a permanent stimulation by the partial agonist would be reasonable. Also, CLN, CPX and OLA, a thienobenzodiazepine and advancement of CLO that was developed to reduce the risk of agranulocytosis, are partial agonists at H_4R. The high incidence of agranulocytosis by CLO was at least reduced in OLA (Beasley *et al*., 1997; Tolosa-Vilella *et al*., 2002). This may be due to the fact, that therapeutic plasma concentration of OLA are too low for an interaction with H_4R (K_i = 17 µM). Strikingly, also the H_2R antagonists metiamide (K_i = 3.0 µM; E_{max} = 0.73) and cimetidine (K_i = 12.4 µM; E_{max} = 0.62), both known to cause agranulocytosis (Aymard *et al*., 1988), exhibited partial agonism at H_4R but with low potency. In case of metiamide the thiourea moiety was made responsible for the high incidence of agranulocytosis (Fitchen and Koeffler, 1980). CLO, however, contains no such equivocal partial structure that cannot be found also in other common substances without elevated prevalence for agranulocytosis. Arguing also against this theory is the fact that the remaining substances which are also known for decreasing the white blood cell count, in contrary, acted as antagonists/inverse agonists at H_4R. For this reason a relationship between the H_4R and agranulocytosis is not consistent.

D.5 Comparison of medication: mavericks or gregarious creatures?

Although the examined drugs are structurally closely related and are all deployed in the therapy of either depression or schizophrenia, they may be assigned as mavericks. The smallest change in the molecule may already modify the binding profile for the single receptor. The compounds are further known to bind to up to 20 different receptor families. Due to the number of feasible molecular targets, the result is a unique binding profile for each drug. By means of H_xRs the varying binding performance within only one receptor family can be assessed.

The amino acid sequence of the hH_1R is tolerating these changes best. Apart from CBZ, CLN, HAL, LPM and PRX all drugs bound to this receptor in the low nanomolar range, although the mediated effects, sedation and weight gain, are of a different intensity. At H_2R the affinities are inconsistent even within similar structural types. Affinities for the phenothiazines varied between 197 nM and 16 µM, for the TCAs between 44 nM and 6.3 µM and for the dibenzodiazepines between 528 nM and > 100 µM. Despite structural diversities no

compound displayed a higher affinity for H_3R than 10 µM. A different pattern is found for H_4R: beside the dibenzodiazepines single compounds displayed at least moderate affinities.

D.6 Examples for "new" mechanisms of drug action for antidepressants and antipsychotics

A contribution of H_2R to the therapeutic effects of antidepressants and antipsychotics may appear as follows: Blockade of cerebral H_2R coupled to $G\alpha_s$ by antagonists reduces the synthesis of cAMP *via* AC. Protein kinase A (PKA) is inhibited and, therewith, the alteration of transcription in the cell nucleus by the cAMP response element-binding protein which may have impact on receptor sensitivity. Additionally to the G protein-dependent mechanism, H_2R is directly linked to the phosphoinositide signalling pathway. For H_2R inverse agonists a receptor up-regulation has been observed which may cause hypersensitivity to HA (Del Valle and Gantz, 1997). Some atypical antidepressants are known to have no effect on NE or 5-HT reuptake. Taking the monoamine hypothesis as the molecular basis of depression for granted the deficiency of the neurotransmitters has to be balanced otherwise. Threlfell *et al*. (2008) reported that the blockade of H_2R expressed in the substantia nigra pars reticulata of rats enhanced 5-HT release independently of GABAergic or glutamatergic inputs. Hence, not only H_3R but also H_2R may regulate 5-HT neurotransmission and increase the concentration in the synaptic cleft.

The signalling in GPCRs may also take place in a G protein-independent manner and mediate actions simultaneously through distinct effector systems (Beaulieu *et al*., 2005). The signalling molecules protein kinase B (Akt) and glycogen synthase kinase-3 (GSK3) play an important role in the regulation of DA and 5-HT and may, thus, be implicated in the actions of psychoactive drugs such as antidepressants, antipsychotics and the augmenter lithium. Investigations in mice revealed that the multifunctional scaffolding molecule β-arrestin-2, which is generally regulating desensitization of GPCRs, is involved in the Akt/GSK3 pathway of D_2R (Beaulieu *et al*., 2009). This additional pathway was also shown for H_2R in parietal cells. H_2R inverse agonism may decrease the activity of phosphatidylinositol 3-kinase leading to a reduced activity of Akt and, hence, increase cAMP concentration (Mettler *et al*., 2007). This modulation might also proceed in cerebral H_2R. Beside inhibition of inositol monophosphatases, recent findings suggest that the alkali metal lithium may reduce Akt activity and,

therefore, inhibition of GSK3β by destabilizing a DA receptor regulated signalling complex composed of Akt, protein phosphatase 2A and β-arrestin-2 (Beaulieu and Caron, 2008). Ahmed *et al*. (2008) showed differences for the antipsychotics HAL and CLO in affecting the expression of arrestins and GPCR kinases and in modulating the extracellular signal-regulated kinase pathway, which may explain the discrepancy in their clinical profiles. Further, ligands may modulate the activity of two effector systems *via* AC and MAPK and show complex pluridimensional efficacy profiles as reported for β_1AR and β_2AR (Galandrin and Bouvier, 2006).

Another influence on H_2R-mediated $G\alpha_s$ trafficking and signalling may be exerted by lipid rafts and caveolae, specialized membrane microdomains that compartmentalize cellular processes. The findings of Allen *et al*. (2009) implicate that $G\alpha_s$ is removed from membrane signalling cascades by lipid rafts and caveolins, the integral membrane proteins of the microdomains. This reduces $G\alpha_s$-mediated stimulation of AC and, thus, cAMP signalling. Chronic treatment with escitalopram is, consequently, able to increase AC activity and cAMP concentration independently of 5-HT transporters by translocation of $G\alpha_s$ from lipid rafts back to a non-raft fraction of the plasma membrane (Zhang and Rasenick, 2010). Beside the typical targets, this effect may explain the delayed onset of therapeutic benefit of antidepressants.

D.7 Future studies

Recently, numerous psychotropic drugs entered the market featuring various pharmacological properties. But due to deficiency of selectivity of a single compound for a defined molecular target, in particular for the therapy of schizophrenia, many drugs still exhibit a wide varying spectrum of unwanted side effects.

For verifying a significant involvement of the H_2R in the pathophysiology of psychiatric diseases like depression or schizophrenia a more careful examination with TMP needs to be performed. Its lead structure should, therefore, become optimized for selective H_2R antagonism while maintaining the essential property of penetrating the blood-brain barrier. Positron emission tomography studies may then be performed and shed light to the antagonism of antidepressants and antipsychotics in brain. Also, the initiated analysis of 34 drugs at the four HA receptor subtypes should be completed with more compounds such as butriptyline, a combination of TMP and AMI, dosulepin, noxiptyline, propizepine, the

atypical antidepressants tianeptine, amineptine, iprindole, bupropion and trazodone. Helpful for clarifying the correlation of the unexplained disease patterns could also be the completion of data for 5-HT_xR, D_xRs, αARs or mAChRs and the examination of other receptor families like metabotropic glutamate receptors (mGluR). Further predictions of new off-targets with SEA for diverse substances at receptors other than H_xRs are also possible. The examination of TMP and optimized derivatives in mouse models could give interesting insight into the functionality of H_2R in brain. With the help of H_2R knockout mice (Kobayashi *et al*., 2000) this relation should be studied. Suitable models for the analysis of depression-like symptoms in mice could be forced swim test, tail suspension test, olfactory bulbectomy and chronic mild stress (Cryan and Mombereau, 2004; Pollak *et al*., 2010).

E. Summary/Zusammenfassung

E.1 Summary

Antidepressant and antipsychotic drugs are known to affect multiple molecular targets. Beside their determinant effects on the neurotransmission of serotonin, norepinephrine and dopamine *via* several transporters and receptors, they may also modulate muscarinic acetylcholine receptors and the histamine H_1 receptor (H_1R). Consequently, these drugs do not only yield unique profiles of desired effects but also several unwanted side effects that may impact therapy. In addition to the H_1R, the histamine H_2 receptor (H_2R), histamine H_3 receptor (H_3R) and histamine H_4 receptor (H_4R) belong to the large family of GPCRs and are very important drug targets. All four H_xR subtypes are expressed in brain. An interaction of the highly lipophilic, blood-brain barrier-penetrating compounds with histamine receptors may, thus, not only affect peripheral receptors but also cerebral receptors and contribute to the therapeutic and unwanted effects of the medication.

The aim of this thesis was to investigate possible interactions of 34 antidepressants and antipsychotics with the four histamine receptor subtypes. By comparison of the obtained data with literature-reported therapeutic reference ranges for the compounds, conclusions are drawn regarding their contribution to desired or unwanted effects. Almost all of the antidepressant and antipsychotic drugs displayed high binding affinities to H_1R. We related the clinically relevant sedative effects to the molecular affinities at H_1R while the association with weight gain was not consistent. Several antidepressant and antipsychotic drugs may achieve therapeutically blood concentrations that are sufficiently high to interact with central H_2R. Possible reasons for the discrepancies between the results and literature-obtained data from different tissue preparations are discussed. The highest H_2R affinities were yielded for tricyclic antidepressants, most notably trimipramine. This atypical antidepressant inhibits the reuptake of monoamines only marginally but still possesses high clinical efficacy so that its antidepressive properties may be related to the H_2R receptor. Hence, possible mechanisms of action for this H_2R-mediated contribution to the therapeutic effects of antidepressant and antipsychotic drugs are discussed. Although H_3R is involved in the release of the neurotransmitters serotonin, norepinephrine and dopamine and may, therefore, constitute a potential target to modulate monoamine concentrations in the therapy of psychiatric diseases, none of the examined compounds reaches blood concentra-

tions that are, in comparison to their affinities to H_3R, sufficient to mediate any clinical effect *via* this receptor. Similar properties were observed also for H_4R at which receptor only the atypical antipsychotic clozapine caused a therapeutically significant interaction. In fact, the heterogeneous pharmacological profiles of the examined drugs indicate no involvement in the onset of the potentially lethal side effect agranulocytosis *via* H_4R.

Despite the homology of the histamine receptor subtypes, especially H_3R and H_4R, several compounds exhibit substantial pharmacological differences for the receptor subtypes. These were explored in detailed investigations by construction of active and inactive state models for H_1R, H_2R and H_4R with the most interesting compounds in the binding pocket. On this basis, structure-activity relationships are discussed. A comparison of the experimentally obtained data and the results of the *Similarity Ensemble Approach* showed an insufficient predictability for the determination of new off-targets by the statistics-based chemoinformatics method.

In conclusion, this thesis provides new insights into the molecular interactions of a number of antidepressant and antipsychotic drugs to the histamine receptor subtypes. The pharmacological data for all known histamine receptor subtypes may be used to reduce adverse effects and drug interactions as well as to develop novel optimized and selective drugs with a decreased number of off-targets. Further, this thesis contributes to the exploration of the role of cerebral H_2R in the pathophysiology and therapy of psychiatric diseases.

E.2 Zusammenfassung

Antidepressiva und Neuroleptika entfalten ihre spezifischen Wirkungen durch Interaktionen mit zahlreichen Zielstrukturen. Neben den Wirkungen auf die serotonerge, noradrenerge und dopaminerge Neurotransmission durch verschiedene Transporter und Rezeptoren interagieren sie ebenso mit muskarinischen Acetylcholin-Rezeptoren und dem Histamin H_1 Rezeptor (H_1R). Infolgedessen zeichnen sich diese Arzneistoffe nicht nur durch ein spezifisches Wirkprofil aus, sondern auch durch einige unerwünschte Wirkungen, die die Therapie erheblich beeinflussen können. Neben dem H_1R gehören auch der Histamin H_2 Rezeptor (H_2R), der Histamin H_3 Rezeptor (H_3R) und der Histamin H_4 Rezeptor (H_4R) zur Superfamilie der G Protein-gekoppelten Rezeptoren und stellen wichtige pharmakologische Zielstrukturen dar. Alle vier Subtypen werden u.a. im zentralen Nervensystem exprimiert. Interaktionen von Antidepressiva und Neuroleptika mit Histamin-Rezeptoren betreffen daher nicht nur die Rezeptoren in der Körperperipherie, sondern auch im Gehirn, und könnten somit zur therapeutischen Wirkung ebenso wie zu unerwünschten Arzneimittelwirkungen der sehr lipophilen und damit gehirngängigen Arzneistoffe beitragen.

Ziel dieser Arbeit war es, 34 Antidepressiva und Neuroleptika auf mögliche Interaktionen mit den Histamin-Rezeptor-Subtypen zu untersuchen. Durch einen Vergleich der gewonnenen Daten mit den jeweiligen therapeutischen Referenzbereichen aus der Literatur wurden Schlüsse auf eine Beteiligung an den erwünschten oder unerwünschten Arzneimittelwirkungen gezogen. Fast alle untersuchten Antidepressiva und Neuroleptika zeigten eine hohe Affinität zum H_1R. Diese konnten mit den klinisch-relevanten sedierenden Eigenschaften der Substanzen in Beziehung gesetzt werden, eine Verbindung mit häufig auftretender Gewichtszunahme jedoch konnte nicht hergestellt werden. Einige Antidepressiva und Neuroleptika erreichen therapeutische Plasmakonzentrationen, die auch für eine Interaktion mit zentralen H_2Rs ausreichend sind. Mögliche Ursachen für Diskrepanzen zwischen den gewonnenen Ergebnissen und Daten aus der Literatur von anderen Gewebepräparationen werden diskutiert. Die höchsten H_2R-Affinitäten zeigten trizyklische Antidepressiva, allen voran Trimipramin. Dieses atypische Antidepressivum blockiert die Wiederaufnahme von Monoaminen trotz vergleichbarer klinischer Wirksamkeit nur unwesentlich, so dass eine Interaktion mit dem H_2R ursächlich für die antidepressiven Eigenschaften sein könnte. Daher werden mögliche Wirkmechanismen einer H_2R-Beteiligung an den therapeutischen Effekten

von Antidepressiva und Neuroleptika diskutiert. Obwohl der H_3R an der Freisetzung der Neurotransmitter Serotonin, Noradrenalin und Dopamin beteiligt ist und durch Beeinflussung der Monoaminkonzentrationen daher eine Rolle in der Therapie von psychiatrischen Erkrankungen spielen könnte, erreichte keine der untersuchten Verbindungen im Blut Konzentrationen, die im Vergleich mit den jeweiligen Affinitäten ausreichend wäre, um einen klinische Wirkung über diesen Rezeptor zu vermitteln. Ein ganz ähnliches Verhalten zeigten die Substanzen am H_4R, wo nur für das atypische Neuroleptikum Clozapin eine therapeutisch relevante Interaktionsmöglichkeit besteht. Aufgrund des heterogenen pharmakologischen Profils der Wirksubstanzen kann aber keine Verbindung des H_4R mit Agranulozytose, einer potenziell tödlichen Nebenwirkung, hergestellt werden.

Trotz der Homologie der Histamin-Rezeptor-Subtypen, besonders von H_3R und H_4R, weisen einige der Verbindungen erhebliche pharmakologische Unterschiede an den verschiedenen Rezeptor-Subtypen auf. Für die interessantesten Substanzen wurden an H_1R, H_2R und H_4R Rezeptormodelle in aktivem oder inaktivem Zustand erstellt und genauer untersucht. Darauf basierend werden Struktur-Wirkungsbeziehungen entwickelt und diskutiert. Der Vergleich der experimentell gewonnen Ergebnisse mit den Resultaten des *Similarity Ensemble Approach* ergab nur eine ungenügende Vorhersagekraft von neuen Zielstrukturen durch diese auf Statistiken basierende Methode der Chemoinformatik.

Zusammenfassend gewährt diese Dissertation neue Einblicke in die molekularen Interaktionsmöglichkeiten zahlreicher Antidepressiva und Neuroleptika mit Histamin-Rezeptoren. Die gewonnenen pharmakologischen Daten für alle Histamin-Rezeptor-Subtypen können darin Verwendung finden, Nebenwirkungen und Arzneimittelwechselwirkungen zu verringern sowie neue optimierte und selektive Wirkstoffe mit einer reduzierten Anzahl an Interaktionsmöglichkeiten zu entwickeln. Ferner trägt diese Arbeit zur weiteren Aufklärung der Rolle des zerebralen H_2R in der Pathophysiologie und Therapie von psychiatrischen Erkrankungen bei.

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G. Appendix

G.1 Abstracts and Publications

Prior to submission of this thesis, results were published in part or were presented as short lectures or posters.

G.1.1 Original Publications

2010

Appl H, Holzammer T, Dove S, Straßer A and Seifert R (2010) Interaction of histamine receptors with antidepressant and antipsychotic drugs (*in preparation*).

G.1.2 Short Lectures

2010

Appl H, Holzammer T, Dove S and Seifert R (2010) Interaction of antidepressant and antipsychotic drugs with the four histamine receptor subtypes. *Naunyn Schmiedebergs Arch Pharmacol* **381 Suppl 1**:11. 51. Jahrestagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie (DGPT), Mainz.

G.1.3 Poster Presentations

2009

Appl H and Seifert R (2009) Interaction of antipsychotic drugs with the four histamine receptor subtypes. *Naunyn Schmiedebergs Arch Pharmacol* **379 Suppl 1**:46. 50. Jahrestagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie (DGPT), Mainz.

2008

Appl H and Seifert R (2008) Interaction of histamine receptors with antipsychotics and histamine H_2 receptor antagonists. Symposium “Signal transduction – innovative fountain for pharmacology”, Medizinische Hochschule Hannover und 4th Summer School “Medicinal Chemistry”, Regensburg and Fachgruppentagung der Gesellschaft Deutscher Chemiker (GDCh) und Deutschen Pharmazeutischen Gesellschaft (DPhG) Pharmazeutische/Medizinische Chemie (“Frontiers in Medicinal Chemistry”), Regensburg.

Appl H and Seifert R (2008) Interaction of histamine receptors with antipsychotic drugs. *Naunyn Schmiedebergs Arch Pharmacol* **377 Suppl 1**:15. 49. Jahrestagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie (DGPT), Mainz.

G.2 Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 24. September 2010

Heidrun Appl