Biochemical Pharmacology of the Positive Allosteric Modulation of the $GABA_B$ Receptor *in Vitro* and *in Vivo*

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2. List of abbreviations

5-HT	5-hydroxytryptamine (or serotonin)
7β-forskolin	7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl-forskolin
7TM	7-transmembrane spanning domain
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
APPA	3-aminopropylphosphinic acid
baclofen	β-p-chlorophenyl GABA
CaM	calmodulin
CAM	constitutively active mutant
cAMP	cyclic AMP, cyclic 3',5'-adenosine-monophosphate
Calhex 231	chlorophenylcarboxamide or (1S,2S,1'R)-N^1-(4-clorobenzoyl)-N^2-[1-(1-naphtyl)ethyl]-1,2-diaminocyclohexane
CaSR	calcium-sensing receptor
CGP13501	2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenaldehyde
CGP35348	3-aminopropyl-(diethoxymethyl)-phosphinic acid
CGP47656	3-aminopropyl-(difluoromethyl)-phosphinic acid
CGP52432	3[[(3,4-(dichlorophenyl)methyl]amino)-propyl](diethoxymethyl) phosphinic acid
CGP56999	[3-[1-(R)-[[3-cyclohexylmethyl)hydroxyphosphinyl]-2-(S)-hydroxypropyl]amino]ethyl]-benzoic acid
CGP62349	[3-[1-(R)-[[(2S)-2-hydroxy-3-[hydroxyl[4-methoxyphenyl]methyl]phosphynyl]propyl]amino]ethyl]-benzoic acid
CGP7930	2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol
CHO	Chinese hamster ovary
Cinacalcet	N-2-(1-naphthyl)ethyl-3-(3-trifluormethylphenyl)propylamine
CNS	central nervous system
CPA	N^6-cyclopentyladenosine
CRF1	Corticotropin-releasing factor type I receptor
CRH of CRF	corticotropin-releasing hormone or factor
DA	Dopamine
DFB	3,3'-difluorobenzaldazine
DIV	day *in vitro*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
FLIPR	fluorescence imaging plate reader
G-protein	guanine nucleotide-binding protein
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GHB	γ-hydroxybutyric acid
GIRK (or Kir3)	inwardly rectifying potassium channels

GPCR	G-protein-coupled receptor
GRK	GPCR kinase
GS39783	N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine
GTP(γ)S	guanosine 5'-O-(3-thiophosphate)
HBSS	Hanks' buffered salt solution
HEK	human embryonic kidney
HTS	high-throughput screen
i2 or i3	intracellular loop 2 or intracellular loop 3
IBMX	isobutyl-methylxanthine
IP	inositol-phosphate
IPSC	inhibitory postsynaptic current
Kir3 (or GIRK)	inwardly rectifying potassium channels
LY354740	(1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
LY544344	[(1S,2S,5R,6S)-2-[(2'S)-(2'-amino)propionyl]aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid hydrochloride]
M_1-M_5	muscarinic acetylcholine receptor type 1-5
mGluR	metabotropic glutamate receptor
NPS2143	N-[R-2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl]-1,1-dimethyl-2-(2-naphthyl)ethylamine
PACAP	pituitary adenylate cyclase activating protein
PBP	periplasmic binding protein
PBS	phosphate-buffered saline
PD81'723	2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone
PIP_3	phosphatydilinositol-3,4,5-triphosphate
PLC	phospholypase C
PKA	cAMP dependent protein kinase or protein kinase A
PKC	protein kinase C
PTH	parathyroid hormone
R-121919	3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-a]pyrimidin-7-amine
RGS	regulator of G-protein signaling
RIA	radioimmunoassay
SCH50911	(2S)-(+)-5,5-dimethyl-2-morpholineacetic acid
SPA	scintillation proximity assay
T62	1-amino-4,5,6,7-tetrahydrobenzo(β)thiophen-3-yl
TCM	ternary complex model
VFTM	Venus flytrap module
VTA	ventral tegmental area
WGA	wheat germ agglutinin
WT	wild-type

3. Summary

Allosteric modulators of G-protein coupled receptors (GPCRs) interact with binding sites on the receptor molecule that are topographically distinct from the classic orthosteric site. Having only a marginal effect by themselves, they induce conformational changes of receptors that result in the modulation of agonist-induced function in either a stimulating or an inhibiting way, depending on whether they are positive or negative allosteric modulators, respectively. Their mechanism of action is, thus, in synchrony with the frequency and the magnitude of physiological signaling. This is the main reason why allosteric modulators are considered to have a better side-effect profile and to be less prone to induction of tolerance than classic orthosteric agonists. Allosteric modulators have gained significance in the scientific community in the past decade.

This thesis comprises four parts and focuses on the positive allosteric modulation of the GABA$_B$ receptors. Two prototypal positive allosteric modulators CGP7930 and GS39783 have recently been discovered and characterized in Novartis Pharma (Urwyler et al. 2001 and 2003). A number of questions regarding their further characterization, namely their effects on orthosteric ligands with distinct intrinsic properties, the role allosteric modulation plays in GABA$_B$ receptor desensitization and biochemical effects of GS39783 *in vivo* are addressed in this thesis.

Mechanisms of allosteric modulation at GABA$_B$ receptors by CGP7930 and GS39783: effects on affinities and efficacies of orthosteric ligands with distinct intrinsic properties

The first part of this thesis shows that, as it is predicted by theoretical models of receptor activation, all GABA$_B$ ligand species are amenable to allosteric modulation. A number of

selective GABA$_B$ receptor ligands were tested in the presence and the absence of positive

allosteric modulators CGP7930 and GS39783 in *in vitro* assays, such as radioligand binding,

GTP(γ)S and cellular cyclic AMP (cAMP) measurements. A decrease in affinity of

antagonists was observed in radioligand binding experiments, without a change of the

receptor number, oppositely to increases in affinity of partial agonists. In the GTP(γ)S

experiment the presence of CGP7930 and GS39783 revealed intrinsic efficacies for

CGP35348 and 2-OH-saclofen, two "silent" GABA$_B$ receptor antagonists. In the cAMP

measurements, an even more sensitive experimental system, the two abovementioned

compounds acted as partial agonists, with increased efficacies in the presence of positive

allosteric modulators. Inverse agonistic tendencies were observed with the "silent" antagonist

CGP52432. In this part of the thesis, the positive allosteric modulators GS39783 and

CGP7930 have been shown to be useful experimental tools for elucidating intrinsic properties

of orthosteric ligands. **(Chapter 5, Section 5.1.)**

**Receptor activation involving positive allosteric modulation, unlike full agonism, does
not result in GABA$_B$ receptor desensitization: an *in vitro* study**

To inspect the role of the positive allosteric modulator GS39783 in GABA$_B$ receptor

desensitization, receptor function and cell surface receptor density were examined in a

recombinant GABA$_B$ cell line and in primary neuronal cultures upon persistent treatments

with GABA$_B$ agonists, and combinations of agonists and GS39783. While the GABA$_B$

receptor desensitized after lasting pretreatments with saturating concentrations of GABA$_B$

agonists GABA or R(-)-baclofen, the combined treatment with low concentration of agonists

and GS39738 did not lead to desensitization, despite activating the receptor to the same extent

as desensitization-inducing agonists. These results indicate that it is the degree of occupancy

of the orthosteric binding site that determines desensitization, rather than the degree of

receptor activation. Desensitization experiments with the GABA$_B$ receptor and GS39783 in this study demonstrate that, according to predictions, positive allosteric modulation as a therapeutic principle may indeed be more promising than orthosteric agonism, having less propensity for developing tolerance due to receptor desensitization. **(Chapter 5, Section 5.2.)**

Changes in behavior of allosteric and orthosteric GABA$_B$ receptor ligands after a continuous agonist pretreatment

Investigating the effects of GS39783 on GABA$_B$ receptor desensitization, interesting findings revealed changes in ligand behavior upon receptor desensitization in the GABA$_B$ recombinant cell line. "Silent" antagonists such as CGP62349, CGP52432, CGP56999 and SCH50911 were found to have inverse agonistic properties, the partial agonist 2-OH-saclofen was devoid of positive intrinsic efficacy and the positive allosteric modulator GS39738 was acting in a manner of an allosteric agonist. The possibility of residual GABA present from the pretreatment and responsible for these effects was ruled out. All observed phenomena point toward an increase in constitutive activity of the receptor. Increase of constitutive receptor activity after lasting agonist pretreatments have previously been reported for the β_2-adrenergic and the opioid receptors. This is, however, the first such finding for the GABA$_B$ receptor, which might be important in elucidating the valence of orthosteric ligands as well as their effects upon a chronic drug treatment. It would be interesting to see whether the same phenomena would be observed also for other members of GPCR family 3. **(Chapter 5, Section 5.3.)**

The positive allosteric modulator GS39783 enhances GABA$_B$ receptor-mediated inhibition of cyclic AMP formation in rat striatum *in vivo*

In the last part of this thesis, I provide the first biochemical evidence of *in vivo* activity of a positive allosteric modulator of GPCRs. By using *in vivo* microdialysis in striata of freely moving rats, changes in extracellular levels of cAMP following GABA$_B$ receptor activation were monitored. Locally applied GABA$_B$ receptor agonist R(-)-baclofen inhibited cAMP formation stimulated by 7β-forskolin in a concentration-dependent manner, which was reversed by the co-application of the selective GABA$_B$ antagonist CGP56999. Orally applied positive allosteric modulator GS39783 lacked effects on its own but, together with a threshold concentration of R(-)-baclofen, it significantly decreased cAMP formation in a dose-dependent fashion. Effects of GS39783 were revoked with CGP56999, showing dependence on concomitant GABA$_B$ receptor activation by an agonist and suggesting allosteric modulation as its mechanism of action *in vivo*. **(Chapter 5, Section 5.4.)**

4. Introduction

The focus of this thesis is allosteric modulation of the GABA$_B$ receptor. There are two positive allosteric modulators of the GABA$_B$ receptors, CGP7930 and GS39783, that have recently been discovered and characterized in Novartis Pharma AG (Urwyler *et al.* 2001 and 2003). I was interested in further biochemical characterization of the allosteric mechanism of action of these compounds.

In this introduction, I will address allosteric modulation in general and potential advantages of the use of allosteric modulators over orthosteric ligands in receptor activation. Subsequently, the main points of the GABA$_B$ receptor discovery and its unique structure and function, potential role in central nervous system (CNS) disorders and mechanisms of desensitization will be outlined. Finally, I will describe the actions of GABA$_B$ positive allosteric modulator and summarize the questions addressed in this thesis.

4.1. Allosteric modulation of G-protein-coupled receptors (GPCRs): a novel therapeutic principle

4.1.1. General aspects of allosteric modulation

The term *allosteric* originates from Greek, with άλλος (allos) meaning *other* and στερεός (stereos) meaning *shape*. It describes different mechanisms by which protein functions can be regulated and fine-tuned in either a positive or a negative direction. The initial observation of the phenomenon of allostery was made by C. Bohr in his early studies on hemoglobin in

1904, when he revealed that hemoglobin could simultaneously bind more than one molecule of oxygen and introduced the term cooperativity referring to the interactions between the binding of oxygen molecules (Bohr *et al.* 1904). The term *allosteric* has, however, been coined in 1965 by J. Monod, J. Wyman and J.P. Changeux to explain control of enzyme activity by a regulatory molecule that binds to sites that are distinct and often removed from the catalytic site and that exerts its action through conformational changes (Monod *et al.* 1965). One classical example of an allosteric interaction in pharmacology is the effect of benzodiazepines (*e.g.* diazepam) on $GABA_A$ receptors, which enhance receptor function by binding to a separate site on the receptor.

When it comes to receptor function, allosteric modulators are substances that bind to receptors at the site termed the allosteric binding site (the *alternative* binding site), which is topographically distinct from the orthosteric (Greek ορθός or orthos means *correct*) binding site that binds orthosteric ligands, either orthosteric agonists (*e.g.* the natural ligands) or competitive antagonists. The binding of an allosteric modulator to its binding site induces a conformational change of the receptor. The transmission of this conformational change from the allosteric to the orthosteric binding site and/or directly to effector coupling sites enables allosteric ligands to modulate receptor activity. A reliable proof of a true allosteric mechanism is a demonstration of a change in affinity of an orthosteric ligand in the presence of the alleged allosteric agent by utilizing kinetic (non-equilibrium) radioligand binding experiments (Christopoulos and Kenakin 2002). However, allosteric modulators can either affect affinity (or potency) of orthosteric ligands, their efficacy or both. There are also examples of allosteric agonism or allosteric inverse agonism; these compounds bind to the allosteric binding site on the receptor and stimulate the receptor on their own, independently of orthosteric ligands (Figure 1). This thesis, however, focuses only on modulating compounds depicted by mechanisms (1) and (2) on Figure 1, and not on allosteric agonists (mechanism (3) on Figure 1).
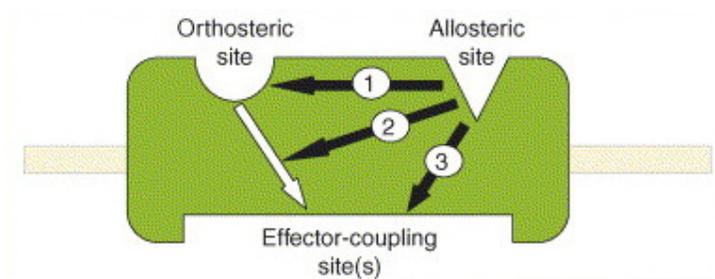
Figure 1. The binding of an allosteric modulator can either affect the binding affinity of an orthosteric ligand (1) and/or the orthosteric ligand efficacy (2). Allosteric agonists can directly activate the receptor on their own (3). Taken from (Langmead and Christopoulos 2006), with permission from Elsevier.

There are numerous advantages of allosteric modulators over conventional orthosteric ligands. To begin with, upon their binding to the allosteric site in the absence of an orthosteric ligand, allosteric modulators on their own usually affect the signaling cascade of the receptor in a limited fashion (which can only be detected in very sensitive experimental systems) or not at all. This means that not the whole population of receptors is affected by the binding of the allosteric species, but only the fraction of receptors that is activated by the endogenous agonist; thus is the action of the allosteric drug in spatial and temporal synchrony with physiological stimulation (Figure 2). In this light, the probability of the target receptor desensitizing (which is one of the mechanisms for acquired tolerance) is smaller even in a continuous presence of an allosteric agent, when compared to continuous activation *via* the orthosteric ligand. In addition, for the same reason allosteric modulators are expected to have a better side-effect profile than agonists and are less likely to elicit toxic effects due to an overdose.

Moreover, since the allosteric binding sites are usually situated in non-conserved regions of a receptor, allosteric agents can often be selective for a certain receptor subtype, which is more unlikely for orthosteric ligands, that bind to highly conserved sites on receptors. This could be explained with less evolutionary pressure for the conservation of allosteric binding sites, most likely due to the lack of endogenous allosteric ligands. An interesting variant of this notion

(a) Action of endogenous agonist

(b) Action of endogenous agonist in the presence of an allosteric enhancer

(c) Action of synthetic agonist
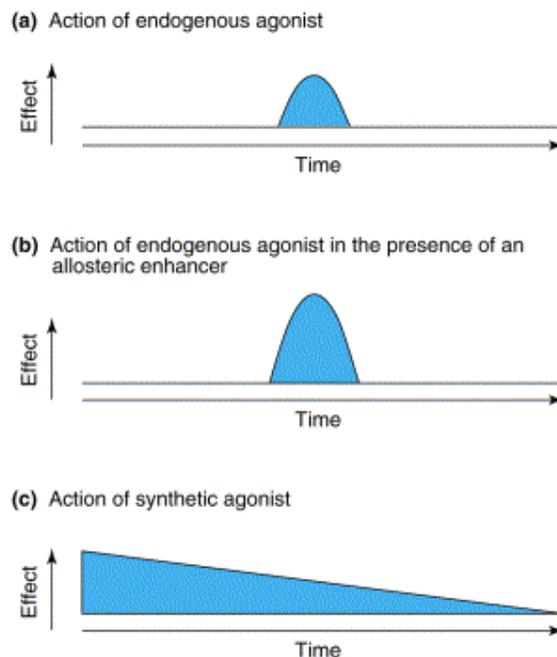
Figure 2. An allosteric modulator is a more physiological alternative when compared to a synthetic agonist. An allosteric modulator acts only in the presence of an endogenous agonist, mimicking the duration (and the spatial organization) of the natural signaling. Taken from (Soudijn *et al.* 2004), with permission from Elsevier.

implies different degrees of cooperativity between the orthosteric and the allosteric site at different receptor subtypes leading to *absolute subtype selectivity*, introduced by the group of N.J.M. Birdsall (Lazareno *et al.* 1998). A remarkable example of this phenomenon was demonstrated at the muscarinic acetylcholine (ACh) receptors, which exist in five subtypes (M_1-M_5). Whereas thiochrome was shown to bind to all subtypes, it was absolutely selective for M_4, by increasing the affinity for ACh 3- to 5-fold, while having only negligible effects (neutral cooperativity) on ACh binding at the other four muscarinic receptor subtypes (Lazareno *et al.* 2004).

Further, it has been shown that different GPCR conformations can stimulate distinct signaling pathways (see Kenakin 2003; Perez and Karnik 2005; Maudsley *et al.* 2005 for reviews). It is thus possible for the activation involving an allosteric agent to activate a particular signaling pathway. In fact, agonist-directed trafficking has recently been demonstrated for the allosteric

agonist of the metabotropic glutamate receptor (mGluR) type 7 AMN082. Namely, Suzuki *et al.* (2007) have shown that while AMN082 inhibited cAMP formation by activating the mGluR7, it failed to induce intracellular Ca^{2+} mobilization when the receptor was artificially coupled to the phospholipase C (PLC) pathway. In addition, one part of the work presented in this thesis, regarding continuous exposure of the $GABA_B$ receptor to the positive allosteric modulator GS39783, goes in this direction (see Chapter 5, Section 5.3.).

Finally, allosteric ligands offer new opportunities to medicinal chemistry aiming at receptors considered to be problematic drug targets. Such are for example large molecular weight ligand (*e.g.* peptide) receptors, regarded as difficult targets for small molecules due to the size of their binding sites or the calcium-sensing receptor (CaSR), orthosteric agonists of which are inorganic cations (see Christopoulos 2002; Jensen and Spalding 2004; May *et al.* 2007; Langmead and Christopoulos 2006 for reviews).

Another example where allosteric modulation provides new possibilities for medicinal chemistry are the mGluRs. There are eight known mGluRs divided into three families, with L-glutamate being a natural ligand for all the receptor subtypes (Conn and Pin 1997). There are two main hurdles for developing orthosteric ligands for mGluRs, namely subtype selectivity and physicochemical properties of ligands. As mentioned above, because of the highly conserved orthosteric binding site, it is extremely difficult to develop subtype specific orthosteric ligands. Moreover, all the known orthosteric ligands of mGluRs are amino acid derivatives and it seems that this structural element is crucial for their binding and efficacy. Unfortunately, amino acid-like structures consist of charged and polar moieties, which is the reason for both their limited absorption from the gut and their poor brain penetrability. The development of allosteric modulators has thus proven to be a promising alternative solution for specific targeting of mGluRs subtypes and elucidation of their therapeutical potential in diseases (Ritzen *et al.* 2005).

To summarize, the fact that there are less structural constraints for compounds with allosteric properties has opened new avenues in medicinal chemistry. The attractiveness of GPCR allosteric modulators as novel drug targets has resulted in an increasing body of literature on the subject in recent years, which is represented by a large number of review articles (Christopoulos 2002; Christopoulos and Kenakin 2002; Rees *et al.* 2002; Soudijn *et al.* 2002; Conigrave and Franks 2003; Birdsall *et al.* 2004; Christopoulos *et al.* 2004; Jensen and Spalding 2004; May *et al.* 2004 and 2007; Soudijn *et al.* 2004; Bowery (ed), 2006; Gao and Jacobson 2006; Langmead and Christopoulos 2006; Noeske *et al.* 2006; Schwartz and Holst 2006).

It is worthwhile looking at mechanisms of allosteric modulation in the light of classical and more recent receptor models.

4.1.2. Theoretical receptor models

a) The two state model of receptor activation (Figure 3)

The two-state model of receptor activation illustrates the intrinsic efficacies of orthosteric ligands, but does not account for allosteric modulation (Leff 1995). The major assumption in the two state model is that there are two interchangeable conformations of a receptor: the
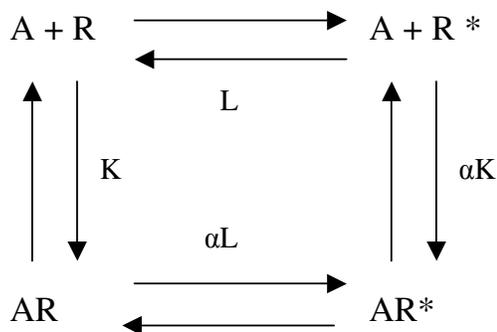
Figure 3. The two-state model of receptor activation. R: resting state of the receptor, R*: active state of the receptor, A: ligand, K: binding constant of A, L: receptor isomerisation constant, α: intrinsic efficacy of A. Taken from (Urwyler, Gjoni *et al.* 2005) with permission from Elsevier.

resting (R) and the active state (R*) (Figure 3). The exchange between the two states is

characterized by an equilibrium constant L. The existence of R* in an absence of the ligand

(A) is the basis for constitutive activity. The constant K describes the binding affinity of the

ligand (A) to the receptor (R). If the ligand bound stabilizes the active (R*) over the resting

state (R), the equilibrium is shifted toward the active state ($\alpha > 1$) and the ligand is an agonist.

Contrarily, an inverse agonist prefers and stabilizes the inactive form of the receptor and has

an α value smaller than 1. A silent antagonist binds to both states of the receptor with equal

affinity and does not affect the proportions of the active and the resting state ($\alpha = 1$), but

inhibits agonist binding by blocking the binding site.

b) The ternary complex model (TCM) (Figure 4)

The TCM was originally developed to describe the changes in agonist affinity induced by the

receptor-G-protein coupling, a prototype example of allosteric interactions (De Lean *et al.*

1980). It can, however, also be applied to allosteric modulation by small molecules. The TCM

takes into consideration the influence on binding affinity that the allosteric ligand (B) has on
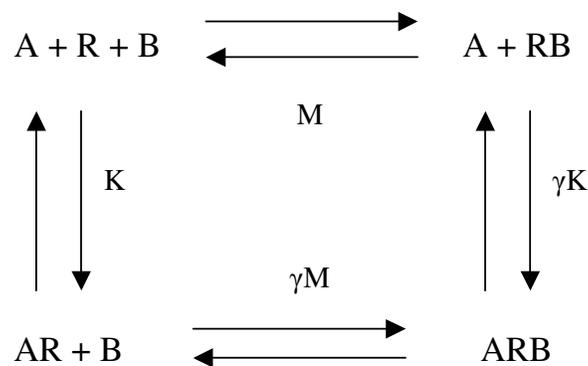
Figure 4. The ternary complex model of receptor activation. R: receptor, A: orthosteric ligand, B: allosteric ligand, K: binding constant of A, M: binding constant of B, γ: binding cooperativity between A and B. Taken from (Urwyler, Gjoni *et al.* 2005) with permission from Elsevier.

the orthosteric ligand (A) when it binds to the receptor (R) to a distinct binding site. The

constant K depicts the binding affinity of the orthosteric ligand (A) for the receptor and M is

the affinity constant of the allosteric ligand B toward the receptor R. The binding

cooperativity between A and B is described by the factor γ. This model, unlike the two-state

model, takes into account allosteric interactions, but it does not account for the intrinsic

efficacy of the orthosteric ligand, *i.e.* the activation of the receptor by ligand binding.

c) The allosteric two-state model (Figure 5)
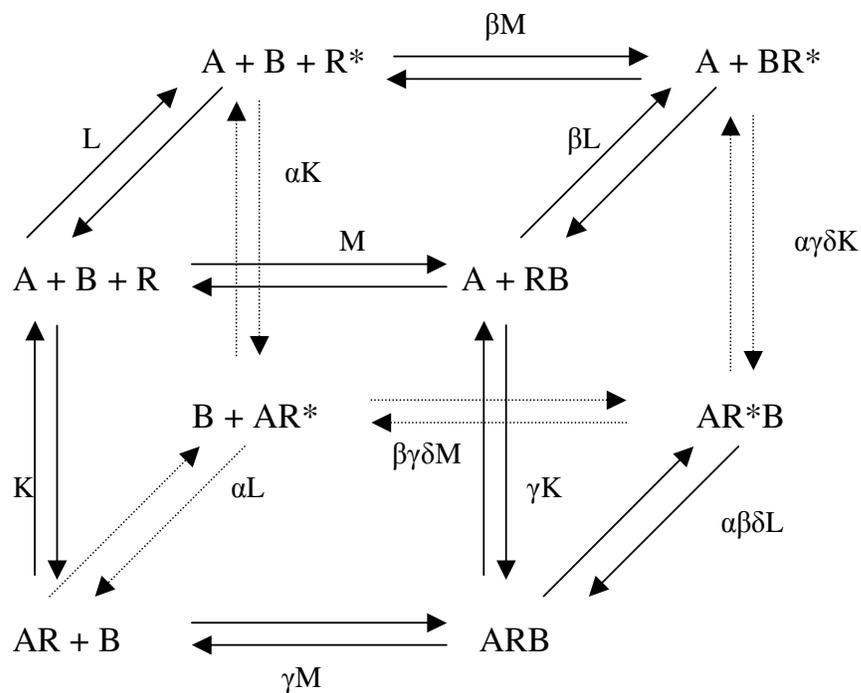
Figure 5. The allosteric two-state model of receptor activation. R: resting state of the receptor, R*: active state of the receptor, A: orthosteric ligand, B: allosteric ligand, K: binding constant of A, L: receptor isomerisation constant, M: binding constant of B, α: intrinsic efficacy of A, β: intrinsic efficacy of B, γ: binding cooperativity between A and B, δ: activation cooperativity between A and B. Taken from (Urwyler, Gjoni *et al*. 2005) with permission from Elsevier.

Although the "allosteric ternary complex model" (ATCM) (Lefkowitz *et al.* 1993) was the first attempt to combine the two previous models, the allosteric two-state model described by Hall (2000), allows for the first time the allosteric modulator to simultaneously affect the affinity of orthosteric ligands as well as their efficacy. The extension of the two-state model of receptor activation introduces the allosteric constant δ, by which the intrinsic efficacy of the orthosteric ligand α is modified. This model treats the allosteric interactions strictly in numerical terms, *i.e.* the constants γ and δ are independent of the qualitative nature of α. This means that the orthosteric agonist (intrinsic efficacy of which is described by α) can be a partial or full agonist, a silent antagonist or an inverse agonist, with and all the chemical entities equally amenable to allosteric modulation. This topic is addressed and further discussed in Chapter 5, Section 5.1. of this thesis or (Urwyler, Gjoni *et al.* 2005).

4.2. The GABA$_B$ receptor

GABA is the main inhibiting neurotransmitter in the CNS. It modulates the neuronal activity by mediating its action *via* GABA$_A$, GABA$_B$ and GABA$_C$ receptors. GABA$_A$ and GABA$_C$ receptors are ligand-gated ion channels while the GABA$_B$ receptor is a metabotropic receptor coupled to heterotrimeric G-proteins. The GABA$_A$ receptor, a pentameric ligand-gated ion channel that mediates a fast neuronal inhibition (hyperpolarization) by enabling the influx of chloride ions into the postsynaptic terminal, is known longest. There are many drugs currently on the market that target the GABA$_A$ receptor, namely benzodiazepines (*e.g.* diazepam) or barbiturates (such as pentobarbital) which are widely used in the clinical practice as anticonvulsants, myorelaxants, sedatives and anesthetics. It was believed that the GABA$_A$ receptor was the only GABA receptor until the late 1970ies, when the existence of the GABA$_B$ receptor was first proposed (Bowery *et al.* 1980). The GABA$_C$ receptors were

postulated even later, in the 1990s, as mediators of the GABA response that is insensitive to

$GABA_A$ and $GABA_B$ antagonism (Johnston 1996).

4.2.1. The discovery and structure of the $GABA_B$ receptor

The prototypical $GABA_B$ agonist baclofen (β-p-chlorophenyl-GABA) has been in clinical

practice as an antispastic agent under the name of *Lioresal* for more than thirty years, long

before $GABA_B$ receptors were known as a distinct entity (see more in Subsection 4.2.4.).

In the late seventies of the 20th century the group of N. G. Bowery observed that the actions of

GABA and baclofen to inhibit noradrenaline, dopamine and serotonin release were not

blocked by the known GABA antagonist bicuculline, nor mimicked by GABA-mimetics such

as isoguvacine or 3-aminopropanesulphonic acid. Moreover, they were independent of the

concentrations of chloride ions, but not of Mg^{2+} and Ca^{2+}. As a consequence the novel

baclofen-sensitive bicuculline-insensitive receptor termed the $GABA_B$ receptor was

postulated (Bowery *et al.* 1980).

The main breakthrough in the $GABA_B$ receptor research occurred 17 years later, with its

cloning by the group of B. Bettler (Kaupmann *et al.* 1997). The delay in the cloning of the

receptor was due to the fact that there were difficulties in coupling of the receptor to its

effector systems in heterologous cells and the lack of pharmacological tools suitable for

expression cloning at the time (see Bettler *et al.* 2004). Only after an iodinated high-affinity

$GABA_B$ ligand was finally available, two isoforms of the same protein, structurally similar to

the mGluRs, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ were discovered using a radioligand-binding

screening approach (Kaupmann *et al.* 1997). Rat $GABA_{B(1a)}$ and $GABA_{B(1b)}$ proteins are

composed of 960 and 844 amino acids, respectively, with the only difference being the

presence or the absence of the so-called "Sushi repeats" (or "short consensus repeats") at their

extracellular NH_2-terminal domain (N-terminus), respectively. It was found later that the

human $GABA_{B(1a/b)}$ proteins share 99% sequence identity with the rat $GABA_{B(1a/b)}$ proteins (Kaupmann *et al.* 1998b). Despite the success in isolating two $GABA_B$ receptor proteins, it was noted that there was a hundred-fold decrease in binding affinities of $GABA_B$ agonists when compared to the wild-type (WT) receptors. In addition, there was little functional response upon agonist binding and only at agonist concentrations which were saturating in native tissues. This aroused an interest of the scientific community, which soon afterward resulted in the cloning of another $GABA_B$ receptor protein, termed $GABA_{B(2)}$, that shared 35% homology to the first two $GABA_B$ proteins. The discovery was made by six groups simultaneously (Kaupmann *et al.* 1998a; Jones *et al.* 1998; Kuner *et al.* 1999; Martin *et al.* 1999; Ng *et al.* 1999; White *et al.* 1998).
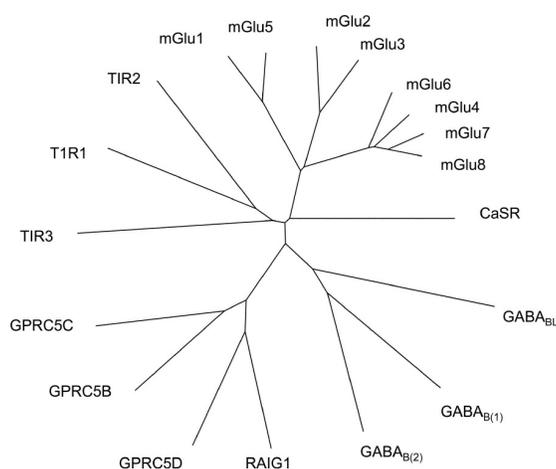
Figure 6. Phylogenetic analysis of human family C GPCRs. Taken from (Bettler *et al.* 2004), with permission of the American Physiological Society.

The $GABA_B$ receptor belongs to the GPCR family 3 (or C), together with eight mGluRs, the CaSR, taste and pheromone receptors and five orphan receptors (Figure 6) (Pin *et al.* 2003). The peculiarity of the $GABA_B$ receptor is the fact that in order to be functional it needs to be a heterodimeric complex, composed of the $GABA_{B(1a)}$ or the $GABA_{B(1b)}$ and the $GABA_{B(2)}$ subunit (Figure 7).

As the other members of GPCR family 3, the $GABA_B$ receptors possess the seven transmembrane spanning domain (7TM), an intracellular COOH-terminal tail (C-terminus),

which has been shown to bind many proteins that regulate the $GABA_B$ receptor function (see

Bettler *et al.* 2004 and Emson 2007 for reviews) and a large extracellular NH_2-terminal

domain (N-terminus) that contains the orthosteric site for agonist/competitive antagonist

binding, which, like it is the case with mGluRs, is related to the bacterial periplasmic binding

proteins (PBP) (O'Hara *et al.* 1993). However, in contrast to the mGluRs, the N-terminal part

of the $GABA_B$ subunits lacks a cysteine-rich region that connects the PBP-like domain to the

TM1 (Malitschek *et al.* 1999). The agonist-binding site consists of two large globular lobes

connected by a hinge region. A conformational change occurs in the hinge region upon

agonist binding which brings the two lobes closer together trapping the agonist similarly to

the trapping of the insect by a carnivorous plant called the Venus flytrap (Galvez *et al.* 1999;

Bessis *et al.* 2000; Galvez *et al.* 2000a; Bernard *et al.* 2001; Kniazeff *et al.* 2004). Even
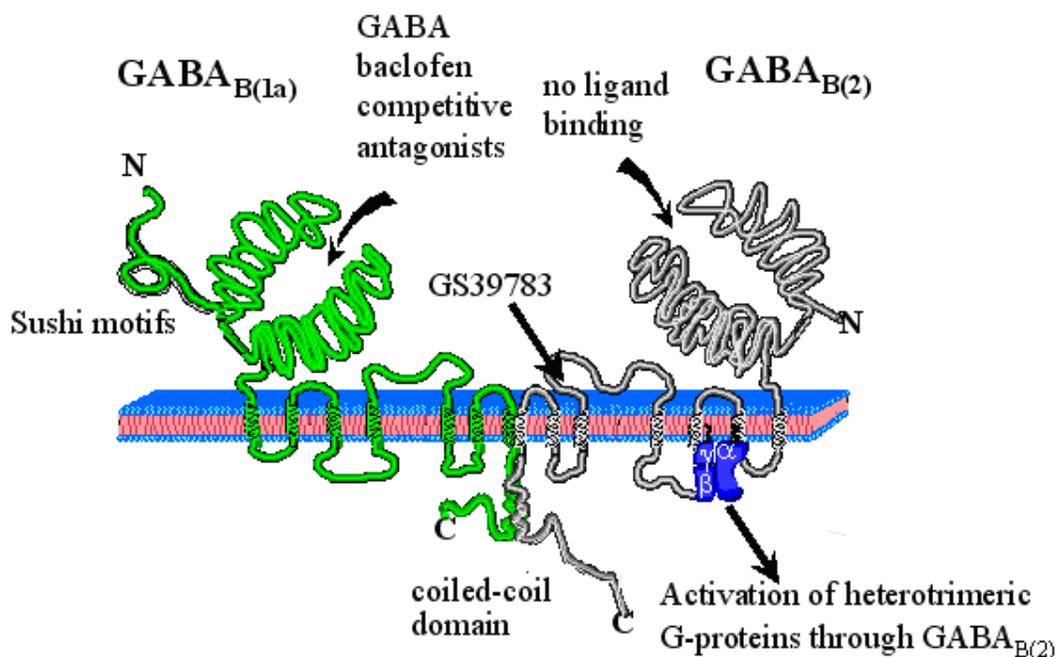
Figure 7. A cartoon of the $GABA_B$ receptor. $GABA_{B(1a)}$ (green) and $GABA_{B(2)}$ (grey) form a heterodimer mainly interacting *via* the C-terminal tail forming a coiled-coil domain. Both $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits contain a VFTM, but only the VFTM of $GABA_{B(1)}$ is able to bind orthosteric ligands. In addition, the N-terminus of $GABA_{B(1a)}$ has two Sushi motifs, which are missing in the $GABA_{B(1b)}$ isoform. The $GABA_{B(2)}$ subunit is crucial for the interaction with the G-protein (blue) and it also contains the binding site for the positive allosteric modulator GS39783 (Dupuis *et al.* 2006).

though both the $GABA_{B(1)}$ and the $GABA_{B(2)}$ subunits contain the "Venus flytrap module" (VFTM), only the $GABA_{B(1)}$ subunit is able to bind orthosteric ligands. The amino acids critical for ligand binding in the VFTM of the $GABA_{B(2)}$ subunit have not been conserved (Galvez *et al.* 2000a).

The functional importance of the presence of the $GABA_{B(2)}$ receptor subunit lies in the surface trafficking of the $GABA_{B(1)}$ receptor subunit and the $GABA_B$ receptor coupling to effector systems and signal transduction. The $GABA_B$ heterodimer is assembled mainly through the interaction of the two subunits at the C-terminal tail forming a coiled-coil domain. Although there is evidence that other parts of the two receptor subunits can interact with each other, it has been shown that the formation of the coiled-coil domain is crucial for $GABA_B$ receptor surface trafficking. In the absence of the $GABA_{B(2)}$ subunit the $GABA_{B(1)}$ does not reach the cell surface but remains in the endoplasmatic reticulum because of the amino acid sequence of four amino acids (RSRR) known as the retention signal at its cytoplasmic tail (Margeta-Mitrovic *et al.* 2000; Pagano *et al.* 2001). In the presence of the $GABA_{B(2)}$ receptor subunit, by the formation of the coiled-coil domain, the retention signal of the $GABA_{B(1)}$ is masked and the receptor is successfully expressed at the cell surface (Couve *et al.* 1998). $GABA_{B(2)}$, however, does not need the co-expression of the $GABA_{B(1)}$ subunit to reach the cell surface. Moreover, it has been shown that there is an allosteric interaction between the VFTMs of both subunits, which results in a higher affinity of agonists for the VFTM of $GABA_{B(1)}$. This is an explanation for the lower affinity for agonist binding when only the $GABA_{B(1)}$ subunit was cloned in 1997 (see Kaupmann *et al.* 1997 or Bettler *et al.* 2004 for a review). Finally, the coupling of the $GABA_B$ receptor to its effector systems happens exclusively *via* the $GABA_{B(2)}$ receptor subunit. Namely, it is the intracellular loop 2 (i2) of the $GABA_{B(2)}$ that is crucial for G-protein coupling (Margeta-Mitrovic *et al.* 2001; Robbins *et al.* 2001; Havlickova *et al.* 2002; Grünewald *et al.* 2003; Thuault *et al.* 2004; Duthey *et al.* 2002). This "sideways" signal

transduction from GABA$_{B(1)}$ subunit, that binds orthosteric ligands, to GABA$_{B(2)}$ subunits, that couples to the effector systems, is a unique feature of the GABA$_B$ receptor.

4.2.2. The anatomical expression pattern of the GABA$_B$ receptor

The GABA$_B$ receptor is abundantly expressed in the mammalian CNS. It has been found that GABA$_B$ binding sites are present in almost all neuronal populations (Bischoff *et al.* 1999), as well as in glial cells (Hosli *et al.* 1990; Oka *et al.* 2006), with the highest expression levels in the thalamic nuclei, the molecular layer of the cerebellum, the cerebral cortex, the interpeduncular nucleus and the dorsal horn of the spinal cord (Bowery *et al.* 1987; Kaupmann *et al.* 1997; Bischoff *et al.* 1999; Fritschy *et al.* 1999; Charles *et al.* 2001; Chu *et al.* 1990; Liang *et al.* 2000).

Functional GABA$_B$ receptors are not only confined to the CNS. They are also expressed in peripheral tissues, for example heart, spleen, lung, liver, small intestine, large intestine, kidney, stomach, adrenal gland, testis, ovary and urinary bladder (see Bettler *et al.* 2004 for a review).

4.2.3. The GABA$_B$ receptor and its effector systems

The GABA$_B$ receptors are coupled to many different effector systems. They mostly couple to the Gα_i and Gα_o proteins (Asano and Ogasawara 1986; Morishita *et al.* 1990; Campbell *et al.* 1993; Menon-Johansson *et al.* 1993; Greif *et al.* 2000).This mainly results in an inhibition of the adenylyl cyclase activity, as has been shown for many native experimental setups *in vitr*o (Wojcik and Neff 1984; Cunningham and Enna 1996; Knight and Bowery 1996; Olianas *et al.* 2005) and *in vivo* (Hashimoto and Kuriyama 1997; Gjoni *et al.* 2006), as well as in recombinant systems *in vitro* (Wise *et al.* 1999; Hirst *et al.* 2003; Urwyler, Gjoni *et al.* 2005). However, there are also reports of GABA$_B$-mediated stimulation of adenylyl cyclase activity

in conjunction with activation of noradrenalin, pituitary adenylate cyclase activating protein (PACAP) or corticotrophin-releasing hormone (CRH) receptors in native preparations. These effects are most likely a result of a receptor-receptor crosstalk and have been postulated to involve the Gβγ subunits released upon $GABA_B$ receptor activation, which stimulate certain types of adenylyl cyclase in the presence of $Gα_s$ that originated from G_s coupled-GPCR activation (Cunningham and Enna 1996; Knight and Bowery 1996; Olianas and Onali 1999; Onali and Olianas 2001).

The function of $GABA_B$ receptors in the CNS mainly depends on their pre- or post-synaptic localization (Figure 8). When expressed presynaptically, they act as either autoreceptors or heteroreceptors, inhibiting the release of GABA or other neurotransmitters, *e.g.* glutamate, various neuropeptides, catecholamines, serotonin or acetylcholine, into the synaptic cleft (Bowery *et al.* 1980; Taniyama *et al.* 1992; Waldmeier *et al.* 1994; Teoh *et al.* 1996; Bonanno *et al.* 1998; Bonanno *et al.* 1999). These effects are mainly mediated *via* the inhibition of the high-voltage activated calcium channels of the N-type or P/Q type which are both expressed in presynaptic terminals and shown to trigger neurotransmitter release (Takahashi *et al.* 1998). There is also evidence of interaction between presynaptically expressed $GABA_B$ receptors and L-type and T-type Ca^{2+} channels (for review see Bettler *et al.* 2004). Postsynaptic $GABA_B$ receptors, on the other hand, activate inwardly rectifying potassium channels (GIRK or $K_{ir}3$) resulting in a prolongation of the slow inhibitory postsynaptic current (also known as late IPSC). This regulation involves Gβγ signaling (Lüscher *et al.* 1997).
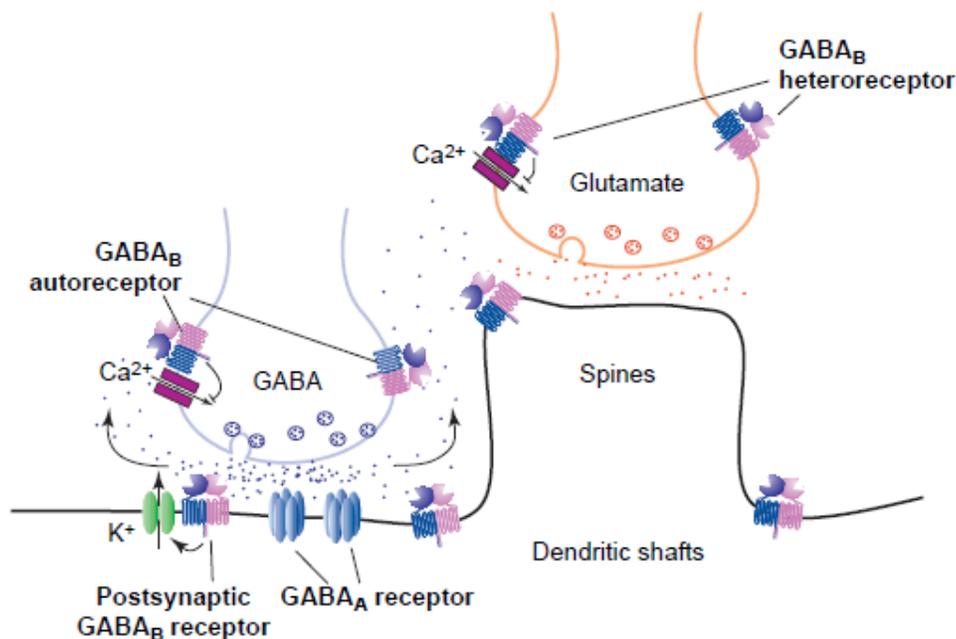
Figure 8. Presynaptic and postsynaptic localization of the GABA$_B$ receptor in the CNS. Presynaptic autoreceptors (regulating the release of GABA) and heteroreceptors (regulating the release of other neurotransmitters, in this case glutamate) mediate their effect by inhibiting the influx of Ca^{2+} into the presynaptic terminals. Postsynaptically expressed GABA$_B$ receptors induce hyperpolarization of the postsynaptic terminal (late IPSC) by activating K$_{ir}$3 channels. From (Cryan and Kaupmann 2005), with permission from Elsevier.

4.2.4. The potential role of GABA$_B$ receptors in disease

The GABA$_B$ receptor has been implicated in many neurological disorders, namely spasticity, pain, drug addiction, anxiety and depression, absence epilepsy and cognition (see Vacher and Bettler 2003; Bettler *et al.* 2004; Ong and Kerr 2005; Bowery 2006 for reviews). The widespread peripheral expression in mammalian organisms (see Subsection 4.2.2.) points to intestinal, pulmonary and bladder dysfunction as possible therapeutic applications for GABA$_B$ drugs. Insights in the potential role of the GABA$_B$ receptor in diseases were mostly obtained from experiments performed with baclofen and recently with GABA$_B$ knock-out mice (Schuler *et al.* 2001; Prosser *et al.* 2001).

The selective GABA$_B$ receptor agonist baclofen is a lipophilic brain-penetrable derivative of GABA with central muscle-relaxant properties. It was synthesized in 1962 and has been in

clinical use in its racemic form under the trade name *Lioresal* since 1972 as a drug of choice for treating spinal spasticity and skeletal muscle rigidity, associated with cerebral palsy, multiple sclerosis, stiff-man syndrome and tetanus (Bowery 1993), even though its mechanism of action was unclear at the time of its release. The anti-spastic effects of baclofen are most likely mediated by the activation of the presynaptic GABA$_B$ receptors in the spinal cord, which inhibit the release of excitatory neurotransmitters onto the motoneurons in the monosynaptic reflex arc, resulting in relaxation of the contracted muscles. Additionally, baclofen has also been used in the treatment of chronic pain, *e.g.* neuropathic pain (see Fromm 1994 or Vacher and Bettler 2003; Bowery 2006; Bettler *et al.* 2004; Ong and Kerr 2005 for reviews). The mechanism underlying GABA$_B$-mediated antinociceptive effects is mediated *via* both the spinal cord (Malan *et al.* 2002) and higher brain centers (Ipponi *et al.* 1999; Jasmin *et al.* 2003).

A growing preclinical and clinical literature implicates the GABA$_B$ receptors in drug addiction. GABA$_B$ agonists were found to promote abstinence and reduce the use of cocaine, heroin, alcohol and nicotine (reviewed in Cousins *et al.* 2002) by modulating the mesolimbic dopamine system, also known as the reward and reinforcement circuitry (Robbins and Everitt 1999). Efficiency of GABA$_B$ agonists to diminish cocaine self-administration and reinforcement has been observed in rats (Roberts *et al.* 1996; Roberts and Andrews 1997; Brebner *et al.* 1999, 2000 and 2002; Shoaib *et al.* 1998; Campbell *et al.* 1999) and clinical studies demonstrated effectiveness of baclofen in reducing cocaine craving in cocaine addicts (Ling and Shoptaw 1998; Shoptaw *et al.* 2003; Kaplan *et al.* 2004). Further, baclofen has not only been found to reduce self-administration of alcohol in rats (Colombo *et al.* 2002 and 2004), but it was also found to be palliative against alcohol withdrawal and craving in humans patients, albeit in high doses (Ameisen 2005; Bucknam 2007; Addolorato *et al.* 2002a and 2002b). GABA$_B$ receptor activation has also been found to block the locomotor stimulatory effect of amphetamine and reduce its self-administration in animals (Bartoletti *et*

al. 2004; Brebner *et al.* 2005). Decrease in the self-administration of heroin (Xi and Stein 1999; Di Ciano and Everitt 2003) and nicotine (Paterson *et al.* 2004a and 2004b) were also demonstrated upon administration of baclofen and the GABA$_B$ agonist CGP44532 in rats. Moreover, a significant association between variants of the human GABA$_{B(2)}$ gene and nicotine dependence has been reported that is thought to play a crucial role in the etiology of nicotine addiction (Beuten *et al.* 2005).

GABAergic neurotransmission has been implicated in several psychiatric and emotional disorders, including anxiety and depression (Cryan and Kaupmann 2005). The involvement of the GABA$_B$ receptor in depression was first suggested by (Lloyd *et al.* 1985). Later, GABA$_B$ receptor antagonists were demonstrated to be effective in animal models for depression (Nakagawa *et al.* 1999; Slattery *et al.* 2005a), which could be linked with the finding that GABA$_B$ antagonists produced a rapid increase in the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Heese *et al.* 2000; Froestl *et al.* 2004). The anti-depressant effects of GABA$_B$ antagonists are in line with the findings that the genetic deletion of either GABA$_{B(1)}$ or GABA$_{B(2)}$ subunits in mice resulted in their antidepressant-like behavior in the forced swim test (FST), a model for the assessing of antidepressant action of drugs (Mombereau *et al.* 2004 and 2005).

 Baclofen was demonstrated effective in panic disorders in human patients (Breslow *et al.* 1989), which is in alignment with the fact that GABA$_{B(1)}$-deficient mice display a more anxious phenotype than the WT animals (Mombereau *et al.* 2004). In addition, anxiolytic effects of baclofen were also shown in several animal models (see Cryan and Kaupmann 2005 for a review).

GABA$_B$ receptors appear to play a role in absence epilepsy, which is thought to be related to a predominance of inhibitory activity in the reticular thalamic nucleus, a part of the thalamocortical circuit responsible for development of seizures, that comprises mainly GABA-containing neurons (see Manning *et al.* 2003 for a review). Namely, GABA$_B$ receptor

antagonists inhibited spike and wave discharges in genetically modified animals, that are used as animal models for absence epilepsy, while administration of R(-)-baclofen aggravated the spontaneous seizures (Marescaux *et al.* 1992; Hosford *et al.* 1992). In addition, bilateral injections of R(-)-baclofen into specific relay nuclei and reticular nuclei of the thalamus increased spike and wave discharges in a concentration dependent fashion in rats with spontaneous absence seizures (Liu *et al.* 1992). Although this seems to be in contradiction with the fact that mice lacking functional $GABA_B$ receptors were more prone to spontaneous seizures (Schuler *et al.* 2001; Prosser *et al.* 2001), it must be noted that $GABA_{B(1)}$-deficient mice suffer from a different type of absence seizures than observed in genetic animal models of absence epilepsy (see Bettler *et al.* 2004 for a review).

Further, $GABA_B$ receptor antagonism has also been shown to improve spatial memory (Helm *et al.* 2005). The cognitive enhancer SGS742 is the first $GABA_B$ antagonist in clinical trials (Froestl *et al.* 2004).

Peripherally, baclofen inhibited lower esophageal sphincter relaxation in dogs (Lehmann *et al.* 1999), healthy humans (Lidums *et al.* 2000) and esophageal reflux disease patients (Zhang *et al.* 2002).

Despite these numerous implications of the $GABA_B$ receptor in neurological and non-neurological disorders, the only drug on the market that targets the $GABA_B$ receptor is baclofen. Its main shortcomings are deleterious side-effects such as sedation, motor impairment, hypothermia and fast onset of tolerance, observed in several animal models (Wang *et al.* 2002; Cryan *et al.* 2004; Lobina *et al.* 2005; Jacobson and Cryan 2005), as well as in human patients (Fromm 1994; Loubser and Akman 1996), which limit its widespread utility in preclinical and clinical settings. This is the main reason for the interest the discovery of positive allosteric modulators of the $GABA_B$ receptors CGP7930 and GS39783 arose within the scientific community (see Subsection 4.2.6.).

4.2.5. GABA$_B$ receptor desensitization

The phenomenon of receptor desensitization plays an important physiological role acting as the feedback mechanism limiting both acute and lasting (chronic) overstimulation of GPCR signaling cascades. Desensitization of GPCRs has been extensively explored. There seems to be a "universal" mechanism by which most GPCRs desensitize. This "canonical" pathway mainly involves agonist-induced receptor phosphorylation of serine/threonine residues (usually in the i3 and/or the C-terminus) by intracellular kinases, usually GPCR kinases (GRKs). Phosphorylation is followed by the recruitment of cytoplasmic accessory proteins such as β-arrestins, which sterically hinder further coupling of the receptor to the G-protein thus acting as a turn-off switch of the signal. Subsequently most GPCRs internalize into clathrin-coated vesicles. Once internalized, receptors are targeted to specialized compartments where they are either dephosphorylated and recycled back to the plasma membrane (resensitization) or targeted to lysosomes for degradation (see Ferguson 2001; Tsao *et al.* 2001; Clark and Rich 2003; Gainetdinov *et al.* 2004 for reviews).

Desensitization of the GABA$_B$ receptor has been studied for a long time. It has been observed in *in vivo* studies in which chronic baclofen treatments resulted in the loss of its antinociceptive effects (Malcangio *et al.* 1992), the absence of GABA$_B$-mediated induction of late IPSCs (Malcangio *et al.* 1995) and the loss of baclofen-induced hypothermic effects in rats (Lehmann *et al.* 2003). Although early *in vitro* studies have implicated phosphorylation by protein kinase C (PKC) (Taniyama *et al.* 1992) and protein kinase A (PKA) (Yoshimura *et al.* 1995) as a key step in GABA$_B$ receptor desensitization, several lines of evidence suggest that the GABA$_B$ receptor does not follow the β-arrestin-mediated desensitization pathway described above. Couve *et al.* (2002) have shown that the PKA-mediated phosphorylation of a single serine residue (Ser892) in the cytoplasmic tail of the GABA$_{B(2)}$ subunit enhanced the stability of the receptor at the cell surface. GABA$_B$ agonists thus reduced PKA activity, by

inhibiting cAMP formation, resulting in a lesser degree of phosphorylation, thereby leading to desensitization of the receptor. Soon afterwards another study proposing an atypical mechanism of receptor desensitization came out, in which GRK4 was implicated as crucial for GABA$_B$ receptor desensitization in cerebellar granule cells (Perroy *et al.* 2003). Surprisingly, GRK4-mediated desensitization was found to be phosphorylation-independent, as it was promoted even in the absence of agonist-induced phosphorylation as well as by a mutant GRK4 lacking its kinase domain. Another report recently linked both GRK4 and GRK5 to GABA$_B$ receptor desensitization (Kanaide *et al.* 2006). However, this process is probably not generalized as GRK4 is absent from many brain regions that express high level of the GABA$_B$ receptor such as the cerebral cortex or the hippocampus (Sallese *et al.* 2000). Fairfax *et al.* (2004) provided evidence for endocytosis-independent degradation of the receptor at the cell surface as a mechanism of GABA$_B$ receptor desensitization (there was no proof of internal receptor pools, but the decrease in the cell surface receptor number was evident). Receptor degradation was found to correlate with a reduced phosphorylation at the Ser892 residue of the GABA$_{B(2)}$ receptor subunit, which is in alliance with the previously mentioned finding by Couve *et al.* (2002). In a recent report by Pontier *et al.* (2006) the phosphorylation by PKC was found to induce GABA$_B$ receptor desensitization, the pre-association of the NEM sensitive fusion protein (NSF) with the GABA$_B$ receptor being a critical step in its phosphorylation by PKC. Further mechanisms that were proposed for GABA$_B$ receptor desensitization comprise regulation of receptor-G-protein coupling by endogenous regulators of G-protein signaling (RGS) proteins (Mutneja *et al.* 2005) and agonist-induced endocytosis (Gonzales-Maeso *et al.* 2003; Laffray *et al.* 2007). Interestingly, Grampp *et al.* (2007) recently observed a high constitutive clathrin-mediated internalization of the receptor, which was not altered in the lasting presence of GABA$_B$ agonists/antagonists. Apparent divergences among findings in abovementioned studies might be due to different experimental systems employed to study receptor desensitization. It is possible that varying

expression levels of intracellular regulatory proteins, which serve as parts of the "desensitization machinery", give rise to different desensitization pathways in different cellular contexts (see Chapter 5, Section 5.3. for further discussion).

4.2.6. Positive allosteric modulators of the GABA$_B$ receptor

The development of functional and cell-based assays represents an important progress in discovery of GPCR drugs. In contrast to radioligand binding experiments, which are based on the displacement of a known (orthosteric) labeled ligand, the use of functional assays in high throughput screening (HTS) facilitates the identification of compounds with different mechanisms of activation, *e.g.* allosteric modulators (Rees *et al.* 2002; Christopoulos *et al.* 2004). One functional assay, that is frequently used to measure GPCR function, is agonist-promoted GTP$(\gamma)^{35}$S binding in cell membrane preparations. This experimental system measures the first step in the signaling cascade of a GPCR (Hilf *et al.* 1989; Harrison and Traynor 2003). As a result of a GTP$(\gamma)^{35}$S screen in membranes of a recombinant Chinese hamster ovary (CHO) cell line stably expressing the GABA$_B$ heterodimer the two positive allosteric modulators CGP7930 and GS39783 came out (Urwyler *et al.* 2001 and 2003). These two compounds are structurally different, but they exerted similar actions with similar potencies (in the low micromolar range) on both native and recombinant receptors. Without effects on their own in the absence of agonists, CGP7930 and GS39783 increased not only the potencies of the GABA$_B$ agonists GABA and R(-)-baclofen in the GTP$(\gamma)^{35}$S assay system, but also their maximal efficacies (Urwyler *et al.* 2001 and 2003). Together with allosteric enhancers of the mGluR1 receptor (Knoflach *et al.* 2001), CGP7930 was the first example of a compound with a dual mechanism of action (an effect on both agonist potency and the maximal effect, as predicted by the allosteric two-state model of receptor activation, see Subsection 4.1.2.). For example, benzodiazepines, possibly the best-described class of

positive allosteric modulators, act only by increasing the potency of $GABA_A$ agonists, without influencing their maximal effects. The dual mode of action of CGP7930, an enhancement of both the potencies and the maximal effects of the agonists GABA, APPA and the active R(-) enantiomer of baclofen in the $GTP(\gamma)^{35}S$ assay in membranes from human cortex, was confirmed by others (Olianas *et al.* 2005).

An increase of agonist affinity by CGP7930 and GS39783 also became apparent in radioligand binding experiments. A saturation experiment with the selective $GABA_B$ agonist $[^3H]$-APPA in the presence of CGP7930 revealed an increase in affinity, without a change in the B_{max} value (Urwyler *et al.* 2001). Radioligand kinetic experiments in rat brain cortex membranes, examining the rates of association and dissociation of $[^3H]$-APPA, in the presence of GS39783 have yielded surprising results. The rate of association of the radioligand in the presence of GS39783 was lower than in its absence. However, this effect was overcompensated by an even greater effect on slowing down the dissociation, resulting in a net increase of affinity (Urwyler *et al.* 2003). An increase in agonist affinity was also observed in displacement experiments. The curves describing the displacement of the radiolabeled $GABA_B$ antagonist CGP62349 from rat brain membranes by GABA were fitted better when a two-site model was used, rather than a one-site model (Figure 9). The two states
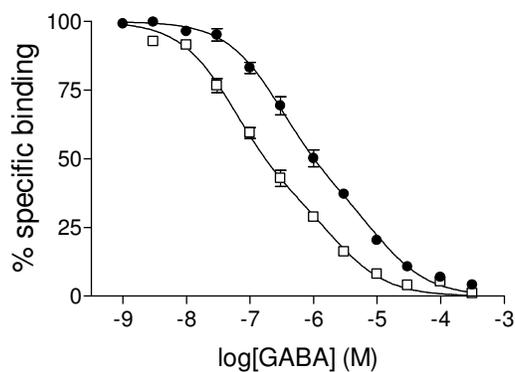

Figure 9. Effects of CGP7930 on the displacement of the $GABA_B$ antagonist $[^3H]$-CGP62349 by GABA from native $GABA_B$ receptors in rat cortical membranes. Filled circles: control curve with GABA alone, open squares: concentration-response curve of GABA in the presence of 30 μM CGP7930. Adapted from (Urwyler, Gjoni *et al.* 2004), with permission from Elsevier.

correspond to the receptor being either coupled or uncoupled from its G-protein which results in different affinities for the two states (Hill *et al.* 1984; Parmentier *et al.* 2002). The positive allosteric modulators CGP7930 and GS39783 increased the affinities for both receptor states (Urwyler *et al.* 2001 and 2003). Moreover, the proportion of receptors in the high affinity state was increased in their presence, suggesting that CGP7930 and GS39783 also promoted the coupling of the receptor to G-proteins.

Concerning other *in vitro* experimental setups, increases of GABA effects by CGP7930 and GS39783 were also seen measuring the activation of $K_{ir}3$ channels co-transfected in *Xenopus laevis* oocytes together with the $GABA_B$ receptor. No potassium currents were elicited in the presence of either modulator in the absence of GABA (Urwyler *et al.* 2001 and 2003). Further, the modulators were found to increase the potency and the maximal effects of agonists to inhibit 7β-forskolin-induced cAMP production, only with marginal effects on their own (Onali *et al.* 2003; Olianas *et al.* 2005; Urwyler, Gjoni *et al.* 2005). CGP7930 also increased the potencies and the maximal efficacies of R(-)-baclofen or GABA to stimulate either basal or CRH-stimulated cAMP production in the membranes of rat frontal cortex and the granule cell layer of rat olfactory bulb (Onali *et al.* 2003). This effect is likely mediated *via* the stimulatory action of βγ subunits of the G_i/G_o proteins on adenylyl cyclase type II and IV (see Tang and Gilman 1992 for a minireview). Moreover, in a more physiological setting (Chen *et al.* 2005) have observed effects of CGP7930 on baclofen-induced depression of the spontaneous activity in dopamine (DA) cells of the ventral tegmental area (VTA) in rat brain slices, which probably occurs *via* the activation of $K_{ir}3$ channels. CGP7930 increased the potency of baclofen to inhibit the spontaneous spiking. Since baclofen fully blocked the firing of DA neurons in the VTA at its highest concentrations, no further enhancement of its maximal effect was observed in the presence of CGP7930. This kind of a ceiling effect was also present when $GABA_B$-mediated inhibition of calcium fluctuations was measured in neuronal cortical cultures (Urwyler *et al.* 2001).

To summarize, the *in vitro* studies mentioned above have found positive allosteric modulators of the $GABA_B$ receptor CGP7930 and GS39783 to increase both the affinities and the efficacies of agonists, without having a significant effect on their own in their absence. On the other hand, there was one study by (Binet *et al.* 2004), in which the binding site of CGP7930 was identified to lay in 7TM of $GABA_{B(2)}$, that showed a direct activation of the receptor by CGP7930 in a manner of a partial agonist. It must be noted that a very sensitive system was used in this study, seemingly with a high degree of receptor reserve, which is most likely the reason why the low efficacy partial agonism of CGP7930 alone was detected. Another mapping study by (Dupuis *et al.* 2006) discovered that the binding site for GS39783 was also located in the 7TM of $GABA_{B(2)}$.

So far there has only been one study, that is a part of this thesis (Chapter 5, Section 5.4.), in which the principles of allosteric modulation of the $GABA_B$ receptor were shown on a biochemical level *in vivo* (Gjoni *et al.* 2006). *In vivo* microdialysis in the rat striatum was employed to measure cAMP, the second messenger of $GABA_B$ receptor activation, the results of which strongly suggested an allosteric mechanism of action of GS39783 *in vivo* (see Chapter 5, Section 5.4. or Gjoni *et al.* 2006).

On the other hand, behavioral effects of CGP7930 and GS39783 have been extensively studied in animal models. CGP7930 synergistically increased the sedative/hypnotic effects of baclofen and γ-hydroxybutiric acid (GHB) in mice, without having an effect on its own (Carai *et al.* 2004), which again supports the notion of an allosteric enhancement. GS39738 was found to have anxiolytic properties when tested in the classical behavioral paradigms, for example in the elevated zero maze or the light-dark box (Cryan *et al.* 2004; Mombereau *et al.* 2004). However, it had no antidepressant action in the forced swim test (Cryan *et al.* 2004; Slattery *et al.* 2005a). Importantly, GS39783 lacked all baclofen- and benzodiazepine-related

side-effects, namely sedation, hypothermia, muscle relaxation, cognitive impairment, impairment of motor abilities and the potentiation of ethanol effects (Cryan *et al.* 2004). Because the implications of GABA$_B$ receptors in drug addiction have been extensively studied (see Subsection 4.2.4.), effects of the positive allosteric modulators CGP7930 and GS39783 have also been assessed in animal models of drug abuse. Smith *et al.* (2004) demonstrated that CGP7930 and GS39783 decreased cocaine-self administration in rats, with no evidence of inducing sedation or motor ataxia. In addition, Slattery *et al.* (2005b) showed that GS39783 attenuated the reward-facilitating effects of cocaine by using intracranial self-stimulation procedure in rats. GS39783 attenuated chronic cocaine-induced locomotor sensitization in mice, without affecting the basal locomotor activity. It also blunted ΔFosB upregulation in dorsal striatum and blocked the upregulation and activation of dopamine- and cAMP-regulated phosphoprotein of 32kDa (DARPP-32) and cAMP-response-element-binding protein (CREB), all associated to chronic cocaine (Lhuillier *et al.* 2006). Similarly, GS39783 reduced the nicotine-induced accumulation of ΔFosB in rat dorsal striatum (Mombereau *et al.* 2007). Decreases of ethanol-intake mediated CGP7930 and GS39783 in rats have also been reported (Orru *et al.* 2005; Liang *et al.* 2006).

In conclusion, although it is difficult to definitely confirm allosteric mechanisms *in vivo*, the findings outlined above indicate that CGP7930 and GS39783 act *via* similar mechanisms as they do *in vitro*. The positive allosteric modulators of the GABA$_B$ receptor mimic/potentiate the effects of baclofen in behavioral animal models, but lack the severe side-effects of baclofen, which makes them valuable tools in GABA$_B$ receptor research.

Other members of the family 3 GPCR, namely the CaSR and certain mGluRs, have been shown to be either directly activated or modulated by extracellular calcium, respectively (Kubo *et al.* 1998; Saunders *et al.* 1998). GABA$_B$ receptor function was also demonstrated to be susceptible to Ca^{2+} ions in two independent studies (Wise *et al.* 1999; Galvez *et al.* 2000b).

Sensitivity to a regulation by Ca^{2+}-ions was observed in both native and recombinant $GABA_B$ receptors. In the $GTP(\gamma)^{35}S$ assay system, Ca^{2+} increased the potency, but not the maximal effect of GABA. Similar observations originated from other experimental systems, such as potassium channel regulation in *Xenopus* oocytes, measurement of IP turnover (*via* artificial coupling to the PLC pathway) or inhibition of forskolin-amplified adenylyl cyclase activity (cAMP). In all the experimental systems, apart from cAMP, baclofen was insensitive to the potentiation mediated by Ca^{2+} ions. Point mutation experiments have identified the Ser269 residue on the $GABA_{B(1)}$, which lies in the close vicinity of the orthosteric ligand binding site (Galvez *et al.* 1999; Bernard *et al.* 2001), as being responsible for the effects of Ca^{2+} ions on $GABA_B$ receptor function (Galvez *et al.* 2000b). If a true allosteric mechanism underlies the effects of calcium ions on $GABA_B$ receptor function, the presence of Ca^{2+} should change the conformation of the receptor protein, possibly to optimize the binding of GABA in the binding pocket (see Galvez *et al.* 2000b for further discussion). Alternatively, it has been proposed that Ca^{2+} acts as a chelator of the carboxylic group of GABA and residues of Ser269 and Tyr366 (Costantino *et al.* 2001). Whereas this alternative hypothesis provides an explanation for the insensitivity of baclofen to modulation by Ca^{2+}, it is difficult to distinguish the two mechanisms experimentally. The facts that the EC_{50} of calcium, that is responsible for its enhancing actions, lies in the low micromolar range (37 µM) (Galvez *et al.* 2000b) and the concentrations of calcium in the cerebrospinal fluid being in the milimolar range, make it unclear whether calcium-mediated modulation of $GABA_B$ receptor function is of any physiological relevance, as in normal conditions the calcium site in the $GABA_B$ binding pocket is most likely saturated. However, under pathophysiological conditions such as epileptic seizures or ischemia, synaptic calcium concentrations might drop significantly (Pumain *et al.* 1983; Heinemann *et al.* 1986; Lazarewicz 1996).

Arylalkylamine-like compounds and certain L-amino acids and dipeptides, which are known to allosterically modulate the CaSR (Hammerland *et al.* 1998; Nemeth *et al.* 1998; Conigrave

et al. 2000) have also been claimed to be positive allosteric enhancers of GABA$_B$ receptors (Kerr *et al.* 2002; Kerr and Ong 2003). Electrophysiological recordings in brain slices were used to demonstrate their action. The abovementioned compounds increased the potency and the maximal effect of baclofen to induce field potentials, without inducing field potentials by themselves when applied alone. However, in such an electrophysiological setup the enhancing effects of these compounds could well have been due to other mechanisms, not necessarily of allosteric nature, for example receptor-receptor cross-talk or downstream effects. Since we did not observe any effects of these compounds on the affinity or efficacy of GABA in various assay systems, more suitable for detecting true allosteric interactions (Urwyler, Gjoni *et al.* 2004), it is unlikely that the mechanism of action of these compounds is of allosteric nature. The mechanism of action of these compounds, thus, remains unclear and more investigations are needed to answer that question.

4.3. The questions addressed in this thesis

During this PhD project, I have focused mostly on the positive allosteric modulators of GABA$_B$ receptors, evaluating their effects biochemically both *in vitro* and *in vivo*. Despite the comprehensive previous *in vitro* characterization of the positive allosteric modulators of GABA$_B$ receptors CGP7930 and GS39783, the effects these compounds exert upon ligands with different intrinsic properties (competitive antagonists and partial agonists) were not known. According to the theoretical models of receptor activation (see Subsection 4.1.2.), all chemical species can be influenced by allosteric modulators. To address the question of the effects that positive allosteric modulators CGP7930 and GS39783 have on ligands other than agonists, I have examined both affinities and the efficacies of a number of GABA$_B$ receptor ligands and confirmed the theoretical predictions that antagonists and partial

agonists are also amenable to positive allosteric modulation. Moreover, CGP7930 and GS39783 have also been demonstrated to be excellent tools for elucidating the intrinsic properties of orthosteric ligands. See Chapter 5, Section 5.1.

Secondly, as discussed in Section 4.1., allosteric modulators act only upon receptors stimulated by endogenous agonists and not upon the whole receptor population. This means that they affect the strength of the physiological signaling, while preserving the pattern of activation (*e.g.* patterns of neuronal signaling), unlike exogenous orthosteric agonists, which activate all their receptors, indiscriminately of the temporal and the spatial organization (see Figure 2). For this reason, one of the postulated advantages of allosteric over orthosteric ligands is that continuous activation involving positive allosteric modulation is less likely to induce receptor desensitization, compared to a lasting exposure to an orthosteric ligand. This assumption was tested in Chapter 5, Section 5.2. The approach used in this thesis was to continuously activate (both native and recombinant) $GABA_B$ receptors *in vitro* to the same extent, using either an orthosteric agonist or a combination of an orthosteric agonist and GS39783, and subsequently assess their functional responses and expression levels at the cell surface.

Further, while studying $GABA_B$ receptor desensitization, surprising changes in behavior of ligands were observed in the recombinant cell line stably expressing the $GABA_B$ receptor upon receptor desensitization. A number of selective $GABA_B$ antagonists were found to have inverse agonistic efficacy, the partial agonist 2-OH-saclofen lost its efficacy and the positive modulator GS39783 now had an agonistic effect on its own. Nothing similar has been observed for the $GABA_B$ receptor so far, but there are a few reports on the μ-, δ- and κ-opioid and β_2-adrenergic receptors describing similar phenomena. See Chapter 5, Section 5.3.

Finally, effects of positive allosteric $GABA_B$ receptor modulators have been extensively studied in behavioral models (see Subsection 4.2.6.). While they were effective in, for example, many anxiety paradigms, the positive allosteric modulators were devoid of side-

effects that are usually displayed by baclofen, such as sedation, muscle relaxation or locomotor impairment. Although, this is compatible with an allosteric enhancement, there were no studies demonstrating the allosteric mechanism of action *in vivo*. The final part of this thesis describes *in vivo* microdialysis experiments in rat striatum, measuring the second messenger cAMP as an assessment of $GABA_B$ receptor activation. It demonstrates that the effects of the positive allosteric modulator GS39783 on the $GABA_B$ receptor *in vivo* is similar to the one observed *in vitro*, strongly suggesting an allosteric mechanism of action in living animals (Chapter 5, Section 5.4.).

5. Results and discussions

5.1. Mechanisms of allosteric modulation at GABA$_B$ receptors by CGP7930 and GS39783: effects on affinities and efficacies of orthosteric ligands with distinct intrinsic properties

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5.1.1. Abstract

We determined the effects of the allosteric γ-aminobutyric acid B receptor modulators CGP7930 and GS39783 on binding and function of orthosteric ligands with distinct intrinsic properties. In radioligand binding (saturation or displacement) experiments, the affinities of a number of competitive antagonists were decreased by the modulators, with no change in receptor number. The binding curves of the partial agonist CGP47656 comprised a high and a low affinity component; the affinity of the former was increased by the allosteric agents. The maximal stimulation of $GTP(\gamma)^{35}S$ binding *via* recombinant $GABA_B$ receptors by CGP47656 was increased 4-fold in the presence of 30 μM CGP7930 or GS39783. Two compounds known so far as "silent" competitive $GABA_B$ receptor antagonists, CGP35348 and 2-OH-saclofen, did not stimulate $GTP(\gamma)^{35}S$ binding on their own, but became low efficacy partial agonists in the presence of the two modulators. The potency of GABA to inhibit the formation of cAMP induced by a forskolin analog in a recombinant CHO cell line expressing $GABA_B$ receptors was increased by the modulators. CGP35348 and 2-OH-saclofen, like CGP47656, were partial agonists on their own in this assay, and the allosteric modulators increased the potency as well as the efficacy of all three compounds. With CGP52432, there was a trend toward inverse agonism in the cAMP assay. These results show that the intrinsic properties of orthosteric ligands are highly dependent on the characteristics of the assay system used and that allosteric modulators are useful tools for elucidating these properties.

Abbreviations: cAMP, cyclic 3',5'-adenosine-monophosphate; CHO, Chinese hamster ovary; 7β-forskolin, 7-deacetyl-7-(*O-N*-methylpiperazino)-γ-butyryl-forskolin, dihydrochloride; GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor; GTP(γ)S, guanosine 5'-*O*-(3-thiotriphosphate); IBMX, isobutyl-methylxanthine; mGluR, metabotropic glutamate receptor; RIA, radioimmunoassay; SPA, scintillation proximity assay; TCM, ternary complex model; VFTM, Venus flytrap module; WGA, wheat germ agglutinin

5.1.2. Introduction

Allosteric modulators are molecules that bind to a site on a neurotransmitter or hormone receptor which is topographically distinct from the orthosteric binding pocket for agonists or competitive antagonists. Allosteric agents usually have little or no intrinsic agonistic activity of their own, but induce conformational changes in the receptor protein which affect its interaction with orthosteric ligands. The therapeutically widely used benzodiazepines are well-known examples of such drugs. They enhance the sensitivity of the ionotropic inhibitory GABA$_A$ receptor without stimulating it directly themselves and without affecting the magnitude of the maximal response. A number of allosteric enhancers are also known for G protein - coupled receptors (GPCRs) (for reviews see Christopoulos 2002; Christopoulos and Kenakin 2002; Jensen and Spalding 2004). Interestingly, several examples of allosteric drugs which not only enhance the potency, but also the intrinsic efficacy of agonists at GPCRs have been recently described for GABA$_B$ (Urwyler *et al.* 2001 and 2003), metabotropic glutamate mGluR1 (Knoflach *et al.* 2001), mGluR2 (Schaffhauser *et al.* 2003) and mGluR4 (Maj *et al.* 2003; Mathiesen *et al.* 2003), as well as adenosine A$_3$ receptors (Gao *et al.* 2002).

Several theoretical receptor models describe various aspects of ligand binding and receptor activation. The two state - model (reviewed by Leff 1995; Fig. 3 in Chapter 4) asserts that a

receptor exists in two interconvertible conformations, a resting (R) and an activated (R*) state. The spontaneous formation of R* without agonist binding is the basis for constitutive receptor activity. The binding of an agonist stabilizes the activated form R* ($\alpha > 1$), whereas an inverse agonist preferentially binds to and shifts the equilibrium to the resting state ($\alpha < 1$). Competitive antagonists block the access of agonists to their binding site, but are themselves neutral in terms of receptor activation ($\alpha = 1$). This model thus accounts for intrinsic efficacies (α) of orthosteric ligands, but does not allow understanding the effects of allosteric drugs. Changes in the binding affinity of orthosteric ligands induced by allosteric agents have traditionally been described by a ternary complex model (TCM) (Fig. 4 in Chapter 4), which was originally designed for the regulation of agonist binding by receptor - G-protein coupling (De Lean *et al.* 1980), but also encompasses mechanisms of reciprocal regulation of the binding of two low molecular weight compounds to distinct sites on the same receptor protein. However, this model does not take into account the degree of agonist-induced receptor activation. Although the "allosteric ternary complex model" (Lefkowitz *et al.* 1993) was a first attempt to combine the two previous models, the newly observed phenomenon that allosteric modulators in some cases enhance both agonist affinity and efficacy (see above), is accommodated only by more recently developed theoretical concepts (Hall 2000; Christopoulos and Kenakin 2002). An extension of the two state model of receptor activation (Hall 2000; Fig. 5 in Chapter 4), introduces the allosteric constant δ, by which the intrinsic efficacy α of a ligand is modified. This model treats the allosteric interactions in purely numerical terms, *i.e.* the constants γ and δ are *a priori* independent of the qualitative (agonistic or antagonistic) nature (*i.e.*, the values of α) of the orthosteric ligand. Therefore, antagonists or inverse agonists should be amenable as much as agonists to allosteric modulation. Recent concepts state that compounds devoid of any intrinsic activity (α exactly

equaling one) are likely to be rare, and that most compounds considered being "silent"

competitive antagonists are in fact low efficacy partial agonists or inverse agonists
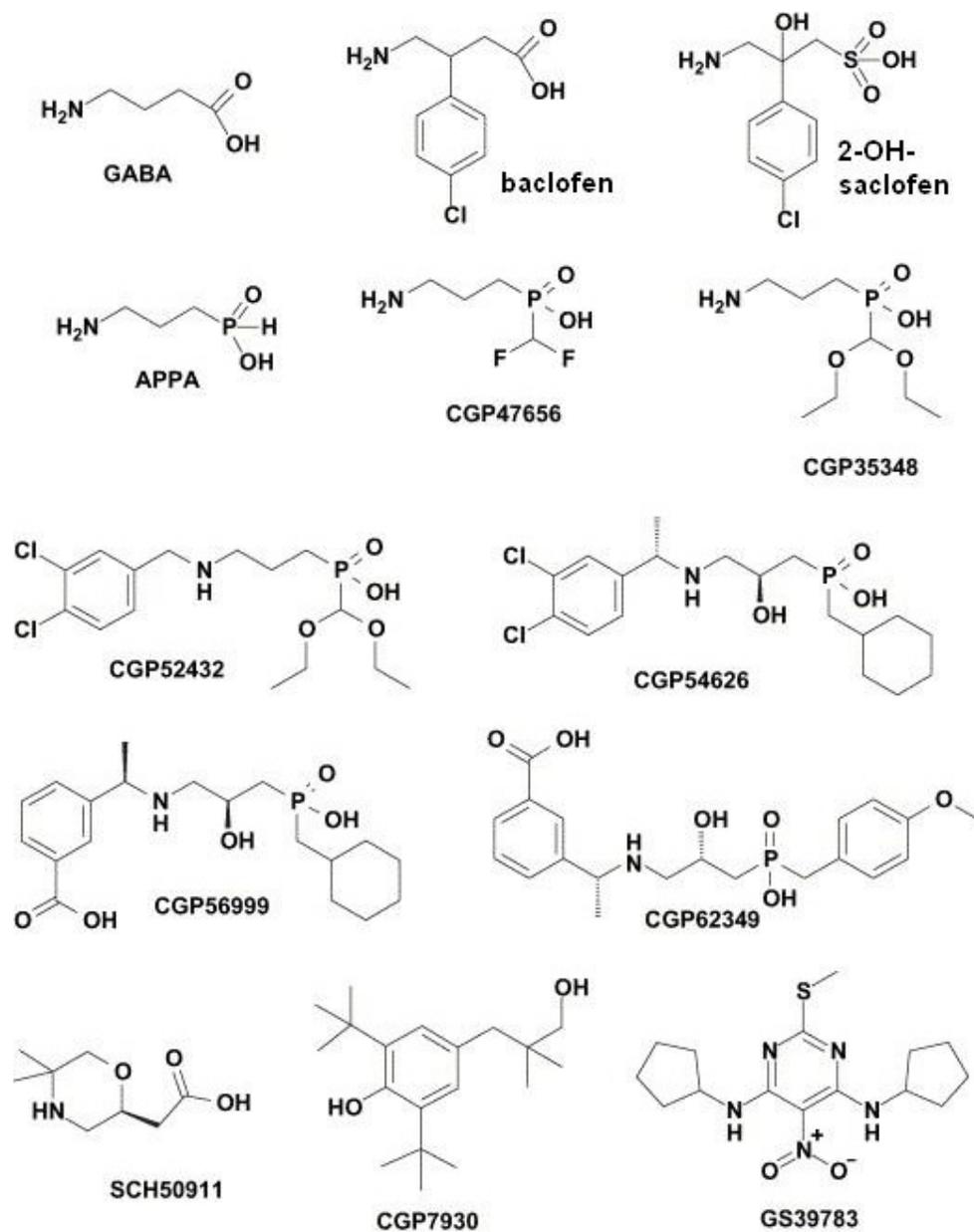
**Fig. 1.** Chemical structures of the compounds tested and referred to in this study. Agonists: GABA, baclofen, APPA. Partial agonist: CGP47656. Allosteric modulators: CGP7930, GS39783. The other compounds shown are known from the literature (Bowery *et al.* 2002) as competitive antagonists, but some of them were found in this study to be partial (CGP35348, 2-OH-saclofen) or inverse (CGP52432) agonists.

(Strange 2002; Milligan 2003; Kenakin 2004). The detection of these properties is highly dependent on the assay system used. In this study, we have tested and confirmed these various predictions by examining the effects of the two $GABA_B$ receptor modulators CGP7930 and GS39783 (Urwyler *et al.* 2001 and 2003) on a number of well known orthosteric ligands (Bowery *et al.* 2002) with distinct intrinsic efficacies (Fig. 1). In particular, we show that allosteric modulators decrease the affinities of competitive antagonists and strongly increase the efficacies of partial agonists, thus revealing intrinsic activities of compounds previously believed to be "silent" antagonists.

5.1.3. Materials and methods

Radioligand binding experiments: The binding of [^{3}H]-CGP62349 (85Ci/mMol, American Radiolabeled Chemicals Inc., St. Louis, MO) to rat cortical membranes was measured in the scintillation proximity assay (SPA) format as described previously (Urwyler *et al.* 2003). The assay mixture in a final volume of 250 µl contained 20 mM Tris-HCl buffer (pH 7.4), 118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM D-glucose, 1 nM [^{3}H]-CGP62349 (or differing concentrations in saturation experiments), the test compounds at the desired concentrations, rat cortical membranes (*ca.* 15 µg protein), and 1.5 mg wheat germ agglutinin (WGA) - coated SPA beads (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Non-specific binding was assessed in the presence of 5 µM CGP56999. The samples were incubated for 90 min at room temperature, before being counted in a Wallac 1450 Microbeta liquid scintillation counter. Saturation and displacement curves were analyzed with nonlinear regression using Prism 3.0 (GraphPad software, San Diego, CA).

GTP(γ)^{35}S assay: These experiments using membranes from Chinese hamster ovary (CHO) cells stably expressing GABA$_B$ receptors were also performed as described previously (Urwyler *et al.* 2001). The composition of the assay mixtures (in a final volume of 250µl in 96-well clear-bottom microtiter plates [WALLAC Isoplates^{TM}]) was as follows: 50 mM Tris-HCl buffer, pH 7.7; 10 mM MgCl$_2$; 0.2 mM EGTA; 2 mM CaCl$_2$; 100 mM NaCl; 10 µM guanosine 5'-diphosphate (Sigma Chemical, Buchs, Switzerland), 50 µl of the membrane suspension (approximately 10-20 µg of protein), 1.5 mg WGA-coated SPA beads (Amersham Biosciences), 0.3 nM GTP(γ)^{35}S (ca 1000 Ci/mmol, stabilized solution, Amersham Biosciences), and the test compounds at the appropriate concentrations. Non-specific binding was measured in the presence of unlabelled GTP(γ)S (10 µM, Sigma). The samples were incubated at room temperature for 60 min, before the SPA beads were sedimented by centrifugation at 2600 rpm for 10 min. The plates were then counted in a Wallac 1450 Microbeta liquid scintillation counter. For data analysis, basal activity, measured in the absence of agonist, was subtracted from all the other values. The effects of test compounds were calculated relative to the stimulation above baseline obtained with a maximally active concentration (100µM) of GABA. Concentration - response curves were analyzed by non-linear regression using GraphPad Prism 3.0.

Adenylyl cyclase assays: A stable CHO cell line co-expressing human GABA$_{B(1b)}$ together with rat GABA$_{B(2)}$ (Urwyler *et al.* 2001) was used for these experiments. The cells were cultured in Dulbecco's modified eagle medium (DMEM, glutamine-free, Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 20µg/ml L-proline, 500µM glutamine, 1mg/ml geneticin, 250µg/ml zeocin. The cells were grown to 80-90 % confluency in 15 cm cell culture dishes. For the cyclic 3',5'-adenosine-monophosphate (cAMP) measurements, the cells were treated with DMEM supplemented with 1 mM 3-isobutyl-1-methylxanthine

(IBMX) (Fluka, Buchs, Switzerland) for 15 minutes at 37°C and 5% CO_2. The cells were

harvested by trypsinization, resuspended in Hepes buffer (Invitrogen) supplemented with 1

mM IBMX and 2 mM $CaCl_2$ and splitted into 96-well clear-bottom microtiter plates

[WALLAC Isoplates^{TM}]) at a density of 15'000 – 30'000 cells per well. The cell suspension

was then incubated for 20 minutes at 37°C in a total volume of 45µl with Hepes buffer

containing IBMX (1 mM), $CaCl_2$ (2 mM), the water-soluble forskolin analog 7-deacetyl-7-(O-

N-methylpiperazino)-γ-butyryl-forskolin dihydrochloride (7β-forskolin, Calbiochem, Juro

Supply, Lucerne, Switzerland) (100µM), and the test compounds at the concentrations given

in the Subsection 5.1.4. The amount of cAMP formed was quantified by radioimmunoassay

(RIA) following the cAMP SPA Biotrak direct screening assay system protocol from

Amersham Biosciences: At the end of the incubation period, 5µl of lysis reagent (containing

dodecytrimethylammonium bromide [10%]) were added to each well. After 5 minutes of

shaking, 150µl of immunoreagent solution (containing rabbit anti-succinyl cAMP serum,

donkey anti-rabbit IgG second antibodies coated at the surface of SPA beads, and adenosine

3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I]iodotyrosine methyl ester as a tracer) were

added to each sample. The plates were incubated at room temperature for 15-20 hours and

then counted on a WALLAC 1450 microbeta Trilux scintillation counter. The quantification

of the cAMP formed was performed with the aid of an appropriate RIA cAMP standard curve.

Protein concentrations were measured with the Bradford assay method, using the Bio-Rad

protein assay kit and bovine serum albumin as a standard.

Chemicals: The sources of commercially obtained chemicals are given above. The GABA$_B$

receptor modulators GS39783 and CGP7930, as well as the orthosteric ligands tested in this

study (Fig. 1) were all available in house. Stock solutions were usually prepared in DMSO

and subsequently diluted in the respective assay buffers. The final DMSO concentrations usually did not exceed 0.3% and did not interfere with the measured parameters.

5.1.4. Results

Saturation binding experiments with [³H]-CGP62349. The effects of the allosteric modulators CGP7930 and GS39783 in saturation binding experiments with [³H]-CGP62349 are shown in Fig. 2. Both compounds slightly, but significantly and consistently, decreased the affinity of this antagonist radioligand for native $GABA_B$ receptors in rat cortical membranes. In fact, the K_d was significantly increased from 0.54 ± 0.04 nM (without modulator, mean $\pm$ SEM, N = 4) to 0.83 ± 0.04 nM ($p < 0.05$) or to 0.92 ± 0.11 nM ($p < 0.01$) in the presence of 30 μM CGP7930 or 30 μM GS39783, respectively. These K_d values were used accordingly for the conversion of IC_{50} values obtained in subsequent displacement
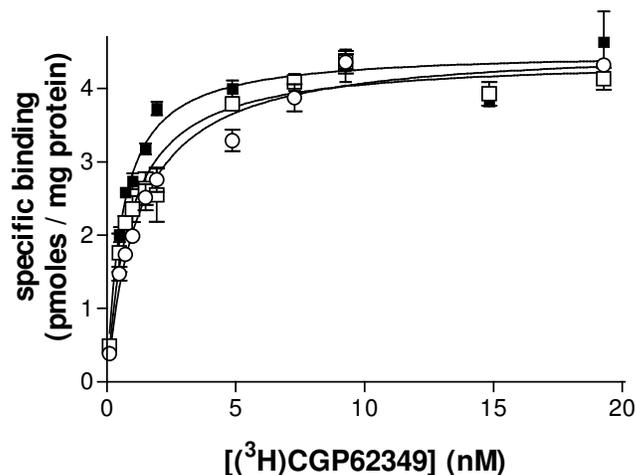
Fig. 2. Effects of allosteric modulators on the binding of the antagonist radioligand [³H]-CGP62349 to native $GABA_B$ receptors in rat cortical membranes. Saturation curves were measured in the absence of modulators (filled squares) or in the presence of CGP7930 (30 μM, squares) or GS39783 (30 μM, circles). The mean K_d and B_{max}-values from four such experiments are given in the text.

experiments into their corresponding K_i-values. On the other hand, B_{max}-values for [³H]-

CGP62349 binding remained unchanged upon the addition of the two modulators (in

pmol/mg protein: control 4.5 ± 0.4; with CGP7930: 4.4 ± 0.3; with GS39783: 4.6 ± 0.4).

Effects of allosteric modulators on the displacement of [³H]-CGP62349 by orthosteric

GABA_B receptor ligands. Some representative curves illustrating the displacement of the

antagonist radioligand [³H]-CGP62349 from native GABA_B receptors in rat cortical

membranes are shown in Fig. 3.
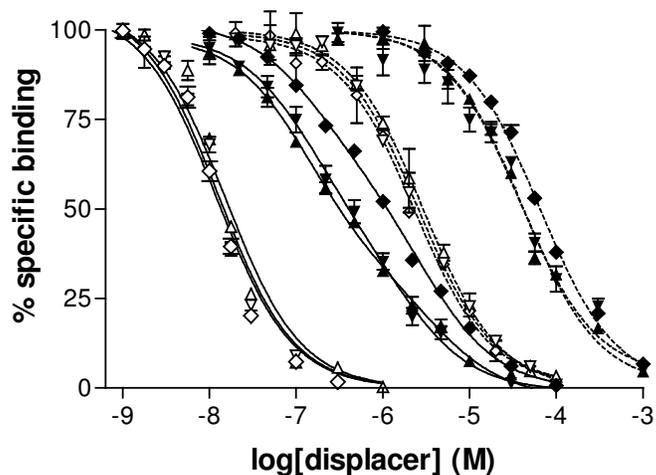
Fig. 3. Displacement of [³H]-CGP62349 from native GABA_B receptors in rat cortical membranes
by, from left to right, CGP54626 (open symbols, solid lines), CGP47656 (filled symbols, solid lines),
SCH50911 (open symbols, dotted lines) and 2-OH-saclofen (filled symbols, dotted lines). Diamonds:
control curves; triangles: curves measured in the presence of 30 μM GS39783; inverted triangles:
curves with 30 μM CGP7930. The graph shows typical curves from a single experiment; a summary of
the data from a number of such experiments is given in Table 1.

The curve fit parameters for all the compounds tested are given in Table 1. Best curve fits were obtained with a one-site model in all cases, with the exception of CGP47656. The displacement curves with this partial agonist were shallow (Fig. 3), consisting of a high affinity and a low affinity component (Table 1). The affinities of the high affinity, but not of the low affinity, sites were significantly increased by the allosteric modulators CGP7930 and GS39783 (Table 1). The relative proportion of high affinity sites was also increased, although this change did not quite reach statistical significance (ANOVA: $p = 0.07$). The inhibition

Displacing ligand	pK_i control (-log M)	K_i	pK_i with 30µM CGP7930 (-log M)	K_i	pK_i with 30µM GS39783 (-log M)	K_i
CGP35348	5.11 ± 0.05 (6)	7.7 µM	5.18 ± 0.08 (6)	6.6 µM	5.20 ± 0.07 (6)	6.3 µM
CGP47656	7.15 ± 0.05	71 nM	7.39 ± 0.10 *	41 nM	7.45 ± 0.10 *	35n M
	(44 ± 3.4 %)		(54 ± 2.9 %)		(53 ± 2.6 %)	
	5.97 ± 0.12 (6)	1.07 µM	6.01 ± 0.18 (6)	0.98 µM	6.06 ± 0.10 (6)	0.87 µM
CGP52432	7.25 ± 0.10 (4)	57 nM	7.09 ± 0.12 (4) *	81 nM	7.04 ± 0.08 (4) **	91 nM
CGP54626	8.44 ± 0.05 (4)	3.6 nM	8.39 ± 0.09 (4)	4.1 nM	8.23 ± 0.04 (4) *	5.9 nM
CGP56999	9.60 ± 0.06 (4)	0.25 nM	9.37 ± 0.04 (3) *	0.43 nM	9.39 ± 0.03 (3) *	0.41 nM
CGP62349	9.26 ± 0.05 (8)	0.55 nM	9.07 ± 0.05 (8) **	0.85 nM	8.99 ± 0.06 (8) **	1.02 nM
2-OH-saclofen	4.62 ± 0.05 (8)	24 µM	4.66 ± 0.05 (8)	21.9 µM	4.70 ± 0.07 (8)	20 µM
SCH50911	6.30 ± 0.08 (4)	0.5 µM	6.17 ± 0.09 (4) **	0.68 µM	6.11 ± 0.11 (4) **	0.78 µM

Table 1. Effects of CGP7930 and GS39783 on the affinities of orthosteric ligands for native GABA$_B$ receptors from rat cortical membranes in radioligand binding assays. The binding of [^{3}H]-CGP62349 to membranes from rat brain cortex was measured as described in the Subsection 5.1.3. Inhibition curves with the ligands shown as displacers were constructed as illustrated in Fig. 3. Best curve fits were obtained with a one-site model in all cases, with the exception of CGP47656 (the relative proportion of high affinity sites is given in percent). The results shown are means ± SEM from (N) independent experiments. *: $p<0.05$, **: $p<0.01$ compared to the corresponding control values (ANOVA / Dunnett's test).

curves of the other compounds, previously described as competitive GABA$_B$ receptor antagonists, seemed to be little affected or, in some cases, slightly shifted toward lower concentrations by the two allosteric modulators (Fig. 3). However, a precise calculation of the corresponding K_i-values, taking into account the modulator effects on the affinity of the radioligand (Cheng and Prusoff 1973), revealed a slight, but significant decrease in binding affinities for most orthosteric ligands (Table 1). The only noteworthy exceptions were CGP35348 and 2-OH-saclofen. The affinities of these two compounds were slightly, but not significantly, increased in the presence of the allosteric modulators.

Effects on allosteric modulators on orthosteric ligands in GTP(γ)^{35}S assays (experiments performed by scientific associates D. Monna and M. Horvath). The compound CGP47656 stimulated GTP(γ)^{35}S binding to recombinant GABA$_B$ receptors to maximally 25% of the maximal effect of GABA, and thus was found to be a partial agonist (Fig. 4, Table 2). In the presence of the allosteric modulators CGP7930 or GS39783 (30 μM each), the maximal stimulation obtained by CGP47656 was substantially increased by about 4-fold, to about the same maximal effect as the one obtained with a saturating concentration of GABA alone (Fig. 4). The potency of CGP47656 in this assay was increased by 2- to 3-fold by the two modulators (Table 2).

Some compounds previously described as competitive GABA$_B$ receptor antagonists (CGP35348, CGP52432, CGP54626, CGP56999, CGP62349, 2-OH-saclofen and SCH50911) were also tested in the GTP(γ)^{35}S assay. As expected, when applied alone at different concentrations in the range of their antagonist potencies (or binding affinities), none of them stimulated GTP(γ)^{35}S binding, confirming that these compounds are devoid of intrinsic agonistic activity in this assay (see Fig. 4 for two representative examples). However, in the presence of 30 μM GS39783 or CGP7930, two of the antagonists, CGP35348 and
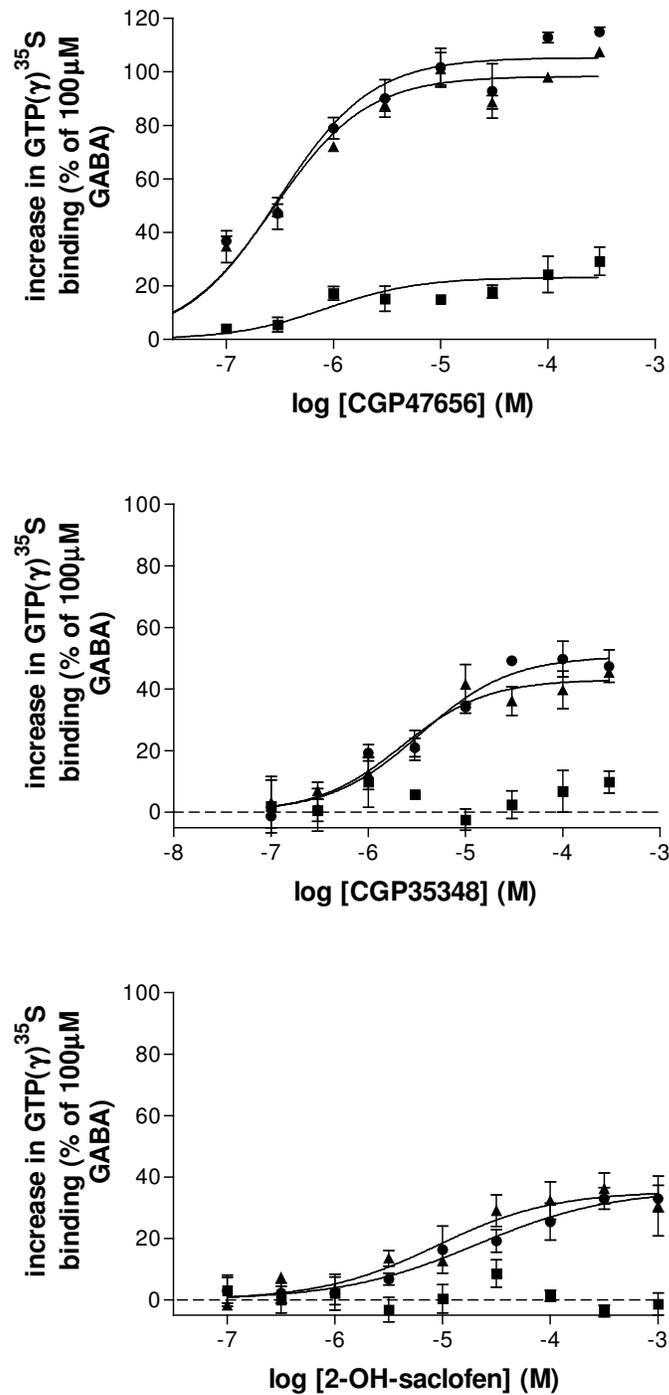
Fig. 4. GTP(γ)³⁵S binding to membranes from a recombinant CHO cell line stably expressing GABA_B receptors. The stimulation of GTP(γ)³⁵S binding by CGP47656 (top), CGP35348 (middle) and 2-OH-saclofen (bottom) was measured in the absence of modulators (squares) or in the presence of 30 μM CGP7930 (triangles) or 30 μM GS39783 (circles). The data represent the stimulation above basal activity relative to the effect of a maximally active concentration of GABA (100 μM) alone. The data points are means ± SEM from quadruplicate determinations.

compound	Control			+ 30µM CGP7930			+30µM GS39783		
	pEC_{50} (-log M)	EC_{50} (µM)	Max. Effect (%)	pEC_{50} (-log M)	EC_{50} (µM)	Max. Effect (%)	pEC_{50} (-log M)	EC_{50} (µM)	Max. Effect (%)
CGP47656	5.98 ± 0.08 (4)	1.11	25 ± 2.7	6.43 ± 0.07 (5) **	0.39	105 ± 12 **	6.33 ± 0.07 (5) *	0.50	109 ± 10 **
CGP35348	--	--	0	5.7 ± 0.04 (7)	2.1	36 ± 3 **	5.5 ± 0.07 (7)	3.0	37 ± 5 **
2-OH-saclofen	--	--	0	5.11 ± 0.18 (4)	7.76	31 ± 2.9 **	4.96 ± 0.10 (4)	11	35 ± 4 **

Table 2. Effects of allosteric modulators on potencies and efficacies of a partial agonist and neutral antagonists to enhance GTP$(\gamma)^{35}$S binding stimulated by recombinant GABA$_B$ receptors expressed in CHO cells. Concentration-response curves for CGP47656, CGP35348 and 2-OH-saclofen (Fig. 4) were measured in membranes from CHO cells stably expressing GABA$_B$ receptors, in the absence and in the presence of a fixed concentration (30µM) of allosteric modulators. The maximal stimulation above basal activity (*i.e.*, the relative intrinsic efficacy of partial agonists) is expressed in percent of the effects obtained with a saturating concentration of GABA (100µM) alone. The Hill-coefficients of the concentration-response curves were not significantly different from one. The data shown are means ± SEM from (N) independent experiments. *: $p<0.05$, **: $p<0.01$ compared to the corresponding control values (ANOVA / Dunnett's test). Because CGP35348 and 2-OH-saclofen were inactive under control conditions, this statistical analysis could not be performed for the pEC$_{50}$ values of these two compounds. Their maximal effects, however, were significantly different from zero (** $p < 0.01$, one sample t-test).

2-OH-saclofen, stimulated GTP$(\gamma)^{35}$S binding to maximally 30–40% of the full GABA effect, *i.e.*, they became partial agonists at the GABA$_B$ receptor (Fig. 5 and Table 2). The other antagonists tested remained "neutral" or "silent" in the GTP$(\gamma)^{35}$S binding experiments, also in the presence of the two allosteric modulators (data not shown).

Regulation of cAMP formation in a recombinant cell line stably expressing GABA$_B$ receptors. The stimulation of adenylyl cyclase activity by the forskolin analog 7β-forskolin (Laurenza *et al.* 1987) in a recombinant GABA$_B$ receptor expressing CHO cell line was inhibited by GABA in a concentration-dependent manner (Fig. 5a). The inhibition of cAMP formation by GABA was reverted by the competitive antagonist CGP56999, and no effect of

GABA was seen in CHO cells which had not been transfected with cDNA encoding the GABA$_B$ receptor (data not shown). The potency of GABA was enhanced by CGP7930 and GS39783; although GABA produced a close to full inhibition of adenylyl cyclase activity by itself, a small further increase in the maximal inhibition by the modulators was seen as a trend (Fig. 5a, Table 3). As can be seen from the respective control levels in Fig. 5, CGP7930 and GS39783 produced a small inhibition of cAMP formation by themselves. Although this effect was observed quite consistently in our experiments, its small magnitude precluded determining EC$_{50}$ values for the two modulators. With the partial agonist CGP47656, an increase of both the potency and the maximal effect of adenylyl cyclase inhibition was observed in the presence of the modulators. In this assay system, CGP35348 and 2-OH-saclofen, applied alone, turned out to be low efficacy partial agonists. Again, both
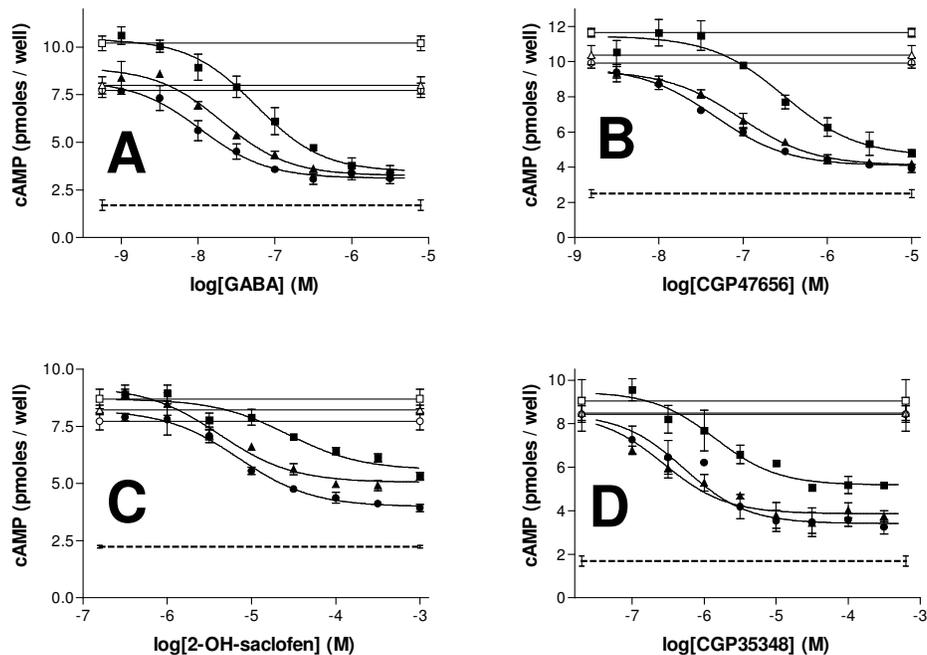
Fig. 5. 7β-forskolin stimulated adenylyl cyclase activity in a recombinant CHO cell line stably expressing GABA$_B$ receptors. The inhibition of cAMP formation by GABA **(a)**, CGP47656 **(b)**, 2-OH-saclofen **(c)** and CGP35348 **(d)** was measured in the absence (filled squares) or in the presence of 10μM CGP7930 (filled triangles) or 10 μM GS39783 (filled circles). The control values in the absence or presence of the modulators are indicated by the corresponding open symbols. The dotted lines represent basal cAMP levels. The data shown represent means ± SEM from triplicate (controls: quadruplicate) determinations. The results from several such experiments are summarized in Table 3.

Compound	Control			+ 10 µM CGP7930			+10 µM GS39783		
	pEC$_{50}$ (-log M)	EC$_{50}$	Max.effect (% inhibition)	pEC$_{50}$ (-log M)	EC$_{50}$	Max. effect (% inhibition)	pEC$_{50}$ (-log M)	EC$_{50}$	Max. effect (% inhibition)
GABA	7.08 ± 0.05 (7)	83 nM	83 ± 1	7.45 ± 0.09 (7) **	35 nM	86 ± 1.8	7.64 ± 0.09 (6) **	23 nM	85 ± 1.4
CGP47656	6.62 ± 0.09 (4)	240 nM	70 ± 4.9	6.95 ± 0.07 (4) *	112 nM	78 ± 2.8 *	7.04 ± 0.11 (4) *	91 nM	79 ± 3.2 *
CGP35348	5.41 ± 0.05 (5)	3.9 µM	55 ± 4.7	5.72 ± 0.16 (4)	1.9 µM	71 ± 1.9 *	5.83 ± 0.08 (4) *	1.5 µM	67 ± 5.3
2-OH-saclofen	4.65 ± 0.08 (6)	22.4 µM	46 ± 8.9	4.97 ± 0.35	10.8 µM	55 ± 6	5.13 ± 0.29 (6) *	7.5 µM	65 ± 8.4 **

Table 3. Effects of allosteric modulators on the potencies and efficacies of partial and full agonists to inhibit adenylyl cyclase activity in a recombinant GABA$_B$ receptor expressing cell line. Concentration-response curves for GABA, CGP47656, CGP35348 and 2-OH-saclofen (Fig. 5) were measured in CHO cells stably expressing GABA$_B$ receptors, in the absence and in the presence of a fixed concentration (10µM) of allosteric modulators. The maximal effects are expressed in percent inhibition of the control stimulation obtained with 100 µM 7β-forskolin above basal cAMP levels. The Hill-coefficients of the concentration-response curves were not significantly different from one. The data shown are means ± SEM from (N) independent experiments. *: p<0.05, **: p<0.01 compared to the corresponding control values (ANOVA / Dunnett's test).

their potencies and maximal efficacies were enhanced by CGP7930 and GS39783 (Fig. 5, Table 3). With CGP52432, results suggestive of inverse agonism were seen as a trend (data not shown); it marginally increased the formation of cAMP induced by 7β-forskolin by maximally 25 ± 5%. The inverse agonistic potency of CGP52432 seemed to be decreased by the allosteric modulators (pEC$_{50}$ = 6.6 ± 0.2 and 6.8 ± 0.2 in the presence of 10µM CGP7930 or GS39783, respectively, compared to 7.5 ± 0.1 (N=7) in the absence of modulators). The

other compounds tested (CGP54626, CGP56999, CGP62349 and SCH50911) did not affect

7β-forskolin stimulated cAMP formation (data not shown).

5.1.5. Discussion

According to the TCM (Fig. 4 in Chapter 4) an orthosteric and an allosteric ligand mutually

influence each other's binding affinities to the same extent, which is determined by the

allosteric constant γ. The formalism of the TCM does not at all take into account the intrinsic

efficacies of the compounds, *i.e.* whether the orthosteric ligand is an agonist or an antagonist.

Therefore, the binding affinities of competitive antagonists are expected to be amenable to

allosteric modulation like those of agonists. This prediction has been entirely confirmed in the

present study. In saturation experiments, it has been shown that the affinity of the antagonist

radioligand [^{3}H]-CGP62349 for native $GABA_B$ receptors is decreased by the allosteric

modulators CGP7930 and GS39783, whereas the maximal binding capacity remains

unchanged (Fig. 2). The affinities of several other compounds previously known to be

$GABA_B$ receptor antagonists CGP52432, CGP54626, CGP56999, CGP62349 (Froestl and

Mickel 1997; Bowery *et al.* 2002) and SCH50911 (Bolser *et al.* 1995) were also decreased in

displacement experiments (Table 1). Only with 2-OH-saclofen (Kerr *et al.* 1988) and

CGP35348 (Olpe *et al.* 1990) a small, but not significant, trend toward an increase in binding

affinity was observed in the presence of the two modulators (Table 1). However, these two

compounds were found in our functional assays not to be completely devoid of intrinsic

agonist activity (see below). Previously, we have observed that CGP7930 and GS39783

produced increases in the affinities of both high affinity (G protein – coupled) and low affinity

states of native $GABA_B$ receptors for agonists (Urwyler *et al.* 2003; Urwyler, Gjoni *et al.*

2004). At the same time, the relative proportion of high agonist affinity states was increased

by the modulators. In the experiments shown here, we also found biphasic binding curves

with CGP47656, but only the high affinity component was affected by the modulators (Table 1). It seems possible that the somewhat lesser effects of the two modulators on the binding of CGP47656 to $GABA_B$ receptors are related to the partial agonistic nature of this compound.

Similar to the allosteric constant γ in the TCM (affecting binding affinities), the extended two-state model of receptor activation (Hall 2000) introduces a factor δ by which the intrinsic efficacy of an orthosteric ligand (α) may change under the influence of an allosteric agent. This concept explains the finding, quite unique at the time of the discovery of CGP7930, that allosteric modulators are able to increase the maximal $GABA_B$ receptor stimulation produced by agonists. Here we show that the same phenomenon occurs with the partial agonist CGP47656 (Froestl *et al.* 1995; Knight and Bowery 1996). The efficacy of this compound was modulated by CGP7930 and GS39783 even more strongly than that of GABA. In fact, in the $GTP(\gamma)^{35}S$ assay its maximal effect was increased by about a four-fold by both modulators (Fig. 4, Table 2), whereas 1.5 to 2-fold increases were reported previously for their effects on GABA (Urwyler *et al.* 2001 and 2003). Apparently, the less efficacious receptor activation with a partial agonist leaves more room for positive modulation. Different degrees of increases in efficacies produced by an allosteric modulator with different partial or full agonists have also recently been reported for the mGluR2 receptor (Schaffhauser *et al.* 2003).

We have found two compounds, CGP35348 and 2-OH-saclofen, which did not stimulate $GTP(\gamma)^{35}S$ binding at all on their own, but became partial $GABA_B$ receptor agonists in the presence of the allosteric modulators (Fig. 4). These compounds are both structurally closely related to two well known $GABA_B$ receptor agonists, APPA and baclofen (Fig. 1). Like that of other "family 3" GPCRs, the orthosteric binding site of $GABA_B$ receptors consists of a "Venus flytrap module" (VFTM), two hinge lobes in the large extracellular domain, which

oscillate between open and closed states. The binding of an agonist in the cleft between the lobes causes them to close, whereby the agonist is trapped (Galvez *et al.* 1999 and 2000a; Pin *et al.* 2003). It seems that although CGP35348 and 2-OH-saclofen are still able to bind to the receptor pocket, the chemical modifications which distinguish them from APPA and baclofen prevent them from activating the receptor by closing the VFTM (smaller modifications in CGP47656, compared to CGP35348 [Fig. 1], result only in a partial loss of efficacy). However, the conformational change induced in the receptor by the allosteric modulators obviously results in a better fit of these molecules, not only increasing their affinities, but to a certain extent also their intrinsic efficacies. Another example of changes in the receptor-ligand interaction at the orthosteric binding site of a family 3 GPCR, which convert antagonists into agonists has been reported by Bessis *et al.* (2002). In that case, structural changes in the VFTM (site directed mutations of two critical amino acids) converted two mGluR8 antagonists into agonists. In our case, it does not become clear from the $GTP(\gamma)^{35}S$ experiments whether the modulators confer partial agonistic activity to otherwise completely silent antagonists ($\alpha = 1$, $\delta > 1$), or whether CGP35348 and 2-OH-saclofen possess some marginal agonistic properties, which cannot be detected in this assay under normal circumstances but are unmasked by the allosteric modulators. According to recent concepts, neutral antagonists that bind to receptors without altering the equilibrium between their active and inactive states at all, *i.e.* having an intrinsic efficacy constant α of exactly one (Figs 3, 4 and 5 in Chapter 4) are likely to be rare (Kenakin 2004). Compounds appearing at first as "silent" antagonists may in reality be low efficacy partial agonists or inverse agonists (Strange 2002; Kenakin 2004). The detection of inverse agonism depends on the availability of an assay system with measurable constitutive receptor activity f. On the other hand, the detection of low efficacy partial agonism is facilitated in a system with a high degree of receptor reserve.

GABA$_B$ receptor agonists inhibit forskolin-stimulated activity of adenylyl cyclase in native and recombinant assay systems (Wojcik and Neff 1984; Karbon and Enna 1985; Hirst *et al.* 2003). Recently, it has been shown that CGP7930 enhances this effect in rat brain preparations (Onali *et al.* 2003), and here we show the same to occur with both modulators in a recombinant cell line expressing GABA$_B$ receptors (Fig. 5, Table 3). In this assay system, the partial agonist CGP47656 had an intrinsic efficacy which was higher than that found in GTP(γ)^{35}S experiments (Fig. 4). This discrepancy is likely due to the existence of a large receptor reserve in the cAMP assay system, as suggested by the much higher potency of GABA (EC$_{50}$ = 83 nM) when compared to the GTP(γ)^{35}S assay (EC$_{50}$ = 4.9 μM, Urwyler *et al.* 2001). This interpretation is further corroborated by the finding that the partial agonistic nature of CGP35348 and 2-OH-saclofen, which was hidden in the GTP(γ)S assay and only revealed by the allosteric modulators, was visible in the cAMP assay even when the compounds were applied on their own. Both the potencies and the maximal inhibitions produced by CGP47656, CGP35348 and 2-OH-saclofen were enhanced by GS39783 and CGP7930. Interestingly, the allosteric modulators CGP7930 and GS39783 also had some small inhibitory effect on cAMP formation on their own, whereas they had been found previously to be devoid of any intrinsic activity at GABA$_B$ receptors in various assay systems (Urwyler *et al.* 2001 and 2003). This is again in line with the existence of a large receptor reserve in the cAMP signaling pathway. Recently, Binet *et al.* (2004) have also reported that CGP7930 is a partial agonist in a seemingly even more sensitive assay system with a high degree of receptor reserve, by directly activating the heptahelical domain of GABA$_{B(2)}$. This is not at variance with the concept of allosteric modulation, since the extended ternary complex model allows for allosteric modulators having some efficacy on their own *via* the allosteric binding site (β, see Fig. 5 in Chapter 4). SCH50911 and CGP54626 have recently been

reported to be inverse agonists in cAMP assays with recombinant $GABA_B$ receptors (Grünewald *et al.* 2003; Hirst *et al.* 2003). We found inverse agonism as a trend only with CGP52432. It is not clear whether low intrinsic inverse agonist efficacies or a small constitutive activity of the $GABA_B$ receptor in our recombinant cell line explain why we have not detected any inverse agonism with SCH50911 and CGP54626, and possibly also with CGP56999 and CGP62349.

In conclusion, this study shows that agonists and competitive antagonists at $GABA_B$ receptors are amenable to allosteric modulation by CGP7930 and GS39783. Whereas the positive modulators enhance agonist affinity by further stabilizing the closed conformation of the VFTM, they decrease the affinities of silent competitive antagonists or inverse agonists, which bind to or even stabilize the open state of the VFTM. They also enhance the intrinsic efficacies of partial and full agonists, thus making possible the detection of partial agonism in little sensitive systems such as the $GTP(\gamma)^{35}S$ assay. The cAMP signaling cascade in a recombinant cell line, due to its high degree of receptor reserve, also is a very sensitive assay system to detect partial agonism at the $GABA_B$ receptor. Compounds believed to be "silent" competitive antagonists are often used as tools for pharmacological studies *in vitro* and *in vivo.* In a physiological context, especially *in vivo* and unlike in a recombinant cell line, important variables such as constitutive receptor activity and receptor reserve are largely unknown, and hidden partial or inverse agonistic effects of such compounds might introduce unpredictable confounding effects. Therefore, powerful tools, such as suitable cellular assay systems in conjunction with allosteric modulators, to unmask the true intrinsic properties of orthosteric ligands, should be of great value.

Acknowledgments

5.2. Receptor activation involving positive allosteric modulation, unlike full agonism, does not result in GABA$_B$ receptor desensitization: an *in vitro* study

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5.2.1. Abstract

Allosteric modulators act more physiologically than orthosteric ligands, targeting only

endogenously activated receptors and not their whole population, which is why they are

expected to produce less side-effects and tolerance. To inspect the role of the positive

allosteric modulator GS39783 in $GABA_B$ receptor desensitization, we examined receptor

function and cell surface expression in a recombinant $GABA_B$ cell line and in primary

neuronal cultures upon persistent treatments with $GABA_B$ agonists, and combinations of

agonists and GS39783. The potency of GABA to inhibit 7β-forskolin-induced cAMP

formation in recombinant cells decreased after the exposure to a saturating GABA

concentration, but not after a combination of a low GABA concentration and GS39783, that

activated the receptor to the same extent. Concordantly, a significant decrease of cell surface

receptors was found after GABA-induced desensitization, unlike after the combined treatment

with GABA and GS39738. Similar observations regarding receptor function were found in

primary neurons for baclofen-induced inhibition of spontaneous Ca^{2+} oscillations. However,

the cell surface receptor density remained unaffected upon baclofen-induced desensitization

in the primary neurons, possibly due to different mechanisms of desensitization in the neurons

and the recombinant cell line. These findings indicate that the degree of occupancy of the

orthosteric site determines desensitization rather than the degree of receptor activation. In

summary, our results conform to predictions that positive allosteric modulators have less

propensity for the development of tolerance due to receptor desensitization than classical

agonists.

Abbreviations: GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor; mGluR

metabotropic glutamate receptor; CHO, Chinese hamster ovary; CNS, central nervous system;

CPA, N^6-cyclopentyladenosine; GTP(γ)S, guanosine 5'-*O*-(3-thiotriphosphate); DIV, day *in*

vitro; FLIPR, fluorescence imaging plate reader; HBSS, Hanks' buffered salt solution; HEK, human embryonic kidney.

5.2.2. Introduction

G-protein coupled receptors (GPCRs) represent the most "popular" drug targets in modern pharmacology: more than 1000 membrane-bound receptors compose the GPCR superfamily and almost 40% of all the pharmaceuticals on the market today act through them, mainly by binding as agonists or antagonists to the same ("orthosteric") site as their endogenous ligands (Hopkins and Groom 2002; Maudsley *et al.* 2005). The use of such drugs is, however, often limited by their side-effects and/or receptor up- and downregulation underlying tolerance (Gainetdinov *et al.* 2004).

Allosteric modulation, an alternative way of acting at GPCRs, is expected to be devoid of abovementioned disadvantages. Allosteric modulators are substances that bind to and act through topographically distinct ("allosteric") sites on receptors than orthosteric ligands. Positive modulators act synergistically with agonist-induced receptor activation by enhancing it. Many of them do not stimulate receptors on their own. Their action is, therefore, in concert with physiological signaling, both temporally and spatially. Because of this use-dependent mechanism, they are expected to produce less side-effects and tolerance than orthosteric drugs (Christopoulos 2002; Christopoulos and Kenakin 2002; Langmead and Christopoulos 2006). Despite the fact that allosteric modulators of GPCRs are presently in the focus of pharmacological research, so far there have been only few studies performed to assess whether they play a role in GPCR desensitization and downregulation (Klaasse *et al.* 2005; May *et al.* 2005; Bhattacharya and Linden 1996).

GABA is the major inhibitory neurotransmitter in the CNS, the action of which is mediated *via* ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptor.

Dysfunctions of the GABAergic neurotransmission in the CNS have been linked with many disorders such as epilepsy, spasticity, anxiety, stress, sleep disorders, depression, addiction and pain. Heterodimeric $GABA_B$ receptors belong to the family 3 of GPCRs together with the metabotropic glutamate receptors, the calcium-sensing receptors and mammalian taste and odorant receptors (Pin *et al.* 2003). They mediate slow synaptic inhibition by negative coupling to adenylyl cyclase and modulating voltage-gated calcium channels and inwardly rectifying potassium channels (reviewed in Bowery *et al.* 2002).

The only marketed drug that acts *via* the $GABA_B$ receptor is its selective agonist baclofen, introduced in the clinical practice as an anti-spastic agent more than thirty years ago. However, side-effects as well as rapid development of tolerance are its main shortcomings (Malcangio *et al.* 1992; Lehmann *et al.* 2003; Malcangio *et al.* 1995, reviewed in Vacher and Bettler 2003).

This explains the interest in the recently discovered positive allosteric modulators of $GABA_B$ receptors CGP7930 and GS39783 (Urwyler *et al.* 2001 and 2003). They were found to increase both the affinity and the efficacy of agonists, with none or very little effect in their absence, in many assay systems *in vitro* (Urwyler *et al.* 2001 and 2003; Urwyler, Gjoni *et al.* 2005; Onali *et al.* 2003; Olianas *et al.* 2005) and *in vivo* (Gjoni *et al.* 2006).

In this study, we aimed to investigate whether receptor activation involving positive allosteric modulation leads to $GABA_B$ receptor desensitization. To this end, we have used both a recombinant (a cell line stably expressing the $GABA_B$ receptor) and a native assay system (mouse primary neuronal cortical cultures). Our key strategy was to persistently activate the receptor either with desensitizing agonist concentrations or with combinations of low agonist concentrations and GS39783 that activate the receptor to the same extent. The functional responses of the receptor as well as the cell-surface receptor expression were then compared after these different pretreatments.

5.2.3. Materials and Methods

Materials: GS39783, R(-)-baclofen, S(+)-baclofen, CGP56999, CGP52432 and CGP54626 were available in-house. Mastoparan was purchased from Tocris bioscience, UK. Stock solutions of R(-)-baclofen, S(+)-baclofen, CGP56999, CGP52432 and CGP54626 were made in appropriate experiment buffers freshly before each experiment. GS39783 was dissolved in DMSO to give a stock solution that was further diluted in appropriate buffers. Sources of other chemicals are given in method descriptions.

Adenylyl cyclase assays in a recombinant cell line stably expressing GABA$_B$ receptors: A stable Chinese hamster ovary (CHO) cell line co-expressing human GABA$_{B(1b)}$ together with rat GABA$_{B(2)}$ (Urwyler *et al.* 2001) was cultured in Dulbecco's modified eagle medium (DMEM, glutamine-free, Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 20 μg/ml L-proline, 500 μM glutamine, 1 mg/ml geneticin, 250 μg/ml zeocin). The cells were grown to 80–90% confluency in 15 cm cell culture dishes. Prior to the cAMP measurement, they were treated with the appropriate concentrations of agonists and GS39783 diluted in the cell growth medium for 2 hours. After the pretreatment, cells were washed twice with PBS. The cAMP accumulation experiment was then performed as has been described previously (see Urwyler, Gjoni *et al.* 2005 for details).

Calcium oscillations in neuronal networks: Primary cultures of cortical neurons were prepared from embryonic (day 16 to 18) BALB/c mice (adapted from Wang and Gruenstein 1997, as described in Urwyler *et al.* 2001). Dissociated cells were plated on poly-D-lysine coated 96-well plates at *ca.* 60'000 cells/well in 200 μL medium and incubated at 37°C in 5% CO$_2$ for 7 to 10 days. Half of the medium was changed every 2 days. Two hours before the

experiment, half of the medium was replaced with fresh medium or fresh medium containing R(-)-baclofen, S(+)-baclofen, GS39783 and CGP52432 or their combinations to give the final concentrations described in the Subsection 5.2.4.

After the preincubation period, the culture medium was removed, cells were washed twice with Hank's buffered saline solution (HBSS, Invitrogen, Switzerland) supplemented with 10 mM HEPES (pH adjusted to 7.4) and loaded with 2 µM fluo-4 AM in the same buffer. After loading (10 min), cells were washed twice with Mg^{2+}-free HBSS/10 mM HEPES and then transferred to the fluorescence imaging plate reader (FLIPR). Fluorescence was measured at room temperature and at a sampling rate of 0.5 Hz for 10 minutes. Drugs were dissolved in Mg^{2+}-free HBSS and added to the cultures during recording. Oscillations were analyzed using IgorPro (Wavemetrics, Lake Oswego, OR) by peak detection and calculation of the ratio of peak frequencies before and after compound addition.

Cell surface [^{3}H]-CGP62349 labeling in the recombinant GABA$_B$ cell line. The CHO-K1 cells stably expressing the GABA$_B$ receptor (*vide supra*) were grown to confluency, treated, washed and trypsinized as described above. The cells were counted, resuspended in Krebs-Tris 7.4 buffer (20 mM Tris HCl pH 7.4, 118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.6 mM D(+)-glucose) and 200'000 cells were dispensed per well. A high-affinity GABA$_B$ selective radioligand [^{3}H]-CGP62349 (85 Ci/mMol, American Radiolabeled Chemicals Inc., St. Louis, MO) was added at a saturating concentration (15 nM) and incubated with the cells while mildly shaking for 20 min. Nonspecific binding was measured in the presence of the GABA$_B$ specific antagonist CGP54626 (1 µM). After the incubation, the cell suspension was filtered on a Packard Filtermate Harvester and 50 µL/well of scintillation fluid was added. The plate was shaken for 1 hour and counted on a Packard TopCount NXT (counting time: 20 min/well).

Protein concentrations for the samples in each treatment group were measured with the Bradford assay method, using the Bio-Rad protein assay kit and bovine serum albumin as a standard, and the specific binding was expressed relative to protein content.

To verify that the radioligand [^{3}H]-CGP62349 does not cross the cell membrane, Human embryonic kidney (HEK-FT) cells were transfected either with human GABA$_{B(1a)}$ subunit alone (8 μg) or with human GABA$_{B(1a)}$ (8 μg) and rat GABA$_{B(2)}$ (16 μg) subunits together (Fugene 6, Roche, Switzerland). 48h post-transfection, the binding experiment with whole cells was performed as described above. Also, the same procedure was applied to membranes prepared (as described previously in Urwyler *et al.* 2001) from cells transfected with human GABA$_{B(1a)}$ or human GABA$_{B(1a)}$ and rat GABA$_{B(2)}$.

Cell surface [^{3}H]-CGP62349 labeling in mouse cortical neuronal cultures: Cortical neuronal cultures were prepared from BALB/c mice, seeded and maintained as described above. Two hours before the experiment, half of the medium was replaced either with fresh medium or fresh medium containing R(-)-baclofen. The cells were then washed twice with HBSS supplemented with 10 mM HEPES (pH adjusted to 7.4), a saturating concentration of [^{3}H]-CGP62349 (15 nM) in Krebs-Tris buffer, or the mix of the radioligand with 1 μM CGP54626 for the assessment of nonspecific binding, was added to each well and the plate was shaking for 20 minutes. The plate was put on ice and cells were washed three times with cold Krebs-Tris buffer to remove the unbound radioligand. 200 μL of 0.1 M NaOH was added to each well and the plate was left to shake for 20 minutes. The contents of three wells were pooled into one vial and an aliquot was removed for protein content analysis. Scintillation fluid was added to each vial, vials were shaken for 1h and measured on a Packard TRI-CARB 3100TR Liquid Scintillation Analyzer (counting time 20 min per vial).

5.2.4. Results

cAMP measurement in CHO-K1 cells stably expressing the GABA$_B$ receptor. To

examine the effect of prolonged pretreatments of GABA$_B$ receptor-expressing cells with

GABA, GS39783 and their combinations, inhibition of cAMP formation stimulated by 7β-

forskolin was measured as a readout of GABA$_B$ receptor activation. The concentrations for

the pretreatments were chosen on the basis of the previously obtained GTP(γ)S assay data

(Urwyler *et al.* 2003). In the GTP(γ)S experimental system, receptor activation corresponds to

occupancy (same agonist potencies as binding affinities) and thus seems to be devoid of

receptor reserve, which would have had confounding effects in our experimental design. As

has been shown previously (Gonzales-Maeso *et al.* 2003), after a two-hour exposure to a

saturating GABA concentration (100 μM), the potency of GABA to inhibit cAMP production

significantly decreased as well as its maximal effect (Fig. 1).
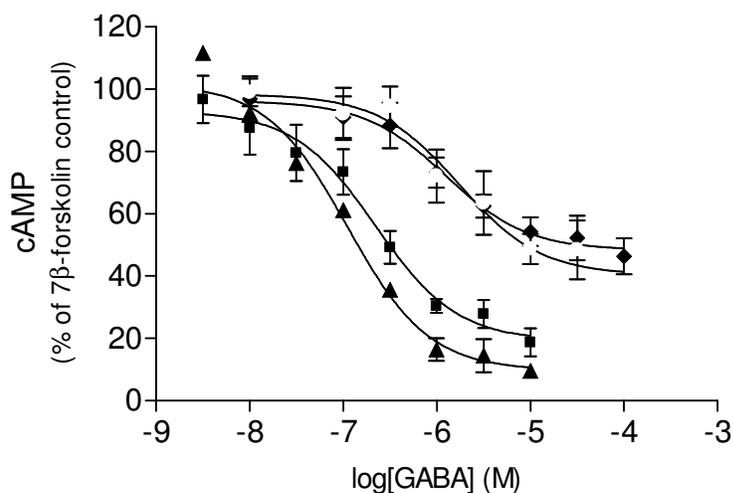
Fig. 1. cAMP measurement in a recombinant cell line stably expressing GABA$_B$ receptors.
Comparison of the effects of different cell pretreatments (2h) on agonist potency and efficacy. Control:
squares, 100 μM GABA: circles, 0.3 μM GABA + 10 μM GS39783: triangles, 100 μM GABA + 10
μM GS39783: diamonds. These are representative curves done in triplicates (means ± SEM). The data
from several such experiments are summarized in Table 1.

Pretreatment conditions (2h):	GABA			
	pEC$_{50}$	EC$_{50}$	Maximal inhibition (%)	N
Control (no pretreatment)	6.95 ± 0.03	118 nM	84.4 ± 1.2	25
GABA, 100 µM	6.25 ± 0.06**	653 nM	71.6 ± 3.4*	16
GABA, 0.3 µM + GS39783, 10 µM	7.08 ± 0.06	90 nM	90.4 ± 2.1	9
GABA, 100 µM + GS39783, 10 µM	6.21 ± 0.04**	622 nM	70.6 ± 8.3**	4

Table 1. Effects of different pretreatments (2h) on the potency and maximal effect of GABA to inhibit 7β-forskolin-induced cAMP production. Concentration-response curves with GABA were measured in CHO cells stably expressing GABA$_B$ receptors after different pretreatments. The maximal effects are expressed in percent inhibition of the control stimulation obtained with 100 µM 7β-forskolin above basal cAMP values. The data shown are means ± SEM from (N) independent experiments. * p<0.05, ** p<0.01 *vs.* control group (no pretreatment), one-way ANOVA, *Dunnett's* test.

Similar effects were observed with R(-)-baclofen, but not with its inactive S(+)-enantiomer (data not shown). However, the exposure to a combination of a low GABA concentration (0.3 µM) and GS39783 (10 µM), that activated the receptor to the same extent as 100 µM GABA alone, did not lead to desensitization. On the other hand, when the cells were incubated with a saturating GABA concentration and the positive allosteric modulator GS39783 (100 and 10 µM, respectively), desensitization of the GABA$_B$ receptors still occurred (Table 1, Fig. 1). The pretreatment with GS39783 alone, did not lead to receptor desensitization (data not shown).

The responsiveness of adenylyl cyclase to mastoparan, a direct activator of G$_{i/o}$ signaling, to inhibit cAMP, was the same in the control and the pretreated cells (data not shown).

To verify that there were no confounding effects of GS39783 accidentally carried over from the pretreatment containing GS39783, LC/MS analysis of washing and assay buffers was

performed and showed that there was no GS39783 left in the assay buffer (data not shown, courtesy of Dr. M. Koller, internal communication).

GABA$_B$ receptor cell surface labeling with [^{3}H]-CGP62349 in the recombinant cell line.
Radioligand binding with a saturating concentration of the selective GABA$_B$ receptor antagonist [^{3}H]-CGP62349 was employed to investigate cell-surface receptor expression upon different two-hour pretreatments (*vide supra*). B$_{max}$ values were calculated for each pretreatment. Firstly, to verify that the radioligand does not cross the cell-membrane, we took advantage of the fact that the GABA$_{B(1)}$ subunit containing the ligand binding site, is not expressed at the cell surface in the absence of the GABA$_{B(2)}$ subunit (Pagano *et al.* 2001). Binding experiments were performed on intact cells transiently expressing the human GABA$_{B(1a)}$ subunit alone, or the human GABA$_{B(1a)}$ and rat GABA$_{B(2)}$ subunit together. Additionally, binding was measured on membranes prepared from cells transiently expressing the human GABA$_{B(1a)}$ subunit alone. There was substantial specific binding either when both receptor subunits were expressed in intact cells or when membranes were prepared from cells expressing the human GABA$_{B(1a)}$ subunit alone, but there was no specific binding on intact cells expressing only the human GABA$_{B(1a)}$ subunit (data not shown).
In line with the functional readout, a two-hour preincubation with the saturating concentration (100 μM) of GABA led to a significant decrease of cell surface receptor labeling by 23 ± 2%**. In contrast, exposing the receptor to a combination of low GABA and the allosteric modulator GS39783 (0.3 and 10 μM, respectively) that activate the receptor to the same extent as a desensitizing GABA concentration (100 μM), the cell-surface binding of the radioligand remained same as in the control group. On the other hand, when the cells were incubated with a saturating GABA concentration and the positive allosteric modulator GS39783 (100 and 10 μM, respectively), there was a reduction of 24 ± 2 %** in the cell surface binding (means ± SEM, N=8, **p<0.01, One-way ANOVA, Dunnett's test) (Fig. 2).
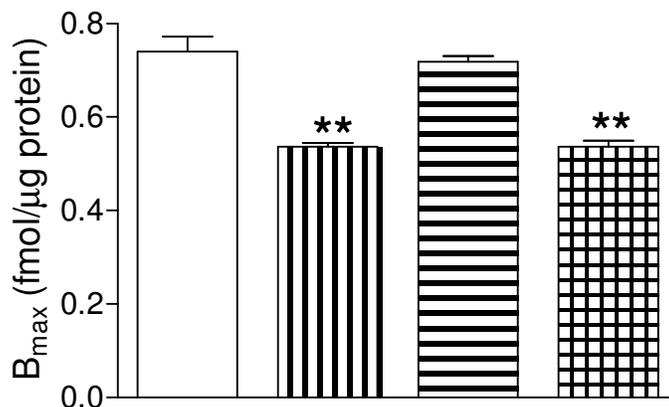
Figure 2. Assessment of GABA$_B$ receptor cell-surface expression upon different two-hour pretreatments. Their effects on receptor number were measured by radiolabeling with a saturating concentration of [^{3}H]-CGP62349 (15 nM). Control: white bar, 100 μM GABA: vertical stripes, 100 μM GABA + 10 μM GS39783: checkered bar, 0.3 μM GABA + 10 μM GS39783: horizontal stripes. Data are means ± SEM of a typical experiment done in sextuplicates. ** p<0.01, One-way ANOVA *vs.* control followed by *Dunnett's* test.

R(-)-baclofen-mediated inhibition of spontaneous Ca^{2+} oscillations in primary mouse neuronal cultures is enhanced by GS39783. In order to observe these phenomena in a native system, experiments were carried out in mouse primary cortical neurons. As has been shown previously, when dissociated neurons are plated densely and left for sufficient time (DIV 6-10) in culture, they form a network. In the absence of Mg^{2+} in the medium, spontaneous oscillations of intracellular Ca^{2+} concentrations are observed in this network reflecting the net outcome in excitatory and inhibitory signaling (Wang and Gruenstein 1997). We have shown previously that these spontaneous oscillations of Ca^{2+} are inhibited when the GABA$_B$ receptor is activated (Urwyler *et al.* 2001). In the first series of experiments establishing a concentration-response relationship for R(-)-baclofen to inhibit spontaneous Ca^{2+} spikes, the pEC$_{50}$ of the agonist was found to be 6.01 ± 0.04 (means ± SEM, N=12) (Fig. 3a). This inhibition was antagonized by the potent and GABA$_B$ selective antagonist CGP56999 and the relative Ca^{2+} spiking frequency was brought back to the control level (Fig. 3a). In the presence of the GABA$_B$ positive allosteric modulator GS39783 at 1 and
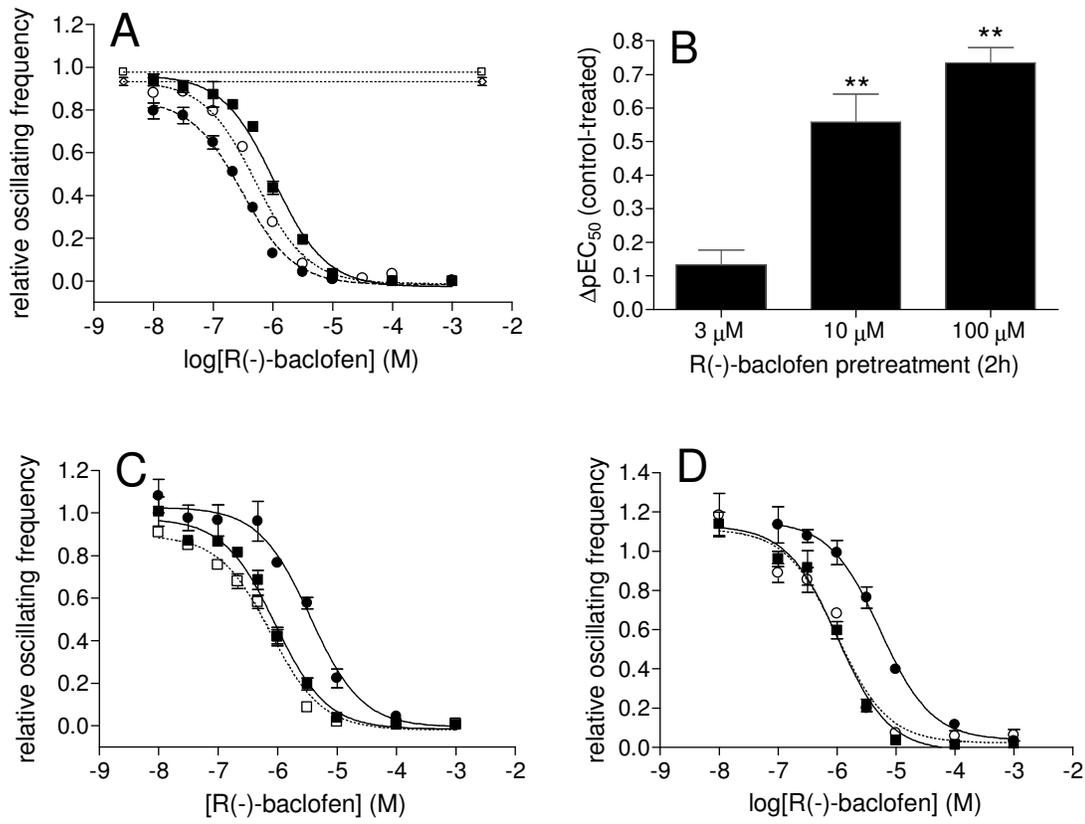
Fig. 3. (a) Inhibition of spontaneous Ca^{2+} oscillations (no drug added: open squares connected by a dotted line) in mouse primary cortical neurons by R(-)-baclofen (filled squares, full lines) enhanced in the presence of 1 (open circles) and 3 μM (filled squares) GS39783. $GABA_B$ competitive antagonist CGP56999 (1 μM) brings the R(-)-baclofen-inhibited (10 μM) spiking frequency back to the control level (open diamonds connected by a dotted line). The graph shows typical curves done in quadruplicates as means ± SEM Corresponding pEC_{50} values are indicated in the text. **(b)** Two-hour pretreatments with 10 (N=3) and 100 μM R(-)-baclofen (N=11), but not 3 μM (N=3), induce $GABA_B$ receptor desensitization. Data on the graph are differences in pEC_{50} between control and treated cells presented as means ± SEM One way ANOVA followed by Dunnett's test, **$p<0.01$. **(c)** Desensitization of the $GABA_B$ response with prolonged activation (2h) with a saturating concentration (100 μM) R(-)-baclofen can be inhibited with a selective $GABA_B$ antagonist CGP52432. Concentration-response curve of R(-)-baclofen inhibiting spontaneous Ca^{2+} spiking in unpretreated (control) cultures (filled squares, full line), 100 μM R(-)-baclofen-pretreated cultures (for 2h; desensitized cultures: filled circles, full line) and 100 μM R(-)-baclofen + 100 μM CGP52432-pretreated cultures (antagonized desensitization: open squares, dotted line). Data are means ± SEM of representative curves done in quadruplicates. **(d)** Stereospecificity of the $GABA_B$ receptor desensitization. Concentration-response curve of R(-)-baclofen in non pretreated (control) cultures (filled squares, full line), 100 μM R(-)-baclofen-pretreated cultures (for 2h; desensitized cultures: filled circles, full line) and 100 μM S(+)-baclofen-pretreated cultures (for 2h; open circles, dotted line). Data are means ± SEM of one typical experiment done in quadruplicates.

3 μM, the potency of R(-)-baclofen to inhibit Ca^{2+} spikes significantly increased to pEC_{50} values of $6.29 \pm 0.07^{**}$ (means ± SEM, N=6) and $6.46 \pm 0.07^{**}$ (means ± SEM, N=6, ** p<0.01 one way ANOVA followed by Tukey's test), respectively (Fig. 3a).

Desensitization of the $GABA_B$ receptors in primary cortical cultures. The ability of R(-)-baclofen to inhibit spontaneous Ca^{2+} oscillations in primary cortical neurons was significantly decreased after a two-hour exposure to 10 and 100 μM R(-)-baclofen, but not to 3 μM (Fig. 3b), *i.e.* the response of the $GABA_B$ receptors desensitized. Differences in R(-)-baclofen pEC_{50} values between control cells and cells treated with 3, 10 and 100 μM R(-)-baclofen were 0.13 ± 0.04 (N=3), $0.56 \pm 0.08^{**}$ (N=4) and $0.73 \pm 0.05^{**}$ (N=11) (**p<0.01, one-way ANOVA *vs.* control followed by Dunnett's test), respectively (Fig. 3b). Desensitization induced with a 2h 100 μM R(-)-baclofen preincubation was successfully antagonized by a co-pretreatment with the selective high affinity $GABA_B$ antagonist CGP52432 (Fig. 3c, Table 2).

Pretreatment conditions (2h):	R(-)-baclofen		N
	pEC_{50}	EC_{50}	
Control (no pretreatment)	6.12 ± 0.02	821 nM	44
R(-)-baclofen, 100 μM	$5.47 \pm 0.04^{**}$	3.85 μM	24
R(-)-baclofen, 100 μM + CGP52432, 100 μM	6.28 ± 0.15	623 nM	5
S(+)-baclofen, 100 μM	5.98 ± 0.02	1.05 μM	2
R(-)-baclofen, 3 μM + GS39783, 1 μM	6.19 ± 0.08	790 nM	8
R(-)-baclofen, 100 μM + GS39783, 1 μM	5.99 ± 0.09	1.03 μM	6

Table 2. Effects of different pretreatments (2h) on the potency of R(-)-baclofen to inhibit spontaneous Ca^{2+} oscillations in mouse primary neuronal cultures. The potencies of R(-)-baclofen were obtained from concentration-response curves measured after the indicated pretreatments. The data shown are means ± SEM from (N) independent experiments. ** p<0.01, compared *vs.* control group, one-way ANOVA *vs.* control (no pretreatment) and *Tukey's* test.

Moreover, when the cultures were preincubated with the inactive enantiomer S(+)-baclofen,

the potency of R(-)-baclofen stayed the same as in the control cultures (Fig. 3d), which further

supports the conclusion that desensitization of the R(-)-baclofen response was $GABA_B$

receptor specific. When the cultures were preincubated with a combination of R(-)-baclofen

(3 μM) and GS39783 (1 μM), that activated the $GABA_B$ receptor to the same extent as a

saturating R(-)-baclofen concentration (100 μM), which desensitized the receptor, there was

no desensitization of the $GABA_B$ receptors (Fig. 4, Table 2). Further, in contrast to our

finding in the $GABA_B$ stably expressing cell-line, the presence of 1 μM GS39783 diminished

the $GABA_B$ receptor desensitization which 100 μM R(-)-baclofen normally produced (Fig. 4,

Table 2). The preincubation of the cultures with 1 μM GS39783 alone (2h) had no effects on

either the R(-)-baclofen potency or the Ca^{2+} spiking pattern (data not shown).
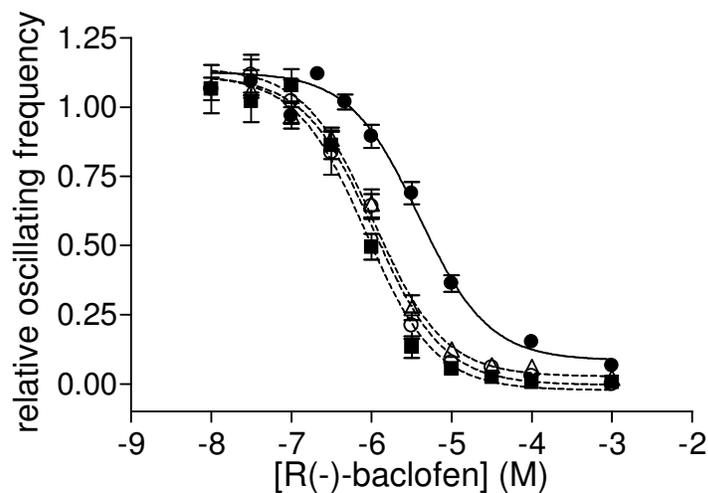
Fig. 4. The role of GS39783 in the R(-)-baclofen-induced $GABA_B$ receptor desensitization in mouse primary cortical neurons. Concentration-response curve of R(-)-baclofen in non pretreated (control: filled squares, full line) cultures, 100 μM R(-)-baclofen- (filled circles, full line), 3 μM R(-)-baclofen + 1 μM GS39783- (open triangles, dotted line) and 100 μM R(-)-baclofen + 1 μM GS39783-pretreated (2h) cultures (open circles, dotted line). Data on the graph are means ± SEM of typical curves done in quadruplicates.

Cell-surface GABA$_B$ receptor labeling in primary cortical neurons with [^{3}H]-CGP62349.

Contrary to the recombinant GABA$_B$ cell-line, there was no decrease in the cell-surface receptor expression in cultured primary neurons upon desensitization with 100 μM R(-)-baclofen. The GABA$_B$ receptors at the cell surface amounted to 0.79 ± 0.02 fmol/μg protein (N=24) in the control group (no pretreatment) and 0.77 ± 0.02 fmol/μg protein (N=24) in the 100 μM R(-)-baclofen pretreated group. The data are pooled from 5 experiments performed in pentuplicates, means ± SEM.

5.2.5. Discussion

Receptor desensitization, the most common cause underlying tolerance or lack of sustained efficacy, has been shown for many GPCRs after a persistent high degree of receptor activation (reviewed in Gainetdinov *et al.* 2004). Because of its use-dependent mechanism, it is expected that positive allosteric modulation has less potential to induce receptor desensitization. We have therefore set out to investigate whether this holds true in the case of the GABA$_B$ receptor and its positive allosteric modulator, GS39783, as predicted earlier (Pin *et al.* 2001). Our major finding is that GABA$_B$ receptor activation involving positive allosteric modulation by GS39783 did not lead to receptor desensitization.

The approach used was to compare different two-hour pretreatments corresponding to equal degrees of receptor activation. They consisted of either a desensitization-inducing concentration of a GABA$_B$ agonist alone or of a combination of an agonist and the positive allosteric modulator GS39783. Assuming that it might be agonist-occupancy of the orthosteric binding site that drives desensitization, the choice of pretreatment concentrations was made on the basis of the GTP(γ)S assay system (Urwyler *et al.* 2003). Since the GTP(γ)S experimental paradigm seems to be devoid of receptor reserve, levels of receptor activation essentially correspond to receptor occupancy (further discussed in Urwyler, Gjoni *et al.* 2005

or Section 5.1.). Had the choice been based on a system with a high receptor reserve (*e.g.* cAMP measurement), induction of desensitization could have failed, as in such systems the receptor is maximally active even with low agonist occupancy. $GABA_B$ receptor function and the cell-surface receptor number were examined in both a recombinant and a native assay systems following the described pretreatments.

Our results confirm that in both, the recombinant (inhibition of cAMP formation in the $GABA_B$ stable cell line) and the native (inhibition of Ca^{2+} oscillations in primary mouse cortical neurons) systems, there was no functional desensitization after the pretreatment with a low concentration of agonist together with GS39783 (Figs 1 and 4) as opposed to the desensitization occurring following pretreatments with saturating concentrations of agonists. Given that the degrees of receptor activation were the same in the different pretreatments used, we conclude that it is indeed the degree of occupancy of the orthosteric binding site that determines whether the receptor desensitizes or not. From the known affinities of GABA alone and in the presence of GS39783 (Urwyler *et al.* 2003) it can be calculated that the occupancy of the orthosteric binding site by 100 μM GABA alone, that desensitized the receptor in the recombinant cell line, amounts to about 97%, whereas it is only approximately 8% in the case of 0.3 μM GABA alone, which is the low agonist concentration used for the pretreatment combination with GS39783 (Fig. 1, Table 1). However, because the positive allosteric modulator GS39783 increases the binding affinity of agonists toward the receptor (Urwyler *et al.* 2003), the occupancy of the orthosteric site by 0.3 μM GABA in the presence of 10 μM GS39783 rose to *ca.* 35% which is apparently still not sufficient to lead to receptor desensitization.

As predicted by the allosteric two-state model (Hall 2000), the enhancing influence of a positive allosteric modulator can be a result of its effect on agonist affinity, efficacy or a combination of both. The positive allosteric modulator GS39783 enhances both the affinity and the efficacy of $GABA_B$ agonists (Urwyler *et al.* 2003). However, it has been calculated

that it is the effect on agonist efficacy, rather than affinity, which is predominantly responsible for its positive modulatory action (May *et al.* 2007). Exertion of positive allosteric effects by primarily affecting the efficacy of agonists, seems to perfectly match the profile an ideal positive allosteric modulator should have. Oppositely, a positive allosteric modulator that acts by enhancing primarily the affinity of agonists, instead of their efficacy, will increase the occupancy of the orthosteric site, potentially leading to receptor desensitization. Although these mechanisms may differ from one receptor to another, a comparison with other GPCRs is worthwhile. In fact, little has been investigated regarding allosteric modulation and its effect on GPCR desensitization and no study pursuing a strategy similar to ours has been, to our knowledge, published to date. Together with a report on the lasting effects of negative allosteric modulators on M_2 muscarinic acetylcholine receptor (May *et al.* 2005), only studies examining long term receptor activation involving positive allosteric modulation were conducted on the adenosine A_1 receptors and its allosteric enhancers PD81'723 and T62. Bhattacharya and Linden (1996) reported desensitization after a pretreatment with a saturating concentration of A_1 receptor agonist N^6-cyclopentyladenosine (CPA) alone and in combination with the positive enhancer PD81'723. Moreover, the degree of receptor internalization, produced by CPA concentration at, or close to, a saturation level, was magnified in the presence of PD81'723 (Klaasse *et al.* 2005). It is known that PD81'723 exhibits its allosteric effects primarily by increasing agonist affinities and thereby increasing receptor occupancy (Bruns and Fergus 1990). However, the high intrinsic efficacy of the compound that has also been reported, seems to be confounding in this context (Bruns and Fergus 1990). In line with these findings, an *in vivo* study by Li *et al.* (2004) showed that repeated oral administration of the oral allosteric modulator T62, which belongs to the same chemical class as PD71'723, produces tolerance in rats with neuropathic pain.

It could be argued that desensitization of the functional response observed in our experiments happens downstream of the receptor at the effector level and not at the level of the receptor

itself. In our case, we find this highly unlikely, because desensitization of the effector system should be activation-dependent. Since the pretreatments used differ only in the way (allosterism *vs.* orthosterism), but not the degree of receptor activation, the decreased response to agonism should be seen in both treatments, were it due to desensitization of the effector, which is not the case. This is further corroborated by the fact that we did not see a change in responsiveness of adenylyl cyclase to direct $G_{i/o}$-protein activation by mastoparan in desensitized cells, when compared to control.

Although the main findings, showing that GS39783 is not prone to inducing receptor desensitization, are the same in both assay systems, we have also come across differences between the two experimental setups. Namely, in the recombinant cell line, 10 µM GS39783 has no influence on the 100 µM GABA-induced desensitization of the $GABA_B$ response, regarding both the decrease in GABA potency as well as its maximal inhibition (Fig. 1, Table 1). However, in the primary neurons it seems that 1 µM GS39783 prevents desensitization induced by a saturating concentration of R(-)-baclofen (Fig. 4, Table 2). Another divergence between the two systems is a decrease in the number of receptors at the cell surface (B_{max}) that correlates with functional desensitization found in the recombinant cell line (Fig. 2) and no such decrease in the B_{max} in the neurons upon receptor desensitization. This is in agreement with previous findings, Gonzales-Maeso *et al.* (2003) having shown receptor internalization to underlie functional desensitization of the $GABA_B$ receptor in a recombinant cell line similar to ours. Also, our inability to observe a decrease in receptor number at the cell surface in the cultured cortical neurons upon desensitization is in line with a report by Perroy *et al.* (2003). These differences might reflect distinctive processes of desensitization in the two examined systems. Several diverse mechanisms of $GABA_B$ receptor desensitization have been proposed in the literature. So far it has been undisputed that it does not involve β-arrestin recruitment, which is a part of desensitization pathways for most GPCRs. Numerous mechanisms of $GABA_B$ receptor desensitization have, however, been proposed: degradation

of the receptors at the cell surface (Fairfax *et al.* 2004), regulation of G-protein-receptor coupling by endogenous regulator of G-protein signaling proteins (Mutneja *et al.* 2005), internalization (Gonzales-Maeso *et al.* 2003; Laffray *et al.* 2007), phosphorylation-independent G-protein receptor kinase 4 regulation without changes in the receptor number (Perroy *et al.* 2003), protein kinase A-mediated phosphorylation as stabilization of the receptor at the cell surface (Couve *et al.* 2002) and a co-regulation mechanism by NEM sensitive factor and protein kinase C (Pontier *et al.* 2006). It is possible that the varying expression levels of intracellular regulatory proteins (that are a part of the "desensitization machinery") in the two types of cells examined in this study give rise to the abovementioned differences.

The main finding in this study *i.e.* that activation involving allosteric modulation by GS39783 enhances $GABA_B$ receptor signaling but does not promote desensitization, is reminiscent of agonist directed trafficking, a phenomenon described for various GPCRs, which involves the ability of different agonists activating different transduction pathways by acting upon the same receptor (reviewed in Urban *et al.* 2007). The prolonged presence of an orthosteric agonist both stimulates the receptor and promotes receptor desensitization. However, the change in the receptor conformation induced by the binding of an allosteric modulator apparently enhances only the signaling of the $GABA_B$ receptor, but does not lead to receptor desensitization.

To conclude, in this study we have shown that, according to the expectations, the positive allosteric modulator GS39783 has no propensity for producing desensitization of the $GABA_B$ receptor, a key mechanism that underlies tolerance. To our knowledge, this is the first study demonstrating that it is indeed possible to enhance receptor function with allosteric modulation, to the same extent as with agonists alone, without inducing receptor desensitization. This confirms that positive allosteric modulation as a therapeutic principle may be advantageous over classical receptor activation by agonists.

Acknowledgements

5.3. Changes in behavior of allosteric and orthosteric GABA$_B$ receptor ligands after a continuous agonist pretreatment

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5.3.1. Abstract

It has been estimated that only 15% of the compounds classified as silent GPCR antagonists are indeed devoid of either positive or negative intrinsic efficacy. Considering that 40% of all drugs on the market target GPCRs mainly as orthosteric ligands, elucidating their true nature is becoming increasingly important. While agonism can be demonstrated using appropriately sensitive experimental setups, the detection of inverse agonism can be limited by a low degree of constitutive activity in many assay systems.

In this study, changes in ligand behavior upon a lasting pretreatment with GABA, that induced receptor desensitization, were observed, measuring the second messenger cAMP in a $GABA_B$ receptor-expressing recombinant cell line. The $GABA_B$ partial agonist 2-OH-saclofen lost its ability to inhibit 7β-forskolin-induced cAMP production upon GABA-pretreatment. The "silent" antagonists CGP62349, CGP52432, CGP56999 and SCH50911, on the other hand, stimulated 7β-forskolin-induced cAMP production under these conditions. The inverse agonism of CGP56999 was inhibited by the efficacy-deficient 2-OH-saclofen, proving it was truly mediated through the orthosteric site of the $GABA_B$ receptor. Finally, the positive allosteric modulator GS39783, which previously only marginally inhibited cAMP production, suppressed it by 60% both alone and in the presence of the competitive antagonist 2-OH-saclofen, thus GS39783 became an allosteric agonist at desensitized $GABA_B$ receptors. These changes likely reflect adaptations in the mechanisms of $GABA_B$ receptor desensitization and may be important in the elucidation of intrinsic ligand efficacies as well as for the consequences of chronic drug treatment.

5.3.2. Introduction

Chronic drug treatment often brings about not only receptor desensitization (the main cause of tolerance), but also other adaptations and changes as a response to it. In this study, we report apparent changes in efficacies of $GABA_B$ receptor ligands after a lasting desensitizing pretreatment with a saturating concentration of GABA.

According to the two-state model of receptor activation (reviewed by Leff 1995), it is assumed that a given receptor exists in at least two interchangeable conformations: the resting (R) and activated (R*) state. The equilibrium between the two states can be described with an equilibrium constant L. Spontaneous conversion from R to R* without agonist binding is the basis for constitutive receptor activity, also known as basal or ligand-independent signaling, a concept that was first proposed by (Costa and Herz 1989). An agonist has a high preferential affinity for the activated state R* and upon binding to the receptor shifts the equilibrium toward it. Partial agonists do the same, only to a lesser extent (due to a smaller intrinsic efficacy). On the other hand, compounds with inverse agonistic properties favor binding to the inactive state of the receptor, thus pushing the $R \leftrightarrow R^*$ equilibrium toward the inactive state R. The prerequisite for detecting inverse agonism is a certain degree of constitutive receptor activity. Neutral (or silent) antagonists do not distinguish R from R* and therefore do not affect the equilibrium between the resting and the active state. It is important to note that the display of inverse agonism is conditional and not a molecular property in itself. Although there is a molecular mechanism that underlies inverse agonism, not observing it experimentally does not mean that the examined compound does not have negative efficacy. Instead, it is often the case that the experimental system used for elucidating the "true" valence of a ligand lacks constitutive activity, precluding the detection of negative efficacy.

Constitutively active mutant receptors (CAMs) mimic, at least to some extent, conformational changes induced by agonist binding to a WT receptor. It is suggested, that a structural "constraint" keeps WT receptors in the resting state in the absence of an agonist, whereas in CAMs a mutation releases such a constraint and brings about receptor activation. These mutations in CAMs can be either artificially inserted or they can occur spontaneously in diseases (Seifert and Wenzel-Seifert 2002). Generation of CAMs has been shown to be very useful in detecting the negative efficacy of many inverse agonists (for reviews see Seifert and Wenzel-Seifert 2002; Cotecchia *et al.* 2003). It has been estimated that only 15% of all the compounds usually considered silent antagonists are indeed silent, the other ones having either a low partial agonistic or inverse agonistic properties (Strange 2002; Milligan 2003; Kenakin 2004).

Considering a large number of clinically used inverse agonists acting upon GPCRs, a broad pharmacotherapeutic relevance of inverse agonism is implicated, including the treatment of hypertension, heart failure, type I allergies, opiate overdose, depression and schizophrenia (see Seifert and Wenzel-Seifert 2002 for a review). For example, it is known that true silent antagonists of μ-opioid receptors produce fewer adverse effects such as withdrawal symptoms, compared to inverse agonists (Sadee *et al.* 2005). In similar lines, atypical antipsychotic agents, which mostly act as inverse agonists on serotonin 5-HT_{2C} and $5\text{-HT}_{1A/B/D}$, are generally considered to be more effective in treating symptoms of schizophrenia than the typical antipsychotic drugs, that are mainly found to be neutral antagonists (Greasley and Clapham 2006).

For these reasons and since the intrinsic efficacies of ligands are conditional, it is important to test them in adequate experimental settings. In this study, we report changes in ligand behavior after persistent activation of the $GABA_B$ receptor with GABA, which induced desensitization. Measuring cAMP, the second messenger of $GABA_B$ receptor activation, in a

recombinant cell line, we have observed compounds that were previously believed to be silent (or neutral) antagonists having inverse agonistic properties, a partial agonist became a silent antagonist and the positive allosteric modulator GS39783, that was previously shown to only marginally inhibit cAMP accumulation (Urwyler, Gjoni *et al.* 2005), significantly decreased cAMP in a manner of an allosteric agonist. Such changes are of potential importance because of functional consequences following a continuous drug treatment.

5.3.3. Method description

Materials: GS39783, CGP56999, CGP52432, CGP54626, SCH50911 and 2-OH-saclofen were available in-house. CGP56999, CGP52432, CGP54626, SCH50911 and 2-OH-saclofen were freshly dissolved in the assay buffer before each experiment. The stock solution of GS39783 was made up in DMSO and was further diluted in the assay buffer.

Adenylyl cyclase assays in a recombinant cell line stably expressing GABA$_B$ receptors: A stable CHO cell line co-expressing human GABA$_{B(1b)}$ together with rat GABA$_{B(2)}$ (Urwyler *et al.* 2001) was cultured in Dulbecco's modified eagle medium (DMEM, glutamine-free, Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum, 20 μg/ml L-proline, 500 μM glutamine, 1 mg/ml geneticin, 250 μg/ml zeocin. The cells were grown to 80–90% confluency in 15 cm cell culture dishes. Prior to the cAMP measurement, they were treated with 100 μM GABA diluted in the cell growth medium for 2 hours. After the pretreatment, cells were washed twice with PBS. The cAMP accumulation experiment was then performed as has been described previously (see Sections 5.1. and 5.2. for details).

5.3.4. Results

The partial agonist 2-OH-saclofen becomes a silent antagonist at desensitized GABA$_B$ receptors. It was shown previously that the GABA$_B$ receptor desensitized after a 2h incubation with a saturating concentration of GABA (see Gonzales-Maeso *et al.* 2003 and
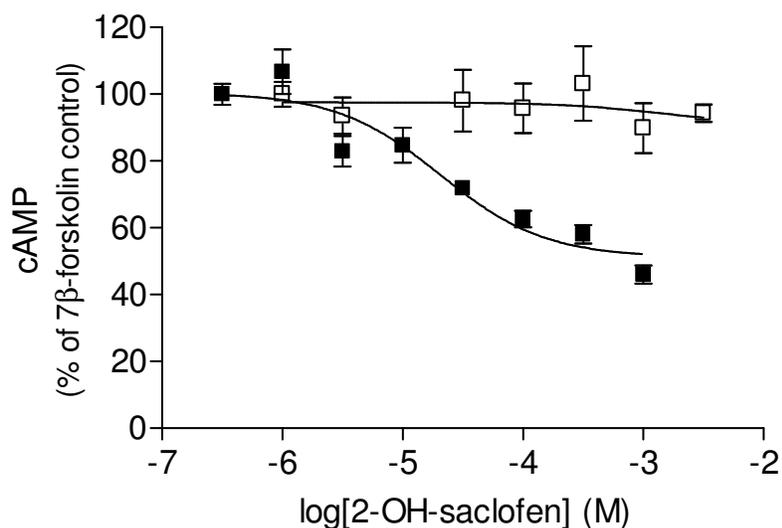

Fig. 1. The partial agonist 2-OH-saclofen loses its efficacy at desensitized GABA$_B$ receptors. Measurement of the inhibition of 7β-forskolin-stimulated cAMP formation in recombinant cells stably expressing the GABA$_B$ receptor: a comparison between control (no pretreatment, filled squares) and desensitized conditions (2h pretreatment with 100 μM GABA, open squares). Data on the graph represent mean ± SEM (quadruplicates) of a typical experiment that was performed three times.

Section 5.2.), which was apparent as a decrease in agonist potency to inhibit 7β-forskolin-induced cAMP production as well as its maximal inhibitory effect. In this assay system 2-OH-saclofen was found to be a partial agonist *i.e.* it inhibited 7β-forskolin-stimulated cAMP production to a maximal extent that was less than the one obtained by GABA (Urwyler, Gjoni *et al.* 2005). However, upon GABA$_B$ receptor desensitization, induced with a two-hour

pretreatment with a saturating concentration of GABA, the compound completely lost its

ability to inhibit production of cAMP, suggesting that it became a silent antagonist (Fig. 1).

The positive allosteric modulator GS39738 becomes an allosteric agonist in a

desensitized recombinant system. Interestingly, the intrinsic efficacy of the allosteric

modulator GS39783 increased after $GABA_B$ receptor desensitization, *i.e.* the compound

inhibited cAMP production on its own (Fig. 2). The pEC_{50} of GS39783 was found to be 5.88

$\pm$ 0.11 (mean $\pm$ SEM, N=8), which is in alignment with the previously measured pEC_{50} of
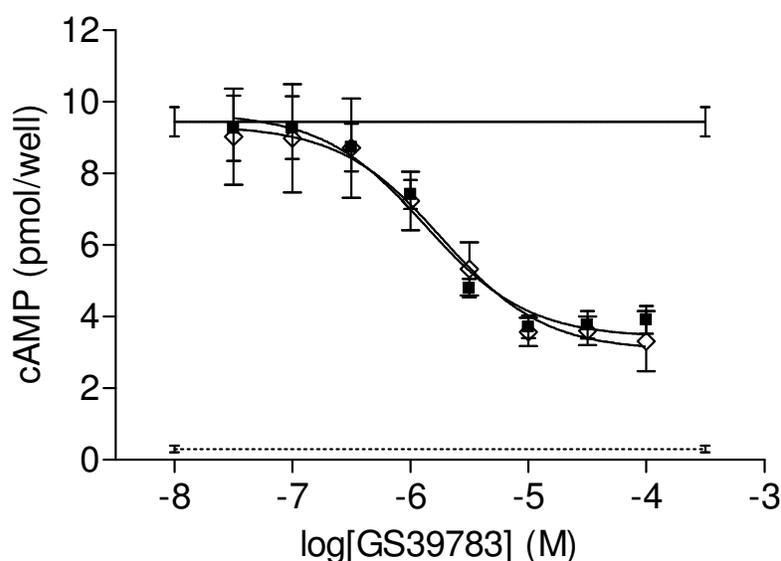
Fig. 2. The positive allosteric modulator GS39783 acts like an allosteric agonist upon desensitized $GABA_B$ receptors (2h pretreatment with 100 μM GABA). 2-OH-saclofen has no effect on the concentration-response curve of GS39783. Measurement of the inhibition of 7β-forskolin-stimulated cAMP formation in recombinant cells stably expressing the $GABA_B$ receptor. Filled squares: GS39783 alone, open diamonds: GS39783 + 100 μM 2-OH-saclofen, filled line: 100 μM 7β-forskolin control, dotted line: basal cAMP level. Data on the graph represent mean $\pm$ SEM (quadruplicates) of a typical experiment that was repeated four times.

GS39783 as a positive modulator under normal conditions (Urwyler *et al.* 2003). Unlike in non-pretreated cells, where GS39783 only slightly inhibited 7β-forskolin-stimulated cAMP production (Urwyler, Gjoni *et al.* 2005), in desensitized cells GS39783 maximally inhibited cAMP production by 60 ± 4.7 % (mean $\pm$ SEM, N=8).

To make sure that the increase in efficacy of GS39783 was not a result of an effect of GABA left from the pretreatment due to insufficient washing, the concentration-response curve of GS39783 was measured in the presence of 100 μM 2-OH-saclofen, which could now be used as a (silent) competitive antagonist under these conditions (see above). This concentration of 2-OH-saclofen is close to saturating (Urwyler, Gjoni *et al.* 2005) and sufficient to antagonize potential residues of GABA in the assay buffer. The concentration-response curve of GS39783 in desensitized cells was the same in the presence and the absence of 2-OH-saclofen, confirming that the phenomenon of increased efficacy of GS39783 was indeed due to the compound only and demonstrating that it was not mediated *via* the orthosteric binding site (Fig. 2).

Compounds known as silent antagonists become inverse agonists at desensitized GABA$_B$ receptors. The compounds CGP56999, CGP62349, CGP52432, CGP54626 and SCH50911, that were previously shown to be either devoid of any intrinsic efficacy or, in the case of CGP52432, to marginally increase cAMP stimulation in the same recombinant cell line that was used in this study (Urwyler, Gjoni *et al.* 2005), acted as inverse agonists upon a continuing exposure to GABA leading to GABA$_B$ receptor desensitization (Fig. 3). The potencies of these compounds and their maximal increase of cAMP stimulation are given in Table 1. The inverse agonistic efficacy of CGP56999 was antagonized with increasing concentrations of 2-OH-saclofen (Fig. 4), which proves that the effects were mediated *via* its binding to the orthosteric site of the GABA$_B$ receptor.
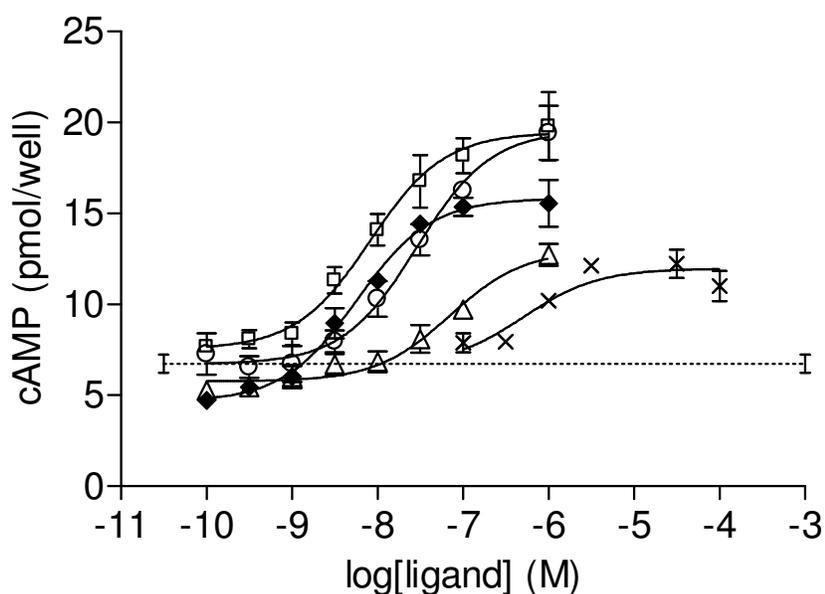
**Fig. 3.** CGP56999 (open squares), CGP62349 (filled diamonds), CGP52432 (open triangles) and CGP54626 (open circles) act as inverse agonists upon desensitized $GABA_B$ receptors (2h pretreatment with 100 μM GABA). cAMP measurement in a stable cell line expressing the $GABA_B$ receptor. Dotted line: 100 μM 7β-forskolin control. Data on the graph represent mean ± SEM (quadruplicates) of typical curves done at least three times; see Table 1 for potencies and maximal effects.

ligand	pEC_{50}	EC_{50}	maximal effect (% of 7β-forskolin control)	N
CGP56999	8.40 ± 0.09	4.0 nM	161 ± 24	9
CGP54626	8.43 ± 0.48	3.7 nM	259 ± 41	3
CGP62349	8.41 ± 0.17	3.9 nM	279 ± 51	3
CGP52432	7.14 ± 0.01	72 nM	216 ± 26	3
SCH50911	6.01 ± 0.17	977nM	208 ± 28	3

Table 1. The silent antagonists CGP56999, CGP54626, CGP62349 and CGP52432 behaved like inverse agonists in the desensitized $GABA_B$ stable cell line potentiating 7β-forskolin-stimulated cAMP production in desensitized recombinant cells stably expressing the $GABA_B$ receptor. Desensitization was induced by a two-hour pretreatment with 100 μM GABA. Typical curves are shown in Fig. 3.
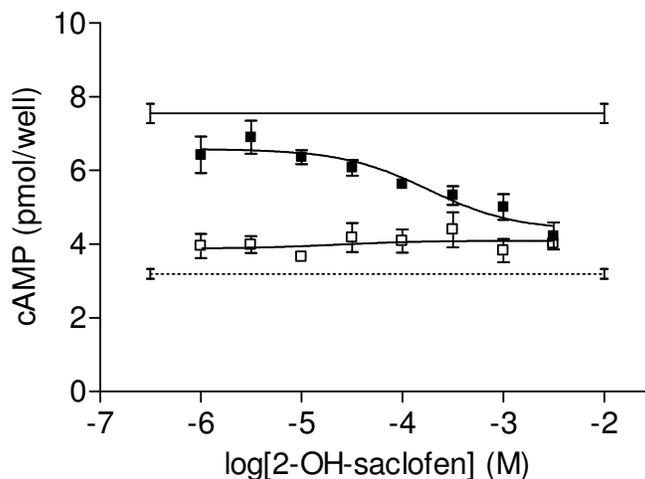
Fig. 4. 2-OH-saclofen, which is devoid of intrinsic efficacy at desensitized receptors (open squares), antagonizes the inverse agonistic effect of 3 nM CGP56999 (filled squares). cAMP measurement in a recombinant cell line stably expressing the GABA$_B$ receptor. Desensitization was induced by a two-hour pretreatment with 100 μM GABA. Dotted line: 7β-forskolin control (100 μM); full line: 7β-forskolin 100 μM + 3 nM CGP56999. Data on the graph represent mean ± SEM (quadruplicates) of a typical experiment repeated three times.

5.3.5. Discussion

In this study, we report changes in ligand behavior of different GABA$_B$ receptor ligands upon persistent agonist treatment leading to receptor desensitization (Gonzales-Maeso *et al.* 2003 and see Section 5.2.). We demonstrate that the compounds CGP62349, CGP52432, CGP56999, CGP54626 and SCH50911, originally considered as silent (or neutral) antagonists at the GABA$_B$ receptor, became inverse agonists, *i.e.* they stimulated cAMP production. 2-OH-saclofen, a partial agonist in this assay system, lost its intrinsic efficacy and became a silent antagonist. Moreover, the positive allosteric modulator GS39783, which was previously shown to only slightly decrease cAMP on its own (Urwyler, Gjoni *et al.* 2005), significantly inhibited cAMP production in a manner of an allosteric agonist.

There were other reports in which silent $GABA_B$ receptor antagonists were claimed to be inverse agonists. Grünewald *et al.* (2003) have found that CGP54626 and SCH50911 increased cAMP formation and Hirst *et al.* (2003) have seen it with CGP54626, SCH50911 and CGP62349. In a recently published publication on constitutively active $GABA_B$ receptor mutants inverse agonistic properties were observed for CGP54626, CGP52432 and CGP55845 (Mukherjee *et al.* 2006). In addition, we have previously observed a trend toward inverse agonistic activity for CGP52432 in the same recombinant cell line, that was used in the present study, but under non-desensitized conditions (Urwyler, Gjoni *et al.* 2005).

To prove that the increase in cAMP production in the presence of these compounds was a specific $GABA_B$-mediated phenomenon, we took advantage of the loss of efficacy of 2-OH-saclofen in desensitized cells (Fig. 1) and used it as a neutral antagonist to bring the CGP56999-stimulated increase of cAMP back to the level of 7β-forskolin control (Fig. 4). According to the measured potencies of inverse agonists (see Table 1), all the compounds were weaker when compared to their binding affinities in control (non pretreated) membranes (Urwyler, Gjoni *et al.* 2005). This is suggestive of an increase in constitutive receptor activity in the sense that, according to the two-state model (Leff 1995), inverse agonists stabilize and prefer binding to the inactive receptor conformation, while agonists favor the activated receptor. Similar trends, with apparent lowering of inverse agonist potencies in response to an elevation of the degree of constitutive activity of the receptor, were also observed in reports mentioned above by Grünewald *et al.* (2003) and Mukherjee *et al.* (2006). An additional factor contributing to a decrease in inverse agonist potency might also be the decrease in receptor density (loss of receptor reserve) concomitant with receptor desensitization (Gonzales-Maeso *et al.* 2003 and see Section 5.2.).

The detection of inverse agonism is possible only in an experimental system with a sufficient degree of constitutive activity (ligand-independent receptor activation). Apparently, upon a

continuous agonist treatment (100 μM GABA, 2h), which has previously been demonstrated to lead to $GABA_B$ receptor desensitization (see Gonzales-Maeso *et al.* 2003 and Section 5.2.), a phenomenon reminiscent of an increase in constitutive activity occurs, that enables detecting inverse agonistic properties of the abovementioned ligands. It seems likely that the increased constitutive activity reflects a change in receptor conformation after a prolonged agonist treatment. This finding is in concordance with a report on a constitutively active $GABA_B$ receptor mutant (Mukherjee *et al.* 2006), where it was found that a mutation of a single amino-acid residue, either near the binding pocket for orthosteric ligands on $GABA_{B(1)}$ subunit or near the binding site for the G-protein on $GABA_{B(2)}$ subunit, increased the constitutive activity of the receptor.

Comparable effects of prolonged agonist treatments increasing constitutive receptor activity were reported for β_2-adrenergic (Chidiac *et al.* 1996) and μ-, δ- and κ-opioid receptors (Liu and Prather 2001 and 2002; Wang *et al.* 2000 and 2007).

Research on the μ-opioid receptor (MOR) revealed changes in ligand behavior upon receptor desensitization similar to ours, namely a loss of maximal efficacy of agonists upon morphine and DAMGO-induced desensitization. At the same time, the authors noted a more pronounced negative efficacy of inverse agonists (Wang *et al.* 2007). These effects were explained by interactions of the receptor with calmodulin (CaM). Apparently, CaM and the G-protein both bind to the intracellular loop 3 (i3) of MOR (Wang *et al.* 1999). In the absence of an agonist, CaM is predominantly bound to i3, sterically hindering MOR-G-protein coupling, which keeps the receptor in an inactive (resting) state. Upon activation, however, and especially upon MOR desensitization, CaM dissociates from the receptor, enabling a more efficient receptor-G-protein coupling. This results in an enhancement of the constitutive activity of the receptor (Wang *et al.* 2000). This phenomenon, an increase of basal receptor signaling after prolonged exposure to agonists, was also proposed to be one of the

mechanisms responsible for development of opioid dependence (Wang *et al.* 1994; Wang *et al.* 2001).

There is no evidence of the GABA$_B$ receptor binding to CaM. However, a similar mechanism as described above for MOR might be involved with other intracellular proteins interacting with the GABA$_B$ receptor (Bettler *et al.* 2004). On the other hand, it can be speculated that regulator of G-protein signaling (RGS) proteins, that were shown to play a role in determining the level of constitutive activity for other receptors, *e.g.* the 5-hydroxytryptamine receptors type 1A and 2A (Welsby *et al.* 2002; Ghavami *et al.* 2004, reviewed in Milligan 2003), might also play a role in the regulation of the GABA$_B$ receptor function. RGS proteins are GTPase activating proteins (GAPs). They increase the GTPase activity of the Gα subunit, *i.e.* they accelerate hydrolysis of Gα-GTP upon GPCR activation and thus act as a "switch" that controls the intensity, duration and specificity of the G-protein-mediated signal, also keeping the basal receptor activity low (see De Vries *et al.* 2000 for review). GAP activity of most RGS proteins is inhibited by phosphatydilinositol-3,4,5-triphosphate (PIP$_3$) (Popov *et al.* 2000). Another mechanism inhibiting the GTPase activity of RGS proteins involves a scaffolding protein 14-3-3 (Benzing *et al.* 2000), a protein that also binds to the C-terminal part of the GABA$_{B(1)}$ receptor subunit (Couve *et al.* 2001). In analogy to PIP$_3$-dependent inhibition of RGS GAP activity, the inhibition by 14-3-3 could also result in an increase of basal receptor signaling. Findings by Grünewald *et al.* (2003), indicating that the coiled-coil domain of the GABA$_B$ receptor is crucial for maintaining an active receptor conformation in the absence of an agonist, are in line with the speculation that RGS proteins might play a role in determining constitutive activity of the GABA$_B$ receptor.

The loss of efficacy of 2-OH-saclofen to inhibit 7β-forskolin-stimulated cAMP accumulation is also in line with the increase of constitutive activity following desensitization. In this case, the "window" for detecting partial agonism becomes smaller, while the one for inverse

agonism broadens (Fig. 5). The proportion of receptors in the inactive (R) and the activated

state (R*) determines the width of the "window" between the bottom and the ceiling for

detecting agonism and inverse agonism, respectively (reviewed in Milligan and Bond 1997;

Negus 2006).

The allosteric modulator GS39783 became an allosteric agonist at the desensitized $GABA_B$

receptor, *i.e.* it significantly inhibited 7β-forskolin-stimulated cAMP formation. This

inhibition was shown to be an effect mediated *via* the allosteric binding site: it was not

antagonized in the presence of 2-OH-saclofen, which could be used as a silent competitive
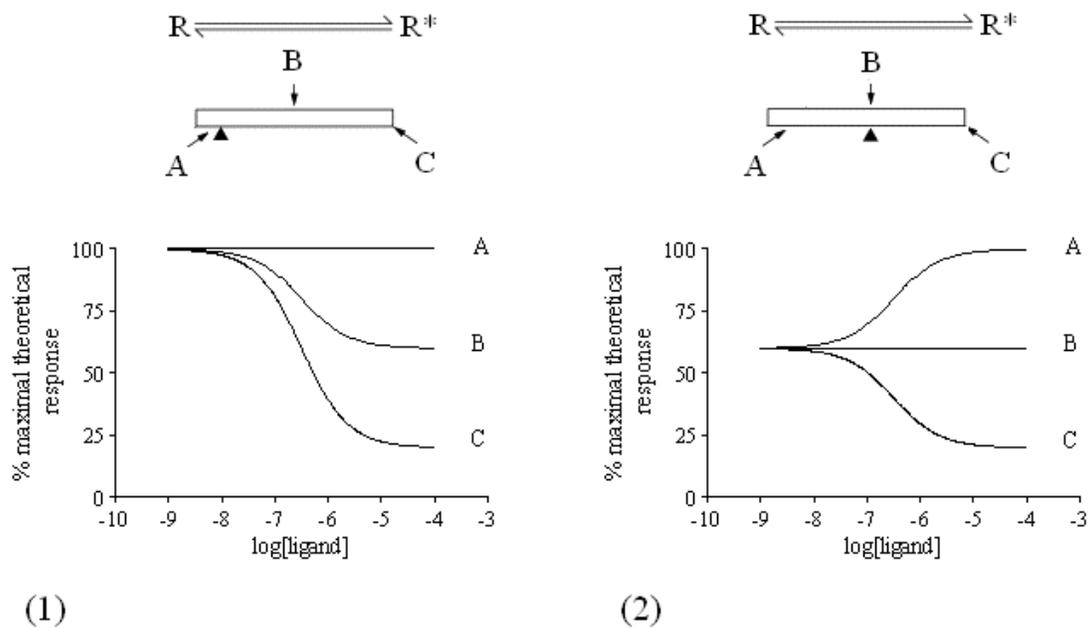
Fig. 5. The window-shift for the detection of agonism, antagonism and inverse agonism upon a lasting agonist treatment is reminiscent of an increase of constitutive receptor activity. Silent antagonists become inverse agonists (A), partial agonists lose their efficacy to become silent antagonists (B) and full agonists lose their efficacy to become partial agonists (C) upon a lasting agonist treatment. Panel (1): control condition (low constitutive receptor activity); panel (2): after a lasting agonist pretreatment (higher constitutive receptor activity). Adapted from (Milligan and Bond 1997) and (Negus 2006), with permission from Elsevier.

antagonist tool under these conditions. It should be noted that the other "silent" antagonists, which had inverse agonistic effects, could not be used for that purpose because their stimulatory effects on cAMP production would have been confounding. This shows the importance of choosing adequate tools for addressing such situations.

The allosteric two-state model allows activation of the receptor by the binding of an allosteric agent alone (Hall 2000). This can be detected in cases of high intrinsic efficacy of the allosteric agent, for example the activation of the mGluR7 by its allosteric agonist AMN082 in its own right (Mitsukawa *et al.* 2005). Another possibility for detecting receptor activation by an allosteric agent alone is using very sensitive experimental systems, *e.g.* due to a high degree of receptor reserve, in which even effects of ligands with low intrinsic efficacies can be discerned. For instance, the other positive allosteric modulator of the GABA$_B$ receptor CGP7930 (Urwyler *et al.* 2001), that has similar *in vitro* activity as GS39783, was found to significantly activate the receptor in the absence of an agonist in a system with a seemingly high receptor reserve (IP turnover) (Binet *et al.* 2004), while its inhibition of cAMP formation was only minor in our hands (Urwyler, Gjoni *et al.* 2005).

However, the fact that GS39783 in this study significantly inhibited cAMP production (by 60%), whereas it did so only marginally under non-desensitized conditions in the same assay system (Urwyler, Gjoni *et al.* 2005), requires an additional explanation. According to a model for the functioning of family 3 GPCRs proposed by Parmentier *et al.* (2002), GPCRs are "allosteric machines" that consist of two parts: the VFTM (that comprises the orthosteric binding site) and the 7TM (that usually contains the allosteric binding site). The two domains can both adopt either inactive or active conformations, allosterically interacting with each other. The adoption of the active state by the VFTM (for instance due to agonist binding) will increase the possibility for the 7TM to also assume the active conformation, leading to G-protein stimulation. These allosteric interactions between domains can either be "loose", as in

the case of mGluRs, or "tight", as in the case of the GABA$_B$ receptor, possibly due to the lack of the cysteine-rich region between the VFTM and the 7TM.

It was recently shown, in a study on deletion mutants of the mGluR5 lacking the VFTM that the positive allosteric modulator 3,3'-difluorobenzaldazine (DFB) directly activated the 7TM as an allosteric agonist. As DFB is usually devoid of intrinsic efficacy, the fact that it displayed allosteric agonistic properties was explained by the loss of the allosteric inhibition of 7TM by the non agonist-occupied VFTM, present at the WT receptor (Goudet *et al.* 2004).

It can be speculated that, in our case, the long-term activation by GABA disrupted or weakened allosteric interactions between the orthosteric and the allosteric binding sites, allowing GS39783 to have an effect in its own right and activate the receptor directly *via* the 7TM. In line with this assumption is a recent finding by (Dupuis *et al.* 2006), where it is shown that GS39783 is able to activate a point-mutated GABA$_{B(2)}$ subunit alone, by binding to a site in its 7TM.

In conclusion, this is the first demonstration of apparent changes in behavior of both orthosteric and allosteric GABA$_B$ receptor ligands upon continuous agonist treatment, most likely due to conformational changes and an increase of constitutive activity of the GABA$_B$ receptor. Although the mechanism responsible for these actions needs to be clarified, this assay system can be useful for observing properties of GABA$_B$ receptor ligands under conditions mimicking a chronic drug treatment *in vitro*.

5.4. The positive allosteric modulator GS39783 enhances GABA$_B$ receptor-mediated inhibition of cyclic AMP formation in rat striatum *in vivo*

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5.4.1. Abstract

In this work we studied effects of the positive allosteric modulator GS39783 on $GABA_B$ receptors at a biochemical level *in vivo.* Changes in extracellular levels of cAMP following $GABA_B$ receptor activation were monitored in the striatum of freely-moving rats using microdialysis. The locally applied $GABA_B$ agonist R(-)-baclofen concentration-dependently inhibited cAMP formation stimulated by a water-soluble forskolin analog ($EC_{50} = 7.3$ μM, maximal inhibition = 40%). The selective $GABA_B$ antagonist CGP56999 reversed R(-)-baclofen-induced cAMP inhibition to control levels, but not higher. Orally applied GS39783 lacked effects on its own, but together with a threshold concentration of R(-)-baclofen (1 μM), it significantly and dose-dependently decreased cAMP formation. Effects of GS39783 were revoked with CGP56999 showing dependence on $GABA_B$ receptor activation and suggesting allosteric modulation as a mechanism of action *in vivo*. Administered with a maximally active R(-)-baclofen dose, GS39783 failed to further inhibit cAMP formation. The data obtained with CGP56999 and the lack of effect of GS39783 alone suggest there is no detectable endogenous activation of the $GABA_B$ receptors controlling cAMP formation in rat striatum. To our knowledge, these results provide the first biochemical demonstration of *in vivo* activity of a G-protein coupled receptor positive allosteric modulator.

Keywords: $GABA_B$ receptor, GS39783, R(-)-baclofen, allosteric modulation, cyclic AMP, *in vivo* microdialysis

Abbreviations used: aCSF, artificial cerebrospinal fluid; cAMP, cyclic AMP; 7β-forskolin, 7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl-forskolin; GPCR, G-protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; ns, not significant; RIA, radioimmunoassay; SPA, scintillation proximity assay.

5.4.2. Introduction

G protein-coupled receptors (GPCRs) comprise one of the largest protein superfamilies and the most diverse form of transmembrane signalling proteins. It has been estimated that 1% of the mammalian genome encodes GPCRs and about 450 of 950 predicted human GPCRs are expected to be receptors for endogenous ligands (Takeda *et al.* 2002). They are most important drug targets, as almost 40% of all current therapeutic agents, mostly identified in ligand-binding assays, act upon GPCRs in a competitive manner to the natural ligand, i.e. as orthosteric drugs (agonists and competitive antagonists) (Hopkins and Groom 2002; Maudsley *et al.* 2005). However, more recent concepts of drug action and modern drug discovery technologies have raised interest in molecules acting at GPCRs through other sites or other mechanisms (Rees *et al.* 2002; Brink *et al.* 2004).

Allosteric modulators are molecules that modify receptor function by binding to a site on a receptor that is distinct from the orthosteric site, which binds agonists and competitive antagonists. Positive modulators usually do not stimulate receptors by themselves, but synergistically enhance receptor activation produced by agonists. This means that they would act mainly in concert with physiological signalling in its temporal and spatial organization in vivo, i.e. act only when and where endogenous transmitter has been released. Therefore, positive allosteric modulators are expected to have a better side-effect profile than agonists which activate receptors independently of synaptic activity. They should also have less propensity for developing tolerance subsequent to desensitization, which is observed following continuous receptor activation with agonists. For these reasons, allosteric modulation of GPCRs as a therapeutic principle has been attracting considerable attention lately (Christopoulos and Kenakin 2002; Christopoulos 2002; Jensen and Spalding 2004; Soudijn *et al.* 2004).

GABA$_B$ receptors are metabotropic GABA receptors which belong to the class III GPCR group, together with the metabotropic glutamate receptors, the calcium-sensing receptor, and mammalian taste and odorant receptors (Pin *et al.* 2003). They directly couple negatively to adenylyl cyclase (Wojcik and Neff 1984) and modulate voltage-gated calcium channels and inwardly rectifying potassium channels (Lüscher *et al.* 1997). They are located both presynaptically and postsynaptically to inhibit neurotransmitter release and neuronal excitability, respectively (reviewed in Bowery *et al.* 2002).

The selective GABA$_B$ receptor agonist baclofen was introduced into clinical practice more than 30 years ago for treating spasticity, for example that originating from spinal injuries and multiple sclerosis. Its use as a therapeutic agent or a tool in preclinical studies is, however, limited on account of its side-effects, such as sedative/hypnotic effects and muscle atonia (a severe side-effect for potential indications other than spasticity), as well as the development of tolerance upon chronic treatment.

The first GABA$_B$ receptor-positive allosteric modulators, CGP7930 and GS39783, were discovered only recently (Urwyler *et al.* 2001 and 2003). They have been characterized extensively *in vitro* and were found to increase both the affinity and efficacy of agonists in many different assays (Urwyler *et al.* 2001 and 2003; Urwyler, Gjoni *et al.* 2005; Onali *et al.* 2003; Binet *et al.* 2004; Chen *et al.* 2005; Olianas *et al.* 2005). CGP7930 and GS39783 have also been tested in behavioural models in vivo in which, according to expectations, they lacked the side-effects seen with baclofen (Cryan *et al.* 2004). They were found to synergistically increase the sedative/hypnotic effects of baclofen (with no such effect of the modulator alone) (Carai *et al.* 2004), to have anxiolytic properties (Cryan *et al.* 2004; Mombereau *et al.* 2004) and to reduce cocaine self-administration in rats (Smith *et al.* 2004).

However, so far the enhancement of $GABA_B$ receptor function by allosteric drugs has not been demonstrated at a mechanistic (signal transduction) level *in vivo*. The activity of GPCRs coupled to G_i proteins can best be monitored by measuring the inhibition of cyclic AMP (cAMP) formation stimulated by forskolin, which directly activates the enzyme adenylyl cyclase. Hashimoto and Kuriyama (1997) have demonstrated that measuring extracellular cAMP levels in the striatum of freely moving rats by microdialysis is a suitable means of monitoring the activation of $GABA_B$ receptors *in vivo*. We have therefore chosen this approach to show, for the first time, the biochemical effects of the positive allosteric modulator GS39783 on $GABA_B$ receptors *in vivo*.

5.4.3. Materials and methods

All experiments complied with the Swiss law on animal experimentation and were approved by the relevant local authorities.

Chemicals: The sources of commercially obtained chemicals are given below. GS39783, R(–)-baclofen and CGP56999 were available in house. R(–)-baclofen and CGP56999 were dissolved and diluted in aCSF containing 3-isobutyl-1-methylxanthine (IBMX) freshly before each experiment. GS39783 was suspended in cremophor EL, propylenglycol and H_2O in a ratio of 15 : 10 : 75 (vehicle) to give a fine suspension after ultrasonication (20 min).

Surgery: Male Wistar rats (280–350 g) were anaesthetized with isoflurane inhalation (3–5% for induction and 0.5–1% for maintenance; Forene[®]; Abbott AG, Baar, Switzerland) using a mixture of N_2O and O_2 (1/3 and 2/3 respectively) (Carbagas, Basel, Switzerland). The anaesthetized animal's head was shaved and the animal was placed in a Kopf stereotaxic apparatus. A midline incision was made to expose the skull. The stereotaxic coordinates of the

caudate–putamen (striatum) were determined from a rat stereotaxic atlas (Paxinos and Watson 1986). All anterior–posterior and lateral measurements were made relative to bregma and the ventral measurements were taken from the dura surface (flat head, i.e. with bregma and lambda in the same horizontal plane). One home-made 4-mm probe was placed at $+0.2$ mm anterior, $+3.0$ mm lateral and -7.4 mm ventral, and secured in place with two skull screws and acrylic dental cement. Additionally, a long screw was fixed in the cement by which to later connect the animal to the freely moving microdialysis system. In preliminary experiments in separate animals, the coordinates were confirmed by histological verification of the probe placement. Microdialysis probes were constructed in a concentric design using 23-G stainless steel tubing (Coopers Needles Works, Birmingham, UK), vitreous silica tubing (Schmidlin AG, Neuheim, Switzerland) and Hospal AN69 hydrogel-type dialysis membrane (Hospal, Lyon, France) with an active length of 4 mm. They were implanted while perfused with artificial CSF (aCSF) (141 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.4 mM Na_2HPO_4, 0.25 mM NaH_2PO_4, pH 7.4) at a flow rate of 5 µL/min. After implantation, the perfusion was stopped, and the inlet and outlet arms were heat-sealed using plastic connectors. After surgery the rats were housed individually in plastic cages and allowed to recover for 24 h with free access to food and water, in a room with a 12 h–12 h dark–light cycle.

Experimental procedure: Some 24h after surgery, the conscious rat was connected to the microdialysis system allowing free movement. The probe was perfused with aCSF containing 1 mM of the phosphodiesterase inhibitor IBMX (Fluka, Buchs, Switzerland), at a flow rate of 2 µL/min. After a 1h waiting period, samples were collected every 20 min over 400 min (total of 20 fractions), and then immediately frozen in dry-ice and stored at $-80°C$ pending analysis.

We employed an experimental paradigm with two cAMP-increasing stimuli, consisting of local application (through the microdialysis probe) of 30 µM of the water-soluble forskolin analogue 7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl-forskolin dihydrochloride (7β-forskolin) (Calbiochem, VWR International, Luzern, Switzerland) over 10 min. The first (control) stimulus (S1), which was the same for all rats, was given at 80 min after the start of the baseline period. Subsequently, the perfusion was changed back to aCSF containing IBMX. The second stimulus (S2) was applied at 240 min (eight fractions later). S2 was either the same as S1 (control rats), or it served to measure effects of drugs on cAMP formation (treated rats). R(–)-baclofen and CGP56999 (10 or 30 µM) were applied locally through the microdialysis probe for 20 min before and for 10 min during S2. At 60 min before S2 (i.e. at 180 min), animals received either vehicle or GS39783 applied orally. After S2, the perfusion fluid was changed back to aCSF containing IBMX and seven more samples were collected. Animals were killed at the end of the experiment.

Quantification of cAMP levels: The amount of cAMP formed was quantified by radioimmunoassay (RIA) in a scintillation proximity assay (SPA) format in 96-well microtitre plates. A 10-µL sample was diluted with 25 µL aCSF containing IBMX and acetylated with 4 µL acetic acid anhydride and triethylamine (1 : 3) prepared shortly before use. Then, 87 µL immunoreagent solution was added to each sample. This solution contained 17 µL rabbit anti-succinyl cAMP serum (anti-cAMP (rabbit); Calbiochem) diluted 1 : 3000, 35 µL donkey anti-rabbit IgG secondary antibodies coated at the surface of SPA beads, diluted according to the instructions for use (SPA PVT antibody-binding beads, anti-rabbit; Amersham Biosciences, Little Chalfont, UK), and 35 µL adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I]iodotyrosine methyl ester as a tracer (approximately 185Bq; Amersham Biosciences). The plates were incubated at 20–25 °C for 15–20 h and then counted on a Wallac 1450

microbeta Trilux scintillation counter (Wallac Oy, Turku, Finland). Quantification of the cAMP formed was performed with the aid of an appropriate RIA cAMP standard curve.

Data analysis: The results were analysed with Prism 3.03 software (GraphPad Software Inc., San Diego, CA, USA). To account for possible drifts in baseline, linear regression of the baseline fractions was calculated using fractions 1–4, 11, 12, 19 and 20. Baseline values acquired in this fashion were deducted from all 20 fractions. The cAMP levels (in fmol) in fractions 5–10 and 13–18 were used to calculate the area under the curves for stimulations S1 and S2. S2 was divided by S1 to calculate the S2/S1 ratio. Using two cAMP-raising stimuli and calculating S2/S1 ratios enabled each animal to be its own control and thus compensated for interanimal variability, therefore increasing the accuracy of the method. The data obtained were expressed as mean ± SEM values and statistical analysis was performed using unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's test.

Recovery of cAMP in vitro: To determine the recovery of cAMP by microdialysis *in vitro*, the efficiencies of five microdialysis probes were tested at room temperature in a 0.5% methylcellulose bath containing 10 µM cAMP with constant aCSF perfusion at a flow rate of 2 µL/min for 2 h. Dialysate fractions were collected every 20 min and cAMP concentrations were quantified as described above. Under these experimental conditions, the mean ± SEM *in vitro* dialysis recovery of cAMP was 12.0 ± 2.1%. However, *in vivo* values were not corrected for the *in vitro* recovery of cAMP.

R(–)-baclofen probe recovery in vivo: In order to assess the R(–)-baclofen concentration that reached the extracellular striatal space, the *in vivo* retrodialysis recovery of R(–)-baclofen was determined for each experiment in which R(–)-baclofen (and no other drug) was applied, by measuring its concentration in aliquots of fraction 12. To this end, a GABA$_B$ radioligand binding assay using rat cortical membranes (described in detail in Urwyler *et al.* 2003) was

modified into a radioreceptor assay format. The assay mixture had a final volume of 100 µL. It contained 25 µL of the unknown sample or R(–)-baclofen standard, 1 nM [^{3}H]-CGP62349 (3.15TBq/mmol; American Radiolabeled Chemicals, St Louis, MO, USA) in 20 mM Tris-HCl buffer (pH 7.4, containing 118 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 5 mM D-glucose), rat cortical membranes (approximately 15 µg protein) and 1.5 mg wheat germ agglutinin-coated SPA beads (Amersham Biosciences). The samples were incubated for 90 min at room temperature, before being counted in a Wallac 1450 Microbeta liquid scintillation counter. R(–)-baclofen concentrations in unknown samples were determined from the inhibition of radioligand binding with the R(–)-baclofen standard curve. The *in vivo* retrodialysis recovery of R(–)-baclofen was calculated on the basis of the difference between the concentrations in the perfusate and fraction 12.

5.4.4. Results

Effects of R(–)-baclofen on cAMP formation. To assess the activation of the GABA$_B$ receptors *in vivo*, we established a concentration–response curve for the inhibition of 7β-forskolin-stimulated cAMP formation by the agonist R(–)-baclofen. Baseline cAMP levels amounted to 27.2 ± 1.8 fmol ($n = 94$) in the first fraction collected (the first 20 min). 7β-Forskolin (30 µM) applied through the microdialysis probe in the striatum induced peaks corresponding to an approximately 3-fold increase in cAMP levels. Control animals, which received two challenges of 30 µM 7β-forskolin through the probe and vehicle applied orally, elicited the same response for both S1 and S2, *i.e.* the S2/S1 ratio was 1 ± 0.05 ($n = 10$). Traces of cAMP efflux are shown in Fig. 1. Pre-infusion of R(–)-baclofen (1 µM to 2 mM) for 20 min and oral application of vehicle did not induce a change in the basal extracellular levels of cAMP in the striatum. However, co-application of R(–)-baclofen through the probe with
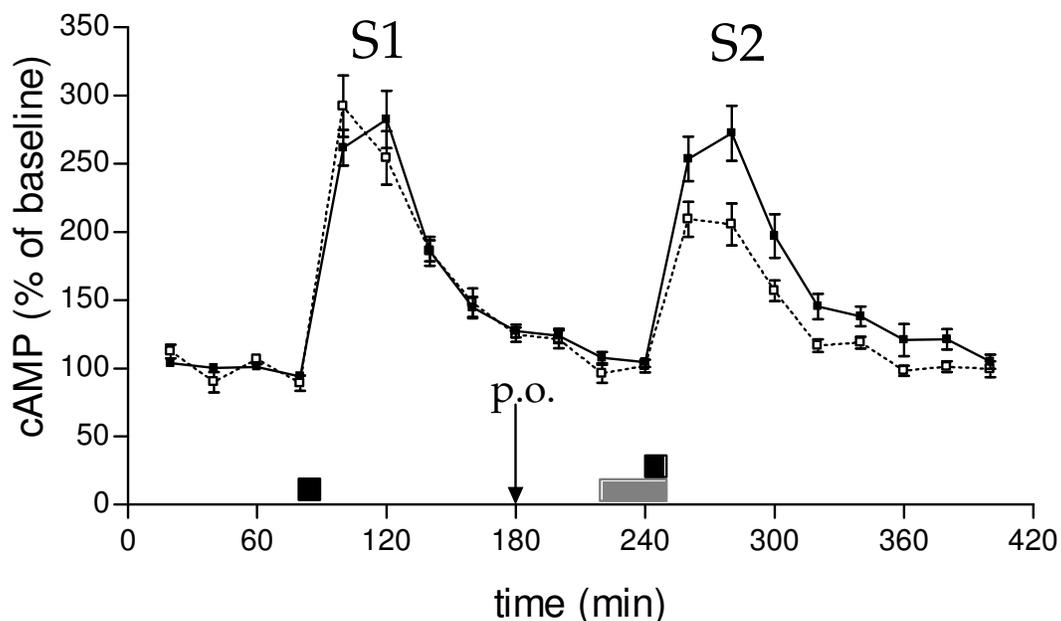
Fig. 1 Traces of extracellular cAMP efflux in rat striatum *in vivo*. Control rats (solid line), mean ± SEM S2/S1 ratio: 1 ± 0.05 (*n* = 10 animals). Effect of 500 µM R(–)-baclofen (dotted line), S2/S1 ratio: 0.61 ± 0.03 (*n* = 6 animals). 7β-Forskolin was perfused through the microdialysis probe twice for 10 min (S1 and S2, black bar) and *R*(–)-baclofen for 20 min before and 10 min during S2 (grey bar). The vehicle [used for oral (p.o.) application of GS39783 in other experiments] was administered at 180 min (arrow). To account for differences in basal values among animals, cAMP extracellular levels were expressed as a percentage of baseline (for the purpose of this graph only).

30 µM 7β-forskolin inhibited the 7β-forskolin-induced cAMP increase in a concentration-dependent manner (Figs 1 and 2). The maximal inhibition was about 40% and the EC_{50} was 7.3 µM (Fig. 2). This effect was antagonized by co-perfusion of 30 µM CGP56999 (Fig. 2, inset). The concentrations of R(–)-baclofen recovered in fraction 12 were 23.4 ± 1.8% (*n* = 27) of the applied concentrations, indicating that approximately 80% of the drug perfused entered the surrounding striatal tissue.
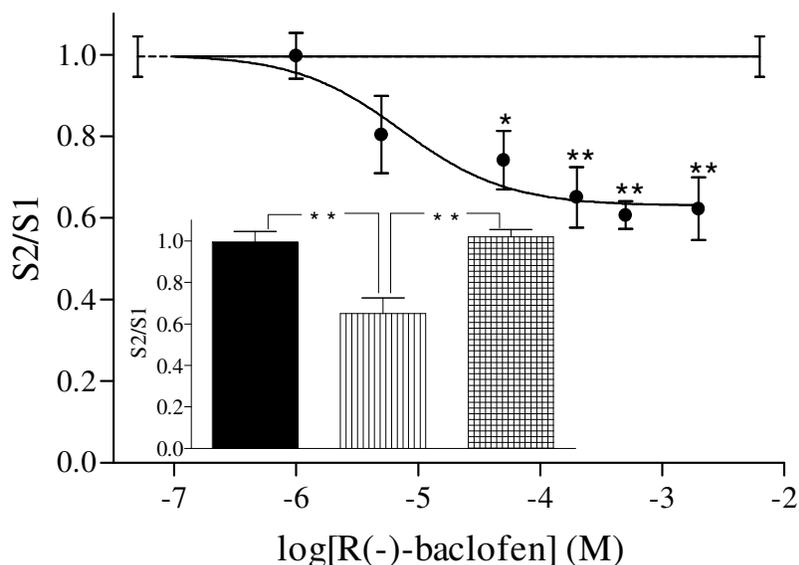
Fig. 2 Concentration–response curve showing effects of $R(–)$-baclofen on extracellular cAMP levels in rat striatum *in vivo*. $R(–)$-baclofen was perfused through the microdialysis probe for 20 min before and 10 min during S2. Data are mean ± SEM S2/S1 ratios obtained from 4–6 animals per treated group and 10 animals in the control group. *$p < 0.05$, **$p < 0.01$ versus control group (horizontal line) (one-way ANOVA followed by Dunnett's test). Inset shows effect of the GABA$_B$ antagonist CGP56999 on $R(–)$-baclofen-induced inhibition of cAMP formation in rat striatum (checkered bar). CGP56999 (30 μM) was co-infused with $R(–)$-baclofen (200 μM) for 30 min before and during S2. Black bar, control; striped bar, 200 μM $R(–)$-baclofen alone. Values are mean ± SEM S2/S1 ratios obtained from five animals per treated group. **$p < 0.01$ (unpaired two-tailed *t*-test).

Effect of GS39783. GS39783 applied orally at 100 mg/kg, without local R(–)-baclofen perfusion, failed to inhibit the 7β-forskolin-induced increase in cAMP levels (Fig. 3). However, when given at 10, 30 and 100 mg/kg together with a threshold concentration of R(–)-baclofen (1 μM) applied locally through the probe, GS39783 inhibited the extracellular cAMP levels in a dose-dependent fashion (significantly by 23% at the highest dose tested) (Fig. 4a). This effect was successfully antagonized by co-perfusion of CGP56999 (10 μM) (Fig 4a). When applied simultaneously with a maximally active concentration of R(–)-baclofen (500 μM), GS39783 was unable to further augment the maximal inhibition produced by R(–)-baclofen (Fig. 4b).
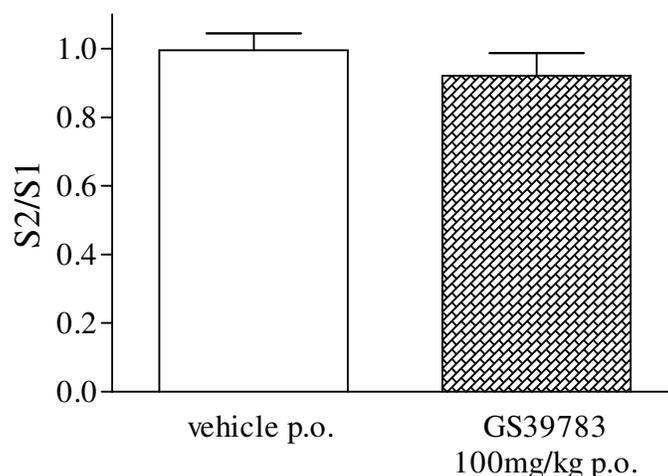
Fig. 3 Lack of effect of GS39783 (100 mg/kg p.o.) alone on 7β-forskolin-induced cAMP formation. Values are mean ± SEM of S2/S1 ratios in controls ($n = 10$) and animals treated with GS39783 100 mg/kg p.o. ($n = 6$). There was no significant difference between the groups (unpaired two-tailed t-test).

5.4.5. Discussion

Benzodiazepines, positive allosteric modulators of ionotropic GABA$_A$ receptors, have proven to be efficacious drugs in over 30 years of clinical use. On the other hand, positive allosteric modulators of metabotropic GABA$_B$ receptors have been discovered only recently (Urwyler *et al.* 2001 and 2003). For the reasons outlined in the introduction, such compounds might be of great therapeutic benefit in clinical indications such as pain, drug dependence and anxiety (Marshall 2000 and 2005; Vacher and Bettler 2003). In the present study we showed for the first time the enhancement of GABA$_B$ receptor function *in vivo* by a GABA$_B$ positive allosteric modulator (GS39783) at a biochemical level. The efflux of cAMP into the extracellular fluid in striatal tissue was monitored using microdialysis in freely moving rats (Hashimoto and Kuriyama 1997). GABA$_B$ receptors are well known to inhibit cAMP formation *via* coupling to G$_i$ proteins. Therefore, the cAMP levels in the

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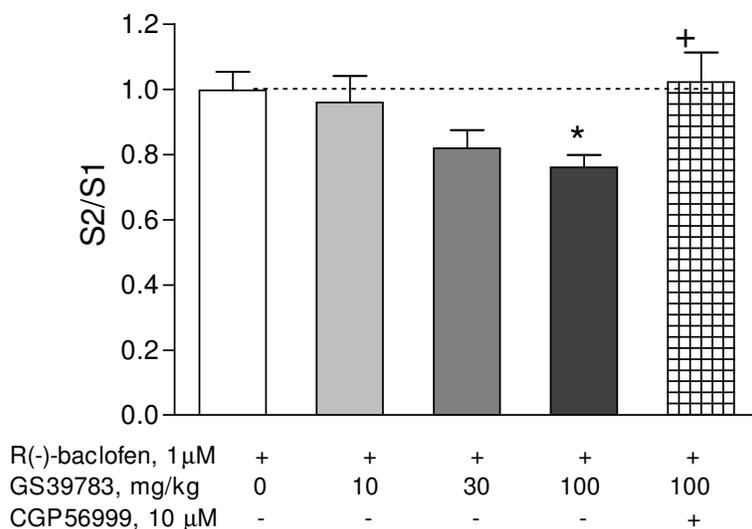
B
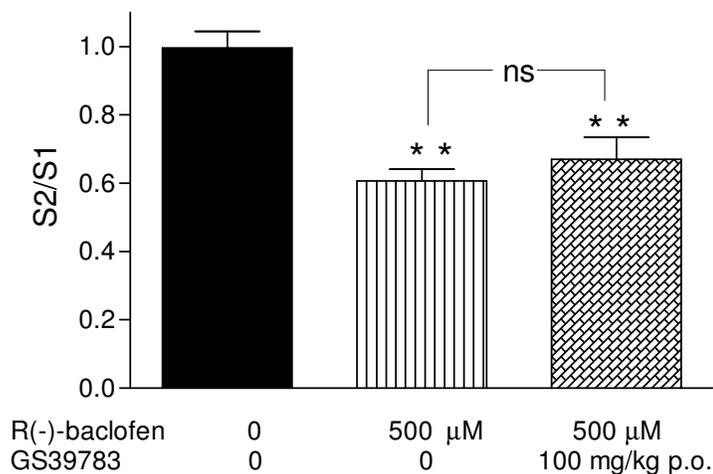
Fig. 4 (a) Effects of GS39783 (10, 30 and 100 mg/kg p.o.) combined with 1 µM*R*(–)-baclofen and antagonization with 10 µM CGP56999 (both applied through the microdialysis probe). Values are mean ±SEM S2/S1 ratios obtained from 5–7 animals per treatment. *$p < 0.05$ versus 1 µM R(–)-baclofen (one-way ANOVA followed by Dunnett's test); +$p < 0.05$ versus 1 µM*R*(–)-baclofen + 100 mg/kg GS39783 (unpaired two-tailed *t*-test). (b) Effects of GS39783 (100 mg/kg p.o.) combined with 500 µM*R*(–)-baclofen. The groups not receiving GS39783 were injected with vehicle. Values are mean ± SEM S2/S1 ratios obtained from five animals per treated group and 10 animals in the control group. **$p < 0.01$ versus control group (one-way ANOVA followed by Dunnett's test); ns, not significant.

extracellular striatal space should represent events of signal transduction that are close to the mechanism of receptor activation. Our experimental paradigm included two cAMP-increasing stimuli (Fig. 1), which enabled each animal to serve as its own control, thus taking into account interanimal variability. The finding that the S2/S1 ratio was exactly 1 in control animals showed that the cAMP-producing capacity of the striatal tissue was not diminished after the first stimulus.

The selective $GABA_B$ receptor agonist R(−)-baclofen significantly suppressed cAMP formation induced by the water-soluble forskolin analogue 7β-forskolin in a concentration-dependent manner (Fig. 2). The EC_{50} obtained for R(−)-baclofen was 7.3 μM, a nominal value corresponding to concentrations in the perfusate. However, in the light of the finding that about 80% of R(−)-baclofen applied through the probe penetrated into the tissue (*in vivo* retrodialysis recovery), this value is in agreement with numerous *in vitro* studies showing that baclofen activates $GABA_B$ receptors in the low micromolar range. Taking into account that Hashimoto and Kuriyama (1997) have used racemic baclofen, the concentration–response curve obtained with the active enantiomer of the drug (Fig. 2) is also in reasonable agreement with their findings. Moreover, in our experiments R(−)-baclofen maximally inhibited cAMP formation by 40% (Fig. 2), which is also in line with the approximately 50% maximal effect reported by Hashimoto and Kuriyama (1997). The effect of R(−)-baclofen, at a concentration that significantly inhibited cAMP formation, was entirely antagonized in the presence of the potent and selective $GABA_B$ antagonist CGP56999 (Fig. 2, inset), further confirming that the inhibition of cAMP production is $GABA_B$ receptor mediated.

To demonstrate modulation of $GABA_B$ receptors by GS39783, a threshold concentration of R(−)-baclofen (1 μM) was chosen, to give a sufficiently large window for detection of the effects of the orally co-applied modulator. Under these conditions, GS39783 significantly inhibited cAMP formation in a dose-dependent fashion (Fig. 4a). This effect was completely

antagonized by CGP56999 co-applied locally with R(–)-baclofen (Fig. 4a). Together with the finding that GS39783 did not inhibit cAMP formation on its own (Fig. 3) this is in line with *in vitro* data showing that the compound is not active in the absence of an agonist or when the agonist site is blocked by a competitive antagonist (Urwyler *et al.* 2003), and indicates an allosteric mechanism of action of the drug.

The positive allosteric modulator GS39783 has been shown previously to enhance not only the affinity of agonists *in vitro*, but also their maximal efficacy (Urwyler *et al.* 2003). To examine whether GS39783 augments the maximal inhibition of cAMP formation by R(–)-baclofen *in vivo*, we tested GS39783 in combination with a maximally active concentration of R(–)-baclofen (500 μM). However, under these conditions, GS39783 failed to further inhibit cAMP formation (Fig. 4b). The most likely interpretation is that production of cAMP in the striatum is under the control of $GABA_B$ receptors only in a limited number of cells, but in these the maximal inhibition is close to complete and therefore not amenable to further enhancement by a modulator. We have recently described a similar situation in a recombinant cell line (Urwyler, Gjoni *et al.* 2005).

Without R(–)-baclofen, GS39783 failed to inhibit cAMP formation at the highest oral dose used (Fig. 3). This finding strongly suggests that striatal $GABA_B$ receptors are not activated by endogenous GABA to any measurable extent. The fact that antagonism of R(–)-baclofen, alone or in combination with GS39783, by CGP56999 brought the S2/S1 ratio to the control level but not higher (Figs 2 and 4) confirms that cAMP production is not detectably inhibited by endogenous GABA in the rat striatum under control conditions. It seems unlikely that $GABA_B$ receptors exist without any activation by endogenous GABA; we therefore conclude that in our paradigm activation by endogenous GABA occurred at a low intermittent level that was not experimentally detectable. Earlier *in vivo* microdialysis studies also failed to detect activation by endogenous GABA of $GABA_B$ receptors controlling striatal acetylcholine

release (Anderson *et al.* 1993; DeBoer and Westerink 1994). On the other hand, physiological (endogenous) activation of GABA$_B$ receptors has been demonstrated in other brain regions, for example in the hippocampus (Moor *et al.* 1998) or in the substantia nigra, where they control the firing rate of nigrostriatal dopaminergic neurones (Erhardt *et al.* 1999). A measurable activation of striatal GABA$_B$ receptors by endogenous GABA would have had confounding effects in our experiments, for example not allowing us to conclude whether GS39783 has any agonistic effect on its own or not. Therefore, its lack made it possible to demonstrate and characterize more clearly the *in vivo* efficacy of GS39783 in conjunction with the exogenously applied agonist R(–)-baclofen.

In conclusion, this is the first time that the enhancement of GABA$_B$ receptor function (and to our knowledge of a GPCR in general) by an allosteric drug has been shown *in vivo* at the signal transduction level, using a biochemical marker (cAMP formation) that is tightly linked to receptor activation. The data obtained strongly suggest that GS39783 acts on GABA$_B$ receptors *in vivo via* the same mechanism that has previously been demonstrated *in vitro*, *i.e.* as a positive allosteric modulator. This study therefore indicates that this appealing therapeutic principle, fairly novel for GPCRs, translates from the *in vitro* situation into that of a living organism.

6. Outlook

Allosteric modulators of GPCRs represent a novel type of ligands with great therapeutic potential and increasing possibilities for medicinal chemistry, especially in cases where the development of orthosteric ligands with sufficient selectivity is elusive (see Chapter 4, Section 4.1.). The field of GPCR allosteric modulation has rapidly expanded, which resulted in a growing number of reviews published on the subject in the past decade and an increasing number of newly characterized positive and negative allosteric modulators (see Chapter 4, Section 4.1.). The discovery of allosteric modulators has been made possible by the development of new HTS technologies *e.g.* functional assays in recombinant cell lines. Their further pharmacological characterization is challenged by the appropriate choice of counter-screening assays, which is absolutely critical.

There are still not many allosteric modulators on the market as therapeutic agents. However, there is an example of an allosteric agent that has been discovered in 1937 and has been in use in man for more than 50 years. It is the non-nutritive artificial sweetener cyclamate, frequently used as an additive in food industry. Cyclamate is an allosteric agonist that binds to the 7TM domain of the T1R3 receptor unit of heterodimeric sweet and umami taste receptors (Bertorelli and Czarnowski-Hill 1990; Xu *et al.* 2004).

There are other examples of allosteric modulators that have entered clinical trials as treatments for several disorders. One such substance is the allosteric enhancer of the adenosine A_1 receptors, which belongs to **GPCR family 1** (or A), T62 (see Chapter 2 for the chemical name) which is potentially useful in the treatment of neuropathic pain (Pan *et al.* 2001). It has so far been tested in healthy volunteers to facilitate dose finding and was planned to enter Phase II clinical trials in the first half of 2007 (source www.kingpharm.com).

Further, according to the corticosteroid receptor hypothesis of depression, an altered function of the hypothalamic-pituitary-adrenocortical (HPA) system results in an overactivity of central corticotropin-releasing factor (CRF) circuits among depressive patients, which can monitored by increased levels of cortisol and corticotropin in their sera. Since the corticotropin-releasing factor type I receptor (CRF1), a member of **GPCR family 2** (or B), has been identified as responsible for conveying the signal into cellular circuitries underlying depression (Holsboer 2000), many pharmaceutical companies were prompted to develop molecules to inhibit the activity of CRF1. One such compound was the negative allosteric modulator of CRF1 R-121919 (see Chapter 2 for the chemical name), an orally active brain-penetrating non-peptide substance, that selectively binds to the CRF1 with high affinity (Grigoriadis *et al.* 2000). R-121919 went into clinical studies for treating stress and depression and has reached Phase II (Holsboer 2003). Although it was found to alleviate depressive symptoms without eliciting undesired endocrine effects, the clinical trials were discontinued due to potential liver toxicity.

Regarding **GPCR family 3** (or C), the positive allosteric modulator of the CaSR cinacalcet (see Chapter 2 for the chemical name), is the only allosteric drug currently on the market. Its calcimimetic actions are utilized in alleviating primary and secondary hyperparathyroidism, a common and life-threatening complication of chronic kidney disease that can lead to cardiovascular calcification. The plasma concentration of ionized calcium (Ca^{2+}) is a primary regulator of bone homeostasis mainly through its action on parathyroid hormone (PTH), which acts on kidney, by increasing renal Ca^{2+} reabsorbtion, and on bone to increase plasma Ca^{2+} levels. Elevated concentrations of plasma Ca^{2+}, in turn, depress the secretion of PTH. This regulation is mediated *via* the CaSR in the parathyroid gland, which is downregulated in chronic renal failure. The positive allosteric modulator of CaSR cinacalcet can counteract the downregulation of CaSR by making the receptor more sensitive to the plasma Ca^{2+} concentrations, thus meeting the needs of patients with renal failure. Negative allosteric

modulators of CaSR such as NPS2143 and Calhex231 (see Chapter 2 for chemical names), in contrast, act as calcilytics and were found to stimulate PTH secretion (Nemeth *et al.* 2001; Petrel *et al.* 2003). However, since PTH in osteoporotic patients has anabolic activity on bone, effects of NPS2143 were tested in ovariectomized rats (an animal model of osteoporosis). When co-administered with 17-β-estradiol, as prevention of bone resorption due to a prolonged increase of PTH in the serum, NPS2143 induced a net bone gain. Other orally active calcilytic agents were also found to increase serum PTH levels in rats, offering a new potential treatment for osteoporosis (Arey *et al.* 2005).

Another example demonstrating a small gap between the clinical use of allosteric modulators and preclinical research concerning **GPCR family 3** are positive allosteric modulators of group II mGluRs (mGluR type 2 and 3). The selective orthosteric agonist of group II mGluRs LY354740 (see Chapter 2 for the chemical name), a structural analogue of glutamate, was in clinical trials for anxiety. Although its anxiolyic effects have been shown in many paradigms in human patients (Pilc 2003; Grillon *et al.* 2003), it had a limited oral availability (Johnson *et al.* 2002). This was circumvented by using a prodrug approach, which resulted in the peptidyl prodrug LY544344 (see Chapter 2 for the chemical name), that also displayed an anxiolytic profile in humans (Kellner *et al.* 2005). An alternative option for circumventing the problem of poor bioavailability of the mGluR2/3 agonist LY354740 was the development of selective positive allosteric modulators. A great number of compounds have been developed (Ritzen *et al.* 2005), some of which have been shown effective in animal models for anxiety and schizophrenia, indicating that clinical efficacy of positive mGluR2 modulators may be a reality in the future.

Considering the continuous extensive use of functional assays in GPCR screening technologies in drug discovery programs of pharmaceutical companies, there is little doubt that even more allosteric ligands will be discovered and characterized in the following years. Subtype-selective allosteric ligands will continue being useful tools in *in vivo* investigations

of the (patho)physiological roles of GPCRs. However, the next challenge for medicinal chemistry will be designing new allosteric ligands with improved properties such as high affinity and bioavailability.

Experiments conducted in this study investigated different aspects of prototypal positive allosteric modulators of the $GABA_B$ receptor. Even though CGP7930 and GS39783 have proven to be useful as tools for investigating $GABA_B$ receptor function both *in vitro* and *in vivo* (see Chapter 4, Section 4.3.), their wider use was restricted to some extent due to their relatively low potencies (in the μM range) and limited pharmacokinetic properties. Improvement of properties of these prototype allosteric ligands, *i.e.* higher potency and bioavailability, are crucial for the development of a new generation of therapeutic agents targeting the $GABA_B$ receptor.

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