New Directions in Receptor Research; Receptor Selectivity and Promiscuity

Ph.D. thesis

by

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List of Abbreviations

[^{35}S]GTPγS:	Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate
7TM:	seven transmembrane
AM251:	N-(pyperidine- 1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrasole-3- carboxamide
Baclofen:	4-amino-3-(4-chlorophenyl)butanoic acid
BSA:	bovine serum albumin
CB_1:	type 1 cannabinoid receptor
CB_2:	type 2 cannabinoid receptor
CCK:	cholecystokinin
CGP54626:	[S-(R*,R*)]-[3-[[1-(3,4- Dichlorophenyl)ethyl]amino]- 2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid
CHO:	Chinese hamster ovary
ChroM:	Morphine-dependent
CNS:	central nervous system
DAMGO:	Tyr-Gly-(NMe)Phe-Gly-ol
DMEM:	Dulbecco's Modified Eagle Medium
EC_{50}:	concentration of the ligand to give half-maximal effect
EGTA:	ethylene-bis(oxyethylenenitrilo) tetraacetic acid
E_{max} :	% maximal stimulation over basal activity
GABA:	γ-aminobutyric acid
GDP:	Guanosine 5′-diphosphate sodium salt
GPCRs:	G-protein coupled receptors
GTP-γ-S-Li_4:	Guanosine 5′-[γ-thio]triphosphate tetralithium salt
IC_{50}:	concentration of ligand required to achieve 50% inhibition
KO:	knock-out
MAPK:	mitogen activated protein kinase
MOR:	μ-opioid receptor
NaCl:	sodium chloride
Naloxone:	(5a)-4,5-Epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one

Phaclofen:	[3-amino-2-(4-chlorophenyl)propyl]phosphonic acid
PI3 kinase:	phosphoinositide 3-kinases
PLC:	phospholipase C
PTX:	pertussis toxin
R-Win55,212-2:	*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl] pyrrolo [1,2,3-de]-1,4 benzo-xazin-yl] -(1-naphthalenyl) methanone mesylate
SKF97541:	3-aminopropyl-methyl-phosphinic acid
SR141716:	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
S-Win55,212-3:	*S*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)- methyl] pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate
Tris:	tris(hydroxymethyl)aminomethane
Wt:	wild type

1. INTRODUCTION

There are four types of protein targets, which drugs can interact with: enzymes, membrane carriers, ion channels and receptors. Among of those protein targets, receptors can be subdivided into four main classes: ligand-gated ion channels, intracellular steroid, tyrosine kinase-coupled and G-protein coupled receptors (GPCRs).

1.1. G-Protein Coupled Receptors (GPCRs)

GPCRs are the largest class of cell-surface receptors. GPCRs can detect a diverse array of stimuli including neurotransmitters, hormones, lipids, photons, odorants, taste ligands, nucleotides and calcium ions, then transduce the signal from these ligand-receptor interactions into intracellular responses. Ligands that activate GPCRs may have therapeutic benefits in many diseases ranging from central nervous system disorders (including pain, schizophrenia and depression) and metabolic disorders, such as cancer, obesity or diabetes (Drews, 2000). GPCRs are considered highly convenient classes of proteins for drug discovery, with more than 50% of all drugs regulating GPCR function, and some 30% of these drugs directly target GPCRs (Jacoby et al., 2006). Approximately 9% of global pharmaceutical sales are realized from drugs targeted against only 40-50 well-characterized GPCRs (Eglen, 2005). As there are encoded by > 1,000 genes in the human genome (Howard et al., 2001), it is likely that many more GPCRs remain to be validated as drug targets. Furthermore, endogenous ligands have been identified for only 200 GPCRs (Jacoby et al., 2006), even though the human genome contains many more GPCR genes. Therefore, there are enormous opportunities for further drug discovery in the field of GPCRs.

1.1.1. Structure and Classification of GPCRs

GPCRs are a large, diverse and highly conserved class of membrane-bound proteins. Based on structure homology with rhodopsin, they possess a single, serpentine-like polypeptide chain with seven transmembrane (7TM) helices, three extracellular loops and three intracellular loops. The amino terminal is located extracellularly and the carboxyl terminal intracellularly.

GPCRs are divided into five broad families, such as *Rhodopsin*, *Secretin*, *Adhesion*, *Glutamate* and *Frizzled/Taste,* based upon the similarity of the transmembrane sequences and the nature of their ligands (George et al., 2002; Lagerström and Schiöth, 2008; Pierce et al., 2002).

Rhodopsin-like receptors is the largest subgroup of GPCRs and contains receptors for odorants, neurotransmitters (dopamine, serotonin, endocannabinoids etc.) as well as neuropeptides, glycoprotein hormones, chemokines and prostanoids. Rhodopsin-like receptors are characterized by several highly conserved amino acids and a disulphide bridge that connects the first and second extracellular loops. Most of these receptors also have a palmitoylated cysteine in the carboxy-terminal tail, which serves as an anchor to the membrane. The diversity is not found in their N-terminals, where most receptors have only a short stretch of amino acids, but within the TM regions. Most Rhodopsin-like receptors are primarily activated by interactions between the ligand and the TM regions and extracellular loops owing to their short N-terminal stretch of amino acids

Secretin-like receptors are activated by ligands including secretin, parathyroid hormone, glucagon, calcitonin gene related peptide, adrenomedullin, calcitonin, etc. The binding profile of the Secretin-like receptors can be illustrated mainly by three binding domains consisting of the proximal region and the juxtamembrane region of the N terminus and the extracellular loops together with TM6. The ligand is thought to activate the receptor by bridging the N-terminal and the TM segments/extracellular loops thereby stabilizing the active conformation of the receptor.

Adhesion receptors; The diverse N-termini of Adhesion GPCRs may contain several domains that can also be found in other proteins, such as cadherin, lectin, laminin, olfactomedin, immunoglobulin and thrombospondin domains. The number and structure of these domains have been shown to have an important role in the specificity of receptor–ligand binding interactions. The *Adhesion* GPCRs are rich in functional domains and most of the receptors have long and diverse N termini, which are thought to be highly glycosylated and form a rigid structure that protrudes from the cell surface

Metabotropic-glutamate-receptor-like receptors are characterized by a long amino terminus and carboxyl tail. The ligand-binding domain is located in the amino terminus.

The Frizzled/Taste2 receptors; The relationship to the GPCR superfamily was further strengthened when sequence comparisons with secretin receptors revealed resemblance in the extracellular regions and the presence of the well-conserved cysteines in the first and second extracellular loops. The extracellular part of the FZDs range from 200 to 320 amino acids in length in which the differences mostly lie in the linker region between the TM part and the extracellular ligand binding domain.

1.1.2. Signaling of GPCRs

Signaling via GPCRs provides multiple ways of communication between cells (Luttrell, 2006; Marinissen and Gutkind, 2001; Pierce et al., 2002). It was shown that different ligands induce either G-protein dependent or G-protein independent signaling of GPCR via β-arrestins, which might result in functional selectivity (Violin and Lefkowitz, 2007). Agonist binding to the GPCR promotes a conformational change in the receptor, specifically in an ionic interchange between the 3rd and 4th transmembrane domain. This induces coupling of the GPCR to the G-protein, initiating signaling to the cell interior. β-arrestins are well known negative regulators of GPCR signaling. Upon GPCR activation, β-arrestins translocate to the cell membrane and bind to the agonist-occupied receptors. This uncouples these receptors from G-proteins and promotes their internalization, thus causing desensitization (Ma and Pei, 2007). Conversely, recent accumulating evidences indicate that β -arrestins also function as scaffold proteins that interact with several cytoplasmic proteins and link GPCRs to intracellular signaling pathways, such as mitogen activated protein kinase (MAPK) cascades (Ma and Pei, 2007).

GPCR signaling induces coupling of the liganded receptor to a heteromeric G-protein. These are composed of α-, β- and γ- subunits, are also a diverse group of proteins comprising 17 Gα, 5 Gβ and 12 Gγ subunits at present (Hur and Kim, 2002) When a ligand activates the GPCR, it induces a conformational change in the receptor that allows the receptor to function as a guanine nucleotide exchange factor that exchanges GDP for GTP on the G_α subunit. In the traditional view of heterotrimeric protein activation, this exchange triggers the dissociation of the G_α subunit, bound to GTP, from the $G_{\beta\gamma}$ dimer and the receptor. The free α- or βγ-subunits then interact with second messengers; the precise nature of which is dependent upon the GPCR type and the G-protein subunits mobilized (Pitcher et al., 1998).

G-proteins are classified into four major classes: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Conklin and Bourn, 1993; Neer, 1995; Rens-Domiano and Hamm, 1995). Stimulation of the G_s subfamily activates adenylyl cyclase, whereas stimulation of the G_i subfamily leads to its inhibition. Stimulation of the G_q subfamily activates phospholipase C (PLC), and the G_{12} family is implicated in the regulation of small GTP binding proteins.

It has now become apparent that not only the α-subunits, but also the βγ -subunits can bind to a great variety of effectors molecules and regulate their activity (Clapham and Neer, 1997; Morris and Malbon, 1999; Schwindinger and Robishaw, 2001). $G_{\beta\gamma}$-subunits mediate signal transduction by interacting with many proteins, including GPCRs, GTPases and various effector molecules. The effector molecules that have been reported to be regulated by $G_{\beta\gamma}$-subunits include adenylyl cyclase, PLC, inwardly rectifying G-protein-gated potassium channels, voltage-sensitive calcium channels, phosphoinositide 3-kinases (PI3 kinase) and molecules in the MAPK pathway.

Recent developments indicate novel levels of complexity in GPCRs functioning (Fredholm et al., 2007). The initial idea of linear signaling pathways, transferring information from the cell membrane to the nucleus, has evolved into a complicated network of signaling pathways. Firstly, cross-talk of the GPCRs on signaling pathways is increasingly more evident (Hur and Kim, 2002). Secondly, some GPCRs may be constitutively active, i.e. active in the absence of its ligand. Particularly, the level of constitutive activity may vary in such a profound way between cells and tissues that this could offer new ways of achieving specificity of drug action (Fredholm et al., 2007; Milligan, 2003). Thirdly, increasing number of evidence showed that many GPCRs can form multimeric ensembles (Fredholm et al., 2007; Rozenfeld et al., 2006). Therefore, regulation of GPCRs at multiple levels causes emergence of specificity and complexity of GPCRs targeting.

1.1.2.1. Cross-talk of GPCR Signaling

The classical paradigm of GPCR signaling was rather linear and sequential. Emerging evidence, however, has revealed that this is only a part of the complex signaling mediated by GPCR (Hur and Kim, 2002). In the classical model of GPCR signaling, stimulation of 7TM spanning GPCR leads to the activation of heterotrimeric G-proteins, which dissociate into α- and βγ-subunits. These subunits activate effector molecules, which include second messenger generating systems, giving rise to various kinds of cellular, physiological, and biological responses. In contrast to the large number of GPCRs, the number of identified effectors is considerably smaller. Because many cells express multiple types of GPCRs that signal through limited types of effectors, it is not surprising that cross-regulation occurs in the signaling pathways of GPCRs, thereby leading to diverse physiological responses. Moreover, there has been growing number of evidence that GPCR stimulation modulates upstream and downstream events of other receptor-mediated signaling pathways, which results in complicated and sometimes unpredictable outcomes (Hur and Kim, 2002).

1.1.2.2. Constitutive Activity and Inverse Agonism

Growing body of evidence suggests that GPCRs may exhibit constitutive activity in the absence of their agonists. A two-state receptor model has been proposed to account for constitutive activity in which GPCRs exist in equilibrium between inactive and active states (Costa et al. 1992). Agonists stabilize the active state and thus display positive intrinsic activity, resulting in an increase in receptor activity. In contrast, inverse agonists stabilize the inactive state and exhibit negative intrinsic activity. Therefore, constitutive activity of GPCRs can be selectively blocked by ligands that are referred to as inverse agonists (for a review, see Milligan, 2003). A variety of human diseases are ascribed to a constitutive activity of GPCRs that is caused by naturally occurring mutations (Spiegel, 1996). Consequently, selective inverse agonists open up new therapeutic strategies for these types of human disorders.

1.1.2.3. GPCR Oligomerization

Traditionally, mechanism of ligand binding and signal transduction by GPCRs were modeled on the assumption that monomeric receptors mediate the processes. However, recent evidences have revealed that GPCRs may exist as homodimers, or may associate with other GPCRs to form heterodimers (Ferre et al., 2007; Franco et al., 2007). This association may alter the function of both receptors, yielding in a distinct functional unit with novel properties (Gomes et al. 2001; Hebert and Bouvier, 1998; Milligan, 2006). Since tissue-selective expression of GPCR heteromers and their differential activation offer exciting perspectives for the development of tissue- and receptor-subtype-selective drugs, these phenomena have promising potential in both basic and clinical research fields (Franco et al., 2007; Rozenfeld et al., 2006).

1.2. $GABA_B$ Receptor System

The main inhibitory neurotransmitter in vertebrates, γ-aminobutyric acid (GABA) was first described in the mammalian brain in 1950 (Awapara et al., 1950; Roberts and Frankel, 1950). GABA activates two classes of receptors, the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors. The ionotropic receptors are postsynaptic chloride ion channels that mediate fast inhibitory responses, while the metabotropic $GABA_B$ receptor is a GPCR that is found both pre- and post-synaptically and mediates slow, long-term inhibition (Chebib and Johnston, 1999). Presynaptic $GABA_B$ receptors can be divided into autoreceptors or heteroreceptors depending on whether or not they control the release of GABA or a different neurotransmitter (Bettler et al., 2004). Although they were first described in 1980, $GABA_B$ receptors were not cloned for many years (Kaupmann et al., 1997). Their molecular structure characterizes them as Class 3 GPCRs (Couve et al., 2000). $GABA_B$ receptors are highly unusual among GPCRs in their requirement for heterodimerization between two subunits, $GABA_{B1}$ and $GABA_{B2}$ for functional expression (Robbins et al., 2001). While ligand binding occurs to $GABA_{B1}$, $GABA_{B2}$ has been shown to play a key role in receptor functioning. $GABA_{B1}$ does not traffic to the cell surface unless $GABA_{B2}$ is present (Couve et al., 1998).

$GABA_B$ receptors mainly couple to $G_{i/o}$-proteins. Upon receptor activation, G-protein α and βγ subunits activate multiple cellular effector systems, that include inhibition of adenylyl cyclase, increase of the potassium current, inhibition of calcium channel activity (for a review, see Bettler et al., 2004).

The distributions of $GABA_B$ receptors are widespread in many brain regions in the vertebrates. High levels of $GABA_{B1}$ and $GABA_{B2}$ protein expression were found in the neocortex, hippocampus, thalamus and cerebellum (Charles et al., 2001). However, recent reports have revealed that the expression of the $GABA_{B1}$ and $GABA_{B2}$ subunits is not regulated in tandem (McCarson and Enna, 1999). For example, $GABA_{B2}$ is not detected, even though $GABA_{B1}$ and a functional $GABA_B$ receptor are present in the caudate putamen (Clark et al., 2000; Durkin et al., 1999; Margeta-Mitrovic et al., 1999).

1.3. Opioid Receptor System

Opioid receptors belong to Class 1 subclass within the GPCRs superfamily (Gether, 2000). They are activated by endogenously produced opioid peptides and exogenously administered opiates. There are at least four types of opioid receptors μ-, δ-, κ-, and nociceptin/orphanin FQ receptors. The human μ-, δ-, κ- and nociceptin/orphanin FQ opioid receptor genes were cloned in early 1990s and the appropriate proteins well characterized since then (Mansson et al., 1994; Meunier et al., 1995; Wang et al., 1994).

Opioid receptors are predominantly coupled to pertussis toxin-sensitive, heterotrimeric $G_{i/o}$-proteins. In addition, their coupling to pertussis toxin-insensitive G_s, G_z, G_q, and G_{12} proteins has also been reported (Chakrabarti et al., 2005; Crain et al., 1990; Garzon et al., 1998; Hendry et al., 2000; Szücs et al., 2004). Upon receptor activation, G-protein α- and βγ-subunits activate multiple cellular effector systems that include inhibition of adenylyl cyclase, increase of the potassium current, inhibition of calcium channel activity, modulation of inositol turnover, and activation of the MAP kinase pathway (Belcheva et al., 2001; Dhawan et al., 1996).

As regards the central nervous system, μ-opioid receptors are widely distributed in the central nervous system and also occur in the peripheral nervous systems. μ-opioid receptors are localized densely in striatum, nucleus accumbens, caudate putamen, thalamus, cortex, and spinal cord (Mansour et al., 1995).

Opioid receptors have been implicated in a broad range of behaviors and functions, including regulation of pain, reinforcement and reward, release of neurotransmitters, and neuroendocrine modulation (Mansour et al., 1995). Opioids are the most commonly used analgesics for severe pain. Morphine, isolated from opium, is one of the widely used analgesics today. However, its clinical use is limited by the development of various unwanted side effects, such as analgesic tolerance and dependence, nausea, vomiting, respiratory depression etc. Morphine binds to opioid receptors with the following order of potency: $\mu >> \delta \sim \kappa$ (for a review, see Eguchi, 2004). In addition, μ-opioid receptors show high propensity to tolerance and dependence upon chronic agonist exposure (for a review, see Waldhoer et al., 2004).

Table 1. Properties of the studied GPCRs

Receptors	**$GABA_{B1}$**	**$GABA_{B2}$**	**µ-opioid**	**CB_1**
Structural Information (human)	960 aa	941 aa	400 aa	472 aa
Gene Location	Chr6: p21.3	Chr9: q22.1-q22.3	Chr6: q25.2	Chro 6. q14-q15
Prototypic Agonist	R-Baclofen	-	Morphine	Δ^9-THC
Prototypic Antagonist	Phaclofen	-	Naloxone	SR141716
Endogenous Ligand	GABA	-	Endomorphins	Anandamide
Physiological Effects		Analgesia neurotransmitter release	Analgesia neurotransmitter release	Analgesia neurotransmitter release Neuroprotection

1.4. Cannabinoid Receptor System

Cannabinoid receptors belong to Class 1 subclass within the GPCRs superfamily. They are activated by endogenously produced lipids, also known as endocannabinoids and exogenous cannabinoids that include the bioactive constituents of the marijuana plant and their synthetic analogs (Howlet et al., 2004). Up to date, two G-protein coupled cannabinoid receptors were identified by molecular cloning in the early 1990s (Howlet et al., 2004). CB_1 receptors are mainly expressed in the brain and mediate most of the neurobehavioral effects of cannabinoids. CB_2 receptors are expressed by immune, hematopoietic tissues and brain, (for a review, see Begg et al., 2005, Gong et al., 2006). In addition, recent findings indicate that some cannabinoid effects are not mediated by either CB_1 or CB_2 receptors that reveal the existence of novel cannabinoid receptors such as GPR55 or nonCB_1/CB_2 hippocampal receptors (Begg et al., 2005; Mackie and Stella, 2006).

CB_1 receptors are predominantly, but not exclusively, coupled to G_i/G_o-proteins (Felder et al., 1998; Howlett, 1985). Neverthless, CB_1 receptors under certain conditions and with certain agonists, also couple via G_s- and $G_{q/11}$-proteins (for a review, see Demuth and Molleman, 2006). Upon receptor activation, G-protein α- and βγ-subunits activate multiple cellular effector systems that include inhibition of adenylyl cyclase, increase of the potassium current, inhibition of calcium channel activity, modulation of inositol turnover, and activation of the MAP kinase pathway (Howlett, 1985; Hill, 1985; Mackie and Hille, 1992; Mackie et al., 1995; Demuth and Molleman, 2006).

Cannabinoid CB_1 receptors are the most abundant GPCRs in the brain, with levels ten-fold higher than other GPCRs (Herkenham et al., 1991). CB_1 receptors are localized in many brain areas, with the regions of densest receptor localization including the cerebellum, hippocampus, cortex and the basal ganglia (Herkenham et al., 1991). This anatomical distribution is consistent with the behavioral and therapeutical effects of cannabinoids, including memory disruption, decreased motor activity, catalepsy, antinociception, hypothermia, attenuation of nausea and vomiting in cancer chemotherapy, appetite stimulation in wasting syndromes, relief from muscle spasms in multiple sclerosis and decreased intestinal motility (Compton et al., 1993; Dewey, 1986; for a review, see Pertwee, 2000).

1.4.1. Constitutive Activity of the CB_1 Receptors

Since the level of constitutive activity is typically proportional to the number of active receptors, inverse agonism is usually most noticeable under conditions of high receptor expression, such as occurs in over expressed systems. However, the high level of CB_1 receptor expression in the CNS also raises the possibility that inverse agonism may be relevant for CB_1 receptors *in vivo*. CB_1 receptors display a significant level of constitutive activity, either when heterologously expressed in non-neuronal cells or in neurons where CB_1 receptors are expressed naturally (for a review, see Pertwee, 2005). The involvement of receptor-mediated G-protein activity in the inverse agonist response is supported by reports that SR141716 (Rimonabant) inhibits [^{35}S]GTPγS binding in CB_1 receptor transfected cell lines (MacLennan et al., 1998), neuronal cells and brain (Breivogel et al., 2001; Sim-Selley et al., 2001). This ligand has been shown to exert a plethora of pharmacological effects in a number of pathological conditions (Bifulco et al., 2007). These effects are mainly attributed to its antagonistic properties at the CB_1 receptors, although the evidence is increasing that it may also behave as an inverse agonist (for a review, see Pertwee, 2005). Recently, European

Medicines Agency (EMEA) has recommended the suspension of marketing authorisation for SR141716 as of 23 October 2008. The reasons given include (a) an approximate doubling of risk of psychiatric disorders; the committee were of the opinion that these side-effects "could not be adequately addressed by further risk minimisation measures", and (b) the effectiveness of rimonabant was lower than in clinical trials because data indicate that patients only take the drug for a short period. Recent studies have revealed the existence of CB_1 receptor-independent actions of CB_1 inverse agonists, SR141716, AM251. High concentrations of SR141716 caused inverse agonism in the CB_1 receptor knock-out (CB_1-KO) mouse brain, mediated by neither CB_1 nor the non-CB_1, non-CB_2 putative cannabinoid receptor type (Breivogel et al., 2001). It has been proposed that the inhibitory effect of SR141716 on the basal receptor activity might occur either via a non-receptor-mediated effect or by binding to a site other than the agonist binding site on the CB_1 receptors, or by binding to GPCRs other than the CB_1 receptors, to which it binds with much lower affinity (Sim-Selley et al., 2001). Although there are data supporting the latter notion; high concentration of SR141716 causes competitive antagonism on adenosine A1 receptors (Savienen et al., 2003) and high concentration of AM251 and Δ9-tetrahydrocannabivarin showed inverse agonism on D2 dopamine receptor expressing D2-CHO cells (Dennis et al., 2008)., the exact mechanism of inverse agonism by SR141716 has not yet been clarified.

1.4.2. Interactions of CB_1 Receptors with Other Receptor Systems

Increasing number of evidence indicate that cannabinoids may modulate the activity of other receptor types, and CB_1 receptors show different levels of interaction with other receptor types (Demuth and Molleman, 2006). The cannabinoid receptors system shares several features with both the μ-opioid and the $GABA_B$ receptor systems. The pattern of expression of the CB_1 receptors strongly overlaps with that of the $GABA_B$ (Hajos et al., 2000; Katona et al., 1999; Katona et al., 2001; Nyiri et al., 2005; Pacheco et al., 1993) and the μ-opioid receptors (Pickel et al., 2004) in certain CNS regions. CB_1, $GABA_B$ and μ-opioid receptors are GPCRs predominantly coupled to $G_{i/o}$-proteins. Several studies have revealed a functional interaction of the CB_1 receptors with the $GABA_B$ (Pacheco et al., 1993) and the μ-opioid receptors (Canals and Milligan, 2008; Hojo et al., 2008; Rios et al., 2006) at the level of G-proteins in certain regions of the CNS. Importantly, CB_1, $GABA_B$ and μ-opioid receptors have been shown to display similar pharmacological effects, in some respect particularly on pain (Bettler et al., 2004; Dhawan et al., 1996; Pertwee, 1997).

2. AIMS OF THE WORK

The present work consists of two distinct, but related studies about the promiscuity of the CB_1 receptor system. In the first part, our goal was to reveal possible interaction between the CB_1 and $GABA_B$ receptors. As outlined in the Introduction, the $GABA_B$ receptors are the only GPCRs that require heterodimerization of their two subunits, $GABA_{B1}$ and $GABA_{B2}$ for functional expression (Robbins et al., 2001). Previous immuno-electron microscopic studies have suggested that the $GABA_{B2}$ subunit may be absent, but electrophysiological data have shown the presence of functional $GABA_B$ autoreceptors in cholecystokinin (CCK)-containing interneurons in rat hippocampus (*T. Freund, personal communication)*. Possible explanations of this phenomenon are that interaction of the $GABA_{B1}$ subunit with another receptor may make it capable of binding and signaling**.** Due to their similar localization and physiological roles (Table 1, section 1.3), we have hypothesized that the CB_1 receptor may substitute for the $GABA_{B2}$ subunit, thereby making the $GABA_B$ receptors functional in rat hippocampus.

Our goals to study:

- if there are functional $GABA_B$ and cannabinoid CB_1 receptors in rat hippocampal membranes;

- whether CB_1 and $GABA_B$ receptors interact on G-protein signaling and if so what are the consequences;

- What may be the mechanism? Cross-talk, hetero-oligomerization, or?

Previous literature data have raised the possibility that the well-known CB_1 receptor antagonist, SR141716 - which is used in the clinics under the name Rimonabant to reduce obesity (Bifulco et al., 2007) - may have some non-CB_1 receptor mediated inverse agonist effects. Thereby, in the second part of our work, we have performed a detailed study on the promiscuous action of SR141716. The aim of our work was to assess the inverse agonist effect of SR141716 in systems containing distinct populations of receptors.

Our goals to study:

- is the inverse agonist effect mediated via__the CB_1 receptors? Under what conditions?
- or does it occur via binding to GPCRs other than the CB_1 receptors, e.g. MOR?
- or is it a non-receptor-mediated effect?

Hence, we have used tissues that:

a) contain both the CB_1 receptors and the MORs (wild-type, wt mouse cerebral cortex);

b) lack the CB_1 receptors (CB_1 receptor knock-out, CB_1-KO mouse cerebral cortex);

c) lack both the CB_1 receptors and the MORs (parental Chinese hamster ovary, CHO cells); or

d) contain a homogeneous population of over-expressed recombinant MORs (MOR-CHO cells), which were either untreated or made morhine-tolerant.

We have utilized the ligand-stimulated [^{35}S]GTPγS functional assay to explore the inverse agonist effects of SR141716 in the above systems. This is a sensitive test of inverse agonism, because such ligands selectively block the basal [^{35}S]GTPγS activity assessed in the absence of agonists, thereby representing constitutive receptor activity.

3. MATERIALS AND METHODS

3.1. Chemicals

Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (37–42 TBq/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary) or Amersham Biosciences (Buckinghamshire, England). [^{3}H]Tyr-Gly-(NMe)Phe-Gly-ol ([^{3}H]DAMGO) (36 Ci/mmol) was synthesized in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary). 4-amino-3-(4-chlorophenyl)butanoic acid (Baclofen), 3-aminopropyl-methyl-phosphinic acid (SKF97541), [S-(R*,R*)]-[3-[[1-(3,4- Dichlorophenyl)ethyl]amino]- 2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid (CGP54626 hydrochloride), N-(pyperidine- 1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrasole-3-carboxamide (AM251), *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1naphthalenyl) methanone mesylate (R-Win55,212-2), (5a)-4,5-epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride (naloxone hydrochloride), and (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (O-2050) were obtained from Tocris (Ellisville, MO, USA). Tris(hydroxymethyl)aminomethane (Tris, free base), sodium chloride (NaCl), ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), Guanosine 5′-diphosphate sodium salt (GDP), Guanosine 5′-[γ-thio]triphosphate tetralithium salt (GTP-γ-S-Li_4), magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O), [3-amino-2-(4-chlorophenyl)propyl]phosphonic acid (phaclofen), *S*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate (S-Win55,212-3), bovine serum albumin (BSA-essentially fatty acid free) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent was from Bio-Rad Laboratories (Hercules, CA, USA). Unlabeled DAMGO was from Bachem AG (Bubendorf, Switzerland). N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide hydrochloride (SR141716) was gift of Dr. Mackie. SR141716 and AM251 were dissolved in ethanol; Win55,212-2 and O-2050 were dissolved in DMSO as 10 mM stock solutions and stored at -20 °C.

3.2. Animals

2-8 Rats (Wistar male, 200-250 g, inbred in the BRC, Szeged, Hungary) and mice (CD1 male, 20-25 g, gift of Dr. Freund, Institute of Experimental Medicine, Budapest, Hungary) were handled in accordance with the European Communities Council Directives (86/609/EEC), and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32). CB_1-KO mutant mice generated as described (Ledent et al., 1999) were provided by Dr. Freund, Institute of Experimental Medicine, Budapest, Hungary). The animals were housed in a temperature- and light-controlled room. Lighting was ensured in a 12-h cycle, and food and water were available *ad libitum.*

3.3. Cell culture and treatment

CHO cells stably transfected with the MORs (MOR-CHO) were cultured as previously described (Szücs et al., 2004). Briefly, the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose with L-glutamine (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Nova-Tech Inc., Grand Island, NE, USA), 1% penicillin/streptomycin (GIBCO, Carlsbad, CA, USA) and 3.6% geneticin (GIBCO, Carlsbad, CA, USA). Cells were grown at 37 ^{0}C in a humidified atmosphere of 10% CO_2, 90% air. One set of cells were treated with 100 ng/ml Pertussis toxin (PTX) (List Biological Labs., Inc., Campbell, CA, USA) for the last 24 h in culture. At the end of PTX exposure, the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells were harvested with PBS containing 1 mM EDTA. Cell suspension was spun at 2,500 rpm for 5 min, after which preparation of the cell membranes commenced.

3.4. Membrane Preparations

3.4.1. Brain membrane preparation

2-8 animals were decapitated, their brains removed, followed by dissection of hippocampi or cortex on ice. The tissues were washed with ice-cold buffer and their weight measured. They were homogenized in 30 volume (v/w) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 5 strokes in a teflon-pestle Braun homogenizer at 1500 U/min. Homogenates were centrifuged at 20,000 x g for 25 min, the resulting pellets suspended in buffer and spun again. Pellets were taken up in the original volume of buffer and incubated for 30 min at 37 ^{0}C, followed by centrifugation at 20,000 x g for 25 min. The supernatants were carefully discarded, and the final pellets taken up in 5 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose. Appropriate membrane aliquots were stored at –80 ^{0}C for several weeks.

3.4.2. Rat spinal cord membrane preparation

Rat spinal cords were dissected and stored at –80 ^{0}C for several weeks. They were thawed before use and homogenized in 10 volume (v/w) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 5 strokes in a teflon-pestle Braun homogenizer at 700 U/min. Homogenates were centrifuged at 5,000 x g for 10 min. The supernatant was carefully decanted and stored on ice. The pellet was suspended in the original volume of buffer and spun as above. The combined supernatants of the two centrifugation steps were spun at 20,000 x g for 25 min. The resulting pellet was taken up in the original volume of buffer and incubated for 30 min at 37 ^{0}C, followed by centrifugation at 20,000 x g for 25 min. The supernatant was carefully discarded. The final pellets were taken up in 20 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4) and used in the functional assay.

3.4.3. Cell membrane preparation

Freshly collected cell pellets were homogenized with a Wheaton teflon-glass homogenizer in 10 vols (v/w) of ice-cold homogenization buffer, pH 7.4, composed of 25 mM HEPES, 1 mM EDTA, 0.5 mg/l aprotinin, 1 mM benzamidine, 100 mg/l bacitracin, 3.2 mg/l leupeptin, 3.2 mg/l soybean trypsin inhibitor and 10% sucrose as reported earlier (Szücs et al., 2004). Homogenates were spun at 1,000 x g for 10 min at 4 ^{o}C, and the supernatant was collected. Pellets were suspended in half of the original volumes of the homogenization buffer and centrifuged as above. Combined supernatants from the two low-speed centrifugations were spun at 20,000 x g for 30 min. The cell pellets were taken up in appropriate volumes of homogenization buffer. Aliquots were stored at -80 ^{o}C until use.

3.5. Protein determination

The protein content of the membrane preparations was determined by the method of Bradford, BSA being used as a standard (Bradford, 1976).

3.6. Ligand-stimulated [^{35}S]GTPγS binding assay

The assay was performed as described (Fabian et al., 2002) except that in preliminary experiments the concentration of GDP (3 μM) and NaCl (100 mM) was optimized for both CB_1 and $GABA_B$ agonists in rat hippocampus, cortex and spinal cord. On the other hand; the concentration of GDP was optimized at 3 and 30 μM for MOR-CHO cells and mouse cortex membranes, respectively. The highest concentrations of the solvents (0.1% ethanol or DMSO) tested in preliminary experiments had no effect on the basal activity in the assay. Briefly, crude membrane fractions (10 μg of protein) were incubated with 0.05 nM [^{35}S]GTPγS and appropriate concentrations of ligands in TEM buffer (50 mM Tris-HCl, 1 mM EGTA and 3 mM $MgCl_2$, pH 7.4) containing 3 μM GDP, 100 mM NaCl and 0.1% (w/v) BSA in a total volume of 1 ml for 60 min at 30 °C . Nonspecific binding was determined with 10 μM GTPγS and subtracted to yield specific binding values. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/F filters with a Brandel Cell Harvester (Gaithersburg, MD, USA). Filters were washed with 3 × 5 ml of ice-cold buffer, and radioactivity of the dried filters was detected in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Wallac, Turku, Finland).

3.7. Radioligand binding assay

Heterologous displacement assays were performed with a constant concentration (1 nM) of [^{3}H]DAMGO (spec. activity 36 Ci/mmol), 11 concentrations (10^{-10}-10^{-5} M) of unlabeled Win55,212-2 or SR141716 and the membrane suspension (10 µg protein) in 50 mM Tris-HCl pH 7.4 buffer containing 0.1% (w/v) BSA in a final volume of 1 ml. Nonspecific binding was defined as the radioactivity bound in the presence of 10 µM unlabeled naloxone, and was subtracted from the total binding to obtain the specific binding. The tubes were incubated at 25 °C for 1 h. The reaction was stopped by vacuum filtration through Whatman GF/C glass fiber filters (Whatman, Maidstone, England), using a Brandel M24-R Cell

Harvester (Brandel, Gaithersburg, MD, USA). Filters were rapidly washed with 3 x 5 ml of ice-cold 50 mM Tris-HCl pH 7.4 buffer, air-dried and counted in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Wallac, Turku, Finland). All assays were performed in duplicate and repeated at least three times.

3.8. Data analysis

To analyze the dose-response curves in the ligand-stimulated [^{35}S]GTPγS binding assay, data were analyzed with the GraphPad Prism 4.0 software (GraphPad Prism Software Inc., San Diego, CA, USA), using nonlinear regression and sigmoidal curve fitting to obtain potency (EC_{50}: the ligand concentration that elicits the half-maximal effect) and efficacy (E_{max}: maximal effect) values. Basal activities were measured in the absence of receptor ligands and defined as 0% in each experiment unless otherwise indicated. All data are expressed as percentages of the basal [^{35}S]GTPγS binding and are the means ± S.E.M. of the result of at least three independent experiments performed in triplicate. IC_{50} (the concentration of ligand required to achieve 50% inhibition) values were obtained from the radioligand displacement curves. All receptor binding data are expressed as percentage inhibition of specific binding and are the means ± S.E.M. of the result of at least three independent experiments performed in duplicate. Statistical analysis was performed with GraphPad Prism, using ANOVA or Student`s t-test analysis. Significance was defined at $p < 0.05$ level.

4. RESULTS

4.1. Cross-talk between CB_1 and $GABA_B$ receptors

4.1.1 G-protein activation of the $GABA_B$ receptors in brain areas with different expression levels of the $GABA_{B1}$ and $GABA_{B2}$ subunits

We have evaluated the effect of $GABA_B$ receptor agonists on G-protein signaling using the ligand-stimulated [^{35}S]GTPγS binding assay in membranes of adult rat hippocampus. Two other tissues, containing distinct expression level of the $GABA_{B1}$ and $GABA_{B2}$ subunits, were used as positive and negative controls. While the cerebral cortex contains high and balanced expression level of the $GABA_{B1}$ and $GABA_{B2}$ (Martin et al., 2004), the spinal cord was shown to have decreased level of the $GABA_{B2}$ subunits in adult rats (Kaupmann et al., 1998; Moran et al., 2001).

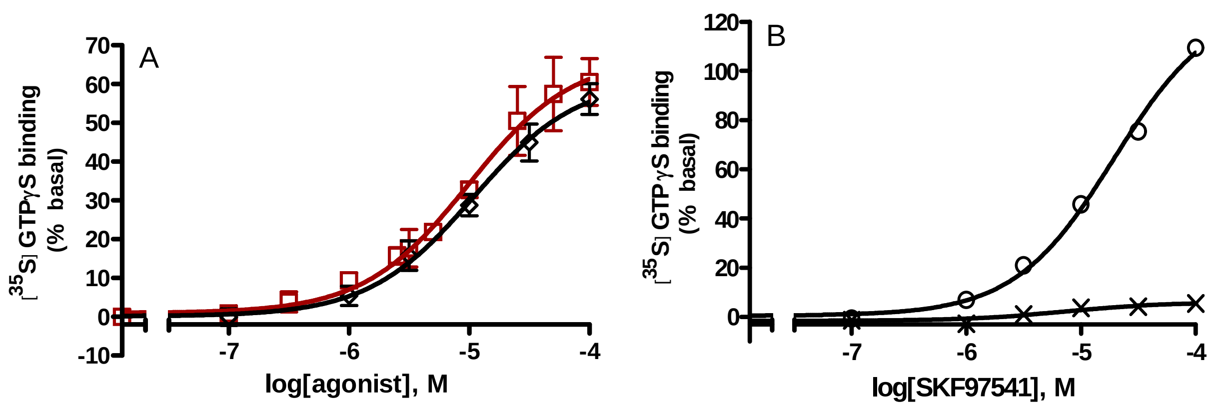

Figure 1. Tissue-specific G-protein activation by $GABA_B$ receptors. A) Dose-response curves of the $GABA_B$ agonists baclofen (□) and SKF97541 (◊) in hippocampal membranes. The data represent means ± S.E.M., n =7-11, all performed in triplicate. B) The effect of SKF97541 in membranes of spinal cord (x) and cortex (○), serving as negative and positive controls, respectively. The data represent means ± S.E.M., n = 3, all performed in triplicate. Non-visible S.E.M. is within the symbol.

In hippocampal membranes, the $GABA_B$ receptor specific agonists, SKF97541 (3-aminopropyl-methyl-phosphinic acid) and baclofen (4-amino-3-(4-chlorophenyl) butanoic acid) resulted in a concentration-dependent stimulation of [^{35}S]GTPγS binding displaying identical potency (EC_{50} = 10 ± 1 μM) and similar efficacy with 62 ± 1 and 67 ± 1% of basal activity, respectively (Figure 1A). As shown in Figure 1B, SKF97541 stimulated [^{35}S]GTPγS binding with 20 ± 2 μM potency and 128 ± 2% efficacy values in cerebral cortex membranes. These data are similar to those published with baclofen in membranes of cerebral cortex (Moran et al., 2001). No significant effect of SKF97541 on G-protein activation was seen in the spinal cord (Figure 1B) that agrees well with literature data (Moran et al., 2001). These results have suggested that there are functional $GABA_B$ receptors that are able to activate G-proteins in the rat hippocampus.

4.1.2. $GABA_B$ receptors show low sensitivity to phaclofen in hippocampus

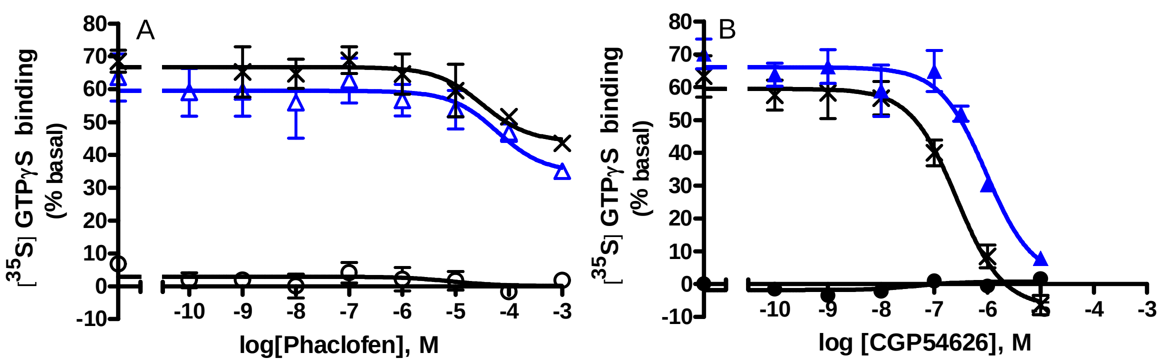

Figure 2. Phaclofen, but not CGP54626, is a low potency $GABA_B$ receptor antagonist in hippocampal membranes. A) Effect of phaclofen in the absence (○), or in the presence of baclofen (100 μM, Δ) or SKF97541 (100 μM, X). The data represent means ± S.E.M., n = 3, all performed in triplicate. B) Effect of CGP54626 in the absence (●), or in the presence of baclofen (100 μM, ▲) or SKF97541 (100 μM, X). The data represent means ± S.E.M., n = 3, all performed in triplicate. Non-visible S.E.M. is within the symbol.

To further characterize G-protein activation via $GABA_B$ receptors, agonist-stimulated [^{35}S]GTPγS binding was probed with two well-known antagonists, phaclofen ([3-amino-2-(4-

chlorophenyl)propyl]phosphonic acid) and CGP54626 ([S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl) ethyl]amino]- 2-hydroxypropyl] (cyclohexylmethyl) phosphinic acid).

The effect of either 100 µM SKF 97541 or baclofen was inhibited by CGP54626 in a concentration-dependent manner resulting in full inhibition at 1 µM (Figure 2B). Conversely, phaclofen showed much lower potency on blocking G-protein activation with either SKF97541 or baclofen, having no significant effect up to 10 µM on either agonists and blocking about 50% of the effect of baclofen and SKF97541 at 1 mM (Figure 2A).

4.1.3. Functional CB_1 receptors in hippocampus

We have also demonstrated that the CB_1 receptors are fully functional with the expected characteristics in rat hippocampal membranes. Accordingly, the CB_1 agonist R-Win55,212-2 dose-dependently stimulated the incorporation of the radioligand (EC_{50} = 33 ± 6 nM, E_{max}= 48 ± 1%), Figure 3A. The CB_1 receptor antagonist AM251 completely blocked the effect of saturating concentrations of R-Win55,212-2 (Figure 3B). It should be noted that AM251 by itself caused about 20% inhibition of the basal [^{35}S]GTPγS activity suggesting that it behaves as an inverse agonist in our system (Figure 3B).

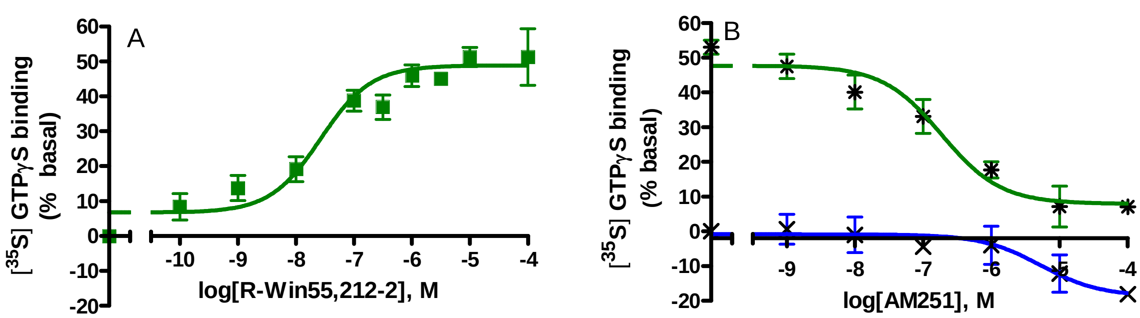

Figure 4. G-protein activation by CB_1 Receptors. A) Dose-response curves of the CB_1 agonist, R-Win55,212-2 (■). The data represent mean ± S.E.M., n = 10, all performed in triplicate. B) Antagonistic effect of AM251 in the absence (X), or in the presence of 10 µM R-Win55,212-2 (*). The data represent means ± S.E.M., n = 3, all performed in triplicate. Non-visible S.E.M. is within the symbol.

4.1.4. Inhibition of CB_1 receptor mediated signaling by the $GABA_B$ antagonist phaclofen in hippocampus

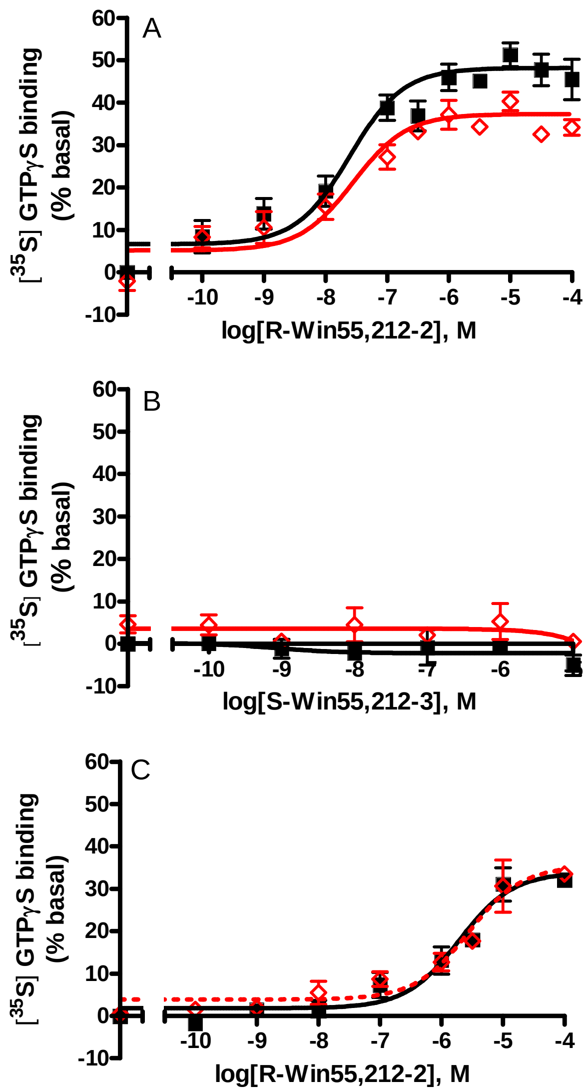

Figure 4. Tissue-specific functional interaction between $GABA_B$ and CB_1 receptors. Dose-response curve of R-Win55,212-2 in the absence (■), or in the presence of 10 nM phaclofen (◊) in membranes of hippocampus, n=9 (A) or spinal cord, n=3 (C). The data represent means ± S.E.M., all performed in triplicate. Dose-response curve of the pharmacologically inactive S-Win55,212-3 in the absence (■), or in the presence of 10 nM phaclofen (◊) in hippocampal membranes (B). Mean ± S.E.M., n = 3, all performed in triplicate. Non-visible S.E.M. is within the symbol.

To investigate whether there is a cross-talk between the two receptors, we assessed the effect of the $GABA_B$ antagonist phaclofen on R-Win55,212-2 stimulated G-protein activity. Importantly, a low dose of phaclofen (10 nM), that had no effect on its respective agonists (Figure 2A), slightly but significantly (two-way Anova, $F_{1,127} = 13.71$, $p < 0.05$) inhibited the dose-response curve of R-Win55,212-2 in stimulating [^{35}S]GTPγS binding in hippocampus (Figure 4A). The pharmacologically inactive stereoisomer S-Win55,212-3 had no effect either alone or in combination with phaclofen establishing that the interaction is stereospecific in hippocampus (Figure 4B). R-Win55,212-2 displayed lower potency (EC_{50} = 1900 ± 18 nM) and efficacy (33 ± 2%) in membranes of spinal cord (Figure 4C) than hippocampus (Figure 4A). Phaclofen at 10 nM had no significant effect on R-Win55,212-5 stimulated [^{35}S]GTPγS binding in spinal cord membranes (Figure 4C).

Table 2. The $GABA_B$ antagonist phaclofen at low doses (1 and 10 nM) significantly decreased the efficacy of R-Win55,212-2-stimulated CB_1 receptor signaling

LIGAND	**E_{max} (% basal)**	**EC_{50} (nM)**
R-Win55,212-2	**48 ± 1**	**33 ± 6**
R-Win55,212-2 + phaclofen (1 nM)	**38 ± 3 ****	**44 ± 6**
R-Win55,212-2 + phaclofen (10 nM)	**37 ± 1 ****	**46 ± 1**

Binding parameters were calculated from the curves shown in Figure 4A with GraphPad Prism computer program as described in Methods. Data represent the mean ± S.E.M. of at least four independent experiments performed in triplicate. Statistically significant effects of phaclofen on the binding parameters of R-Win55,212-2 were calculated using the Student's t-test (two tails, paired) and shown as ** $p < 0.05$.

Significant ($F_{1,92} = 6$, $p < 0.05$) inhibition of the dose-response curve of R-Win55,212-2 was also obtained with 1 nM phaclofen. Table 2 shows that the presence of phaclofen at these concentrations significantly inhibited the efficacy (E_{max}) of CB_1 receptor signaling with a tendency to decrease the potency that was, however, statistically not significant. Contrary, phaclofen at 0.1-10 µM had no significant effect on G-protein activation induced by a maximally effective concentration of R-Win55,212-2 (Table 3) implying that phaclofen does not directly antagonize the population of CB_1 receptors activated by R-Win55,212-2.

Table 3. The $GABA_B$ antagonist phaclofen at higher doses (0.1-10 µM) had no significant effect on the R-Win55,212-2-stimulated CB_1 receptor signaling

LIGAND	(% basal)
R-Win55,212-2	**47 ± 2**
R-Win55,212-2 + phaclofen (0.1 µM)	**46 ± 1**
R-Win55,212-2 + phaclofen (1 µM)	**46 ± 2**
R-Win55,212-2 + phaclofen (10 µM)	**49 ± 6**

R-Win55,212-2 stimulation (% basal of $[^{35}S]GTP\gamma S$ binding) at the maximally effective concentration (100 µM) was assessed in the absence or in the presence of fixed concentrations of phaclofen (0.1, 1, 10 µM). No significant effect of phaclofen on R-Win55,212-2 stimulation was obtained. Data represents the mean ± S.E.M. of three independent experiments performed in triplicate.

4.1.5. The specific CB_1 antagonists AM251 inhibits $GABA_B$ receptor mediated G-protein signaling in hippocampus

The reciprocal experiment showed that a specific CB_1 antagonist at a low dose was also able to modify G-protein activation by a $GABA_B$ receptor agonist. AM251 at 1 nM significantly (two-way Anova, $F_{1,52}$= 26.08, p <0.05) attenuated the dose-response curve of the $GABA_B$ agonist SKF97541 in hippocampal homogenates (Figure 5A).

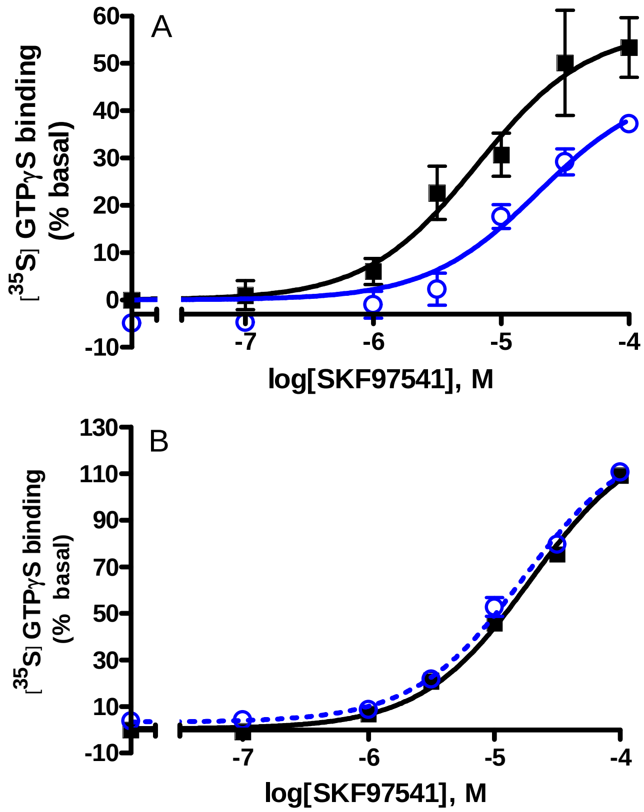

Figure 5. The specific CB_1 antagonist AM251 at 1 nM significantly inhibits G-protein activation by $GABA_B$ receptors in hippocampal membranes. Dose-response curve of SKF97541 was assessed in the absence (■), or in the presence of 1 nM AM251 (○) in membranes of hippocampus (A) and cortex (B). The data represent mean ± S.E.M., n = 5, all performed in triplicate. Non-visible S.E.M. is within the symbol.

Further analysis revealed that the CB_1 antagonist significantly inhibited the E_{max} of $GABA_B$ receptor signaling with a tendency to decrease the potency that, however, was not statistically significant, Table 4. The effect seems to be tissue specific, since no sign of any interaction of the two receptors was detected in cortical membranes (Figure 5B).

Table 4. The CB_1 receptor specific antagonist AM251 at a low dose (1 nM) significantly decreased the efficacy (E_{max}) of SKF97541-stimulated $GABA_B$ receptor signaling

LIGAND	E_{max} (% basal)	EC_{50} (µM)
SKF97541	55 ± 3	10 ± 1
SKF97541 + 1 nM AM251	43 ± 2 **	19 ± 1

Binding parameters were calculated from the curves shown in Figure 5A with GraphPad Prism computer program as described in Methods. Data represents the mean ± S.E.M. of five independent experiments performed in triplicate. Statistically significant effect of AM251 on the dose-response curve of SKF97541 was calculated using the Student's t-test (two tails, paired) and shown as ** $p < 0.05$.

4.2. CB_1 receptor-independent actions of SR141716 on G-protein signaling of opioid receptors

4.2.1. Effects of SR141716 on cannabinoid receptors in wt and CB_1–KO mouse cortical membranes

The potency and efficacy of prototypic cannabinoid receptor ligands on G-protein signaling were measured in ligand-stimulated $[^{35}S]GTP\gamma S$ binding assays. The CB_1,/CB_2 receptor agonist Win55,212-2 significantly stimulated $[^{35}S]GTP\gamma S$ incorporation with a potency of 505 ± 138 nM and efficacy of 230 ± 9% in the wt mouse cortical membranes (Figure 6A). It was noteworthy that, although low concentrations of Win55,212-2 did not exert significant effects in the CB_1-KO mouse cortex, 10 µM of the agonist stimulated $[^{35}S]GTP\gamma S$ binding by 38 ± 5% (Figure 6C).

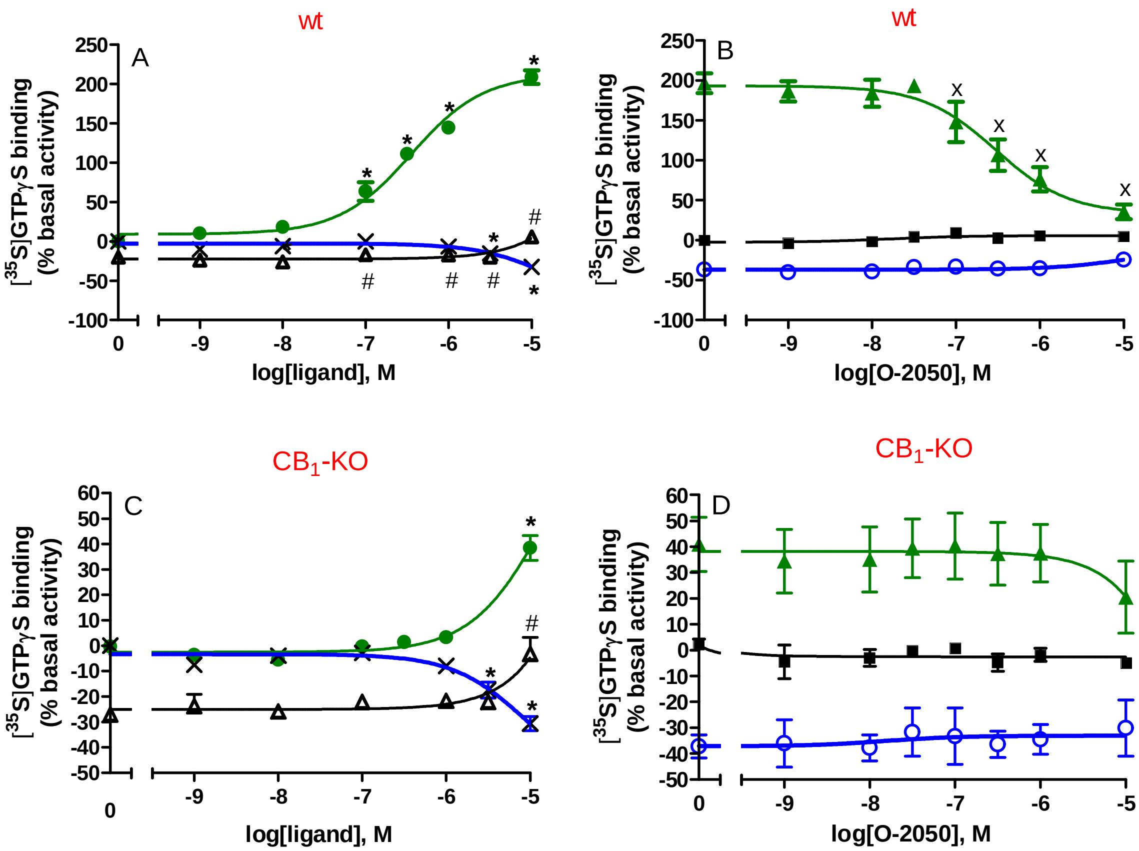

Figure 6. CB$_1$ receptor-independent inverse agonism of SR141716 in mouse cortical membranes. Dose-response curves of SR141716 (X) and Win55,212-2, either alone (●) or in the presence of 10 µM SR141716 (Δ) in membranes from the wt (A) or CB$_1$-KO (C) mouse cortex. Effects of O-2050 in the absence (■) or in the presence of Win55,212-2 (10 µM, ▲) or SR141716 (10 µM, ○) in membranes from the wt (B) or CB$_1$-KO (D) mouse cortex. The data are expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. Means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity, ^{x} indicates significant antagonistic effects of O-2050 on the appropriate ligands, $^{\#}$ indicates significant inhibition of the Win55,212-2 stimulation by SR141716.

O-2050 has been described as a CB_1 receptor neutral antagonist (for a review, see Pertwee, 2005). In accordance with this, O-2050 *per se* did not exhibit any effect on the basal [^{35}S]GTPγS binding: no inverse agonist feature of this ligand at the concentrations tested was observed in either the wt or the CB_1-KO mouse cortical membranes (Figure 6B, D). In contrast, the stimulation induced by Win55,212-2 (10 µM) was concentration-dependently antagonized by O-2050 in the wt cortex (Figure 6B). The Win55,212-2 stimulation was not antagonized by O-2050 in CB_1-KO membranes, and thus it may not be mediated via the CB_1 receptors (Figure 6D).

SR141716 dose-dependently inhibited the basal activity, achieving statistically significant inhibition at > 1 µM in both the wt and the CB_1-KO membranes (Figure 6). However, the SR141716-induced inverse agonistic effects were not reversed by O-2050 in either the wt (Figure 6B) or the CB_1-KO (Figure 6D) membranes, although the effect of an inverse agonist should be blocked by its respective neutral antagonist. SR141716 fully antagonized the effect of Win55,212-2 stimulation in the wt cortex, and inhibited the basal and the Win55,212-2-stimulated (most likely non-CB_1-receptor–mediated) effects to the same extent in the CB_1-KO membranes (Figure 6A, C). These results suggest that SR141716 displays a CB_1 receptor-independent inverse agonist feature in the mouse cortex.

4.2.2. Effects of SR141716 on MORs in wt and CB_1–KO mouse cortical membranes

We next checked the hypothesis that the inverse agonist effect of SR141716 may be manifested at GPCRs other than the CB_1 receptors, e.g. the closely related MORs. The highly specific MOR agonist DAMGO saturably and concentration-dependently stimulated [^{35}S]GTPγS binding with a potency of ~270 nM (log EC50 = -6.5 ± 0.17) and the efficacy of 80 ± 4% (Figure 7A). In combination with 10 µM SR141716 (which completely blocked the Win55,212-2 stimulation of the CB_1 receptor), the basal activity was inhibited by about 25% and the DAMGO dose-response curve was shifted to the right. In order to reflect the net effect of SR141716 on the MOR signaling, we expressed the data by defining the [^{35}S]GTPγS binding in the presence of 10 µM SR141716 *per se* as 0% (Figure 7C). Combination of 10 µM SR141716 with various concentrations of DAMGO significantly ($p < 0.05$) changed the potency of DAMGO, resulting in a log EC_{50} value of -5.8 ± 0.07. The efficacy of DAMGO was not changed by the presence of 10 µM SR141716 (Figure 7C). Overall, therefore, this indicates that SR141716 acts competitively on MORs in mouse cortex. It should be noted that deletion of the CB_1 receptors did not influence the stimulation of [^{35}S]GTPγS by DAMGO in the absence and presence of SR141716 (Figure 7B), further supporting the notion that the inhibitory effect of SR141716 seems to be CB_1 receptor-independent.

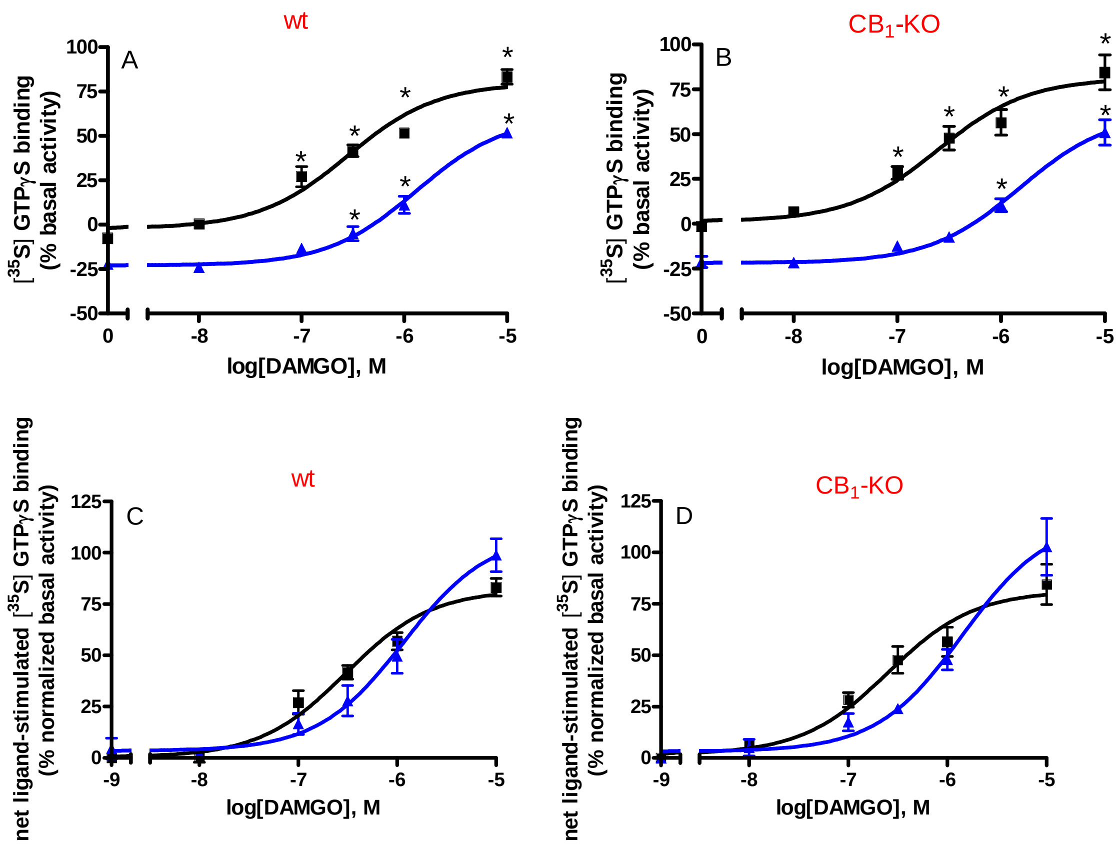

Figure 7. CB_1 receptor-independent inhibition of μ-opioid signaling by SR141716. Dose-response curves of DAMGO either alone (■) or in the presence of 10 μM SR141716 (▲) in membranes from the wt (A) or CB_1-KO (B) mouse cortex. The data are expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. In order to depict the net effect of SR141716 on the μ-opioid signaling, the data are re-plotted and expressed as percentages of the 'normalized basal activity', binding in the presence of 10 μM SR141716 being defined as 0%, in membranes from the wt (C) or CB_1-KO (D) mouse cortex. Means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity.

4.2.3. Effects of SR141716 on MORs in MOR-CHO cell membranes

The mouse brain contains a heterogeneous mixture of receptors, where receptor-receptor interactions (cross-talk, hetero-oligomerization, etc.) might occur. Accordingly, it was of interest to examine the mechanism of action of SR141716 by using a cell line that contains a homogeneous population of MORs at high density. With saturating concentrations of the ligands either alone or in appropriate concentrations, it was found that 10 µM DAMGO resulted in a 501 ± 29% stimulation, which was reduced to 86 ± 7% by the prototypic opioid antagonist naloxone (10 µM), indicating that the effect was mediated via the MORs in the MOR-CHO cell membranes (Figure 8A). SR141716 (10 µM) slightly, but significantly ($p < 0.05$) reduced the basal [^{35}S]GTPγS activity (Figure 8A). Moreover, SR141716 slightly inhibited the effect of DAMGO, resulting in 456 ± 22% of the basal [^{35}S]GTPγS binding; however, this level was not significantly different from that for DAMGO alone. The combination of SR141716 and naloxone displayed the same inhibition of the DAMGO effect as that of naloxone itself (Figure 8A).

Previous reports have demonstrated that PTX-sensitive G-proteins participate in the SR141716-induced inhibition of G-protein activity (Glass and Northup, 1999; Savinainen et al., 2003; Sim-Selley et al., 2001). We therefore, pretreated the cells with PTX to uncouple the receptors from the $G_{i/o}$-proteins. SR141716 did not have any significant effect on the basal G-protein signaling in the PTX-treated MOR-CHO (Figure 8B). Likewise, DAMGO exhibited only a small, naloxone-insensitive effect (~30%) on the [^{35}S]GTPγS binding in the PTX-treated MOR-CHO cell membranes, as expected, since the MORs are predominantly, but not exclusively, coupled to $G_{i/o}$-proteins (Chakrabarti et al., 2005; Childers, 1991; Szücs et al., 2004). The combination of DAMGO and SR141716 (10 µM each) led to a significant ($p < 0.05$) 169 ± 22% stimulation of the G-protein signaling when the MORs were uncoupled from $G_{i/o}$-proteins by PTX (Figure 8B). This novel signaling was totally blocked by naloxone, indicating that it occurs via the MORs (Figure 8B).

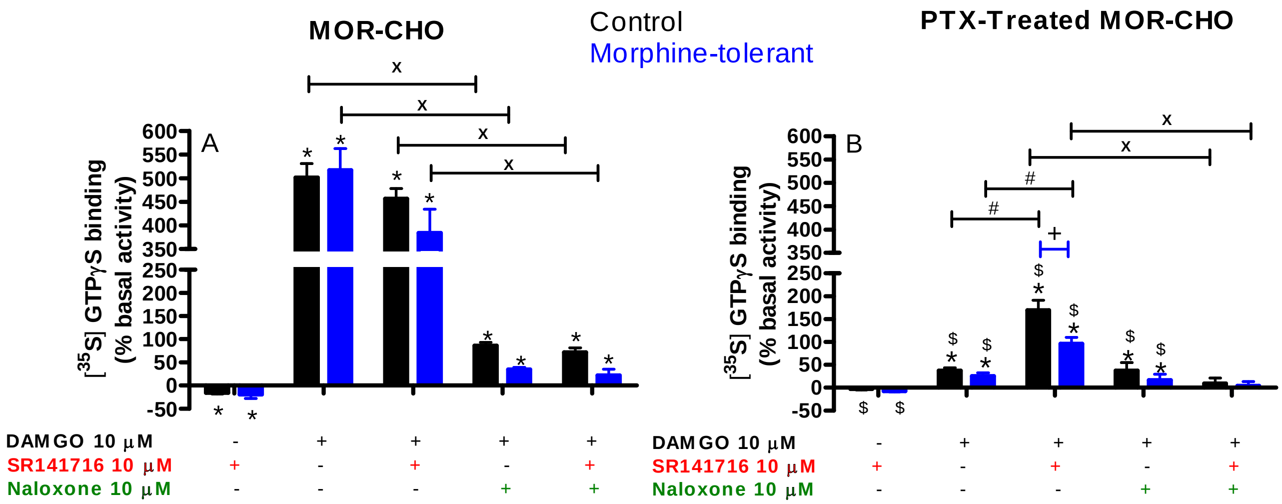

Figure 8. PTX-insensitive opioid signaling is unmasked by the joint application of DAMGO and SR141716 in PTX-treated MOR-CHO membranes. Each ligand was used at 10 μM, either alone or in combination as shown for the MOR-CHO (A) or PTX-treated MOR-CHO (B) membranes. The data are the means ± S.E.M. of the results of at least three independent experiments, all performed in triplicate and expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. Statistical analysis was performed with two-way ANOVA tests followed by Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity. # denotes significant changes of the DAMGO stimulation by SR141716, x indicates significant antagonism of the agonist effects by naloxone. + denotes significant differences between control versus morphine-tolerant membranes determined by unpaired Student`s t-test. $ indicates significant differences between MOR-CHO (A) and PTX-pretreated MOR-CHO (B) membranes determined by unpaired Student`s t-test.

Prolonged exposure of cells to morphine can induce adaptive changes resulting in tolerance (for a review, see Nestler and Aghajanian 1997). We have shown before that the MOR-CHO cells became tolerant after pre-treating them for 48 hours with morphine (Szücs et al., 2004). In cells treated in this fashion, the most notable change was desensitization of the stimulatory effect of co-addition of DAMGO and SR141716 in PTX-treated MOR-CHO membranes, Figure 8B.

We examined the concentration-dependence of the above effects of SR141716 with a view to a better understanding of the underlying mechanism (Figure 9). SR141716 dose-dependently, saturably and significantly ($p < 0.05$) reduced the basal [^{35}S]GTPγS activity, with a potency of 6 ± 0.4 μM, achieving a maximal inhibition of about 25% at 100 μM in the MOR-CHO membranes. PTX treatment completely eliminated the inverse agonist effect of SR141716 (Figure 9A).

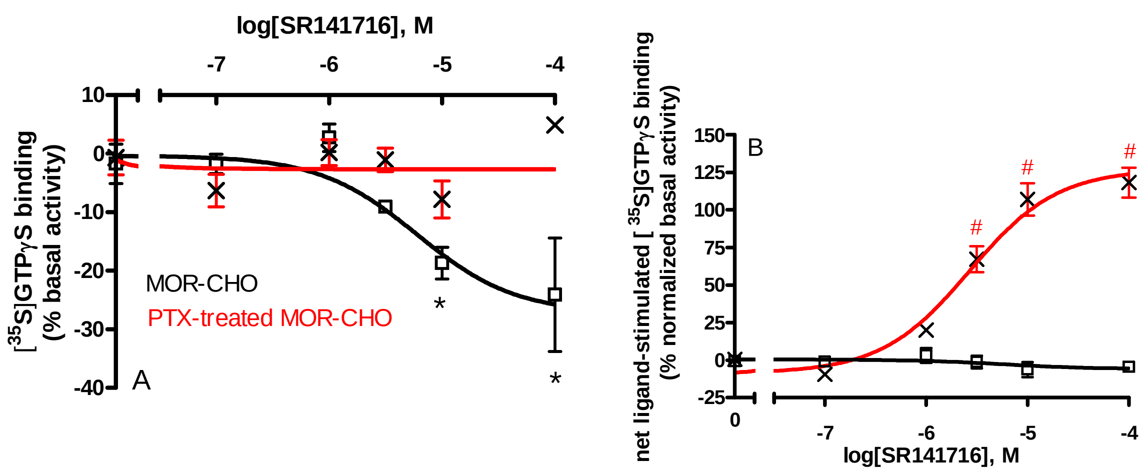

Figure 9. Net effects of SR141716 on basal and DAMGO-stimulated G-protein activity in MOR-CHO membranes. A) Dose-response curve of SR141716 in either MOR-CHO (□) or PTX-treated MOR-CHO (x) membranes. B) In another set, we assessed dose-response curves demonstrating the effects of SR141716 on [^{35}S]GTPγS binding stimulated by 10 μM DAMGO in MOR-CHO membranes without (□) or with PTX treatment (x) and expressed as percentages of the 'normalized basal activity', binding in the presence of 10 μM DAMGO being defined as 0%. The data are means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of SR141716 on the basal activity. # indicates significant changes of the DAMGO stimulation by varying concentrations of SR141716.

In order to determine the net effect of SR141716 on MOR signaling, we expressed the data measured in the joint presence of varying concentrations of SR141716 and a fixed concentration of DAMGO by defining the binding in the presence of 10 µM DAMGO *per se* as 0%. All other data were expressed as percentage stimulation over the normalized basal activity (Figure 9B). SR141716 displayed a slight tendency in a concentration-dependent manner to inhibit the effect of 10 µM DAMGO in MOR-CHO membranes, but this did not reach the level of statistical significance (Figure 9B). PTX treatment resulted in a major effect on the intrinsic efficacy of SR141716; SR141716 in the presence of 10 µM DAMGO induced concentration-dependent, significant PTX-insensitive G-protein activation, with a potency of about 3 µM, which reached 118 ± 10% over the 'normalized basal activity' (Figure 9B).

4.2.4. The inverse agonism of SR141716 persists in parental CHO cell membranes

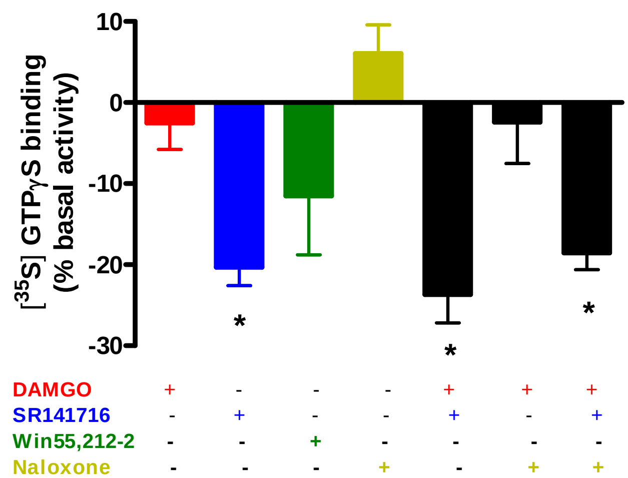

Figure 10. SR141716 inhibits basal G-protein activity in parental CHO membranes. Each ligand was used at 10 µM, either alone or in appropriate combination as shown. The data are means ± S.E.M., n = 3, all performed in triplicate. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant inhibition of the basal activity by SR141716. The presence of DAMGO in the absence or in the presence of naloxone had no significant effect on the inhibitory effect of SR141716.

We also tested another possible explanation for the observed nonspecific inverse agonist effect of SR141716, i.e. that its effect is non-receptor-mediated (Sim-Selley et al., 2001). Neither DAMGO (10 µM) nor Win55,212-2 (10 µM) had any significant effect on G-protein activation in the parental CHO membranes, indicating that neither the MORs nor the CB receptors are endogenously expressed in this cell line (Figure 10). It is important that SR141716 (10 μM) still decreased the basal G-protein activity by 20 ± 2% in parental CHO cell membranes (Figure 10). SR141716 (10 μM) combined with a high concentration of DAMGO, either in the absence or in the presence of naloxone, did not significantly change the inhibitory effect of SR141716 *per se* (Figure 10), further supporting the notion that MORs are not present in these cells. These results confirm the hypothesis that the inverse agonist effect of SR141716 is CB_1 receptor-independent, and possibly even non-receptor-mediated.

4.2.5. SR141716 interacts directly with [^{3}H]DAMGO-binding sites in MOR-CHO cell membranes

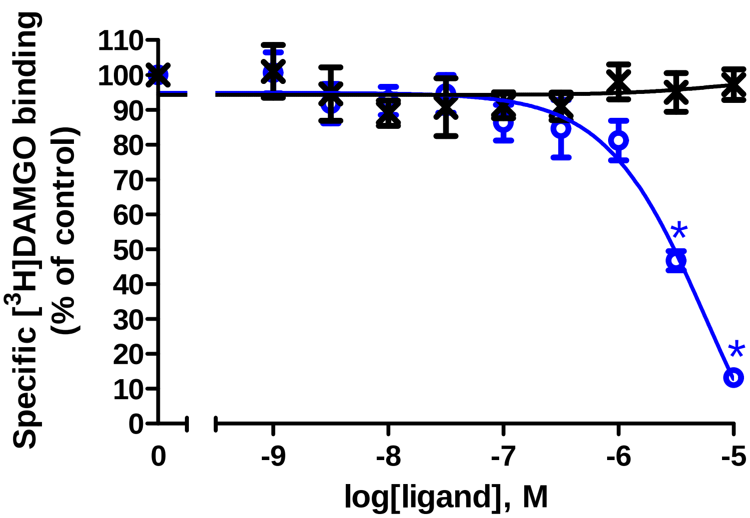

Figure 11. Competition of the CB_1 receptor inverse agonist SR141716 and the CB_1/CB_2 agonist Win55,212-2 for the binding sites of [^{3}H]DAMGO. MOR-CHO cell membranes (10 μg) were incubated with the radioligand (1 nM) in the presence of increasing concentrations of SR141716 (○) or Win55,212-2 (X). The nonspecific binding was measured with 10 μM naloxone and subtracted. Specific binding in the absence of competitors, corresponding to 2286 ± 56 fmol x (mg protein)$^{-1}$, was defined as 100%. Data are expressed as percentages of the specific binding. The data are means ± S.E.M., n = 3, all performed in duplicate. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant inhibition of specific [^{3}H]DAMGO binding by ligands.

The possibility of SR141716 binding with low affinity to GPCRs other than the CB_1 receptors have been proposed (Sim-Selley et al., 2001). Using radioligand competition binding assays in MOR-CHO cell membranes, we tested whether SR141716 is able to bind directly to the MORs. Increasing concentrations (10^{-9}-10^{-5} M) of SR141716 and Win55,212-2 were used against 1 nM [^{3}H]DAMGO, and the inhibition of its specific binding was detected. Although Win55,212-2 had no effect, SR141716 almost fully displaced [^{3}H]DAMGO, with an IC_{50} of 5.7 µM (Figure 11). This result suggested that SR141716 may bind directly to the MORs, albeit with low affinity. It should be noted that the inverse agonist effect of SR141716 is also manifested at low (micromolar) concentrations.

5. DISCUSSION

Classically, GPCRs were considered as a single polypeptide chain consisting of 7TM domains. However, the concept that there may be a significant cross-talk and even physical interaction among different receptors is becoming widely accepted (Milligan and Smith, 2007; Schulte and Levy, 2007; Vazquez-Prado et al., 2003). In addition, it is becoming apparent that the efficacy, i.e. agonist, antagonist or inverse agonist feature of a ligand is not an inherent property, but may vary from tissue to tissue (Fredholm et al., 2007; Milligan, 2003). Promiscuity of the receptors may also manifest at the G-protein level, i.e. the same receptor can be coupled to various G-proteins, resulting in different intracellular effects (Chabre et al., 2009; Hur and Kim, 2003). Allosteric regulation, i.e. regulation of receptors by binding a ligand or an effector molecule at the protein's allosteric site (that is, a site other than the orthosteric ligand binding site) may also greatly contribute to the complexity of receptor-mediated signaling. These interactions may modify the binding and/or signaling properties of the receptor, thereby may open-up new directions for drug discovery. In the present work, cross-talk between CB_1 and $GABA_B$ receptors in rat hippocampus on one site (Cinar et al., 2008), and the multifaceted action of the well-known CB_1 receptor antagonist SR141716 on G-protein signaling on the other site (Cinar and Szűcs, 2009) have been revealed.

5.1. Cross-talk between CB_1 and $GABA_B$ receptors

Using the [^{35}S]GTPγS binding assay stimulated by the respective ligands of either the $GABA_B$ or the CB_1 receptors alone or in their combination to assess receptor function, we have shown cross-inhibition of G-protein signaling between the $GABA_B$ and the CB_1 receptors in rat brain hippocampal membranes. Cross-inhibition of the two receptor systems seemed to be tissue-specific that only manifested in membranes of hippocampus, but not cerebral cortex or spinal cord. The efficacy of the CB_1 receptor agonist R-Win55,212-2 in stimulating [^{35}S]GTPγS binding was significantly decreased by nanomolar concentrations of the $GABA_B$ antagonist, phaclofen (Figure 4A). Importantly, higher concentrations of phaclofen (0.1-10 µM) had no significant effect on R-Win55,212-2 signaling, ruling out the possibility that phaclofen would directly antagonize the binding sites of R-Win55,212-2 (Table 3). The specific CB_1 receptor antagonist AM251 at a low dose (1 nM) also attenuated the efficacy of $GABA_B$ receptor mediated signaling (Figure 5A, Table 4).

One possible explanation for the cross-talk between the CB_1 and $GABA_B$ receptors would be competition of the two receptor systems for a common G-protein pool. Both receptors are known to couple to $G\alpha_{01}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, but not $G\alpha_{i1}$ in the rat hippocampal synapses (Straiker et al., 2002). Previous studies have shown that CB_1 receptors have a high affinity for $G_{i/o}$ proteins and can sequester them from common G-protein pools, thereby preventing signaling by neighboring α2-adrenergic and somatostatin receptors (Vasquez and Lewis, 1999). However, this mechanism would not be able to explain the reciprocal inhibition we observed. Not only the CB_1 antagonist inhibited $GABA_B$ signaling, but the $GABA_B$ antagonist also inhibited CB_1 receptor signaling in hippocampal membranes.

Ligands that are positive or negative allosteric modulators of G-protein coupled receptors have been amply documented (Milligan and Smith, 2007; Prinster et al., 2005; Ross, 2007; Terrillon and Bouvier, 2004). Recently, it has been shown that coactivation of μ-opioid and CB_1 receptors resulted in attenuation of signaling by either receptors and hypothesized to involve allosteric modulations of the receptors by their ligands (Rios et al., 2006). The observation that the $GABA_B$ antagonist at nanomolar, but not higher, concentrations inhibited CB_1 signaling and 1 nM of the CB_1 antagonist inhibited $GABA_B$ signaling suggest that the ligand binding characteristics of the receptors are altered due to their interaction. Altered ligand binding and/or signaling seem to be common features of receptor hetero-oligomers, thus constituting a useful 'biochemical fingerprint' to detect them in natural tissues (Ferre et al., 2007; Franco et al., 2007; Gomes et al. 2001; Hebert and Bouvier, 1998; Milligan, 2006). Opioid receptor complexes composed of the μ- and δ-subtypes showed a substantial increase in the binding of μ-ligands by a low concentration (10 nM) of a variety of δ ligands, including antagonists in heterologous cells (Gomes et al., 2004). Cross-inhibition of G-protein coupled receptors was shown for hetero-oligomers of beta-adrenergic and angiotensin (Barki-Harrington et al., 2003) and μ-opioid and CB_1 receptors (Rios et al., 2006). Very recently, angiotensin AT1 receptor blockers were shown to cause cross-inhibition of homo-oligomerized AT1 receptors (Karip et al., 2007). The CB_1 receptor has been shown to form hetero-oligomers with a variety of other G-protein coupled receptors (for a review see Wager-Miller et al., 2002) and the $GABA_B$ receptor *per se* is a hetero-dimer (Robbins et al., 2001).

Thus, it is an attractive hypothesis to speculate that the CB_1 and $GABA_B$ receptors may form a hetero-oligomer complex in the hippocampus. However, such conclusion can not be verified by the [^{35}S]GTPγS binding assay alone, thus future studies employing additional techniques (e.g. co-immunoprecipitation, co-transfection, fluorescence resonance energy transfer) will be needed to explore this possibility.

Notably, cross-talk of the two receptors may only occur between certain populations of the two receptor systems. The small extent (≈ 20%) of the inhibition of G-protein signaling deriving from the interaction also supports this notion. GABAergic axon terminals were shown to express high levels of CB_1 receptors in the hippocampus (Katona et al., 1999; Tsou et al., 1999). The observation that functional $GABA_B$ receptors may directly control GABA release via CB_1-independent mechanism on GABAergic terminals (Neu et al., 2007) raises the possibility that other axon terminals with less CB_1 receptors such as glutamatergic (Katona et al., 2006, Monory et al., 2006) or cholinergic might play a role in this interaction.

It is known that $G_{i/o}$ proteins represent an essential step in the transduction mechanism underlying the amnesia induced by activation of the GABAergic system (Galeotti et al., 1998). Both $GABA_B$ and CB_1 receptors participate in cognition processes in hippocampus (Ameri, 1999; Brucato et al., 1996; Hampson and Deadwyler, 1999). The two receptor systems mutually influence the action of each other. GABA released from GABAergic presynaptic terminals, suppresses further GABA release via activation of $GABA_B$ autoreceptors on the terminals (Scholz and Miller, 1991). Presynaptic CB_1 cannabinoid receptors, expressed predominantly on axons of CCK-containing interneurons in the hippocampus, reduce GABA release when activated (Katona et al., 1999). Likewise, presynaptic CB_1 (Kawamura et al., 2006; Takahashi and Castillo, 2006) and $GABA_B$ (Dutar and Nicoll, 1988; Kulik et al., 2003) receptors on glutamatergic axon terminals are functionally coupled to inhibition of glutamate release. Interestingly, both $GABA_B$ and CB_1 receptor antagonists were reported to improve cognitive performances in a variety of animal models (Bowery et al., 2002; Mallet and Beninger, 1998**;** Terranova et al., 1996). While low doses of CB_1 antagonists were reported to improve memory in rats, this effect was lost at higher doses (Wolff and Leander, 2003). It is intriguing to correlate these data with ours (Cinar et al., 2008) and speculate that the interaction between CB_1 and $GABA_B$ receptors systems might play a role in cognition in hippocampus.

5.2. CB_1 receptor-independent actions of SR141716 on G-protein signaling of opioid receptors

Our work has demonstrated that the inverse agonist effect of SR141716 is CB_1 receptor-independent (Cinar and Szucs, 2009). This evidence is based on the observation that the extents of SR141716 inhibition of the basal activities were very similar in the wt mouse cortex (containing various kinds of GPCRs including cannabinoid receptors and MORs), MOR-CHO membranes (expressing homogenous MORs), and their counterparts lacking CB_1 receptors, i.e. CB_1-KO cortex and parental CHO membranes (lacking endogenous MORs). Win55,212-2 may bind to CB_1, CB_2 and the non-CB_1, non-CB_2 putative cannabinoid receptors (for a review, see Begg et al., 2005). Since neither the effects of SR141716 nor those of Win55,212-2 were antagonized by the neutral CB_1 antagonist O-2050 in the CB_1-KO mouse cortex (Figure 6D), it might be postulated that the observed effects could be mediated via the CB_2 or the putative CB receptors. However, these possibilities are unlikely to explain inverse agonism by SR141716. As we have shown, while the inverse agonist effect of SR141716 persisted, Win55,212-2 had no significant effect in the parental CHO cell membranes, indicating that CHO cells do not contain significant levels of endogenous CB_1, CB_2 or the putative CB receptors (Figure 10).

The inhibitory effect of SR141716 on the G-protein signaling in the parental CHO cell membranes raises the possibilities that SR141716 may act in a non-receptor-mediated fashion (Dennis et al., 2008), e.g. via a direct membrane effect, by changing the membrane fluidity (Bloom et al., 1997). However, this is unlikely as SR141716 inhibited the basal $[^{35}S]GTP\gamma S$ binding in a concentration-dependent and saturable manner, which implies receptor-mediated action, as we have demonstrated (Figure 9A) and as reported by others (Breivogel et al., 2001; Sim-Selley et al., 2001).

It has also been proposed that SR141716 may act as an inverse agonist at GPCRs other than the CB_1 receptors, binding to these GPCRs with much lower affinity (Sim-Selley et al., 2001). A growing number of orphan GPCRs have been reported to be activated by lipid ligands, such as cannabinoids etc. (Yin et al., 2009). SR141716 behaves as an agonist at the recently discovered GPR55 cannabinoid receptors (Henstridge et al., 2009), and as an antagonist at non-CB_1/non-CB_2 endothelial and CNS cannabinoid receptors (for a rewiew, see Begg et al., 2005). It has been reported that a high concentration of SR141716 exhibits competitive antagonism on the adenosine receptors in the rat brain (Savinainen et al., 2003). Besides the effects of SR141716 on certain GPCRs, it also displays antagonism on TRPV1 (Gibson et al., 2008).

Our data have revealed that SR141716 also influences MOR signaling. SR141716 competitively inhibited DAMGO signaling in the wt and the CB_1-KO mouse cortex (Figure 7) and slightly decreased that in MOR-CHO (Figures 9-10) membranes. Importantly, SR141716 binds directly to MORs, albeit with a low affinity of 5.7 μM (Figure 11). SR141716 shares a piperidine ring and aromatic structures with opioid ligands, such as loperamide and fentanyl (di Bosco et al., 2008; Kane et al., 2006). Since the piperidine ring is important for binding to the MORs, this may give rise to the direct binding of SR141716 to the MORs at high concentrations.

PTX treatment fully abolished the inhibitory effect of SR141716 on the basal G-protein activity (Figure 9A). This confirms that the CB_1-independent inverse agonism of SR141716 is mediated via PTX-sensitive G_i/G_o-proteins. It was intriguing that the combination of DAMGO and SR141716 (10 μM each) led to a significant ($p < 0.05$) 169 ± 22% stimulation of the G-protein signaling when the MORs were uncoupled from $G_{i/o}$-proteins by PTX in MOR-CHO cells (Figure 8B). This novel signaling was totally blocked by naloxone, indicating that it occurs via the MORs (Figure 8B). It may be envisaged that the binding of SR141716 and DAMGO to the MORs may induce a conformational change in the receptors, allowing them to interact more readily with PTX-insensitive G-proteins (e.g. G_s, G_z, G_q or G_{12}, etc). The MORs may have higher affinity for the inhibitory G-proteins than for others. Consequently, when these interactions are inactivated by PTX, the stimulatory component may be manifested. In accordance with this observation, it has been reported that inactivation of G_i/G_o-proteins by PTX unmasks the ability of DAMGO to stimulate adenylyl cyclase activity, which is in contrast with the inhibition observed without PTX treatment in MOR-CHO cells (Szűcs et al., 2004). Increasing evidence indicates that a single receptor type may be linked to the formation of multiple, simultaneous intracellular pathways. Chronic morphine treatment caused desensitization of this novel signaling (Figure 8B). Further studies are required to reveal the G-protein type(s) that participate in this novel MORs-mediated signaling.

The physiological relevance of CB_1 receptor-independent action of SR141716 is open to question in light of the high concentration of SR141716 needed. After the chronic administration of clinically relevant doses, the concentration of SR141716 in human blood plasma is estimated to be 190 nM (Ken Mackie, personal communication). However, due to its inverse agonist activity under physiological conditions, it was shown that SR141716 induces nausea, emesis and mood depression (Sink et al., 2007). Thus, both *in vitro* and *in vivo* data indicate that the antagonist versus the apparent inverse agonist effects of SR141716 in the brain can be differentiated on the basis of potency (Sim-Selley et al., 2001). Our work has revealed that SR141716 exerts multifaceted effects on G-protein signaling (Cinar and Szücs, 2009). It is anticipated that SR141716 may affect the signaling of not only that of MOR but other GPCRs with similar localization and/or function. The revealed multifaceted actions of the drug should be taken into account when applied in high doses.

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8. Summary

G-protein-coupled receptors (GPCRs), the largest class of cell-surface receptors, are one of the major targets for many current and emerging drugs. Recent developments indicate novel levels of regulations in GPCRs functioning, such as cross-talk at the level of signaling,, constitutive activity and oligomerization of GPCRs. The regulations of GPCRs at multiple levels cause emergence of complexity and specificity of GPCRs targeting.

Cannabinoid CB_1 receptors are the most abundant GPCRs in the brain, with levels ten-fold higher than those of other GPCRs. The CB_1 receptor displays a significant level of constitutive activity, either in non-neuronal cells or in neurons. Increasing number of evidences indicate that the CB_1 receptors show different levels of interaction with other receptor types. Particularly; the CB_1 receptors system shares several features with both the μ-opioid and the $GABA_B$ receptor systems. The pattern of expression of the CB_1 receptors strongly overlaps with that of the $GABA_B$ and the μ-opioid receptors in certain CNS regions. Both the $GABA_B$ and the μ-opioid receptors are predominantly coupled to $G_{i/o}$-proteins as well as the CB_1 receptors. Several studies have revealed a functional interaction of the CB_1 receptors with the $GABA_B$ and the μ-opioid receptors at the level of G-proteins in certain regions of the CNS. Importantly; CB_1, $GABA_B$ and μ-opioid receptors have been shown to display similar pharmacological effects, particularly on pain.

The $GABA_B$ receptors are highly unusual among GPCRs in their requirement for heterodimerization between two subunits, the $GABA_{B1}$ and the $GABA_{B2}$ for functional expression. Immuno-electron microscopic studies have suggested that the $GABA_{B2}$ subunit may be absent, but electrophysiological data have shown the presence of functional $GABA_B$ autoreceptors in CCK-containing interneurons in rat hippocampus (*T. Freund, personal communication)*. This observation raises the possibility that the $GABA_{B1}$ may function in association with additional interacting partners, for example a yet unidentified $GABA_B$ receptor subunit, a distinct GPCR, or a chaperoning protein.

The first highly selective CB_1 receptor antagonist, SR141716 (Rimonabant) has been shown to exert a plethora of pharmacological effects in a number of pathological conditions. These effects are mainly attributed to its antagonistic properties at the CB_1 receptors, although there is increasing evidence that it may also behave as an inverse agonist. However, recent studies have revealed the existence of CB_1 receptor-independent actions of CB_1 inverse agonists. It has been proposed that the inhibitory effect of SR141716 on the basal receptor activity might occur either via a non-receptor-mediated effect or by binding to a site other than the agonist binding site on the CB_1 receptors, or by binding to GPCRs other than the CB_1 receptors, to which it binds with much lower affinity. Although there are data supporting these notions, the exact mechanism of inverse agonism by SR141716 has not yet been clarified.

The current work focused on **1)** investigating if there is functional interaction of the CB_1 and $GABA_B$ receptors at the G-protein level in rat hippocampus, and **2)** assessing the inverse agonist effect of SR141716 in systems containing distinct populations of receptors to determine whether its effect is CB_1 receptor-dependent, and if not, whether it is non-receptor-mediated or occurs by binding to GPCRs other than the CB_1 receptor, for example to the closely related the μ-opioid receptors. The main results are the following:

1.1. The $GABA_B$ receptor antagonist, phaclofen at low doses (1 and 10 nM) significantly attenuated maximal stimulation of [^{35}S]GTPγS binding by the CB_1 agonist Win55,212-2 in rat hippocampal membranes.

1.2. The specific CB_1 antagonist AM251 at a low dose (1 nM) also inhibited the efficacy of G-protein signaling of the $GABA_B$ receptor agonist SKF97541 in rat hippocampal membranes.

1.3. Cross-talk of the CB_1 and $GABA_B$ receptor systems was not detected in either spinal cord or cerebral cortex membranes. These results show that interaction between CB_1 and $GABA_B$ receptors is tissue specific.

2.1. 10 μM SR141716 significantly decreased the basal [^{35}S]GTPγS binding in membranes of the wild-type and CB_1 receptor knock-out mouse cortex, parental Chinese hamster ovary (CHO) cells and CHO cells stably transfected with μ-opioid receptors, MOR-CHO. Accordingly, we conclude that the inverse agonism of SR141716 is CB_1 receptor-independent.

2.2. The inverse agonism of SR141716 was abolished, DAMGO alone displayed weak, naloxone-insensitive stimulation, whereas the combination of DAMGO + SR141716 (10 μM each) resulted in a 169 ± 22% stimulation of the basal activity (that was completely inhibited by the prototypic opioid antagonist naloxone) due to pertussis toxin (PTX) treatment to uncouple MORs from G_i/G_o proteins in MOR-CHO membranes.

2.3. In PTX-treated MOR-CHO membranes, chronic morphine treatment caused desensitization of the stimulatory effect on G-protein signaling induced by co-addition of DAMGO and SR141716.

2.4. It was demonstrated that SR141716 directly bind to μ-opioid receptors, albeit with low affinity (IC_{50} = 5.7 μM).

Consequently, these data revealed reciprocal inhibition of G-protein signaling induced by CB_1 and $GABA_B$ receptors in rat hippocampus. It is intriguing that the cross-talk between CB_1 and $GABA_B$ receptors might be involved in balance tuning the endocannabinoid and GABAergic signaling in hippocampus. In addition, CB_1 receptor-independent actions of SR141716 occurred on G-protein signaling. Its co-application with the μ-opioid agonist DAMGO unmasked novel, pertussis toxin-insensitive opioid signaling in MOR-CHO cells. We concluded that SR141716 exerts multifaceted effects on G-protein signaling. It is anticipated that it may also affect the signaling of other GPCRs. The multifaceted actions of the SR141716 should be taken into account when applied in high doses in the clinics.

Receptor promiscuity, such as demonstrated in the present work, may provide not only high degree of selectivity but also broad complexity of the receptor functionality that can be vital in understanding the side effects of receptor ligands. In addition, they may help to develop selective therapeutic agents. Thereby; our work may provide important data for both basic and pharmaceutical research fields.

9. List of Thesis-Related Publications

Publications in International Refereed Journal:

1. **Cinar R**, Freund TF, Katona I, Mackie K, and Szucs M **(2008)** Reciprocal inhibition of G-protein signaling is induced by CB_1 cannabinoid and $GABA_B$ receptor interactions in rat hippocampal membranes. *Neurochem Int* **52**: 1402–1409.

2. **Cinar R** and Szücs M **(2009)** CB_1 receptor-independent actions of SR141716 on G-protein signaling; co-application with the µ-opioid agonist DAMGO unmasks novel, pertussis toxin-insensitive opioid signaling in MOR-CHO cells. *J Pharmacol Exp Ther* 330 (2), 567-574.

Abstracts Published in Referred Journal:

1. Szucs M, and **Cinar R.** CB_1 receptor-independent effects of the CB_1 receptor antagonist/inverse agonist rimonabant (SR141716). **(2009)**. 12th Meeting of the Hungarian Neuroscience Society, Budapest, Hungary published in *Frontiers in Systems Neuroscience* doi: 10.3389/conf.neuro.01.2009.04.242

2. **Cinar R,** Freund T.F, Katona I, Mackie K, Szucs M**.** Cross-talk between cannabinoid CB_1 and $GABA_B$ receptors in rat brain hippocampus. **(2008)**. Szeged University Biology Ph.D. School Seminars, Szeged, Hungary published in *Acta Biologica Szegediensis* 52(2) p.335.

3. **Cinar R,** Mackie K, Freund T.F., Szucs M. Searching for possible interactions between CB_1 and $GABA_B$ receptors in rat brain hippocampal membranes **(2006)** 31st FEBS Congress, Istanbul, Turkey. PP-54; published in *The FEBS Journal*, 273 (Suppl 1.) p.91.