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Relationship Between CB1 and S1P Receptors in the Central Nervous System

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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"The only evil is ignorance, the only good is knowledge."
-Knowledge

Table of Contents

List of Tables

List of Figures

List of Abbreviations

2-AG.....2-arachidonoylglycerol

7TM.....7 transmembrane

cAMP.....cyclic adenosine monophosphate

CB.....cannabinoid

CB_x.....novel cannabinoid receptor

Cblm.....cerebellum

CP.....CP55,940

CPu.....caudate putamen

CNS.....central nervous system

D2.....Dopamine-2-receptor

GAPs.....G-protein associated proteins

GDP.....guanine diphosphate

GTP.....guanine triphosphate

GPCR.....G-protein-coupled receptor

Hip.....hippocampus

KO.....knockout -/-

LPA.....lysophosphatidic acid

MAG.....monoacylglycerols

NO……..nitric oxide

PNS……..…peripheral nervous system

S1P……..……sphingosine-1-phosphate

SEW……..……SEW2871

SN……..……..……substantia nigra

SphK1/SphK2……..sphingosine kinase 1/2

SR1……...……SR141716A

SR2……...…SR144528

WIN……....WIN55,212-2

WT……..…wild type +/+

Abstract

Relationship Between CB_1 and S1P Receptors in the Central Nervous System

By Lauren Michele Collier, MS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters in Pharmacology and Toxicology at Virginia Commonwealth University.

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There is significant sequence homology and anatomical co-distribution between cannabinoid (CB_1) and sphingosine-1-phosphate (S1P) receptors in the CNS, but potential functional relationships between these lysolipid receptors have not been examined. Therefore, to investigate possible relationships between these two systems at the level of G-protein activation, agonist-stimulated [^{35}S]GTPγS binding and autoradiography were conducted. Autoradiographic studies were first performed to localize receptor-mediated G-protein activation in mouse brain. Coronal brain slices were processed for stimulation of [^{35}S]GTPγS binding using the synthetic cannabinoid agonist WIN 55,212-2 (WIN) or S1P. High levels of WIN- and S1P-stimulated [^{35}S]GTPγS binding were observed in the caudate putamen, hippocampus, substantia nigra, and cerebellum. To further characterize the relationship between S1P- and CB_1-mediated G-protein activation, spinal cords from adult

male CB_1 receptor knockout mice, CNS-deleted $S1P_1$ receptor knockout mice and wild type C57 mice were collected, and assessed using agonist-stimulated [^{35}S]GTPγS binding. Results from this experiment revealed that the $S1P_1$ receptor is predominant in mouse spinal cord. To further investigate potential CB_1 and S1P receptor interactions spinal cords were collected from adult male ICR mice. Additivity studies were preformed using agonist-stimulated [^{35}S]GTPγS binding. Results showed significantly less than additive stimulation when spinal cord tissue was treated with both WIN and S1P. These results suggest an interaction between the CB_1 and S1P receptors in the mouse spinal cord. The effect of cannabinoid antagonists, SR141716A (CB_1) and SR144528 (CB_2) on S1P- and WIN-stimulated [^{35}S]GTPγS binding were also examined in mouse spinal cord homogenates. These results showed that there was no significant difference between S1P-stimulated [^{35}S]GTPγS binding in the presence of SR141716A or SR144528 compared to vehicle control. This shows that S1P produced stimulation independent of the CB_1 or CB_2 receptor. In addition WIN-stimulated [^{35}S]GTPγS binding was not affected by SR144528, but was inhibited by SR141716A, confirming that this action is due to the CB_1 receptor. The combined results of this project demonstrate an interaction between CB_1 and S1P receptors in certain CNS regions where they are co-distributed, such as the caudate putamen, hippocampus, substantia nigra, cerebellum and spinal cord. These results may be due to convergence on a common pool of G-proteins via dimerization or co-localization in lipid rafts, or a possible direct ligand-receptor interaction.

Chapter 1 Introduction

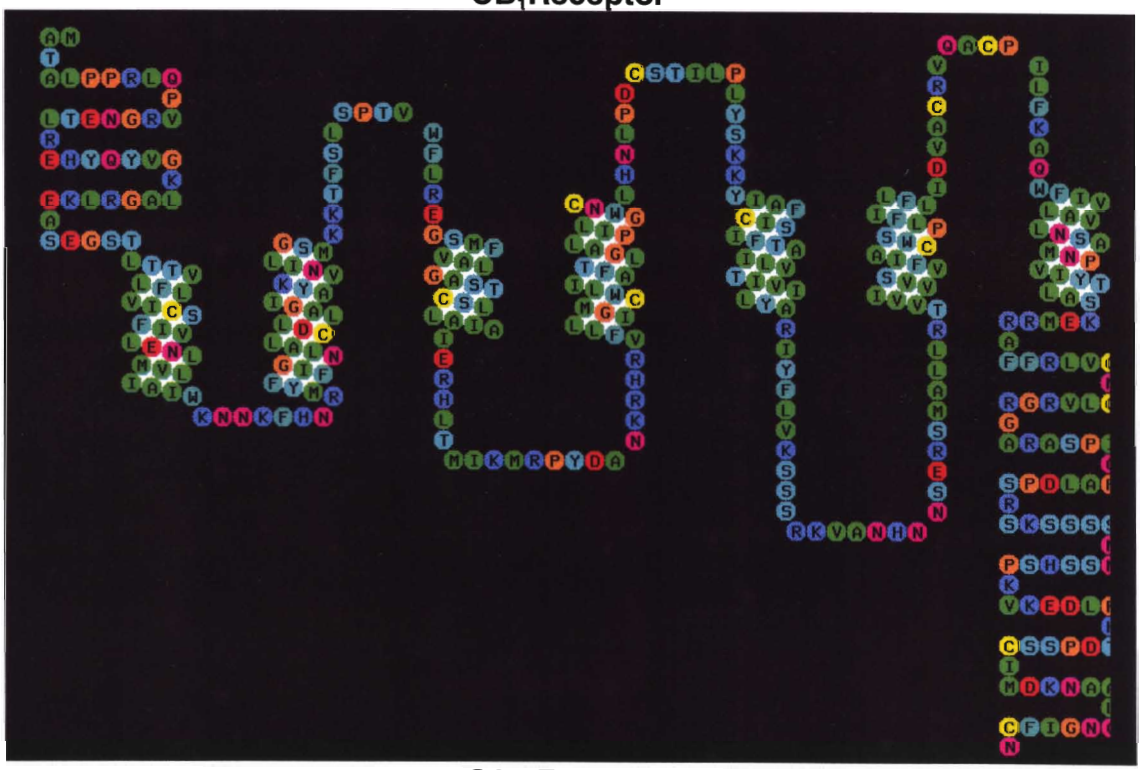
G-protein Coupled receptors

There are over 800 genes in the genome that code for the superfamily of G-protein-coupled receptors (GPCR). These receptors, also known as heptahelical receptors, are characterized by their seven-trans-membrane (7TM) configuration, with an extracellular N-terminus an intracellular C-terminus, and their functional activation of heterotrimeric G-proteins (Lefkowitz *et al.*, 1993; van Neuren *et al.*, 1999). Members of this family include receptors for neurotransmitters, hormones, chemokines and many other endogenous, as well as exogenous, ligands. GPCRs constitute a large and widely distributed superfamily of membrane-bound receptors and are the most common target of therapeutic drugs (van Neuren *et al.*, 1999; Pierce *et al.*, 2002).

In this project we examined two GPCRs in the CNS: the cannabinoid-1-receptor (CB_1 receptor) and the sphingosine-1-phosphate receptors ($S1P_{1\text{-}5}$ receptor). Figure 1 shows the amino acid structures of the CB_1 and S1P receptors (www.wdv.com/CellWorld/Receptors). Both of these receptor systems activate G-proteins (Matsuda *et al.*, 1990; Brambiet *et al.,* 1995; Pyne and Pyne, 2000) and have endogenous ligands that are lysolipids derived from similar precursors (Hla, 2004; DiMarzo *et al.*, 1999; Devane *et al.*, 1992; Stella *et al.*, 1997). The CB_1 and S1P receptors are co-distributed in regions of the CNS and both have been shown to congregate in lipid rafts (Ohanian *et al.,* 2001; Barnett-Norris *et al.*, 2005). Due to the recent advances in the clinical applications using the sphingosine analog FTY720 (2-amino-2-(2-[4-octyphenyl]ethyl)-1,3-propanediol), as an immunosupresant drug; it is

important to examine the possible interaction between the S1P and CB_1 receptors in the CNS (Brinkmann and Lynch, 2002; Brinkmann *et al.*, 2002; Mandala *et al* 2002). These studies might also identify novel receptors that bind endogenous lipid ligands.

CB$_1$Receptor



S1P Receptor

Figure 1. Amino acid sequences of CB1 and S1P receptors (from www.wdv.com/CellWorld/Receptors).

1.1 G-protein coupled receptor structure and families

G-protein coupled receptors have been sequenced and categorized into three distinct families: A, B, C and D. Sequences within families share at least 25% homology in the transmembrane core region, and a distinctive set of highly conserved amino acid residues within the transmembrane regions (Pierce *et al.*, 2002). Family A, the opsin (rhodopsin-like) family is the largest of the GPCR families. CB_1 and S1P receptors belong to family A. Family A also includes receptors for biogenic amines, many small peptide hormones, and neurotransmitters (Dixon *et al.*, 1986; Dohlman, *et al.*, 1991). Family B contains only about 25 members, and includes receptors for the gastrointestinal peptide hormone family, such as secretin and glucagons. Family C is also a small family comprised of metabotropic glutamate receptors as well as $GABA_B$ receptors. Family D is another small family that is comprised of the pheromone receptors.

1.2 G-proteins and signal transduction

GPCR activation is initiated by the interaction of an agonist with the receptor, which causes a conformational change (Sprang, 1997), and stabilizes the receptor in an active state (Lefkowitz *et al.*, 1993; Ulfers *et al.*, 2002). Receptor activation initiates a cascade of cellular responses that begin with the activation of the G-protein. G-proteins

belong to a super family of regulatory GTP hydrolases; they are heterotrimeric guanine nucleotide-binding regulatory proteins (Gilman, 1987; Sprang, 1997). These heterotrimeric G-proteins are comprised of α, β, and γ subunits. Guanine nucleotides (GTP, GDP) bind to the α-subunit of the G-protein (Sprang, 1997). In the inactive state, the α, β, and γ subunits form a complex with GDP bound to the α-subunit of the G-protein (Linder *et al.*, 1992). After ligand binding activates the receptor, the receptor changes conformation and the G-protein is able to interact with the receptor. This interaction increases the disassociation rate of GDP, leading to the release of GDP, and the binding of the more abundant GTP to the α-subunit of the G-protein. The binding of GTP alters the configuration of the α-subunit and leads to its activation (Linder *et al.*, 1992).

Once activated, the GTP-bound α-subunit of the G-protein disassociates from the β/γ-dimer and diffuses along the inner surface of the plasma membrane until it binds with an effector, such as adenylyl cyclase (Sprang, 1997; Pierce *et al.*, 2002). In the case of CB_1 receptors, G-protein activation leads to the inhibition of adenylyl cyclase in a concentration-dependent manner in many regions of the CNS (Matsuda et al., 1990), whereas this inhibition has yet to be observed with S1P receptor activation in the CNS (Selley et al., unpublished). β/γ can also regulate effector functions such as the modulation of N- and P/Q-type Ca^{+2} channels (Koji *et al.*, 1989; Calpham, 1996). Activation of the CB_1 receptor has been shown in the CNS to lead to the inhibition and slowing of the Ca^{+2} current in neurons leading to modulation of neurotransmitter release (Kirby *et al.*, 2000). The signal is terminated when GTP is hydrolyzed back to GDP by the intrinsic GTPase of the α-subunit (Gilman, 1987; Maurine *et al.*, 1992). The rate of

hydrolysis determines the time period between the active and inactive state of the GPCR. Once the GTP is hydrolyzed to GDP, the affinity of the α-GDP subunit for the β/γ-dimer is increased, causing their reassociation and inactivation. This is an example of one G-protein activation cycle (Figure 2). In all experiments for this study, [^{35}S]GTPγS, a nonhydrolyzable, radioactively labeled form of GTP was employed. This technique was used in order to observe G-protein activation by ligands that stimulate CB_1 and S1P receptors both in whole brain slices and spinal cord homogenates (Figure 3).

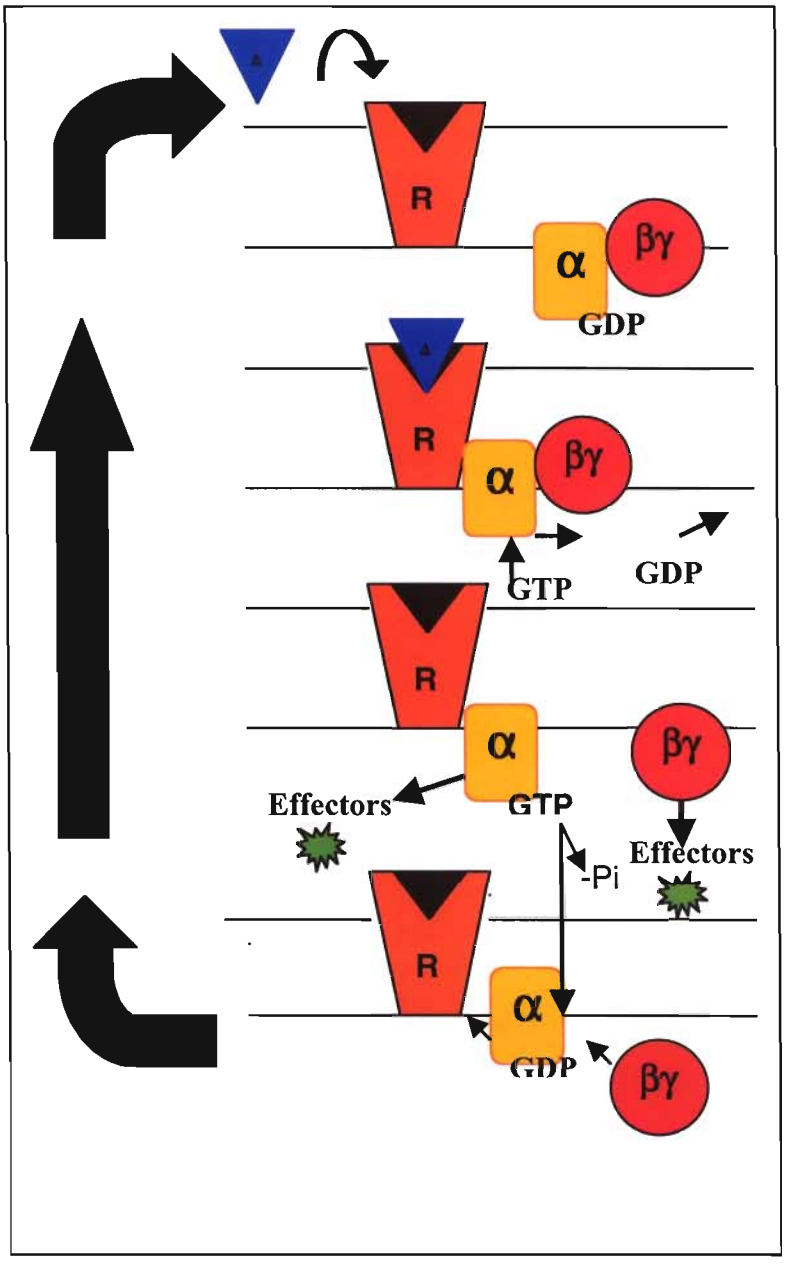


Figure 2. Once activated, the GTP-bound α-subunit of the G-protein disassociates from the β/γ-dimer and diffuses along the inner surface of the plasma membrane until it binds with an effector. β/γ can also regulate effector functions. The signal is terminated when GTP is hydrolyzed back to GDP by the intrinsic GTPase of the α-subunit (Gilman, 1987; Maurine *et al.*, 1992)

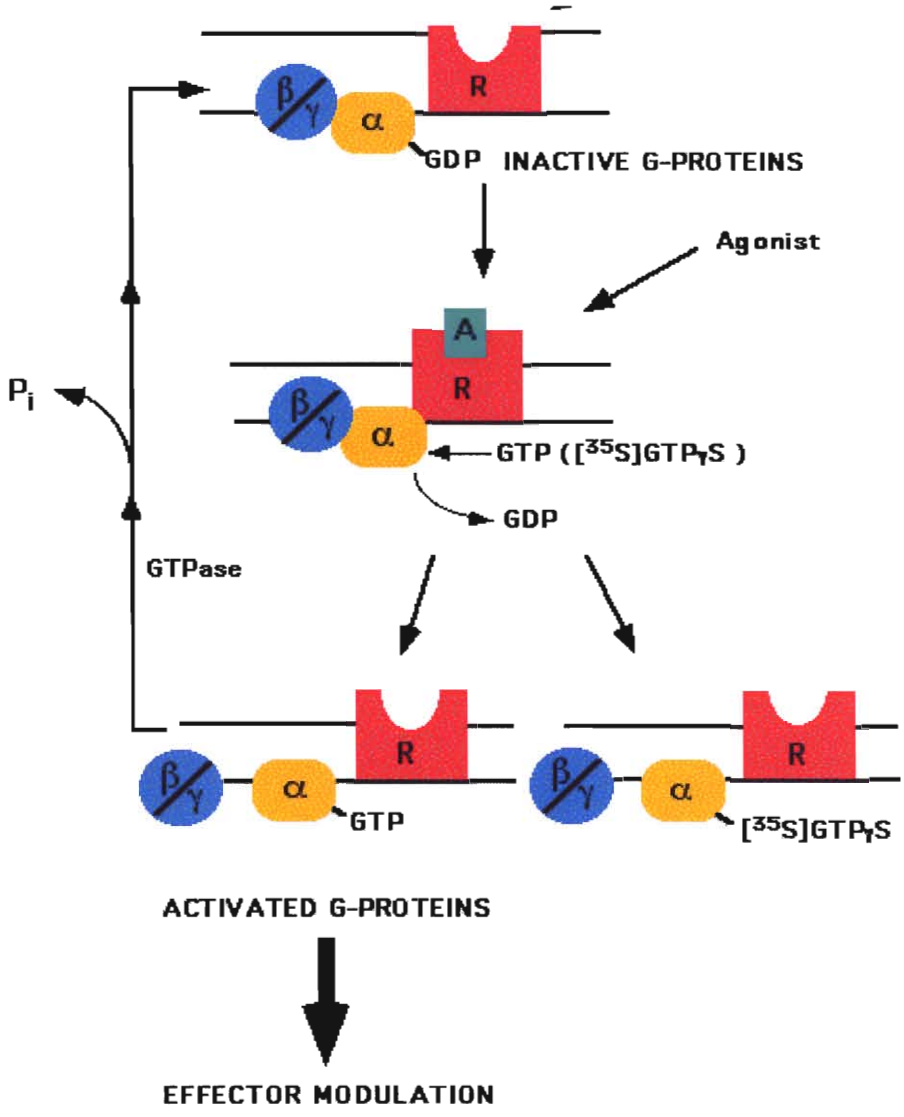


Figure 3. Agonist-stimulated [^{35}S]GTPγS binding compared to the G-protein activation cycle. This technique allows localization of G-protein activation in the mouse CNS and provides a quantitative measure of receptor agonist-stimulated G-protein activation (R=Receptor, A=Agonist).

G-Protein coupling is determined by both the receptor and the Gα subtype. More then 20 different mammalian G-protein α-subunits have been identified and exhibit 60 to 90 percent homology. The Gα subunits have been divided into four major classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (Simon *et al.*, 1991; Pierce *et al.*, 2002). See table 1 for an outline of alpha subunits (Hildebrandt, 1997). Thus far, five types of β-subunits have been identified, as well as twelve γ-subunits, which have approximately 80 percent homology (Hildebrandt, 1997). The possible multiple combinations of the αβγ heterotrimer is one of the reasons for the wide array of effects caused by activation of these receptors.

Table 1. Summary of G_α subtypes. (+)=Stimulate (-)=Inhibit (Hildebrandt, 1997).

Family	α_s	α_{12}	α_i		α_q
Subfamily			α_t	$\alpha_{i/o}$	
Subunit	α_{olf} α_s	α_{12} α_{13}	α_t1 α_t2 α_{gust}	α_o1 α_o2 α_o3 α_i1 α_i2 α_i3 α_z	α_{15} α_{16} α_{11} α_q α_{14}
Effectors	♦AC (+)* ♦ Calcium channel conductance (+)	♦ p115-Rho-GEF* ♦ Na^+/H^+ antiporter (+) ♦ PLA_2 (+)	(1) cGMP-PDE(+)*	♦AC (-)* ♦ (GIR) K^+ channels (+) ♦ (N/PQ) Ca^{+2} channels (-)	♦ PLC_β (+)*

***=direct α subunit effect**

1.3 G-protein coupled receptor interactions

Over the past several years it has been discovered that GPCRs can form dimers or multimers, which alters the function of GPCRs (Wager-Miller *et al.*, 2002; Kearn *et al.*, 2005). Evidence exists for G-protein dimerization of many GPCRs including the CB_1 and S1P receptors. These reports have generated interest in this topic due to the possible pharmacological and clinical applications (Dean *et al.*, 2001; Rodriguez *et al.*, 2001).

Over the past decade the views of cell membranes and their associated proteins have evolved. Many recent studies have indicated that there is sequestering of proteins and lipids that form specific regions of cell membranes called lipid rafts (Simons *et al.*, 2004). The function of lipid rafts is to concentrate GPCRs in order to increase the efficiency and specificity of signal transduction. This concentrating of GPCRs aids in the action of the receptors by facilitating interactions between proteins such as GPCRs and G-proteins, as well as preventing unwanted "cross-talk" between signals (Selbie and Hill, 1998; Moffett *et al.*, 2000; Barnett-Norris *et al.*, 2005). It was proposed by Schroeder *et al.* in 1994 that proteins with high affinity for an ordered lipid environment are selectively recruited to these lipid rafts. It is well documented that heterotrimeric G-proteins, regulators of G-proteins such as G-protein-associated-proteins (GAPs), and GPCRs are targets of lipid rafts due to their high rate of lipid modification, such as fatty-acylation, myristoylation, and palmitylation (Galbiati *et al.*, 1999). Evidence of GPCR interaction, colocalization, and cross talk is important to this project because it suggests a possible mechanism for interactions between the CB_1 and S1P receptors in the CNS.

Cannabinoids

Marijuana has been used for centuries both for its psychoactive and medicinal properties. The potential therapeutic effects include relief of nausea and vomiting, appetite stimulation, pain relief (antinociception) and sedation (Dewey *et al.,* 1996). However these medicinal effects are also accompanied by less desirable effects such as memory impairment and psychoactive effects (Dewey *et al.*, 1986; Hollister *et al.*, 1986). It has also been observed that prolonged use of marijuana leads to tolerance and dependence in many animals, including humans (Jones *et al.*, 1981). In this case animals treated with marijuana would need higher doses of the drug in order to achieve the desired effect (tolerance), as well as physical withdrawal symptoms should they discontinue use of the drug (dependance) (Sim-Selley, 2003; Gonzalez et al., 2005).

Cannabinoids are the group of C_{21} compounds that are typically present in *Cannabis Sativa L*, of which Δ^9-THC has been shown the most psychopharmacologically active constituant (Gaoni and Mechoulam, 1964). The identification of Δ^9-THC led to the development of synthetic ligands that also bind to the CB_1 receptor, such as WIN55,512-2 and CP-55,940. In 1984, it was demonstrated that cannabinoid agonists inhibit cyclic adenosine monophosphate (cAMP) production in a pertussis toxin (PTX)-sensitive manner (Howlett *et al.*, 1985). In 1988 Howlett and co-workers demonstrated the presence of a cannabinoid receptors using [^{3}H]CP-55,940 in membrane homogenates (Devane *et al,* 1988). The existence of cannabinoid receptors was confirmed with the

cloning of the CB_1 receptor from rat cerebral cortex in 1990 by Matsuda and colleagues (Matsuda *et al.*, 1990).

1.5 Cannabinoid Receptors

To date, two subtypes of the cannabinoid receptors have been identified and cloned, CB_1 and CB_2 (Matsuda *et al.*, 1990; Munro *et al.*, 1993). The amino acid sequence of these receptors was consistent with that of a G-protein-coupled receptor (Matsuda *et al.*, 1990). The CB_1 receptor has been cloned from rat, mouse and human tissues and exhibits a 97 to 99% amino acid sequence homology across species. The mouse CB_2 receptor has been cloned and has an 82% sequence identity to the human CB_2 receptor (Matsuda *et al.*, 1990; Gerard *et al.,* 1991; Shire *et al.*, 1995). The CB_2 receptor exhibits 48% homology with the CB_1 receptor.

Radioligand autoradiography, in situ hybridization, and immunohistochemical studies have shown that CB_1 receptors are primarily localized in the central nervous system (CNS) (Herkenham *et al.*, 1991; Devane *et al.*, 1988 Tsou *et al.*, 1997; Sim-Selley, 2003), as well as the testes (Schuel *et al.*, 1998), and vascular and endothelial systems (Sugiara *et al.*, 1998; Wagner *et al.*, 1998). CB_2 receptor distribution is localized primarily to the immune system, particularly in cells such as macrophages and monocytes (Cabral *et al.*, 1998; Hajos *et al.*, 2002), although recent evidence suggests CB_2 receptors are present in brain stem (Gong *et al.*, 2006).

1.6 CB_1 Receptor Pharmacology

CB_1 receptor pharmacology has been well characterized in the past few decades. These studies have been accomplished using Δ^9-THC, as well as synthetic cannabinoid analogs, such as WIN55,212-2 and CP55,940 (Howlett *et al.*, 2002). In 1994 Rinaldi-Carmona *et al.* published results indicating that a pyrazol derivative, SR141716A (SR1), bound to CB_1 receptors with high affinity and fully reversed the effects of potent cannabinoid agonists, suggesting that SR1 acted as an antagonist (Rinaldi-Carmona *et al.*, 1994; Tiziana *et al.*, 2000). SR1 is also reported to exhibit inverse agonist properties (Bauoboula *et al.*, 1997; Landsman *et al.,* 1997, Sim-Selley *et al.*, 2001).

The search for endogenous ligands for the CB_1 receptor, or endocannabinoids, has resulted in the discovery of several lipid compounds that can activate the receptor. N-acyl ethanolamines (NAEs), of which the most widely studied compound is anandamide (N-arachodonoyl-ethanolamine) (Di Marzo *et al.*, 1999; Devane *et al.*, 1992), are produced by a two-step enzymatic pathway and are synthesized and released on demand (Sugiura *et al.*, 1996; Cadas *et al.*, 1997). The monoacylglycerols (MAGs), of which 2-arachidonoylglycerol (2-AG) is the most potent agonist at the CB_1 receptor (Stella *et al.*, 1997), are produced in the CNS via the enzymatic hydrolysis of diacylglycerol, and are also believed to be synthesized and released on demand (Bisogno *et al.*, 2003). The

magnitude and duration of action for both classes of endocannabinoids is tightly controlled by the uptake and catabolism of these precursor lipids (Cravatt and Lichtman, 2002).

The CB_1 receptor has been reported to couple mainly to $G\alpha_{i/o}$ G-proteins (Howlett et al., 1984), although recently there has been some evidence of coupling to $G\alpha_s$ and $G\alpha_{q/11}$ (Rubino *et al.*, 2000; Martin *et al.*, 2004; Kearn *et al.*, 2005; Lauckner *et al.*, 2005). CB_1 receptor activation of $G\alpha_{i/o}$ G-proteins inhibits adenylate cyclase activity (Pacheco *et al.*, 1993; Selley *et al.*, 2004), inhibits Ca^{+2} currents (Mackie *et al.*, 1992), activates G-protein inwardly rectifying K^+ channels (GIRKs) (Felder *et al.*, 1995), and activates certain MAP kinases (Bouaboula *et al.*, 1996). Evidence has shown that CB_1 receptor-mediated activation of $G\alpha_{q/11}$ can increase intracellular Ca^{+2}, and activation of $G\alpha_s$ by CB_1 receptors increases cAMP (Lauckner *et al.*, 2005, Kearn *et al.,* 2005)

1.7 CB_1 Receptor Localization in the CNS

Radioligand autoradiography and immunohistochemistry have been used to demonstrate CB_1 receptor localization within the CNS. CB_1 receptors are located in brain regions whose function is associated with the pharmacological action and behaviors associated with cannabinoid intoxication (Herkenham *et al.*, 1991; Sim *et al.*, 1996; Jansen *et al.*, 1992). For example, disruption of short-term memory been reported with the use of marijuana (Dewey, 1986). In 1991 Herkenham *et al.* reported a high density of CB_1 receptors in the hippocampus. This discovery explains why cannabinoids affect

memory, because the hippocampus is a brain region that is involved with the processing of new information (Robertson *et al.*, 2005).

Autoradiographic studies using ^{3}H-CP55,940 have shown dense CB_1 receptor binding throughout the basal ganglia (caudate putamen, globus pallidus, and substantia nigra), which is consistent with reported effects of cannabinoids such as motor impairment and reward (Herkenham *et al.*, 1991). CB_1 receptors have also been located in the molecular layer of the cerebellum, another important motor center in the CNS that has been associated with the static ataxia effect of cannabinoids (Patel *et al.,* 2001). Modest levels of CB_1 receptors have also been observed in the hypothalamus, which correlates with the hypothermic effects of CB_1 receptor activation by agonists such as WIN and THC (Schmeling *et al.*, 1976). CB_1 receptors have also been located in the PAG and the spinal cord, areas that would be associated with the analgesic effects of activation (Martin *et al.,* 1998; Suplita *et al.,* 2006).

Immunohistochemical studies suggest that CB_1 receptors expressed on neuronal projections in the substanta nigra are derived from neurons within the caudate-putamen and extend throughout the basal ganglia (Herkenham *et al.*, 1991; Tsou *et al.*, 1998). CB_1 immunoreactivity has also been observed primarily in axons and boutons across neocortical regions of the brain (Eggan *et al.,* 2006). Neocortical association regions, such as the prefrontal and cingulate cortices, also demonstrate a higher density of CB_1 receptors compared with primary sensory and motor cortices. The high density, distinctive distribution, and localization to inhibitory terminals of CB_1 receptors in the prefrontal and cingulate cortices suggests that the CB_1 receptor might play a critical role in the circuitry that controls cognitive functions (Eggan *et al.,* 2006). CB_1

immunohistochemical labeling in the molecular layer of the cerebellum has been observed in parallel fibers originating from granule cells, climbing fibers originating in the inferior olive, and inhibitory interneurons in the deep molecular layer. Inhibitory interneurons terminating on Purkinje cell dendrites have been shown to contribute to cannabinoid-mediated cerebellar plasticity (Ashton *et al.,* 2004). These results are consistent with the intense expression of cannabinoid CB_1 receptors in the molecular layer of the cerebellum (Ashton *et al.,* 2004). The brain stem and spinal cord have been shown to have relatively low but detectable and pharmacologically relevant levels of CB_1 receptors (Rice *et al.*, 2002). Recently it was found that the same brain stem circuits that contribute to the analgesic effects of morphine also meditate the antinoceceptive effects of cannabinoids (Meng *et al.*, 2004; Rice *et al.*, 2002). These findings are important to the main topic of this thesis because many of the same CNS circuits also contain S1P receptors (Waeber and Chiu, 1999; Churn, 2004; Toman and Spiegel, 2002).

1.8 CB_1-Induced Behavior and Modulation of Pain

Characterization of the behavioral effects of CB_1 receptor activation by cannabinoids has been advanced by the development of many widely accepted animal models. An excellent example of such a model used in mice and rats to measure cannabinoid-induced behavior is known as the tetrad. Developed by Martin and colleagues in 1986, the tetrad is characterized by analgesia, catalepsy, hypothermia, and

inhibition of spontaneous locomotor activity in response to cannabinoid agonists (Razdan, 1986; Compton *et al.*, 1993).

Cannabis has been promoted as a potential analgesic for many centuries. CB_1 receptor agonists, such as THC, exhibit antinoceceptive activity in animal models of acute pain, such as the tail flick test and the hot plate test (Martin and Lichtman, 1998). This analgesic effect is believed to occur both centrally and peripherally (*ie* inflammation) (Tsou *et al.*, 1998). The antinociceptive properties of CB_1 receptor agonists suggest that these agents might be of therapeutic use in the treatment of pain. However it has proven very difficult to separate the analgesic effects from a number of unwanted side effects, such as psychoactive and cognitive behavioral alterations (Cravatt and Lichtman, 2004).

Sphingosine-1-Phosphate

The S1P receptors are another group of GPCRs that are activated by endogenous lipid ligands. Sphingosine-1-phosphate (S1P) occurs widely in nature and its role as a lipid mediator has been of great interest since the discovery that it acts via distinct GPCRs. In mammals, S1P receptors are expressed throughout the body and are believed to regulate many important physiological actions, such as immune modulation, vascular development, cardiac function, vasoconstriction/dilation, and neonatal development (Hla, 2004).

1.9 S1P Receptors

S1P receptors are part of a larger group of receptors known as the lysophospholipid group of GPCRs (formerly called EDG receptors). There were originally nine EDG (EDG1-9) receptors, four of which we now know mediate lysophosphatidic acid (LPA) signaling and five that mediate signaling through S1P receptor activation. The S1P receptors (1-5) are all GPCRs that exhibit characteristics of such receptors, such as the 7TM domain and coupling to G-proteins. The first S1P receptor identified was $S1P_1$, which is thus far the most well characterized of the five S1P receptor subtypes (Ishii *et al.*, 2004; Pyne and Pyne, 2002).

The $S1P_1$ receptor in both human and mouse contains 382 amino acids, has a widely distributed adult tissue expression, and is coupled to $G\alpha_i$ proteins (Anliker and Chun, 2004). $S1P_2$ receptors also are widely distributed in adult mammals and couple to multiple G-proteins that include $G\alpha_{12}$, $G\alpha_q$, and $G\alpha_i$ (Kupperman *et al.*, 2000). $S1P_3$ receptors exhibit homology and similar tissue distribution to $S1P_1$, but couples to multiple G-proteins like $S1P_2$ receptors (Anliker and Chun, 2004). $S1P_4$ receptors have low homology to the other S1P receptors, and their tissue distribution is limited to the immune system (Fukushima *et al.*, 2001). $S1P_5$ receptors couple to multiple G-proteins including $G\alpha_{12}$, $G\alpha_q$, and $G\alpha_i$, and show intermediate expressions levels when compared to the expression of the other four receptors. $S1P_5$ receptor distribution in the CNS is primarily restricted to white matter (Fukushima *et al.*, 2001; Anliker and Chun, 2004).

1.10 Sphingosine-1-Phosphate Metabolism

Sphingolipids are membrane constituents of all eukaryotic cells, and refer to lipids consisting of a head group attached to the 1-OH of ceramide (Maceyka et al., 2005). In mammalian cells, ceramidases can hydrolyze ceramide to form sphingosine, which can then be phosphorylated by sphingosine kinases (SphK1, SphK2) to form S1P. S1P can then be cleaved by S1P-lyase and degraded or converted back to sphingosine by specific phosphphohydrolases (Maceyka *et al.*, 2005).

S1P levels in cells are relatively low, and tightly regulated. S1P is mainly stored in platelets and is released upon platelet activation (Yatomi *et al.*, 1995). S1P is present in an albumin-bound form at physiologically relevant concentrations in serum (Igarashi

and Yatomi, 1998). S1P is also produced in other cell types in response to growth signals such as cytokines (Pyne and Pyne, 2000). A wide variety of stimuli have been shown to increase SphK activity and induce the formation of S1P in different cell lines, these include growth factors (Pyne *et al.,* 1996), carbachol (Meyer zu Heringdorf *et al.*, 1998), and antigen-stimulated immunoglobulin E receptors (Choi *et al.*, 1996).

1.11Sphingosine-1-phosphate Receptor Activation

Many signaling pathways are activated in response to S1P receptor stimulation leading to widespread physiological actions. S1P affects many different cell types, such as those in the vascular, immune, skeletal, reproductive, and central and peripheral nervous systems (Hla, 2004).

In the vascular system activation of endothelial cells with S1P results in the formation of nitric oxide (NO), leading to endothelial relaxation thereby promoting vasorelaxation (Igarashi *et al.*, 2001). However, in some vascular beds S1P can act as a vasoconstrictor depending on the receptor subtype; such as the $S1P_2$ receptor in the coronary artery smooth muscle cells (Ohmori *et al.*, 2003). These studies show that S1P is important in the regulation of vascular tone (Hla, 2004).

S1P receptors are expressed in many types of immune cells, where they act as immunoregulators. This evidence is derived in part from studies on the immunosuppressive agent FTY720. This compound was originally discovered to inhibit organ rejection by inducing lymphopenia, or the sequestration of lymphocytes to the thymus, lymph nodes and Peyer's patches (Brinkmann and Lynch, 2002). In 2002

Brinkmann *et al.* and Mandala *et al.* reported that FTY720 is phosphorylated by SphK and can then act as an agonist at four of the five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$). These results suggest that the normal function of S1P in the immune system is the regulation of lymphocyte trafficking (Hla, 2004).

Most of the evidence for S1P involvement in skeletal and limb development is provided by the observation that $S1P_1$ -/- mice, although embryologically lethal, display shortened limb bud structure, interdigital sculpting, and cartilage primordium formation between embryonic days 12.5-14.5 (Liu *et al.*, 2000; Chae *et al.*, 2004). S1P receptors are expressed in the reproductive cells such as the testes and the ovaries. It has been observed in various experiments that S1P signaling through the $S1P_1$ receptor is involved in the survival of both the male germ cells and female oocye; although the mechanism by which this is achieved remains elusive (Yatomi *et al.*, 1997; Tilly *et al.*, 2002).

The modulation and manipulation of S1P signaling via its receptor subtypes suggests an enormous potential for a novel class of therapeutics for the treatment of a number of diseases. It is therefore important to understand the CNS effects of this potentially important class of therapeutic drugs.

1.12 Sphingosine-1-Phosphate Receptor Activation in the CNS

Recent evidence has uncovered some roles for S1P receptors in both the CNS and peripheral nervous systems (PNS); the studies in this thesis will focus on the CNS. S1P receptors are abundantly expressed in the CNS; for example the $S1P_1$ receptor is highly expressed in the hippocampus and cerebellum (Chae *et al.*, 2004; Hla, 2004). S1P

receptors mediate many important CNS actions, such as neurite rounding and axonal growth (Chun, 2004). Earlier studies have shown that S1P is a potent inducer of neurite rounding, growth cone collapse and axon collapse which are all very important processes in neuronal development in the CNS (Van Brocklyn *et al.*, 1999; Windh *et al.*, 1999; Hla, 2004). These observations provide evidence that S1P receptor activation is important in the modulation of CNS development and remodeling. S1P receptor activation has also been implicated in the prevention of apoptosis in key cells in the CNS, such as oligodendrocytes (Jaillard *et al.*, 2005). This action shows an important supportive role in the CNS, by helping to maintain efficient neuronal transmission (McGiffert *et al.*, 2002; Jaillard *et al.*, 2005).

Although some research has been conducted on S1P receptor modulation of neuronal development, the role of S1P receptors in the adult CNS remains to be elucidated. For example it is not known whether S1P receptors in the CNS exhibit post-synaptic, pre-synaptic, or cell body location. It has also yet to be reported whether activation of these receptors produces significant behavioral effects. These and other basic questions need to be answered before the role of the S1P receptors can be fully understood in the CNS.

Purpose of Project

Due to the multiple similarities between the CB_1 and S1P receptor systems, our laboratory has begun studies to elucidate the role of S1P in the CNS and determine possible interactions of S1P receptors with CB_1 receptors. In this project, we employed techniques to examine the activation of CB_1 and S1P receptors in the CNS, with a focus on their possible interaction in the spinal cord. Evidence of interactions between the S1P and CB_1 receptors will contribute to elucidating the role of S1P receptors in the CNS, as well reveal potential therapeutic applications through the use of drugs acting at these receptor systems.

1.12 Receptors Similarities

The CB_1 and $S1P_{1\text{-}5}$ receptors are heptahelical GPCRs (Matsuda *et al.*, 1990; Ishii *et al.*, 2004; Pyne and Pyne, 2002). These GPCRs exhibit significant sequence homology (Fukushima et al., 2001). In fact, examination of the phylogenetic tree of lysolipid receptors reveals a close relationship between the CB_1 and S1P receptors (see figure 4, Kostenis, 2004). Both of these receptors are found in the immune and nervous systems (Herkenham *et al.*, 1991; Devane *et al.*, 1988; Hla, 2004). CB_1 and S1P receptors are co-

distributed in the CNS, which could include co-localization in certain regions. In the CNS, both CB_1 and $S1P_{1,2,3,5}$ receptor-mediated G-protein activation are found in high abundance in the striatum, hippocampus, cerebellum, and the cortex as well as white matter tracts (Sim *et al.,* 1996; Sim-Selley, 2002; Waeber *et al.*, 1999).

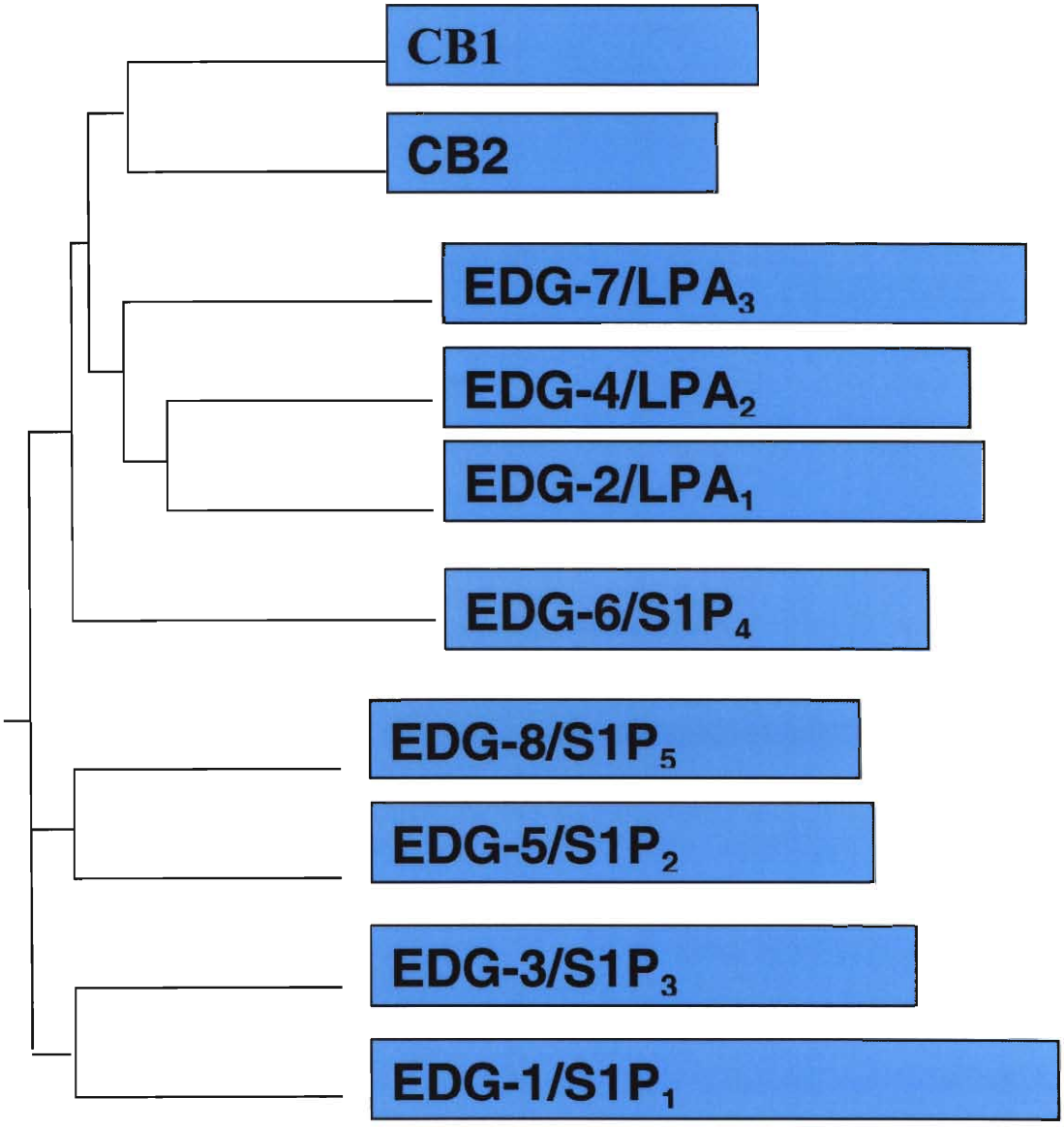


Figure 4. Phylogenetic tree of lysolipid receptors, (Kostenis, 2004).

1.13Ligand Similarity

Ligands for CB_1 and S1P receptors also exhibit similarities. In both systems, receptors are activated by endogenous lipid ligands: sphingosine-1-phosphate for S1P receptors and anandamide and 2-AG for CB_1 receptors (Hla, 2004; Di Marzo *et al.*, 1999; Devane *et al.*, 1992; Stella *et al.*, 1997). Although these lipid ligands are derived from different precursor molecules, (arachidonic acid vs. ceramide), they exhibit similarities in structure (Figure5 Wertz, 1992).

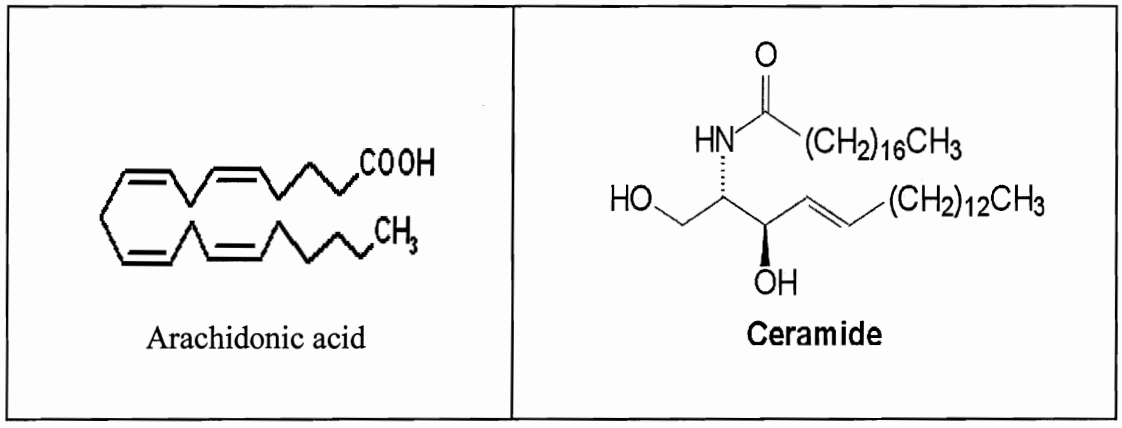


Figure 5. Structure of arachidonic acid and ceramide, the precursors of the endogenous ligands for the CB_1 and S1P receptors (Wertz, 1992).

There are other potential relationships between these ligands. The activation of the CB_1 receptor is coupled to the generation of the S1P precursor molecule ceramide, further connecting these two endogenous ligand systems (Guzman et al., 2001). It is also known that the production of these endogenous ligands is tightly regulated and both have

been implicated in many cellular processes including apoptosis and the regulation of metabolic functions (Chun, 2004; Guzman et al., 2001)

1.14 Hypothesis & Specific objectives

It was hypothesized, based on receptor and ligand similarities in the CB_1 and S1P receptor systems, that an interaction between S1P and CB_1 receptors would be found in the CNS. The purpose of this project was to characterize S1P-mediated G-protein activation in the adult mouse CNS and the possible relationship between the S1P and CB_1 receptors. These questions were addressed by autoradiographic localization of CB_1 and S1P receptors in the CNS and the use of agonist-stimulated [^{35}S]GTPγS binding in membrane homogenates prepared from wild type and knockout mouse spinal cord. In all experiments, agonist-stimulated [^{35}S]GTPγS binding was employed in order to observe G-protein activation by ligands that stimulate CB_1 and S1P receptors in whole brain slices and spinal cord membrane homogenates (see Figure 3 for schematic of [^{35}S]GTPγS technique).

Chapter 2 Materials and Methods

Materials

2.1 Drugs and Chemicals

Sphingosine-1-phosphate (S1P) was purchased from Biomol (Plymouth Meeting, PA). 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3[3-(trifluoromethyl0phenyl]-1-2-4-oxadiazole (SEW2871), the $S1P_1$ receptor agonist, was purchased from Cayman Chemical (Ann Arbor, MI). R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN55,212-2), guanosine-5'-O-thio triphosphate (GTPγS), guanine diphosphate (GDP) and bovine serum albumin (BSA) were purchased from Sigma Chemical Company (St. Louis, MO). N-(piperidinyl)-5-(4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A), (-)-cis-3R-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl)-1R-cyclohexanol (CP 55, 940), N-[(1S)-Endo-1,3,3-trimethylbyclo[2.2.1]heptan-2-y1]-5-(4-chlorro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528), Tyrosyl-D-alanylglycyl-N-methyl-N-(2-hydroxyethyl)phenyalaninamide Trifluoroacetate) (DAMGO), and H-Tyr-D-Pen-Gly-Phe(Cl)-D-Pen-OH (PCl-DPDPE) were provided by the Drug Supply program of the National Institute on Drug Abuse (NIDA). [^{35}S]GTPγS (1150-1300 Ci/mmol) was purchased from Perkin

Elmer Life Sciences (Boston, MA). All other reagent grade chemicals used in these experiments were purchased from Sigma Chemical Co. or Fisher Scientific (Pittsburgh, PA).

2.2 Subjects

Male ICR mice (24-30 g) were obtained from Harlan Labs (Indianapolis, IN). Male CB_1-/- and CB_1 +/+ mice (22-30 g) of C57BL/6 background were born in the Virginia Commonwealth University vivarium. These mice originated from breeding pairs of CB_1 +/- parents created by CB_1 receptor gene mutation in MP12 embryonic stem cells by Zimmer *et al* (Zimmer et al., 1999). $S1P_1$ CNS conditional knockout($S1P_1$-/-) and control mice ($S1P_1$ +/+) were provided by Dr. Richard Proia, of the Genetics of Development and Disease branch of NIDDK at the National Institutes of Health in Bethesda, MD. These mice were from C57BL/6 backgrounds and were developed using a Nestin/CRE system such that the animals are $S1P_1$ deficient only in the CNS, no longer rendering the mutation embryonically lethal.

Methods

2.3 Agonist-stimulated [^{35}S] GTPγS autoradiography

Tissue Preparation. ICR mice were sacrificed by decapitation and brains were removed. The brains were then frozen in isopentane (2-methylbutane) at a temperature of –30 to -35°C for 2-4 minutes. The brains were then left on dry ice to allow isopentane to evaporate for 5 minutes. Brains were stored at -80°C until assay. In these studies unfixed tissue was used because fixation of the tissue would have eliminated G-protein activation in this assay (Sim-Selley, unpublished).

Sectioning of Brains. Tissue was cut on a Leica CM 3050 cryostat at –20°C. Twenty micron sections were collected on gelatin-subbed slides in triplicate. The regions collected included the striatum, hippocampus, substantia nigra, and cerebellum, and were identified according to *The Rat Brain in Stereotaxic Coordinates*, (Paxinos and Watson, 1986). The slides were collected in racks set in a shallow container placed in a chamber containing ice, creating a cold and humid environment for thaw mounting. Slides were desiccated under vacuum and refrigerated overnight at 4°C. The next day, the slides were transferred to a slide box and stored at -80°C until assay.

Autoradiographic Assay. On the day of the assay, slides were removed from the freezer and allowed to return to room temperature under a cool dryer for 30 minutes. Slides were then incubated in TME buffer (50mM Tris, 3mM $MgCl_2$, 0.2mM EGTA, 100mMNaCl, pH of 7.4) for 10 minutes at 25°C. After the incubation in the TME buffer, the slides were transferred to preincubation solution containing 2mM GDP + 10mU/ml adenosine deaminase + TME buffer 0.5% BSA for 15 minutes at 25°C. These conditions decrease basal binding, thereby significantly increasing the signal to noise ratio. The addition of GDP promotes the inactivation of Gα, NaCl decreases spontaneous receptor activation and ADase renders adenosine inactive at its receptor, which is also coupled to $G\alpha_{i/o}$ G-proteins. After the preincubation the slide were separated into two groups (basal and stimulated). The basal solution contained 40pM [^{35}S] GTPγS, 2mM GDP, 10mU/ml adenosine deaminase in BSA/TME buffer. Agonist-stimulated incubation solution contained 40pM of [^{35}S]GTPγS + 2mM GDP + 10mU/ml adenosine deaminase + TME/BSA buffer and either 10μM WIN55,2122-2, 50μM S1P, 10μM WIN55,2122-2 and 50μM S1P, or 50μM S1P and 1μM SR141716A and incubated for two hours at 25°C. These concentrations of agonist have been shown to produce maximal stimulation of [^{35}S]GTPγS binding in concentration effect curves in membranes (Collier, Selley, Sim-Selley, unpublished).

The slides were then rinsed twice for 2 minutes each in 50mM Tris buffer (50mM Tris pH 7.0) at 4°C, and then once for 30 seconds in 4°C ddH_2O. The slides were then dried under a stream of cool air. The next day the slides, as well as ^{14}C microscales, were

exposed to KODAK BioMax MR film for 24 hours, then developed using a KODAK M35A automatic developer.

Analysis. Films were digitized using COHU High Performance CCD camera, and analyzed using National Institutes of Health IMAGE program for Macintosh computers. The quantification of images was obtained by densitometric analysis using the ^{14}C microscales as reference standards. Net agonist-stimulated activity in the brain sections was calculated by subtracting basal activity from agonist-stimulated activity. All autoradiographic results are presented in net-stimulated [^{35}S]GTPγS binding (nCi/g). This set of experiments was performed with an n=5. Significance was determined by analysis of variance using two tailed students t-test, where all $p \leq 0.05$ were considered significant.

3.1 Agonist stimulated [^{35}s]GTPγS Binding in membranes

Spinal Cord Membrane Preparation. Mice were sacrificed by decapitation and whole spinal cords were harvested by high-pressure water ejection from the spinal column. Tissue was stored at -80°C until use. Spinal cords were homogenized in 5ml of TME membrane buffer (50mM Tris-HCl, 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl, pH 7.7) for approximately 10 seconds. The homogenized tissue was then centrifuged at 50,000 x g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of TME buffer and homogenized. The protein concentration was detected using the

method of Bradford (Bradford, 1976) and then the protein was diluted to the desired concentration with TME buffer. Next, 10 mU/ml adenosine deaminase was added and the membrane solution was incubated for 10 minutes at 30°C.

Agonist-stimulated [^{35}s]GTPγS Binding Assays. Test tubes containing 8μg of spinal cord membrane protein in TME buffer, 10μM GDP, 0.1nM [^{35}S]GTPγS, 0.1% BSA (except for the additivity study in which 0.5% BSA was used) and the indicated concentrations of agonist and /or antagonist were then incubated for 90 minutes in at 30°C. Incubations were terminated by vacuum filtration using a Brandel harvester to filter the samples through GF/B glass fiber filters. The radioactivity was then determined by liquid scintillation counting at 95% efficiency for ^{35}S after extraction of the filters in Econo-Safe scintillation fluid. All assays were performed in either duplicate or triplicate and have an n≥4.

Assays performed. Three separate agonist-stimulated [^{35}S]GTPγS binding studies were performed for this study. The first incorporated the use of both CB_1 and $S1P_1$ knockout (KO) mouse spinal cord membranes in which concentration effect curves for WIN55,212, CP55,940, S1P or SEW2871-stimulated [^{35}S]GTPγS binding were generated . This studied was performed in triplicate with an n=4. In the second study the effects of the cannabinoid antagonists SR141716A and SR144528 on agonist-stimulated activity was examined in both WIN- and S1P-stimulated [^{35}S]GTPγS binding assays in ICR mouse spinal cord membranes. This study was also performed in triplicate with an n=6. In the last series of agonist-stimulated [^{35}S]GTPγS membrane studies we examined the additive

effects of WIN, S1P, and opioid agonists (DAMGO and DPDPE) in naïve ICR mouse spinal cord membranes. This study was performed in duplicate with an n=7.

Data analysis. Net-stimulated [^{35}S]GTPγS binding was defined as [^{35}S]GTPγS binding in the presence of drug minus basal binding. Percent stimulation was calculated as (net stimulated [^{35}S]GTPγS binding/basal) x 100%. E_{max} and EC_{50} values were calculated from non-linear regression analysis by iterative fitting of the concentration-effect curves to the Langmuir equation $E=(E_{max}/[EC_{50}$ + agonist concentration] x agonist concentration) using JMP (SAS for Macintosh: Cary, NC). The additivity study results were expressed as actual additivity (net stimulation measured in the assay) vs. theoretical additivity (net stimulation of the drug A alone added to net stimulation of drug B alone). Significance was determined by analysis of variance using the two tailed students t-test, where $p \leq 0.05$ was considered significant. Additivity Studies were also presented as % additivity *([Actual net stimulation - highest actual net stimulation provided by a single agonist] / [Theoretical net stimulation – highest actual net stimulation provided by a single agonist) x 100).* Statistical significance was determined by the distribution of Y method as compared to 100%, and $p \leq 0.05$ was considered significant.

Chapter 3 Results

Previous studies (Sim *et al.*, 1995; Waeber *et al.,* 1999; Paugh *et al.,* 2006) have established that both cannabinoid agonists and S1P stimulate [^{35}S]GTPγS binding in isolated in sections and membranes from the CNS (Waeber *et al.,* 1999). Based on the similarities between CB_1 and S1P receptor systems (see introduction), experiments were conducted to assess CB_1 and S1P receptor-mediated G-protein activity in the CNS. To determine whether CB_1 and S1P receptors interact at the level of receptor and/or G-protein activation, experiments were performed to examine agonist-stimulated [^{35}S]GTPγS binding in brain sections and spinal cord membrane homogenates. CB_1 and $S1P_1$ receptor knockout mice were also used to characterize potential interactions between these receptors in the spinal cord.

3.1 Autoradiographic results

In order to observe the co-distribution of CB_1 and S1P receptor-mediated G-protein activation, agonist stimulated [^{35}S]GTPγS autoradiography was used. Brain sections from male ICR mice were incubated with either WIN or S1P and [^{35}S]GTPγS. Visual analysis revealed that both WIN and S1P showed high levels of [^{35}S]GTPγS stimulation in similar regions, showing co-distribution of $CB_1$and S1P receptors in the mouse brain (Figures 6 and 7). The regions of dark red color are the regions of highest G-protein activation, yellow and green show intermediate activation and blue areas show low

stimulation. Both WIN and S1P show high levels of agonist-stimulated [^{35}S]GTPγS binding in the cortex, as well as moderate stimulation in the hypothalamus, whereas only WIN-stimulated G-protein activation is observed in the entopeduncular nucleus. In order to further assess co-distribution in the regions associated with the cannabinoid system, the caudate putamen, hippocampus, substantia nigra and cerebellum were analyzed densitometrically. The results showed that S1P-stimulated [^{35}S]GTPγS binding was higher than WIN-stimulated [^{35}S]GTPγS binding in regions including the caudate putamen, hippocampus and cerebellum. Interestingly these regions are known to contain high levels of CB_1 receptors and CB_1 receptor-mediated G-protein activity. However, WIN did show a greater G-protein activation in the substantia nigra, a region known for its dense CB_1 receptor localization (Tsou *et al.*, 1998). See Table 2 and Figure 8 for comparative analysis of WIN vs. S1P stimulation of [^{35}S]GTPγS binding and Figures 6 and 7 for representative of autoradiograms.

Drug	Caudate-putamen	Hippocampus	Substantia Nigra	Cerebellum
WIN	202.5 ± 30.07	208.9 ± 21.44	512.1 ± 5.84	148.0 ± 14.77
S1P	393.6 ± 38.07	245.8 ± 28.28	335.1 ± 14.26	372.0 ± 22.52

Table 2. Net WIN- and S1P-stimulated [^{35}S]GTPγS autoradiography. These data show high levels of G-protein activation by produced by WIN and S1P in all regions examined. These experiments were performed in triplicate with an n=5, data is expressed in nCi/g

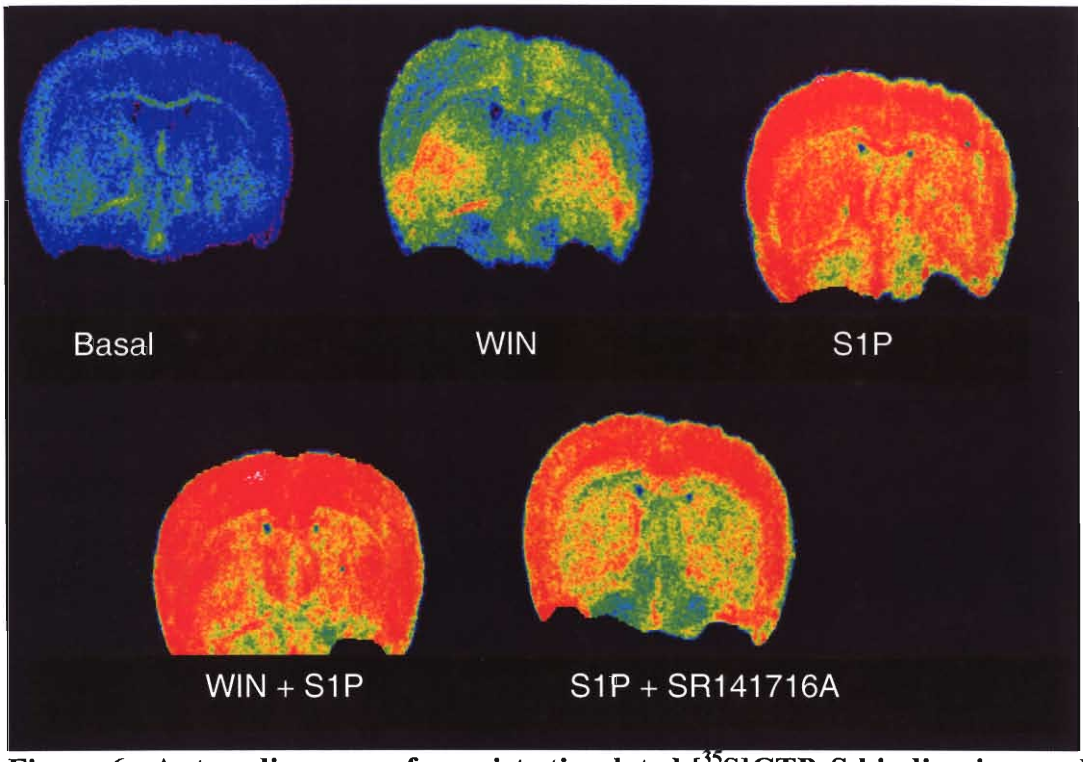


Figure 6. Autoradiograms of agonist-stimulated [^{35}S]GTPγS binding in caudate putamen.

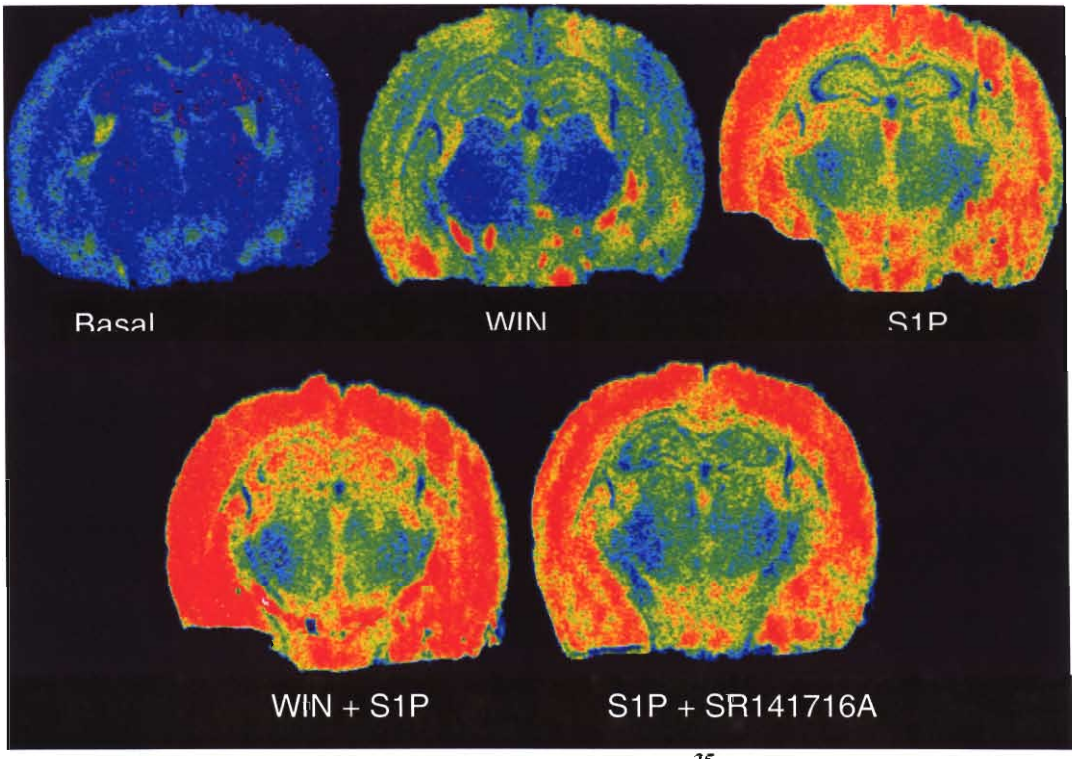


Figure 7. Autoradiograms of agonist-stimulated [^{35}S]GTPγS binding in hippocampus

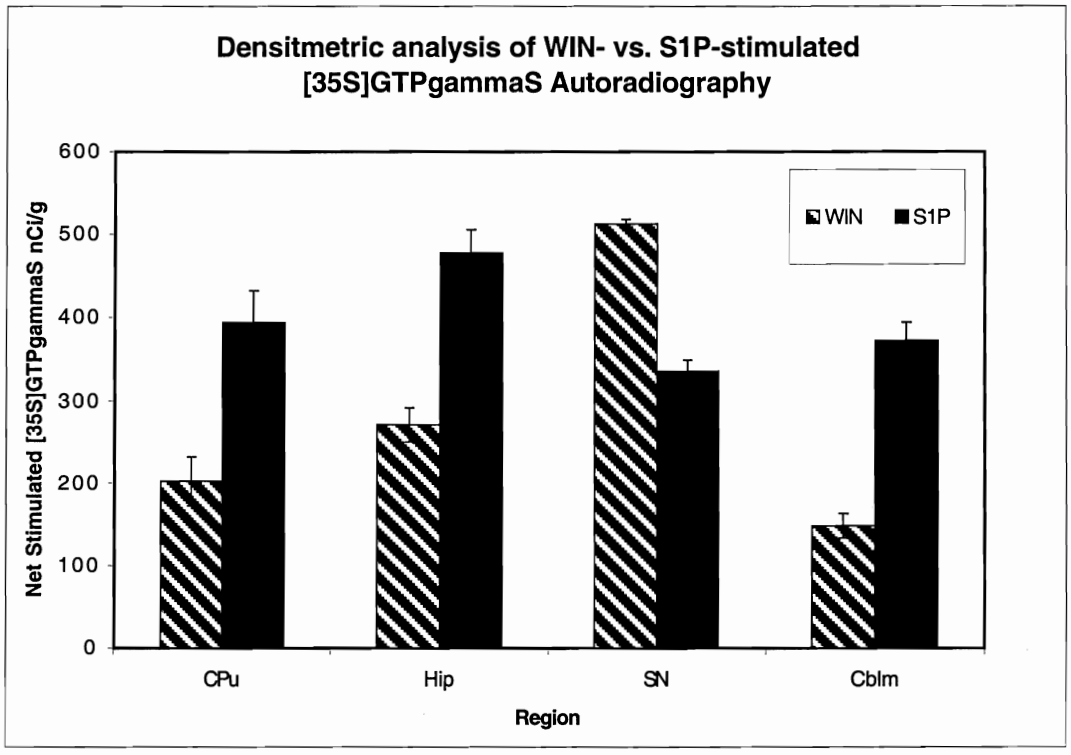


Figure 8. Densitmetric analysis of WIN- Vs. S1P-stimulated[^{35}S]GTPγS autoradiography. Results show high levels of S1P-stimulated [^{35}S]GTPγS activation in the same regions known to have high densities of CB_1 receptors. These regions include the caudate putamen, hippocampus, substantia nigra, and the cerebellum. These experiments were conducted in triplicate using an n=5.

To determine whether there is an interaction between the CB_1 and S1P receptor systems, additive effects of S1P and WIN were also examined in the mouse brain. This was accomplished using autoradiography, in which sections were incubated with S1P and WIN alone or simultaneously, in the presence of [^{35}S]GTPγS. Theoretical additivity of net stimulation was calculated by adding the stimulation produced by S1P + the stimulation produced by WIN and was compared to stimulation measured in S1P+WIN treated sections (actual net stimulation). As shown in Table 3 and Figure 9, there was a significantly less than additive effect in WIN+S1P-stimulated [^{35}S]GTPγS binding in the caudate putamen, substantia nigra and cerebellum. These results suggest that CB_1 and S1P receptors interact in these regions.

Region	**Caudate Putamen**	**Hippocampus**	**Sunstantia Nigra**	**Cerebellum**
Actual	390.4 ± 35.19*	366.5 ± 39.76	601.7 ± 13.23*	413.1 ± 29.58*
Theoretical	596.1 ± 66.01	475.8 ± 47.95	847.2 ± 17.79	520.8 ± 14.71

Table 3. Results of the autoradiography additivity study. Data show a significantly less than additive effect in the caudate putamen, substantia nigra, and cerebellum ($p \leq 0.05$). These experiments were performed in triplicate with n=5.

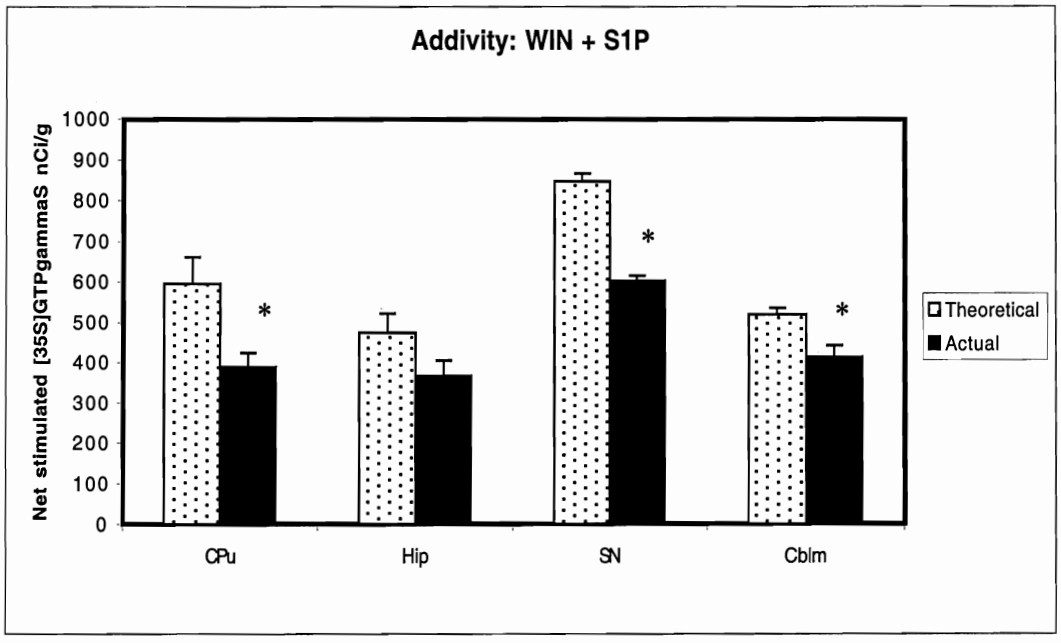


Figure 9. Theoretical Vs. Actual Additivity. Results show that the actual additive net stimulation of WIN + S1P is significantly less than the theoretical additive net stimulation in the caudate putamen, the substantia nigra, and the cerebellum. This experiment was conducted with an n=5, and significance was determined by $p \leq 0.05$.

In order to determine whether S1P binds to or interacts with the CB_1 receptor, or SR141716A might bind to S1P receptors, we examined the effect of SR141716A on S1P-stimulated [^{35}S]GTPγS autoradiography. To examine whether CB_1 receptors contribute to S1P-mediated G-protein activation, sections were treated with S1P + SR141716A. As shown in Figure 10, SR14716A significantly decreased [^{35}S]GTPγS stimulation in the CPu and the SN ($p=.04$ and $p=.02$, respectively), whereas no decrease in [^{35}S]GTPγS stimulation was observed in the hippocampus or cerebellum. These results are consistent with the additivity study, which also indicated that S1P and CB_1 receptors interact in similar regions (caudate putamen and the substantia nigra).

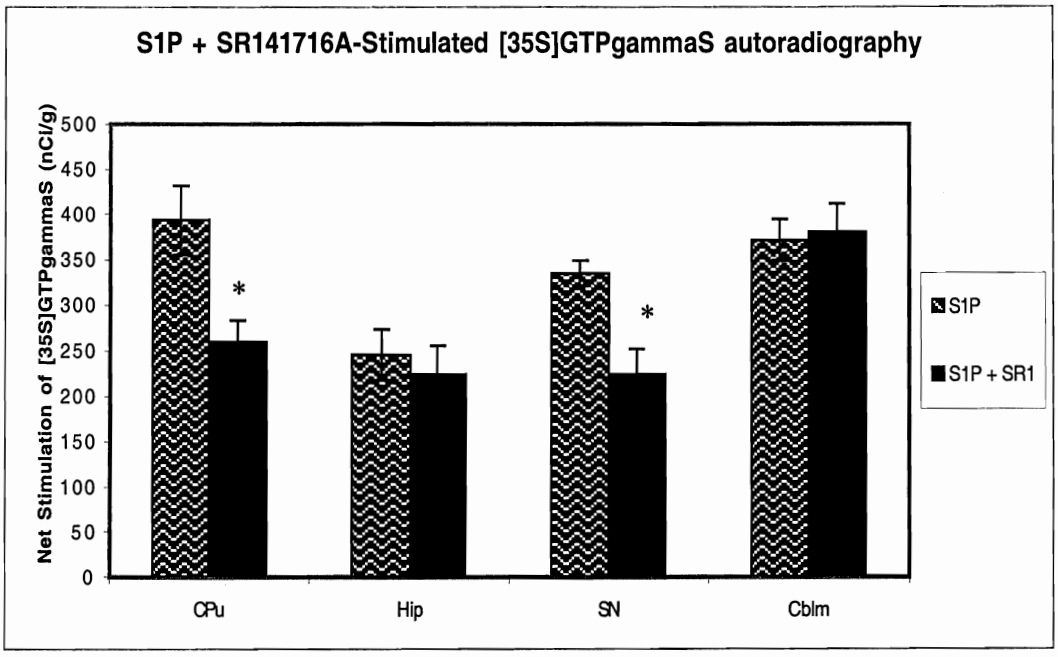


Figure 10. Effect of SR141716A on S1P-stimulated [^{35}S]GTPγS autoradiography. These results show that S1P-stimulated [^{35}S]GTPγS binding in the caudate putamen and the substantia nigra is inhibited by 1μM SR141716A. There is no significant decrease in net stimulation in the hippocampus or cerebellum (*=$p \leq 0.05$).

3.2 Agonist stimulated [^{35}S]GTPγS binding in CB_1 and $S1P_1$ knockout mouse spinal cord

In order to asses the possible changes in CB_1 and S1P receptor-mediated G-protein activation in the spinal cords of mice with targeted genetic deletion of CB_1 and $S1P_1$ receptors, agonist-stimulated [^{35}S]GTPγS binding studies were performed using spinal cords from knockout mice. To compare the results of agonist-stimulated [^{35}S]GTPγS binding in both CB_1 and $S1P_1$ receptor knockout mice, spinal cord membrane homogenates were prepared and incubated with [^{35}S]GTPγS and the CB_1 agonists (WIN and CP) or S1P agonists (S1P or SEW). Results showed that WIN- and CP-stimulated [^{35}S]GTPγS binding was eliminated in the CB_1 knockout mouse spinal cord, but remained robust in wild type mice. S1P- and SEW- (selective $S1P_1$ agonist) stimulated [^{35}S]GTPγS binding was similar in the CB_1 knockout mouse vs. wild type mouse. These results demonstrate that S1P does not appear to activate CB_1 receptors in the mouse spinal cord. Furthermore, S1P-mediated G-protein activation was greatly diminished in spinal cords from $S1P_1$ knockout mice compared to wild type mice. SEW stimulation was completely eliminated in the $S1P_1$ -conditional knockout mouse spinal cord as compared to the wild type mouse spinal cord. These results lead to the conclusion that $S1P_1$ is the primary S1P receptor in mouse spinal cord. WIN- and CP-stimulated [^{35}S]GTPγS binding were similar in $S1P_1$ knockout and wild type mice. See Table 4 for E_{Max} values and Table 5 for EC_{50} values. These results show that there is no significant interaction of cannabinoid agonists with the $S1P_1$ receptor in the mouse spinal cord. Results of these studies are shown in Table 4 and Figures 11-18.

Drug	CB_1 KO E_{MAX} (%stim)	CB_1 WT E_{MAX} (%stim)	$S1P_1$ KO E_{MAX} (%stim)	$S1P_1$ WT E_{MAX} (%stim)
WIN	ND	42.34 ± 5.72	48.75 ± 3.25	52.93±4.63
S1P	102.28 ± 7.62	94.54 ± 7.81	16.36 ± 1.76	66.07±3.66
CP	ND	29.19 ± 3.33	37.23 ± 6.46	40.49±4.51
SEW	70.29 ± 6.05	67.29 ±7 .00	ND	52.58±4.00

Table 4. Results: E_{max} values for cannabinoid and S1P agonist-stimulated [^{35}S]GTPγS binding in spinal cord from CB_1 and $S1P_1$ receptor knock out and wild type mice. These experiments were performed in triplicate with n=4.

Drug	CB_1 KO EC_{50}	CB_1 WT EC_{50}	$S1P_1$ KO EC_{50}	$S1P_1$ WT EC_{50}
WIN	ND	35.64 ± 9.7	1.00 ± 0.7	3.6 ± 1.6
S1P	4.04 ± 0.5	4.12 ± 0.5	6.1 ± 2.0	0.6 ± 0.2
CP	ND	3.33 ± 0.8	0.1 ± 0.09	0.005 ± 0.001
SEW	8.63 ± 1.2	9.15 ± 1.0	ND	2.5 ± 0.7

Table 5. Results: EC_{50} values for cannabinoid and S1P agonist-stimulated [^{35}S]GTPγS binding in spinal cord from CB_1 and $S1P_1$ knock out and wild type mice. These experiments were performed in triplicate with n=4.

ND: Unable to fit data to curve using Langmuir equation due to no significant stimulation.

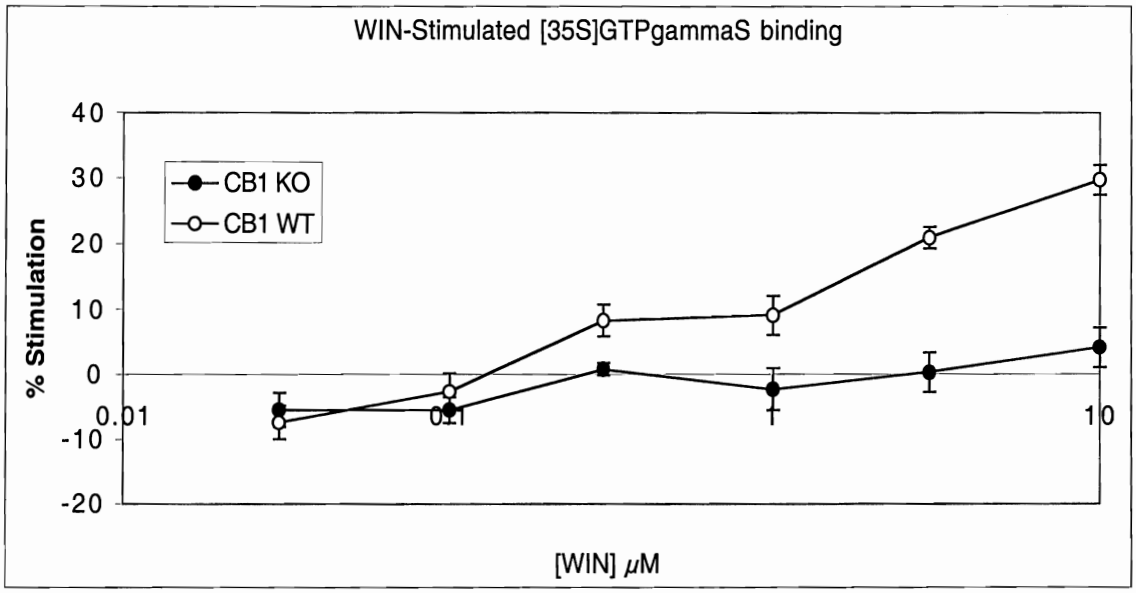


Figure 11. WIN-stimulated [^{35}S]GTPγS binding in CB_1 receptor KO Vs. WT mouse spinal cord. This graph shows that WIN-stimulated [^{35}S]GTPγS binding is eliminated in the CB_1 receptor knockout mouse spinal cords.

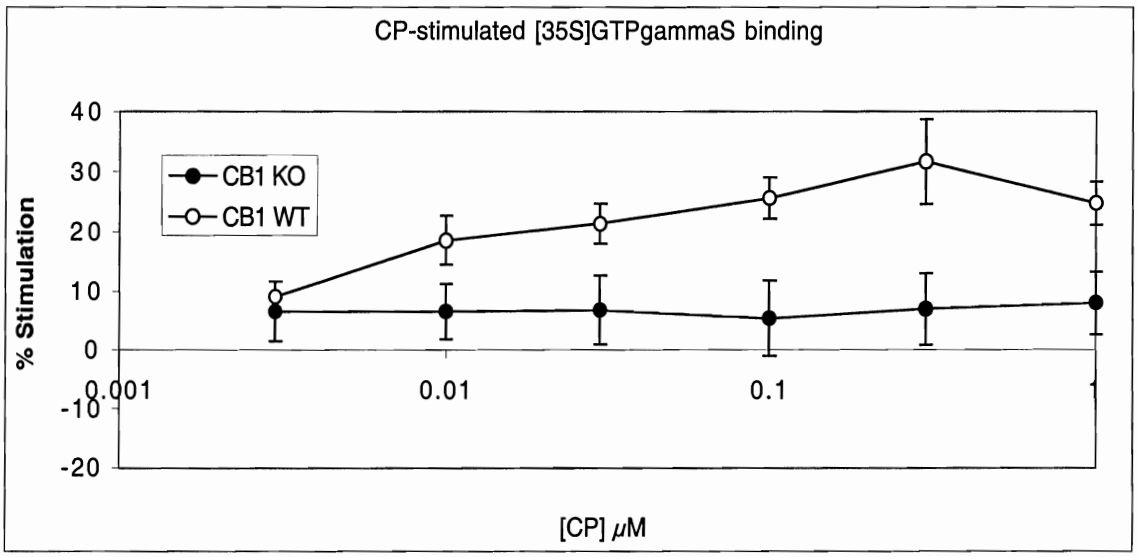


Figure 12. CP-stimulated [^{35}S]GTPγS binding in CB_1 receptor KO Vs. WT mouse spinal cords. This graph shows that CP-stimulated [^{35}S]GTPγS binding is eliminated in the CB_1 knockout mouse spinal cords. These experiments show the elimination of cannabinoid agonist-stimulated G-protein activation in the CB_1 receptor KO mouse spinal cord.

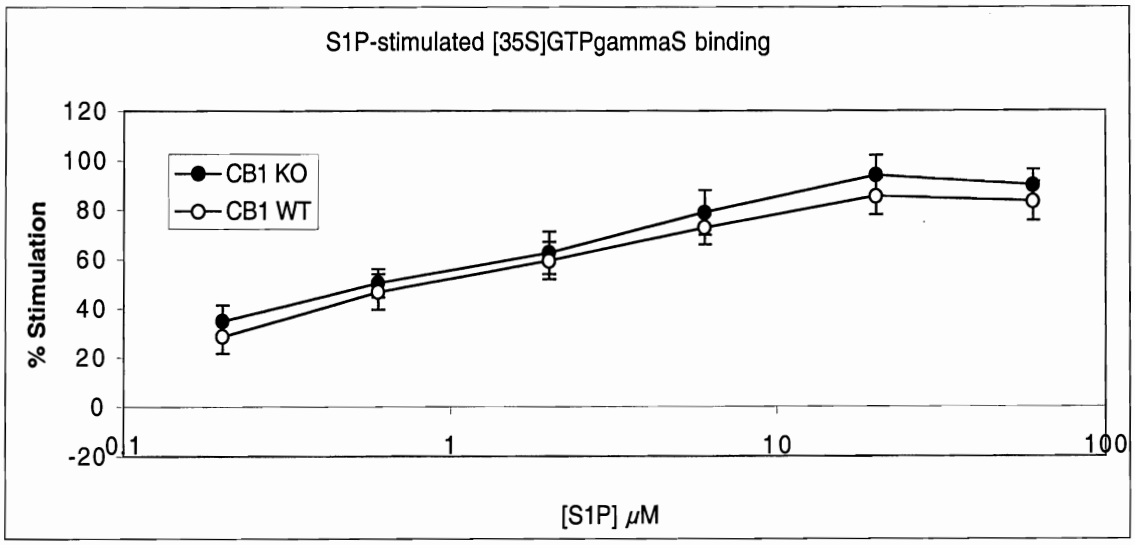


Figure 12. S1P-stimulated [^{35}S]GTPγS binding in CB_1 receptor KO Vs. WT mouse spinal cords. This graph shows that there is no significant difference in the percent stimulation in between CB_1 knockout and wild type mouse when incubated with S1P. These experiments suggest that S1P-mediated [^{35}S]GTPγS stimulation does not act through the CB_1 receptor in the mouse spinal cord.

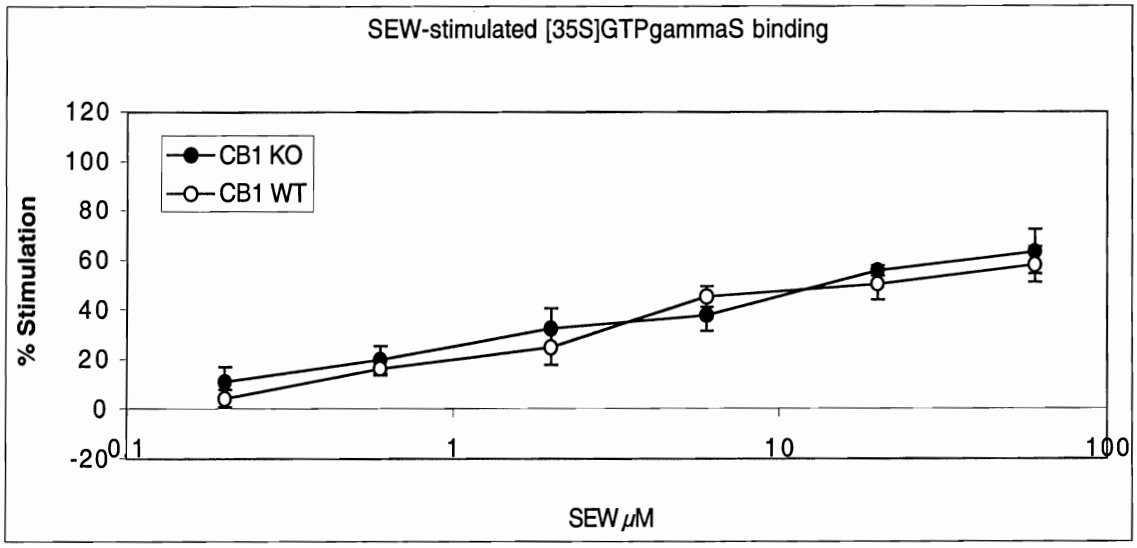


Figure 14. SEW-stimulated [^{35}S]GTPγS binding in CB_1 receptor KO Vs. WT mouse spinal cords. This graph shows that there is no significant difference in the percent stimulation in the CB_1 receptor knockout compared to wild type mouse spinal cord.

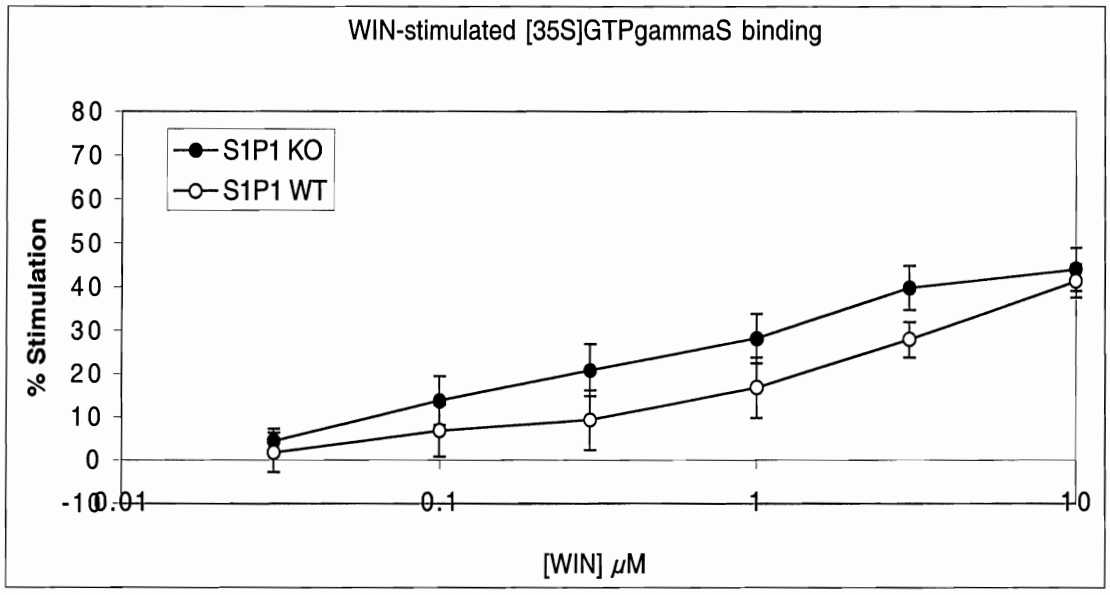


Figure 15. WIN-stimulated [^{35}S]GTPγS binding in $S1P_1$ receptor conditional KO Vs. WT mouse spinal cord. This graph shows that there is no significant difference in stimulation between $S1P_1$ knockout or wild type mouse spinal cord when treated with WIN.

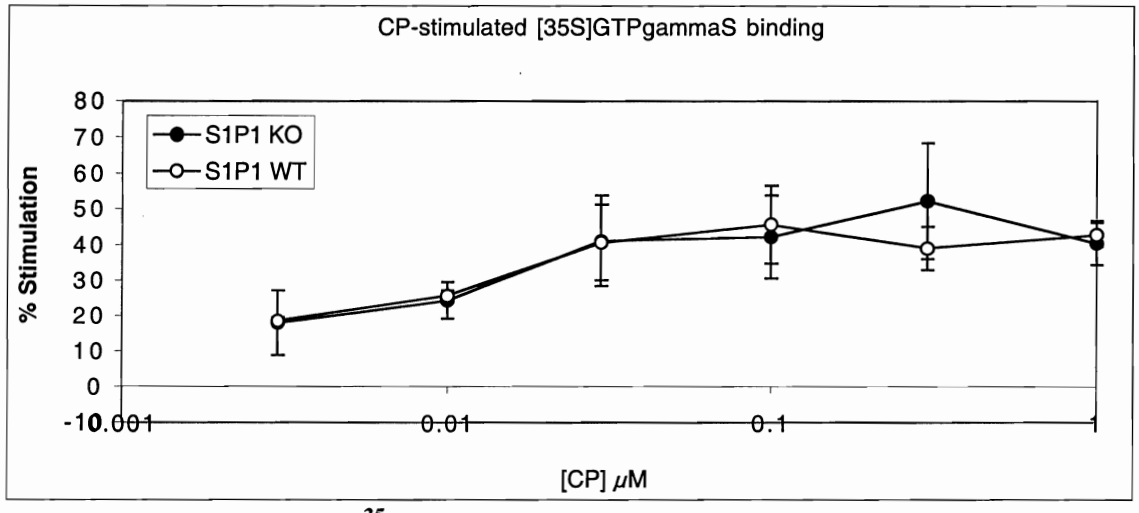


Figure 16. CP-stimulated [^{35}S]GTPγS binding in $S1P_1$ receptorconditional KO Vs. WT mouse spinal cord. This graph shows that there is no significant difference in the percent stimulation in the $S1P_1$ knockout and wild type mouse spinal cords when treated with CP. These experiments show that CP is not activating $S1P_1$ receptors in the mouse spinal cord.

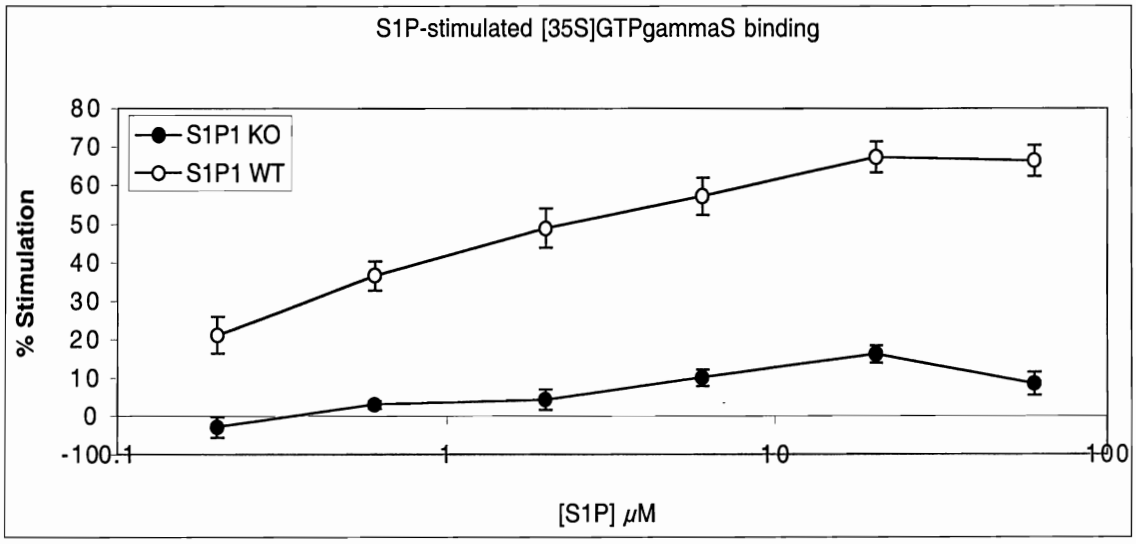


Figure 17. S1P-stimulated [^{35}S]GTPγS binding in $S1P_1$ receptor conditional KO Vs. WT mouse spinal cords. This graph shows that S1P-stimulated [^{35}S]GTPγS binding is greatly diminished in the $S1P_1$ knockout mouse spinal cords, while percent stimulation remains robust in the wild type spinal cords. These experiments show that S1P-stimulated [^{35}S]GTPγS binding in the mouse spinal cord is largely due to the activation of the $S1P_1$ receptors

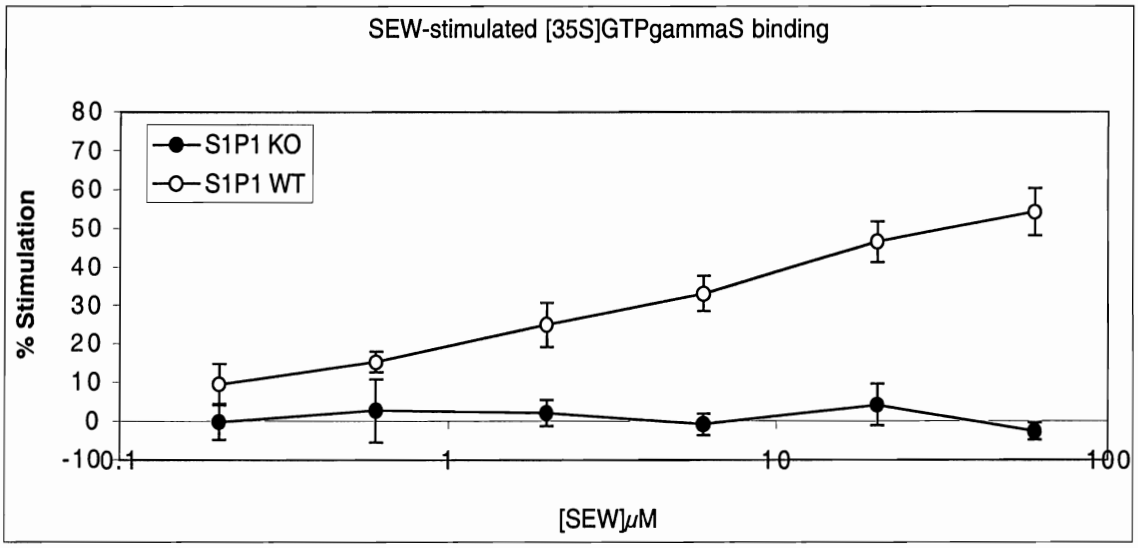


Figure 18. SEW-stimulated [^{35}S]GTPγS binding in $S1P_1$ receptor conditional KO Vs. WT mouse spinal cords. This graph shows that S1P-stimulated [^{35}S]GTPγS binding is eliminated in the $S1P_1$ knockout mouse spinal cords, whereas percent stimulation is robust in the wild type spinal cords. These experiments show that the predominant S1P receptor subtype in the spinal cord is $S1P_1$.

3.3 Effects of SR141716A and SR144528 on CB_1- and S1P-receptor stimulated [^{35}S]GTPγS binding

The effects of the CB_1 and CB_2 receptor antagonists, SR141716A and SR144528, were examined on WIN- and S1P-stimulated [^{35}S]GTPγS binding in ICR mouse spinal cord homogenate. Concentration effect curves were generated, both in the presence and absence of the antagonists. The results showed that neither SR141716A nor SR144528 significantly affected the E_{max} values of either WIN- or S1P-stimulated [^{35}S]GTPγS binding. The results also showed no significant change in EC_{50} values for WIN + SR144528, S1P + SR141716A, and S1P + SR144528-stimulated [^{35}S]GTPγS binding, when compared to WIN or S1P stimulation alone. In contrast, the EC_{50} value for WIN-stimulated [^{35}S]GTPγS binding showed a significant increase in the in the presence of SR141716A (WIN EC_{50} =0.19±.06 and WIN + SR1 EC_{50} =2.49 p=0.05) (see Figures 19 and 20). These results suggest that neither cannabinoid antagonist interacts with the S1P receptor in the ICR mouse spinal cord, and confirms that CB_1 is the cannabinoid receptor in the spinal cord.

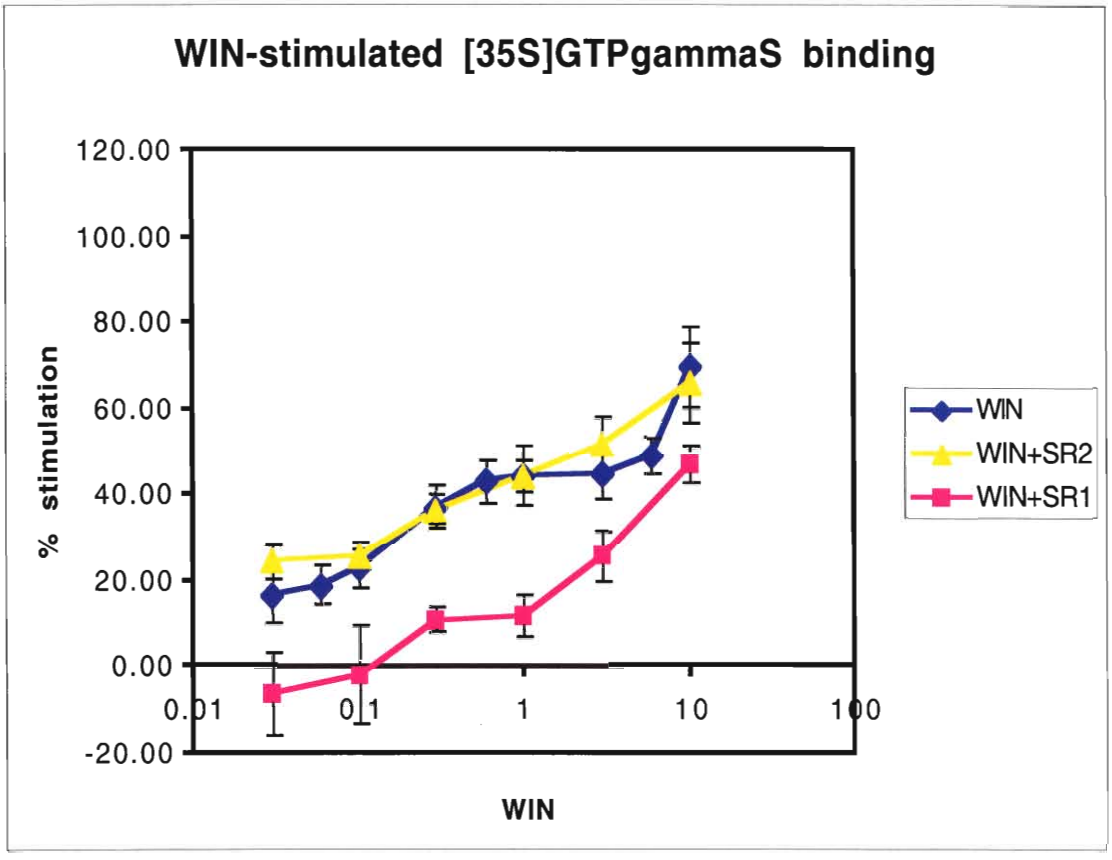


Figure 19. Effect of SR1/SR2 on WIN-stimulated [^{35}S]GTPγS binding in spinal cord. This graph shows that the CB_1 antagonist SR141716A (SR1), but not the CB_2 antagonist SR144528 (SR2), produces a rightward shift in the WIN dose response curve when the mouse spinal cord tissue is treated with 0.05μM SR1 or SR2. These experiments were performed in triplicate with n=6.

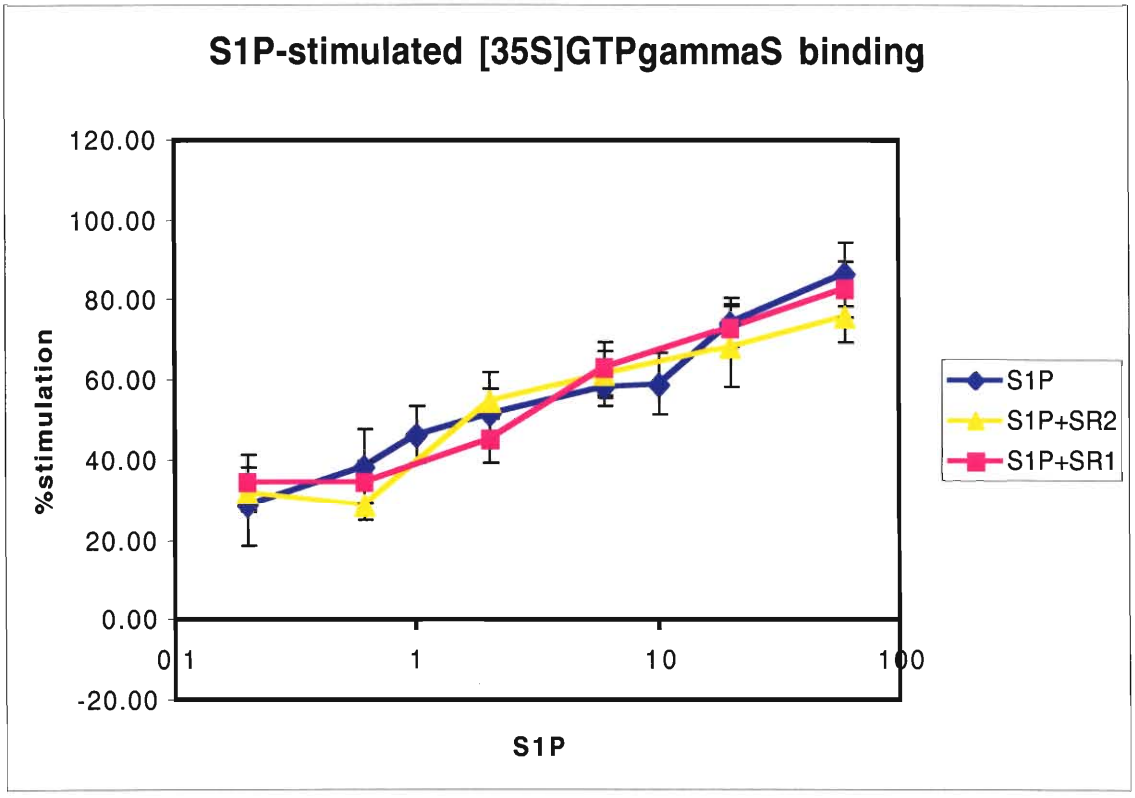


Figure 20. Effect of SR1/SR2 on S1P-stimulated [^{35}S]GTPγS binding in spinal cord. This graph shows that there is no change in the S1P concentration effect curve when the spinal cord tissue is treated with 0.05μM of SR141716A (SR1) or SR144528 (SR2). These experiments were performed in triplicate with n=6.

3.4 Agonist-stimulated [^{35}S]GTPγS binding: Additivity studies

Agonist-stimulated [^{35}S]GTPγS binding assays were performed using CB_1, S1P and mu and delta opioid agonists in mouse spinal cord to determine whether there is an interaction between these systems. The tissue was incubated with agonists alone and different combinations of WIN, S1P, DAMGO (mu-opioid receptor agonist), and PCl-DPDPE (delta-opioid receptor agonist). Results suggested an interaction between the cannabinoid and S1P receptor systems and the cannabinoid and opioid receptor systems (see Table 6 and 7, and Figure 21). The results were expressed as theoretical additivity vs. actual additivity (as previously explained in the methods section). A significantly less than additive effect was observed between the CB_1 and S1P receptor systems, the CB_1 and opioid receptor systems (both mu and delta), but not the S1P and opioid receptor systems (see Table 8 and Figure 22). The results were also presented as percent additivity *([Actual net stimulation - highest actual net stimulation provided by a single agonist] / [Theoretical net stimulation – highest actual net stimulation provided by a single agonist) x 100)*. Both of these data analyses reveal that the cannabinoid and S1P receptor systems exhibit an interaction, whereas S1P and opioid receptor systems do not.

Drug	Theoretical Additivity	Actual Additivity
WIN + S1P	105.58	90.13*
WIN + DAMGO	107.61	94.81
WIN + PCl-DPDPE	88.20	72.82*
S1P + DAMGO	122.26	117.70
S1P + PCl-DPDPE	102.85	97.16
DAMGO + PCl-DPDPE	104.88	82.50*
WIN + S1P + DAMGO	167.73	130.80*
WIN + S1P + PCl-DPDPE	148.31	118.26
WIN + DAMGO + PCl-DPDPE	150.35	99.73*
S1P + DAMGO + PCl-DPDPE	165.00	123.27*

Table 6. Theoretical vs Actual additivity of percent stimulation. These results show that the cannabinoid and S1P receptor systems exhibit an interaction in spinal cord, whereas S1P and opioid receptor systems do not. *$p \leq 0.05$.

Drug	% Additivity
WIN + S1P	65.6%*
WIN + DAMGO	73.2%*
WIN + PCl-DPDPE	60.7%*
S1P + DAMGO	90.65%
S1P + PCl-DPDPE	70.44%
DAMGO + PCl-DPDPE	38.9%*
WIN + S1P + DAMGO	61.2%*
WIN + S1P + PCl-DPDPE	94.21%
WIN + DAMGO + PCl-DPDPE	36.6%*
S1P + DAMGO + PCl-DPDPE	66.9%*

Table 7. Percent additivity: Results from agonist-stimulated [^{35}S]GTPγS binding additivity assays. These results suggest a possible interaction between CB_1 and S1P systems and CB_1 and opioid receptor systems, while results suggest the S1P does not interact with the opioid receptor systems. *$p \leq 0.05$.

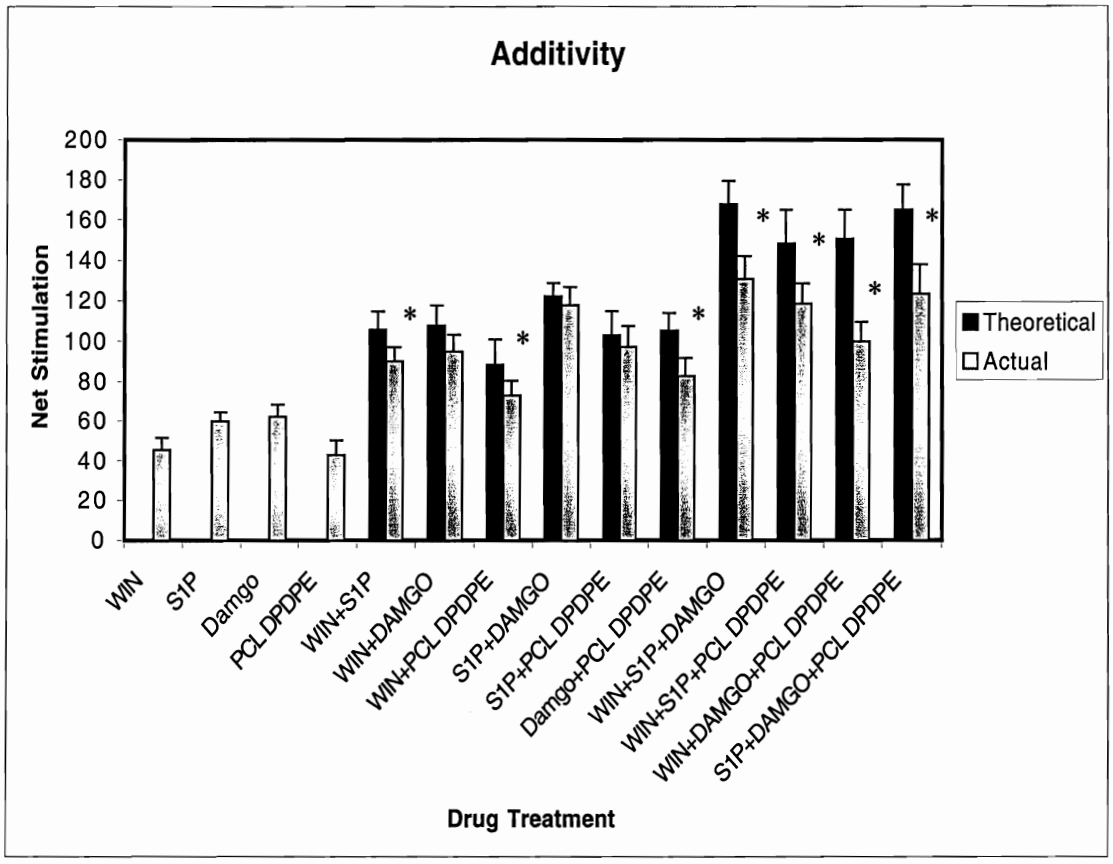


Figure 21. Theoretical vs. Actual Additivity. These results show that there is a significantly less than additive effect between the cannabinoid agonist WIN and S1P. This is evidence that there is an interaction between the cannabinoid and S1P, but not the S1P and opioid receptor systems in the mouse spinal cord. These experiments were performed in duplicate using an n=7.

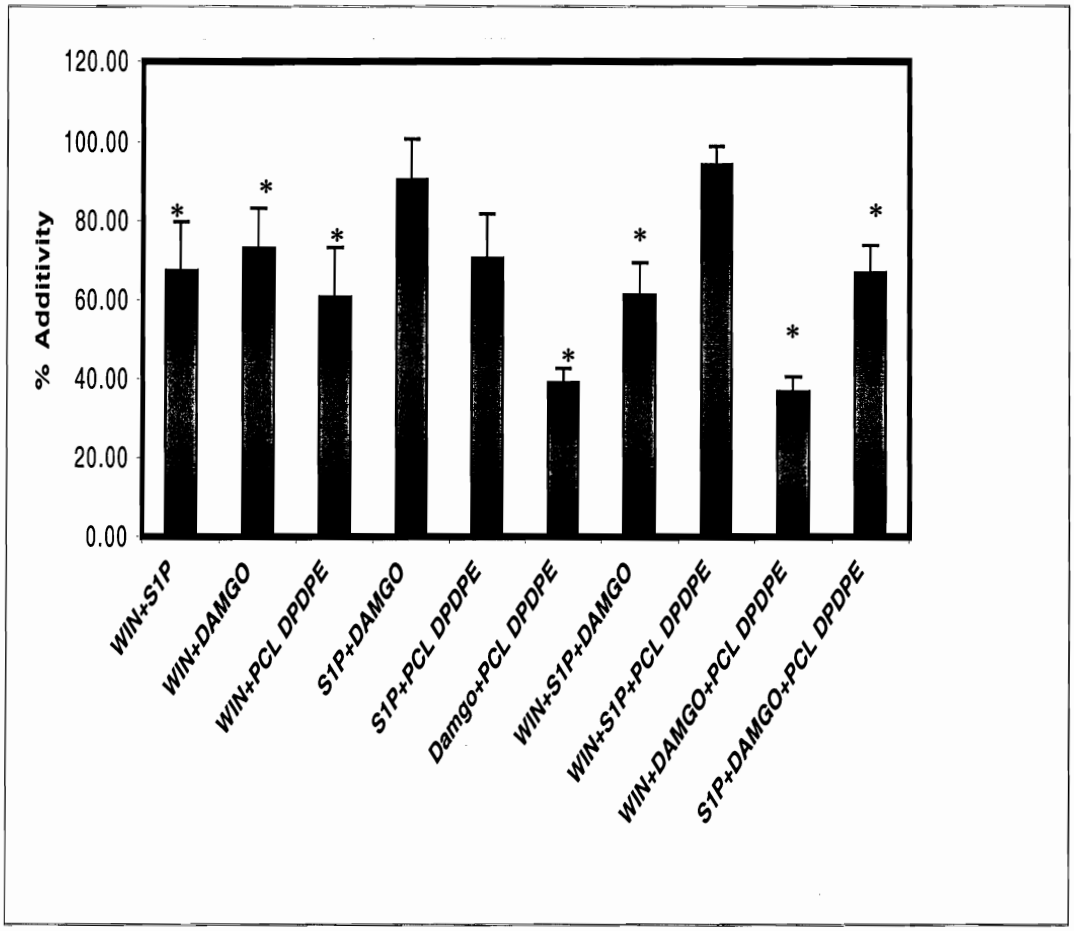


Figure 22. Percent Additivity. These results show that there is a significantly less than additive effect between WIN and S1P, as well as a significantly less than additive effect between WIN and the opioid agonists DAMGO and PCL-DPDPE. This is evidence that there is an interaction between the cannabinoid and S1P, cannabinoid and opiate, but not the S1P and opiate receptor systems in the mouse spinal cord. These experiments were preformed in duplicate using an n=7.

Chapter 4. Discussion

Major Findings of this study

The results of this study suggest that there is an interaction between CB_1 and S1P receptors in the mouse CNS. In the autoradiographic study, CB_1- and S1P-stimulated [^{35}S]GTPγS binding was observed in the same regions in the CNS, both with very high levels of stimulation. These results are consistent with previous studies showing very high levels of CB_1 receptors in regions including the caudate putamen and cerebellum (Herkenham *et al.*, 1991; Tsou *et al.,* 1998; Ashton *et al.*, 2004). It can now be concluded that S1P receptors are located in similar brain regions; possibly in levels even higher than the CB_1 receptor in some regions. These results might be due to higher levels of S1P receptors in the CNS, multiple S1P receptor subtypes localized in the same regions, or an increased ability of S1P receptors to activate G-proteins (efficiency). Due to the close proximity of these receptor systems in the CNS, it is now possible to examine interactions. In the autoradiographic study it was observed that there was a less than additive effect between the CB_1 and S1P receptors the caudate putamen, substantia nigra, and cerebellum. Moreover, SR141716A-mediated inhibition of S1P-stimulated G-protein activity was seen in the caudate putamen and substantia nigra as well. These results might be due to a direct antagonist effect on the S1P receptors by the cannabinoid antagonist, or S1P binding to the CB_1 receptor. The latter is unlikely because deletion of the CB_1 receptors did not affect S1P binding in the spinal cord. However this might be

regionally specific, so more regions must be studied in order to better characterize this finding. When the effects of SR141716A and SR144528 were examined in the mouse spinal cord, no significant decrease in percent stimulation of G-proteins was observed. This is evidence that cannabinoid antagonist-mediated inhibition of S1P receptor activation may be receptor subtype and/ or regionally specific.

The results of this study also demonstrated that there is an interaction between the CB_1 and S1P systems in the mouse spinal cord. It was observed in the knockout mice that there is no direct interaction of CB_1 ligands with the S1P receptors, as well as no direct interaction of the S1P ligands with the CB_1 receptor. It was also shown that the $S1P_1$ receptor subtype is the primary S1P receptor in the mouse spinal cord. These results showed that CB_1 receptor ligands do not interact directly with the $S1P_1$ receptor, and S1P ligands do not directly interact with the CB_1 receptor in the mouse spinal cord. In the additivity studies it was shown that there is a less than additive effect between WIN and S1P in agonist-stimulated [^{35}S]GTPγS binding. This is further evidence for an interaction between these two receptor systems, because if there was no overlap between these receptors there would have been a completely additive effect. In contrast there was no significant change from 100% in the percent additivity for S1P and mu or delta opioid agonists, revealing that these two receptor systems do not exhibit the same interaction as CB_1 and S1P receptors.

Cannabinoid ligand binding to S1P receptors or a CB_x receptor

There has been some evidence collected throughout this study that suggests that cannabinoid ligands might be binding to certain S1P receptor subtypes, or a novel lysolipid receptor (*e.g.* CB_x). It was observed in the autoradiographic experiments that certain regions of the mouse brain show a less than additive effect of S1P and CB_1 agonists on G-protein stimulation, suggesting overlap of receptor systems. These results might be due to an unidentified receptor (CB_x) that binds both S1P and cannabinoid ligands such as WIN. Evidence for this unidentified receptor was reported in 2004 when it was observed by Baskfield and colleagues that Δ^9-THC decreased lever pressing ability in wild type, but not CB_1 receptor knockout mice, whereas methanandamide (a metabolically stable anandamide analog), produced decreased lever pressing ability in both wild type and knockout mice. This data suggested that a possible non-CB_1, non-CB_2 mechanism of action was responsible for the methanandamide-induced decrease in lever pressing ability (Baskfield et al., 2004).

Another explanation for these less than additive results might be the activation of specific S1P receptor subtypes by the cannabinoid agonist WIN, and therefore an overlap leading to less than additive G-protein stimulation. This hypothesis is further supported by autoradiographic results that showed the blockade of S1P-stimulated G-protein activation in specific brain regions by the CB_1 antagonist SR141716A, suggesting that SR141716A might be directly antagonizing a novel receptor, or one of the S1P receptor subtypes in specific brain regions. However the spinal cord, which contains mainly $S1P_1$

receptors, is not a likely target for this ligand interaction because SR141716A and SR144528 did not inhibit S1P-mediated G-protein activation. In these experiments there was no evidence of any cannabinoid agonist or antagonist interaction with a non-CB_1 receptor or an S1P receptor.

Due to the recent findings that indicate promiscuity among lipid ligands for various receptors (Lim and Dey, 2002) it is also possible that cannabinoid ligands might be binding to some S1P receptor subtypes, or perhaps both S1P and CB_1 ligands are binding to a common receptor such as a novel CB receptor. This hypothesis has important implications for drug development, as well as receptor regulation. For example the immunomodulator FTY720, in its phosphorylated form, has been shown to be a potent agonist at four of the S1P receptors (Brinkmann *et al.*, 2002). This drug was in stage three clinical trials for its immunosuppressive effect in transplant and autoimmune disorders patients. In recent studies, FTY720 was reported to act as a copmpetitive antagonist with CB_1 receptor (Paugh *et al.,* 2006). Due to its lipophilicity, FTY720 is predicted to cross the blood brain barrier, were it could affects both CB_1 and S1P receptos, the consequences of which are yet to be characterized.

Dimerization: Implication in CB_1 and S1P receptor interactions

There is evidence that GPCRs form dimers (Hazum *et al.,* 1985; Gomes *et al.,* 2001; Milligan, 2001), which represents a new concept for both the structure and function of these receptors (Brockaert *et al.,* 1999). GPCR dimerization might provide a mechanism by which normal receptor functions and interactions can be explained, such as in the case of the CB_1 and S1P receptors. There is evidence to support the theory that GPCR dimerization occurs early in their biogenesis, and receptors are delivered to the plasma membrane in a dimeric state (White *et al.*, 1998; Yesilaltay and Jenness, 2000).

The dimerization of the CB_1 receptor has been studied over the past few years. The CB_1 receptor, being one of the most abundant GPCRs in the CNS, is of intrest for dimerization studies. In 2002, Wager-Miller *et al.* found evidence supporting the idea that CB_1 receptor homo- or heterodimerization occurs. These data showed that the CB_1 receptor most likely exists as a dimer in vivo, and that the caboxy terminal portion of the receptor might play a role in the assembly of the oligmers (Wager-Miller *et al.*, 2002). It was also reported in 2005, by Kearn *et al.,* that a CB_1/D_2 receptor complex exists, and has the ability to greatly alter CB_1 signaling. For example the formation of CB_1/D_2 receptor dimers results in coupling to $G\alpha_s$ protein in preference to the expected $G\alpha_{i/o}$ proteins, resulting in increased levels of cAMP (Kearn et al., 2005).

There has also been recent evidence of both homo- and heterodimerization of S1P receptors. Several studies over the past decade have indicated that many cells express multiple subtypes of S1P receptors; and in some cases dimerization of these receptors is required for function. For example, in 2001 Paik *et al.* observed that $S1P_1$

and $S1P_3$ dimerization is required for Rho and intergrin activation in endothelial cells (Paik *et al.*, 2001; Van Brocklyn *et al.,* 2002). In fact, it appears that the biological response to S1P in many cell types depends on the expression of more then one S1P receptor subtype, and their possible existence as dimers (Van Brocklyn *et al.,* 2002).

The idea that S1P and CB_1 receptors might form heterodimers raises many interesting possibilities for the modulation and function of these two receptors. One of the properties that might be affected by CB_1 and S1P receptor dimerization is alterations in ligand binding. This has been observed in other instances of heterodimerization, and can lead to alterations in downstream effects (Van Brocklyn *et al.,* 2002; Durroux, 2005). This alteration in ligand binding might be a possible explanation for the finding that G-protein activation was only affected in some brain regions when brain slices were treated simultaneously with SR141716A (CB_1 antagonist) and S1P. In these autoradiographic experiments, a decrease in G-protein activation was only observed in some regions (caudate-putamen and substantia nigra), leading to the hypothesis that dimerization could affect the binding of SR141716A in a region-specific manner.

This phenomenon might be due to S1P receptor subtype ($S1P_1$, $S1P_2$ $S1P_3$ or $S1P_5$) localization within the mouse CNS. It can be hypothesized that CB_1 receptors might only be able to form dimers with certain S1P receptor subtypes. This idea could explain the regional difference in S1P-stimulated [^{35}S]GTPγS activation when the additive effects of the CB_1 agonist WIN and S1P were observed. A less than additive effect was seen in similar regions where the SR141716A reversal was observed, this data might reflect dimerization of the S1P and CB_1 receptors leading to both alterations in ligand binding and conversion of downstream effects on G-protein activation.

This explanation could also explain the results of S1P + SR141716A studies, and additivity observed in the autoradiographic experiments. However, this hypothesis cannot explain the results obtained in the knockout mice or additivity and antagonist studies in the mouse spinal cord binding studies. In the studies conducted in spinal cord of receptor knockout mice, the $S1P_1$ receptor was shown to be the primary receptor in the mouse spinal cord. Due to the lack of difference between the WIN-stimulated [^{35}S]GTPγS binding in $S1P_1$ knockout mouse spinal cords, as compared to wild type, and S1P-stimulated [^{35}S]GTPγS binding in CB_1 knockout mouse spinal cords, it can be hypothesized that there is no alteration in ligand binding, and therefore CB_1 and $S1P_1$ receptors might not form dimers in spinal cord, or CB_1 ligands might not directly interact with $S1P_1$ receptors in the spinal cord.

The discovery that GPCR dimerization results in functional changes in receptor mediated signaling, leads to the question of physiological relevance. Although many studies have investigated the mechanisms by which GPCRs interact with each other, the physiological relevance of this phenomenon remains elusive (Maggio et al., 2005). It has been hypothesized that the heterodimerization of GPCRs, such as CB_1 and S1P receptors, might provide a mechanism to control signaling at the synapse.

GPCR dimerization might also direct the localization of signal transduction pathways, and could be a mechanism to aggregate downstream signaling components leading to convergence of two receptor signaling pathways and the possibility of cross-talk. This has important implications for this study for both pharmacological and functional reasons. It has been suggested that GPCR dimers might be novel targets for

the development of new drugs, as well as new drug administration regimens (Rios et al., 2001).

Over the past decade a growing number of receptors have been shown to form heterodimers/multimers and to exhibit an unexpected level of pharmacological diversity The pharmacological changes that occur with heterodimers are most likely due to the allosteric rearrangements induced by the interaction of the two receptors (Maggio et al., 2005). Although CB_1 agonists have been considered a potential drug for the treatment of pain and other ailments, their psychoactive effects have limited their clinical use (Cravatt and Lichtman, 2004). The potential for new drugs designed to target CB_1/S1P dimers might be a potential therapeutic agent that produces less unwanted side effects. The possibility of developing ligands that are selective for heterodimeric GPCRs is a promising strategy for targeting different tissues of the human body (Maggio et al., 2005). Because it is most likely that the dimerization of CB_1 and S1P receptors is region-specific in the CNS, drugs could be developed to target only certain regions in the CNS, possibly leading to a decrease in unwanted side effects.

Many studies it have shown that GPCR activation by ligands promotes the recruitment of β-arrestin leading to signal termination and endocytosis of the receptor (Krupnick *et al.*, 1998; Zhang *et al.*, 1997). Evidence has now accumulated indicating that dimerization influences the binding of β-arrestin leading to an altered fate of the dimerized receptors (Pfeiffer *et al.,* 2003; Stanasila *et al.,* 2003; Terrillon *et al.,* 2004). This could have important implications for the regulation and trafficking of the CB_1 and S1P receptors in the presence of receptor ligands, such as the immunomodulator FTY720 or the psychoactive component of marijuana, THC.

Lipid Raft: Implication in CB_1 and S1P receptor interactions

Over the past decade, scientific views of cell membrane organization have changed (Barnett-Norris, 2005). It is now known that cell membranes are not a homologous fluid, but rather lipid assemblies, called lipid rafts, that provided a platform to assemble or segregate proteins in the membrane matrix (Simons and Vaz, 2004). These lipid rafts, which are rich in sphingolipids and cholesterol, have been suggested to aid in the organization of many GPCR signal transduction pathways (Moffett *et al.,* 1999). Current evidence indicates that compartmentalization plays an important role in cell signaling, facilitating efficient and rapid flow of signal transduction, as well as contributing to the cross-talk among pathways (Ostrom and Insel, 2004). Such cross-talk might play a role in the interaction observed in these studies between S1P and CB_1 receptors.

Recent findings about the lipid raft environment might be able to help elucidate the relationship between the CB_1 and S1P receptors, as well as aid in the understanding of their possible interaction. Evidence for GPCR targeting to lipid rafts came in 1999 when it was observed that proteins with a high affinity for ordered lipid environments were targeted to these lipid rafts; including proteins such as GPCRs, $G\alpha$ and $G\beta\gamma$ subunits, and other lipid-associated molecules (Melkonian *et al.,* 1999). For example it was observed that endocannabinoids such as anandamide are created in the lipid bilayer of specific lipid rafts that contain embedded CB_1 receptors, where it can then activate the receptor (Barnett-Norris *et al.,* 2005).

The lipid platforms provided by lipid rafts would supply the structure needed for both CB_1 and S1P signaling pathways to take shape. The receptors could be co-localized on

the same lipid raft allowing them to converge on similar pools of G-proteins that are also associated with the lipid raft, like $G\alpha_i$. This convergence would explain the less than additive effect of G-protein stimulation seen in mouse spinal cord homogenates when treated with WIN and S1P simultaneously. This co-localization on the same lipid rafts, and therefore in the same cells, might also be an explanation for the finding that S1P and CB_1 receptor-stimulated [^{35}S]GTPγS binding is seen in many of the same regions of the mouse brain in autoradiographic studies.

From a pharmacological standpoint the possibility that CB_1 and S1P receptors are co-localized on the same lipid rafts in the CNS provides an important advancement in the understanding of interaction between these receptors. This understanding of drug action at these co-localized receptors could lead to the development of new types of drugs. The idea that S1P and CB_1 receptors are colocalized in cells can also aid in the understanding of the tissue-specific responses of agonists, as seen in this study. Lipid raft theory would provide an opportunity to target these specific membrane domains in order to differentially influence regions of S1P and CB_1 receptors in different cell types.

Lipid rafts containing GPCRs are capable of regulating receptors, such as S1P and CB_1, in different ways depending on the cell's metabolic state, differentiation, and stage of cell growth (Chini and Parenti, 2004). These observations have already been shown to occur between the CB_1 and S1P receptor systems by Guzman *et al.* in 2001, who showed that activation of the CB_1 receptor can lead to the generation of the precursor molecule of S1P, ceramide. It is well known that ceramide/S1P metabolism plays an important role in the regulation of neuronal growth and development (Van Brocklyn *et al.*, 1999; Windh *et al.*, 1999; Hla, 2004). It can be postulated that the CB_1/ceramide connection may have

physiological implications and therapeutic possibilities (Guzman *et al.,* 2001). These results show that the interaction between the CB_1 and S1P receptor systems might participate in the control of the balance between cell survival and cell death This has important implications in neuronal development and synaptic plasticity in the CNS, as well as in diseases such as cancer (DePetrocellis et al., 1998; Galve-Roperh et al., 2000).

Future directions

To ensure that the less than additive effect observed in the [^{35}S]GTPγS agonist-stimulated binding is not an artifact due to the lipophilicity of WIN and S1P, thus allowing them to stick to each other and inhibiting G-protein activation, control experiments could be conducted. An example of such an experiment could be performed using S1P-HEK cells that lack any cannabinoid receptors. S1P and S1P + WIN concentration-effect curves could be observed in order to characterize the effect the addition of WIN has on S1P-mediated G-protein activation. If the S1P and WIN do not interact we would expect to see identical stimulation in both the S1P and S1P + WIN treated cells. This experiment could insure that the less than additive effect observed in the [^{35}S]GTPγS agonist-stimulated binding experiments is due to an overlap in the receptor systems, and not a physical interaction of the two ligands.

Immunohistochemical techniques and confocal microscopy could be used to further explore the co-localization between CB_1 and S1P receptors in the CNS. This would allow us to determine whether CB_1 and S1P receptors are co-localized on the same cells in the CNS, and we would be able to better characterize receptor distribution throughout the CNS. These experiments might also begin to clarify mechanisms for S1P and CB_1

receptor interactions, such as dimers or lipid rafts. We could also use this type of study to map S1P receptor subtype localization throughout the CNS. Another technique that could be used to explore the possibility of CB_1/S1P dimerization is immunoprecipitation and western immunoblotting This technique would allow us to observe any possible dimer associations between the two proteins, as could be observed on the western blot showing immunoreactive bands at the predicted molecular weights of possible dimers (Kearn *et al.,* 2005).

In the future we would also like to focus on the functional characterization of CB_1 and S1P receptors in the CNS, and their possible relationship in the modulation of behavior. Due to our observation that S1P receptors are co-distributed in similar regions of the CNS as CB_1 receptors, it is predicted that S1P-activation might be associated with similar cannabinoid-like behaviors. Both CB_1 and S1P receptors activate G-proteins in regions associated with pain perception such as the PAG and spinal cord, therefore antinociceptive effects of S1P might be observed by conducting behavioral tests, such as the tail flick assay and hot plate test. Preliminary data has suggested that intrathecal injections of S1P dose lead to antinociceptive behavior. It has also been observed S1P administration leads to induced hypothermia (Welch and Sim-Selley, unpublished), this might be due to S1P receptor localization in the hypothalamus. S1P and CB_1 also show co-distribution in the cerebellum and basal ganglia, regions associated with motor coordination. This might suggest that an S1P locomotor effect could be observed in behavioral testing using a locomotor box, or during observation of locomotor activity. The effects of S1P on memory could be tested with the use of the Morris-water maze in order to establish whether S1P-stimulated G-protein activation in the hippocampus has a

similar effect to that of cannabinoid stimulation. It might also be useful to observe the effects of both S1P and cannabinoid agonists together in order to establish whether behavioral effects are additive, less then additive, synergistic, or antagonistic. These tests could also be employed using S1P receptor knockout mice to determine S1P subtypes involved in pain modulation, and its interaction with the cannabinoid system in pain related pathways.

WIN- and S1P-stimulated [^{35}S]GTPγS binding in membrane homogenates of adult mouse CNS tissue could be used in order to confirm the activation of G-proteins in important regions related to pain. To follow up our autoradiographic studies from this project, and to support the preliminary behavioral data, we would like to characterize both WIN and S1P receptor-mediated G-protein activation in the regions important to the modulation of pain, such as the spinal cord and the periaqueductal gray (PAG), in order to observe possible co-distribution in these regions. To observe the effect of receptor deletion we could also employ both $S1P_1$ and CB_1 receptor knockout mice in these assays, as well as other S1P receptor subtype-specific knockout mice. These experiments will allow us to observe the changes in WIN and S1P receptor-mediated G-protein activation in specific regions of the CNS related to pain.

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Vita

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PUBLICATIONS

Gerasimov MR, Collier L, Ferrieri A, Alexoff D, Lee D, Gifford AN, Balster RL. 2003. Toluene inhalation produces a conditioned place preference in rats. *The European Journal of Pharmacology* 477:45-52.

POSTER PRESENTATIONS

22nd Annual Daniel T. Watt s Research Poster Symposium, Richmond, VA 10/25-26/2005

The Relationship Between the CB_1 and S1P Receptors in the CNS

35th Annual Meeting of the Society of Neuroscience, Washington, DC 11/2005

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