One step closer to the role of the brain CB_2 cannabinoid receptors: interaction with the μ-opioid system

Ph.D. Thesis

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LIST OF PUBLICATIONS

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2. ***Paldyova, E***., Bereczki, E., Santha, M., Wenger, T., Borsodi, A. & Benyhe, S. (2008). Noladin ether, a putative endocannabinoid, inhibits μ-opioid receptor activity *via* CB2 cannabinoid receptors. *Neurochem Int 52*, 321-328.

3. ***Paldyova, E.,*** Bereczki, E., Santha, M., Wenger, T., Borsodi, A. & Benyhe, S. (2007). Altered gene expression and functional activity of opioid receptors in the cerebellum of CB1 cannabinoid receptor knockout mice after acute treatments with cannabinoids. *Acta Biol Hun 58*, 113-129.

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Gaspar, R., Ducza, E., Mihalyi, A., Marki, A., Kolarovszki-Sipiczki, Z., ***Paldy, E.,*** Benyhe, S., Borsodi, A., Foldesi, I. & Falkay, G. (2005). Pregnancy-induced decrease in the relaxant effect of terbutaline in the late-pregnant rat myometrium: role of G-protein activation and progesterone. *Reproduction 130*, 113-22.

Gaspar, R., Kolarovszki-Sipiczki, Z., Ducza, E., ***Paldy, E.,*** Benyhe, S., Borsodi, A. & Falkay, G. (2005). Terbutalin increases the rat cervical resistance of the pregnant rat uterus in vitro. *Naunyn Schmiedebergs Arch Pharmacol 371*, 61-71.

Minorics, R., Ducza, E., Marki, A., ***Paldy, E.*** & Falkay, G. (2004). Investigation of estrogen receptor alpha and beta mRNA expression in the pregnant rat uterus. *Mol Reprod Dev 68*, 463-468.

ABBREVIATIONS

2-AG .....2-Arachydonoyl glycerol

B_{max} .....Number of maximal binding sites

BSA .....Bovine serum albumin

$CB_1^{+/+}$ .....CB_1 cannabinoid receptor wild-type

$CB_2^{+/+}$ .....CB_2 cannabinoid receptor wild-type

$CB_1^{-/-}$ .....CB_1 cannabinoid receptor knockout

$CB_2^{-/-}$ .....CB_2 cannabinoid receptor knockout

cDNA .....DNA synthesized from a messenger RNA template using reverse transcriptase.

CNS .....Central nervous system

DAMGO .....[D-Ala^2,***N*Me**Phe^4,Gly^5-ol] enkephalin, specific MOR agonist

DNA.....Deoxyribonuceid amino acid

DNase .....Deoxyribonuclease

DMSO .....Dimethyl sulphoxide

EC_{50} .....'Potency'; the agonist molar concentration that produces 50% of the maximal possible effect of that agonist.

EDTA .....Ethylenediamine-tetraacetic acid

EGTA .....Ethyleneglycol-tetraacetate

E_{max}.....'Efficacy'; the maximal effect that an agonist can elicit in a given tissue-preparation.

GDP .....Guanosine 5'-diphosphate

GI .....Gastrointestinal

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

G-protein .....GTP-binding protein

GTP .....Guanosin 5'-triphosphate

GTPγS .....Guanosine-5'-O-[γ-thio] triphosphate

IC_{50} .....'Affinity'; the concentration of the competitor producing 50% displacement in the competion radioligand binding assay.

i.p. .....Intraperitoneal

K_d .....Equilibrium dissociation constant

K_i .....Inhibitory constant

Knockout .....Gene removal to produce null mutation by homologous recombination.

PAG .....Periaqueductal gray

PCR .....Polymerase chain reaction

PI3K .....Phosphatidylinositol 3-kinase

MAP kinase .....Mitogen-activated protein kinase, serine/threonine-specific protein kinase

MOR .....μ-Opioid receptor

NE ..... Noladin ether, 2-arachydonyl glyceryl ether, 2-AGE, putative endocannabinoid

QRT-PCR .....Quantitative Real-time PCR

mRNA .....Messenger RNA

RNA .....Ribonucleid amino acid

Δ^9-THC .....Delta-9-tetrahydrocannabinol, the main psychoactive component of *Cannabis sativa*.

SEM .....Standard errors of means

Tris .....Tris-(hydroxymethyl)-aminomethane

I. REVIEW OF THE LITERATURE

1. Opioids and cannabinoids: brief introduction

1.1 Opioids

Chronology	
2000	CB_2 cannabinoid receptor 'knockout' mice Buckley et al.
2001-2002	Discovery of noladin ether as an endocannabinoid Hanus et al., Fezza et al.
1999	CB_1 cannabinoid receptor 'knockout' mice Ledent et al., Zimmer et al.
1998	Chemical synthesis of noladin ether Mechoulam et al.
1992-1993	Cloning of opioid receptors Kieffer et al., Evans et al., Chen et al., Minami et al., Yasuda et al.
1993	CB_2 cannabinoid receptor cloned Munro et al.
1990	CB_1 cannabinoid receptor cloned Matsuda et al.
1984-1988	Discovery of the cannabinoid receptors Howlett and Fleming, Howlett et al., Devane et al.
1973	Discovery of the opioid receptors Simon et al., Terenius, Pert and Snyder

Figure 1.

Natural defense mechanisms allow organisms to survive under threatening environmental conditions. The sensation of pain for example, serves as a warning of an injury and thus enhances survival chances by promoting the healing process. However, extreme levels of pain can be redundant and could disrupt performance at inopportune times.

Physiological substances that regulate the endogenous release of opioid neuropeptides serve as the natural mechanism for the production of analgesia. The organism's ability to produce analgesia has been exploited by scientists to develop pharmaceuticals that can be used to eliminate aversive sensations of pain. The discovery of analgesic drugs began over 2000 years ago, when it was found that opium could be obtained from the juice of poppy seed capsules (*Papaver somniferum*). In 1805 the active component in opium was isolated and named morphine [206] which provided to be very effective in the control of pain. Even today, morphine like compounds are still the most effective types of analgesics used for the treatment of moderate to severe acute or chronic pain. Opioid agonists include not only the natural (e.g. morphine, codeine) and semisynthetic alkaloid derivatives from opium (e.g. oxymorphone, oxycodone), but also include synthetic surrogates (e.g. phentanyl, DAMGO; Figure 2)

and several endogenous peptides, that interact with the different subtypes of opioid receptors. All their effects can be blocked by the general opioid antagonist naloxone.

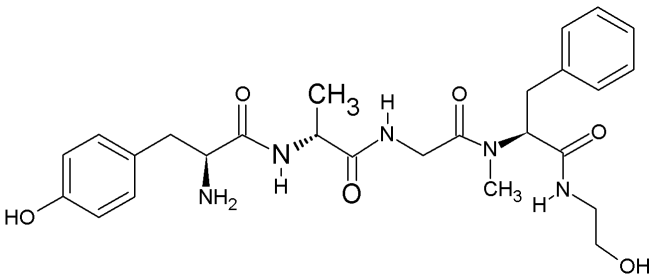

Figure 2: Chemical structure of DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin), a synthetic opioid peptide with high μ-opioid receptor specificity [118], frequently used in radioligand binding assays.

The endogenous opioids [16], a large family of neuropeptides that act as natural analgesics, are derived from three precursor proteins: prepro-opiomelanocortin (POMC), preproenkephalin (proenkephalin A) [162], and preprodynorphin (proenkephalin B) [113]. Posttranslational processing of these three precursor molecules results in the production of various opioids that are included in these families. They are present at several central nervous system (CNS) sites [23, 63, 69, 139, 167] implicated in pain modulation [18, 188]. They function as neurotransmitters, neuromodulators and in some cases, as neurohormones [68]. Moreover they play a role in some forms of stress-induced analgesia and in the analgesia produced by electrical stimulation of discrete brain areas, such as the periaqueductal gray [220].

The major challenge that remains in opioid analgesic research is to develop compounds that do not produce the adverse side effects (e.g. respiratory depression, sedation, constipation, nausea, the development of tolerance, physical dependence and addiction) [143], but still provide the beneficial analgesic effects of morphine like compounds.

1.2 Cannabinoids

Cannabinoids are low molecular weight lipophylic compounds derived from the hemp plant (*Cannabis sativa*), which contains more than 60 different cannabinoids [150, 64, 158]. In 1963 cannabidiol was the first cannabinoid to become purified [144], while the major psychoactive component delta-9-tetrahydrocannabinol (Δ^9-THC) was determined one year later [73]. The plant's name reflects its ancient use – cannabis is composed of the Sanskrit and Hebrew words meaning 'fragrant cane',

while sativa is the Latin word for cultivated. The term „Marijuana" describes the dried cannabis flowers and leaves, which are smoked, while hashish refers to the blocks of cannabis resin, which can be eaten.

1.3 Endocannabinoid system

Recently the endogenous counterparts of Δ^9-THC have been revealed and interest in investigating their pharmacology is increasing. Endocannabinoids are a group of lipid ligands including amides, esters and ethers of long-chain polyunsaturated fatty acids [56]. This system consists of endogenous cannabinoids, cannabinoid receptors and the enzymes responsible for synthesis and degradation of endocannabinoids [99, 131, 57]. The first endocannabinoid identified was arachidonoyl ethanolamide (anandamide) [55]. Since the discovery of anandamide, at least 6 more putative endocannabinoids have been identified: dihomo-γ-linolenoylethanolamide (HEA), docosatetraenoylethanolamine (DEA) [146], 2-arachidonoylglycerol (2-AG) [14, 56], noladin ether (NE) (Figure 3) [147, 87, 67], virodhamine [187] and *N*-arachidonoyldopamine (NADA) [21]. The synthesis, cellular transport and degradation of endocannabinoids are tightly regulated processes [99]. A feature that distinguishes endocannabinoids from many other neuromodulators is that they are not synthesized in advance and stored in vesicles (unlike opioids, ref. 69). Instead, their lipid precursors exist in the cell membranes and are cleaved by specific enzymes during post-synaptic depolarization [5, 13, 107].

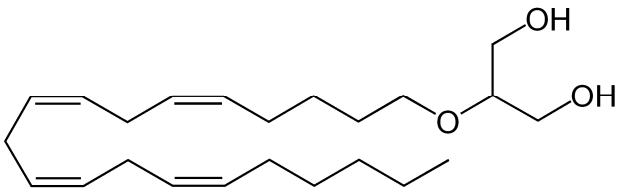

Figure 3: Structure of the putative endocannabinoid noladin ether (2-arachydonyl glyceryl ether, NE, 2-AGE).

2. CNS drugs: receptors and signaling mechanisms

Drugs acting in the central nervous system (CNS) were among the first discovered by primitive humans and are still the most widely used group of bioactive compounds. The mechanisms by which various drugs act in the CNS have not always been clearly understood. In the last three decades however, dramatic advances have been made in the methodology of CNS pharmacology. Therapeutic and toxic effects of drugs result from their interactions with specific molecules in the body. These molecules are called receptors: components of a cell or organism that interact with a drug and initiate the chain of events leading to the drug's observed effects. It is clear, that nearly all drugs with CNS effects act on specific receptors that modulate synaptic transmission. Very few agents such as general anesthetics and alcohol may have nonspecific actions in membranes, but even these nonreceptor-mediated actions result in demonstrable alterations in synaptic transmission. The cellular response to a particular extracellular signaling molecule depends on its binding capacity to a specific receptor protein located on the surface of a target cell, in its cytosol or in the nucleus. We can refer to the signaling molecule (e.g. hormone or neurotransmitter) as the ligand, which binds to a site on the receptor. Binding of a ligand to its receptor causes a conformational change in the receptor that initiates a sequence of reactions. They generate, amplify, coordinate, and terminate post receptor signaling by chemical secondary messengers located in the cytoplasm, leading to a change in cellular function. Being aware of these facts, receptors have become the central focus investigating drug effects.

Like in many areas of science, major progress in the study of CNS drugs depended on the development of new experimental techniques. Histochemical, immunological and radioisotopic methods have made it possible to map the distribution pattern of specific transmitters, their associated enzyme systems and receptors. Mice with mutated genes for specific receptors or enzymes (knockout mice) can serve as valuable models regarding the physiological and pharmacological roles of these components.

3. G-protein-coupled receptors (GPCR) and heterotrimeric G-proteins

Genes encoding for G-protein-coupled receptors (GPCR) represent one of the largest gene family in the human genome. GPCRs are widely distributed in the peripheral and central nervous systems and are one of the most important therapeutic targets in pain medicine [177]. They are, and in the near future are likely to remain, the most tractable and effective set of targets for therapeutic drug design [91].

Despite their molecular and functional diversity, all GPCRs share a similar structure: single polypeptide chain which consists of 7 transmembrane domains linked by alternating intracellular and extracellular loops [156, 126, 119] (Figure 4). The NH_2 terminus is exposed to the extracellular surface and the COOH terminus is located intracellularly (interaction with G-proteins) [77, 232, 233].

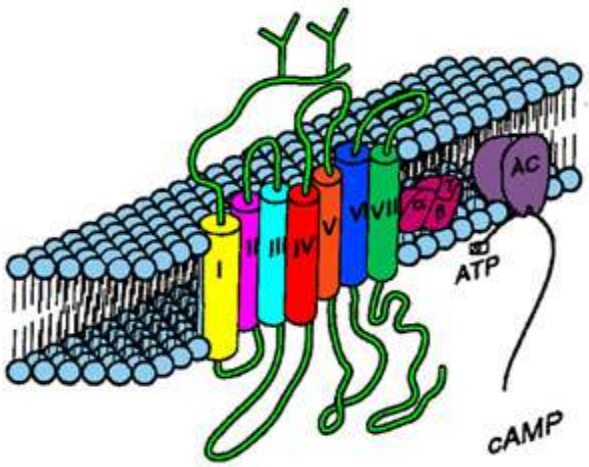

Figure 4: G-protein-coupled receptor with seven transmembrane domain. The receptor's amino (N) terminal is located extracellularly (above the plane of the membrane) and its carboxyl (C) terminal intracellularly. The terminals are connected by a polypeptide chain that crosses the plane of the membrane seven times. The hydrophobic transmembrane segments are designated by roman numerals (I to VII).

A definitive biochemical feature of GPCRs is their interaction with G-proteins and following this, the activation of downstream signaling cascades [4]. Yet, emerging studies have revealed the existence of signaling pathways initiated by these receptors, that are independent from G-protein-coupling [22, 86, 93, 101]. The binding of a transmitter or exogenous agonist alters the conformation of critical domains of the seven-transmembrane helix pocket, which in turn changes the relative positions of the intracellular domains of the receptor. These conformational changes promote the specific association of the receptor with heterotrimeric G-proteins, that are composed of an α-subunit interacting with a βγ complex. Activation of the receptor promotes the exchange of a molecule of GDP with GTP within the active site

of the α-subunit. The binding of GTP results in conformational rearrangements and promotes the dissociation of the heterotrimeric complex, and both the GTP-bound α-subunit and thus the released βγ complex are than able to interact with intracellular or membrane-located effectors. The intrinsic GTPase activity of the α-subunit hydrolyses GTP into GDP, restoring its inactive conformation as well as its affinity for binding the βγ complex (Figure 5). The βγ subunits function as a dimer and can activate a diverse array of effectors, such as enzymes and ion channels [166, 200]. On the other hand, the α-subunits have the key role in determining the receptor coupling specificity and can influence the efficiency of ion channel modulation by βγ subunits [106, 121, 105, 6].

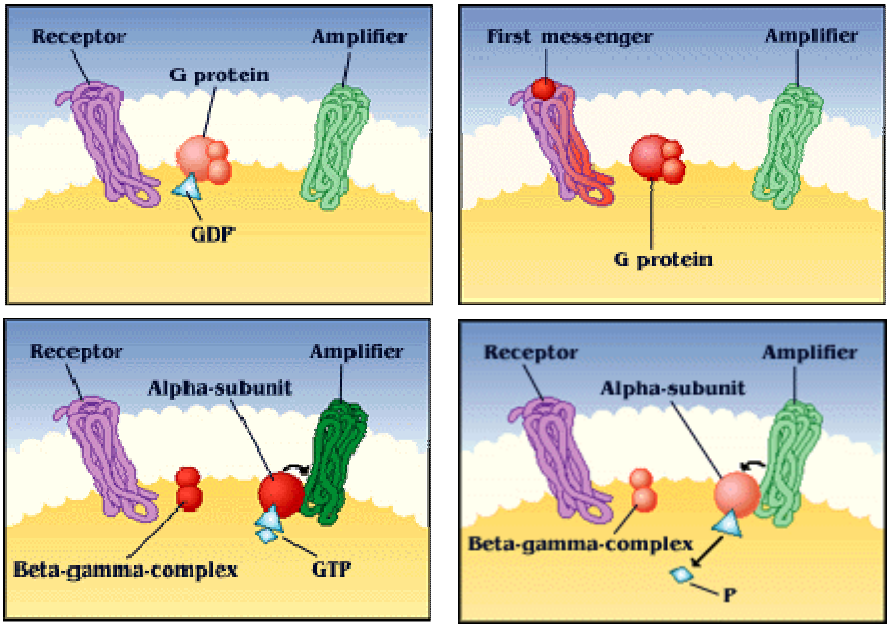

Figure 5: G-protein activation and inactivation. First panel: the G-protein, composed of α, β and γ subunits, in its inactive state with bound GDP. Second panel: the receptor with bound ligand activates the G-protein and replaces GDP with GTP. Third panel: the G-protein is activated. Fourth panel: the GTP bound to the α subunit is hydrolyzed to GDP, the subunits recombine.

The complexity and specificity of GPCR signaling partly relies on the existence of numerous closely related molecular species of the G-protein subunits [58, 94, 155]. Up to now, at least 23 α-subunits have been identified and classified into four major categories (Table 1) [101, 166]. Concerning β- and γ-subunits, at least 6 and 12 different molecular species have been described, respectively [74, 225]. Almost all GPCR agonists that have an analgesic action are coupled to $G_{i/0}$ proteins.

Table 1 G-protein α subunits and their primary effects

Subunit	Primary effects	Tipical example	References
$G_{\alpha s}$	AC ↑	β-Adrenergic receptor D_1 Dopamine receptor Muscarinic (m_1, m_3) receptors	Kilts et al., 2000 Jin et al., 2001; Wang et al., 1995 Akam et al., 2001; Offermanns et al., 1994
$G_{\alpha i/0}$	AC ↓	Opiate receptors Cannabinoid receptors D_2 Dopamine receptor α_2-Adrenergic receptor	Chakrabarti et al., 1995 Bonhaus et al., 1998; Glass & Northup, 1999 Cordeaux et al., 2001; Wiens et al., 1998 Eason et al,. 1992
$G_{\alpha q/11}$	PLC ↑	α_1-Adrenergic receptor	Chen & Minneman, 2005
$G_{\alpha 12}$	?	Substance-P	Barr et al., 1997

AC = Adenylate cyclase ; PLC = Phospholipase C

One of the most widely used method to study receptor activation of heterotrimeric G-protein is measuring the binding of [^{35}S]GTPγS. Accordingly, the nucleotide exchange process can be monitored and the basic pharmacological characteristics and the relative efficacy of compounds coupling to GPCRs can be measured.

4. Opioid and cannabinoid receptors: coupled to $G_{i/o}$ GTP-binding proteins

Opioids and cannabinoids share a number of common features, although in the cell they activate different receptors (μ-, δ- and κ- opioid, and CB_1, CB_2 cannabinoid receptors). They belong to the G-protein-coupled receptor (GPCR) superfamily and transduce signals through activation of pertussis-toxin sensitive $G_{i/o}$ proteins. Both receptor families are targets of drugs of abuse and therefore an understanding of the mechanisms modulating their activities is of significant clinical interest.

Three major classes of opioid receptors have been identified (μ, δ and κ) in various sites of the nervous system and other tissues (Figure 1, Table 2). Acting through the inhibitory $G_{i/o}$ proteins [32, 100] (Table 1) they inhibit adenylyl cyclase activity [109] and decrease calcium ion entry, resulting in a decrease in presynaptic neurotransmitter release [28, 199]. They also enhance potassium ion efflux, resulting in the hyperpolarization of postsynaptic neurons [105], a decrease in synaptic transmission [90] and they also activate mitogen-activated protein kinase (MAP kinase) cascade [48]. Beyond these cellular actions analgesia, euphoria, respiratory depression, tolerance and physical dependence are among the main pharmacological effects of morphine acting on μ–receptors. Moreover, the majority of currently available opioid analgesics also act primarily on μ-opioid receptor.

Table 2 Function, CNS action and endogenous peptide affinities of the opioid receptors subtypes.

Receptor subtype	Function	Sites of action in the CNS	Endogenous opioid peptide affinity
μ (mü)	Supraspinal and spinal analgesia; sedation; inhibition of respiration; slowed GI transmit; modulation of hormone and neurotransmitter release	Primary afferent nociceptors and substantia gelatinosa in spinal cord, locus coeruleus, periaqueductal gray, medullary nuclei, hypothalamus, thalamic nuclei, limbic system, cerebral cortex (Henriksen & Willoch, 2008; Lever, 2007; Inturrisi, 2002)	Endorphins > enkephalins > dynorphins
δ (delta)	Supraspinal and spinal analgesia; modulation of hormone and neurotransmitter release		Enkephalins >endorphins and dynorphins
k (kappa)	Supraspinal and spinal analgesia; psychotomimetic effects; slowed GI transmit		Dynorphins >> endorphins and enkephalins

Because of the lipophylic character of cannabinoids, initially it was assumed that they functioned via non-specific membrane interactions. However, later experiments measuring adenylate cyclase activity and radiolabelled synthetic

cannabinoids, all suggested that their effects were receptor mediated [182]. The existence of cannabinoid receptors were proven by molecular cloning of CB_1 cannabinoid receptor [141]. This was followed by the discovery of a second cannabinoid receptor, designated as CB_2 (Figure 1, Table 3) [157]. Similarly to opioid receptors, cannabinoid receptors are coupled to $G_{i/o}$ GTP-binding proteins (Table 1), but under certain conditions, coupling via G_s and $G_{q/11}$ has also been demonstrated [78, 166].

Table 3 Function and CNS distribution of the cannabinoid receptors.

Receptor	Function	CNS distribution
CB_1	Neuropathic pain, enhancement of appetite, antiemetic effects, role in short term memory and cognition (Reibaud et al., 1999; Darmani et al., 2003; Degroot et al., 2005; Carai et al., 2006)	Cerebral cortex, hippocampus, basal ganglia, cerebellum, striatum, periaqueductal gray (Herkenham et al., 1991; Matsuda et al., 1993; Glass et al., 1997; Biegon & Kerman, 2001)
CB_2	Controlling proliferation, differentiation and survival of neuronal and non-neuronal cells, neuroprotection, suppression of inflammatory and neuropathic pain, modulation of acute and cancer pain (Romero et al., 2002; Mechoulam et al., 2002; Carrier et al., 2004; Palazuelos et al., 2006; Whiteside et al., 2007; Guindon & Hohmann, 2008)	Neuronal and glial cells of: cerebral cortex, striatum, amygdala, thalamic nuclei, hippocampus, substantia nigra, cerebellum, brainstem, spinal cord (Skaper et al., 1996; Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006; Beltramo et al., 2006)

The implications of inhibitory G-protein activation are that stimulation of CB_1 receptors leads to the inhibition of adenylyl cyclase [19, 42, 171], the activation of MAP kinases [26], the inhibition of certain voltage-gated calcium channels [128, 129] and the activation of G-protein-linked inwardly rectifying potassium channels [89, 130]. Stimulation of CB_2 receptors has basically similar consequences, except that the modulation of ion channels by CB_2 receptors is more variable [14, 25, 65]. In addition, the activation of CB_2 receptors has also been linked to the stimulation of additional intracellular pathways, including the phosphatidylinositol 3-kinase (PI3K) / Akt pathway [154, 173, 27], which has been associated with pro-survival effects and the *de novo* synthesis of the sphingolipid messenger ceramide [35], possibly mediating the pro-apoptotic effects of cannabinoids.

5. CB_2 cannabinoid receptors: an update

5.1 CB_2 receptor: pharmacological characteristics

The peripheral cannabinoid receptor was the second cannabinoid receptor discovered (Figure 1). The pharmacology of CB_2 receptors is in part similar to CB_1 receptors (most plant-derived and synthetic cannabinoid agonists activate CB_2 receptors), although the affinity and/or potency at which these agonists bind and/or activate CB_2 receptors present some interesting differences compared to CB_1 receptors [66, 181]. With regard to endocannabinoid ligands, several studies support that 2-arachidonoylglycerol is an endogenous agonist for CB_2 receptors [218]. Anandamide, the first endocannabinoid to be isolated and characterized, was reported to be a less effective agonist for CB_1 receptors and does not practically bind to CB_2 receptors [218]. However, it has recently been demonstrated that anandamide can activate CB_2 receptors under pathological conditions [60]. Noladin ether (Figure 3) has been identified as putative endocannabinoid acting at the CB_1 receptor [87], but more recently Shoemaker et al. [208] showed that noladin ether acts as a full agonist of CB_2 receptors.

5.2 CB_2 receptor: tissue distribution

While human CB_1 receptor and mouse CB_1 receptor share 96% homology [37], the human CB_2 receptor and mouse CB_2 receptor share only 82% homology [207]. CB_1 receptor is expressed at high levels in the brain (Table 3) and to a lesser extent in peripheral tissues such as the adrenal glands, reproductive organs and on immune cells [25, 72, 141]. The first studies that explored the tissue and cell distribution of CB_2 receptors indicated that this cannabinoid receptor type was exclusively present in tissues and cells of the immune system [127, 205], being absent from the CNS [127]. The expression of CB_2 receptor gene in immune tissues has been reported to be 10-100 times higher than that of CB_1 receptors [72]. Further studies suggested that, although absent from the CNS in normal conditions, this receptor might be induced in glial cells, in particular reactive microglia, in response to different damaging conditions associated with local inflammatory events [66]. Lastly, recent evidence has shown that CB_2 receptors can be found in the brain (Table 3) even in healthy conditions [169]. These studies identified CB_2 receptors in glial cells [137,

154, 163, 219] and in certain neuronal subpopulations [15, 173, 224, 214, 237] in different brain structures of various species, including human samples [163].

5.3 CB_2 receptors: involvement in physiological processes and diseases

Most of the physiological functions associated with the CB_2 receptor deal with different types of immunological effects given the predominance of this receptor type over the CB_1 receptor. However, its recent description in certain brain regions allowed to relate this receptor type to neurobiological processes located in the regions described, like in the control of pain, brain reward, emotion and others [169]. An important fact is the implication of the CB_2 receptor in processes related to the control of proliferation [36, 173], differentiation [3, 173] and survival [204] of neuronal cells. Because CB_2 receptor plays a central role in these key cellular processes, it forms the basis for the proposal that selective agonists of this receptor type may act by providing cytoprotection of healthy neuronal cells or by eliciting apoptosis of tumor cells [66].

By using CB_2 receptor deficient transgenic mice [31] investigators have discovered the involvement of CB_2 receptors in many physiological and pathological processes (Figure 6).

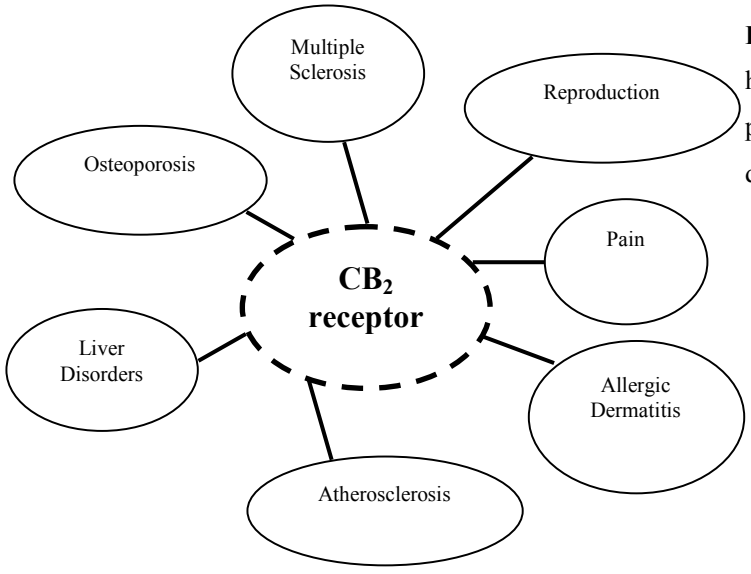

Figure 6: CB_2 receptors have been implicated in diverse physiological processes and diseases.

For example, together with CB_1 receptors, CB_2 receptors are responsible for successful embryonic implantation and that both receptors have a role in

synchronizing embryonic development [178, 229]. CB_2 receptors play a role in bone homeostasis and therefore they are a potential drug target for the treatment of osteoporosis [164] and are also implicated in the antifibrogenic role in the liver [111]. It is known as well, that endocannabinoids synthesized in the CNS [203] have immune suppressive effects and immunosuppression is mediated through CB_2 receptors [138].

Recently the implication of CB_2 receptors in pain modulation is also being recognized. CB_2 receptors have been shown to modulate acute pain, chronic inflammatory pain, post-surgical pain, cancer pain and pain associated with nerve injury [reviewed in ref. 234].

6. Dimerization of G-protein-coupled-receptors: focusing on opioids and cannabinoids

For decades, it has been generally proposed that a given receptor always interacts with a particular GTP-binding-protein (G-protein) or with multiple G-proteins within one family. However, for several GPCRs, it now becomes generally accepted that functional coupling with distinct unrelated G-proteins can be observed, leading to the activation of multiple intracellular effectors with distinct efficacies and potencies [76, 152]. Heterodimerization can generate receptors with novel characteristics, leading to altered pharmacological properties. The first demonstration of receptor heterodimers was performed using chimeric receptor molecules composed of α_{2C} adrenergic and m_3 muscarinic acetylcholine receptors. In this case two otherwise inactive chimeric receptors could physically associate to form a functional heterodimer receptor complex with ligand binding and signaling capabilities [132]. In many cases, the resulting heterodimeric receptor complex has been found to have pharmacological properties different from those of both of the individual partner receptors [11, 194]. Heterodimerization can result in either an enhancement or a reduction in the activity of a fully functional receptor [29, 76].

Coexpression of pairs of GPCRs in cellular systems have been studied most extensively using pairs of opioid receptors. For example, studies with μ-δ opioid receptor heterodimers demonstrated decreased binding affinity to selective synthetic agonists [75]. The rank order of agonist affinities for the heterodimeric receptors has

been shown to be different from that of the individual receptors, suggesting allosteric modulation of the binding pocket [75, 82]. The opioid receptors have also been shown to heterodimerize with distantly related GPCRs such as α_{2A}-adrenergic [110] or substance P [184] receptors. Similarly, CB_1 cannabinoid receptors are able to form homo- [114] and heterodimers [115].

It is well known that opioid and cannabinoid receptor distribution overlaps in several regions involved in the control of pain. Previous studies using selective opioid receptor antagonists suggested that μ- and particularly κ-, but not δ-receptors participate in Δ^9-THC analgesia. The molecular and cellular mechanisms of these interactions are not known, but they may be involved in the release of opioid peptides by Δ^9-THC. Such release has been demonstrated in the spinal cord for dynorphin [189, 96]. Anatomical studies have reported a similar distribution of CB_1 and μ-opioid receptors in the dorsal horn of the spinal cord [95, 202] and in several supraspinal structures of the CNS [195, 196]. Brain areas such as the caudate putamen, dorsal hippocampus, and substantia nigra are rich in both CB_1 cannabinoid and μ-opioid receptors [92, 133, 197] and the co-localization of both receptors is possible. Other brain structures, such as the periaqueductal gray (PAG) contain moderate level of CB_1 cannabinoid and μ-opioid receptor binding sites, but play an important role in antinociception [124].

7. Opioids and cannabinoids: pharmacological and biochemical interactions

Cannabinoids and opioids are separate groups of psychoactive drugs that share a similar pharmacological profile. Compounds in both groups induce analgesia, catalepsy, hypothermia, motor depression, hypotension, immunosupression, sedation and reward effects [136, 140, 226].

There are an increasing number of data available proving the anatomical, biochemical and pharmacological interactions. Illustrative examples are: 1. These drugs can interact in mediating their analgesic effects [43, 45, 213, 231], 2. They affect eachother's long-term activity [212, 221, 230], 3. Cross-dependence between opioid and cannabinoid compounds has also been reported [52, 159, 238], 4. Interactions have been found in drug self-administration studies [50, 112, 160], 5. Cross-sensitization has been reported [185, 186], 6. The endogenous opioid system is involved in different cannabinoid actions like nociception [191], dependence [125] or reward [135], 7. Functional interactions between these two systems have been proved in the feeding behavior as well [49, 50, 236].

Combining opioids with CB_1 cannabinoid agonists has been suggested to be promising therapeutic approach in modulation of pain, but it is important to consider that the nature of cannabinoid and opioid interactions differ in the brain circuits mediating reward and in those mediating other pharmacological properties, such as antinociception [44].

The interactions between opioids and CB_2 receptor-selective agonists have yet to be studied. To date, CB_2 receptor agonists do not exhibit CNS side effects. CNS side effects, such as tolerance or respiratory depression, limit the clinical utility of opioids, currently the most commonly used medications for the treatment of moderate-to-severe pain.

II. AIM OF THE STUDIES

Plant-derived natural, chemically synthesized or endogenous cannabinoid ligands all act on cannabinoid receptors, but there is the possibility that they are not their only target or even the primary one. An example emphasizing this case is the endocannabinoid anandamide, which by some authors would be described primarily as a vanilloid receptor agonist [215]. Also CB_1 and CB_2 cannabinoid receptors have a much more diverse range of signal transduction mechanisms associated with them, than originally thought. This opens up the possibility of a great number of interactions with other signaling systems, like for example opioids. Opioid and cannabinoid receptor distribution overlaps in several regions involved in the control of pain, reward and consequently interactions between the opioid and CB_1 cannabinoid systems have been proposed in many publications. In contrast, there is no such data that links the possible effects of cannabinoids on the opioid system mediated through the brain CB_2 cannabinoid receptor.

It is well known, that GPCRs have the ability to adopt different, complex active conformations [179], which differ in their pharmacological, signaling [10] and regulatory properties [9], thus activating several signaling pathways. The existence of multiple active states of the GPCRs adds an unprecedented diversity to these receptors function and provides a new perspective for the development of longer acting, better tolerated analgesics.

An intriguing question generated by these facts is the examination of interactions between brain cannabinoid and opioid systems, searching for the possible involvement of CB_2 receptors. To answer this we conducted experiments, which included the characterization of each opioid receptor's gene expression levels and measured functional activation after *in vivo* and *in vitro* noladin ether treatments in different parts of CB_1 [122] and CB_2 [30] knockout mice brain. Investigators usually carry out their experiments using synthetic compounds, because of the practical difficulties inherent in natural cannabinoids. However, endocannabinoids may not activate the same repertoire of signaling pathways. It is known that anandamide, an amide and 2-AG, an ester are hydrolyzed rapidly *in vivo* [147], thus we have choosen noladin ether, an ether type endocannabinoid, which binds to both CB_1 and CB_2 receptors ($CB_1 >> CB_2$) and is metabolically stable enough *in vivo*.

Because of the regulated page limit, only a selection of the results are described in the thesis [175, 176] however, another paper was published as a part of this overall study as well [174].

The aims of the study presented here were the following:

► To acutely treat (intraperitoneally) wild-type and CB_1 cannabinoid receptor knockout mice with noladin ether alone and in combinations with CB_1 and CB_2 receptor antagonists.

► To characterize and compare the changes of the μ-opioid receptor (MOR) gene expression level in forebrain and in brainstem, after the *in vivo* cannabinoid treatments by Quantitative Real-time PCR.

► To examine the changes of the μ-opioid receptor G-protein activation in CB_1 wild-type and CB_1 knockout mice forebrain and brainstem membranes, after the *in vivo* cannabinoid treatments by using [^{35}S]GTPγS functional binding assay.

► To investigate the binding properties of the cannabinoids to the MOR in CB_1 wild-type and CB_1 knockout mice forebrain and brainstem membranes in competition binding studies.

► To test the *in vitro* effects of the cannabinoids on MOR G-protein activation in either CB_1 or CB_2 cannabinoid receptor knockout mice brain membranes in [^{35}S]GTPγS binding experiments.

III. MATERIALS AND METHODS

1. Chemicals

1.1 Radiochemicals

[^{3}H]DAMGO ([D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin; 51 Ci/mmol) was purchased from DuPont de Nemours (Wilmington, Del., USA). Guanosine-5'-O-(3-γ[^{35}S]thio) triphosphate ([^{35}S]GTPγS) (37-41 Tbq/mmol) was from the Isotope Institute Ltd. (Budapest, Hungary).

1.2 Receptor ligands and fine chemicals

Unlabelled DAMGO was purchased from Bachem Holding AG (Bubendorf, Switzerland). 2-Arachidonyl glyceryl ether (2-AGE, noladin ether, NE) was from Tocris (Bristol, United Kingdom). SR144528 was provided by SANOFI Research (Montpellier, France). Ethylenediamine-tetraacetic acid (EDTA), bovine serum albumin (BSA), guanosine 5'-diphosphate (GDP), guanosine-5'-O-(3-thiotriphosphate) GTPyS were from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen. DNaseI and reaction buffer, MuLV reverse transcriptase and oligodT primer of Revertaid H-Minus Kit was obtained from Fermentas. Absolute QPCR mix was purchased from ABgene. All other reagents used in this study were of the highest purity available.

2. Animals

CB_1 wild-type ($CB_1^{+/+}$) and CB_1 cannabinoid receptor knockout mice ($CB_1^{-/-}$) were generated in Dr.Ledent's lab (Brussels, Belgium) as desribed [122], and was breed and treated at the Department of Human Morphology and Developmental Biology. The animals were housed in controlled temperature (21 ± 2˚C) and light (on 07^{00} h, off 19^{00} h) and were provided with water and food *ad libitum*. Different treatment groups were composed of 7-10 animals in each group. All housing and experiences were conducted in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in

Research (XXVIII.tv. 32.§). CB_2 wild-type ($CB_2^{+/+}$) and CB_2 cannabinoid receptor knockout mice ($CB_2^{-/-}$) were generated in Dr. Zimmer's lab (Bonn, Germany) [30].

3. Drugs and treatments

Noladin ether (NE) was injected at the dose of 1 mg/kg in DMSO solution. The dose of the CB_2 receptor antagonist SR144528 [193] was 0.1 mg/kg dissolved in the same vehicle as noladin ether. Upon acute *in vivo* treatments animals received a single intraperitoneal (i.p.) injection of noladin ether or SR144528. Control mice were injected with DMSO solution. When used in a combined treatment, the CB_2 antagonist compound SR144528 was delivered 30 min prior to the agonist treatment, as suggested by SANOFI Research Laboratory [193]. All the experiments were carried out with 7-9 animals per treatment groups.

4. Membrane preparations

Forebrain and brainstem membrane fractions from $CB_1^{+/+}$, $CB_2^{+/+}$ and $CB_1^{-/-}$, $CB_2^{-/-}$ mice were prepared according to the method previously described [17]. Briefly, mice were decapitated and the brains were quickly removed, separated (forebrain, cerebellum, brainstem) and homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4˚C and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37˚C. The centrifugation step was repeated, and the final pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at −70˚C until use. Before use membranes were thawed, diluted with fresh buffer and centrifuged again to remove sucrose and used immediately in the binding assays.

5. Radioligand binding assay

Receptor binding studies allow us to analize the interaction of hormones, neurotransmitters and related drugs with the receptors and help us in mapping the distribution of receptors in different areas of the body, as well as the effects of physiological and pathological conditions on the expression of the receptors [33]. The basis of this study is the binding of a ligand to the receptor to form receptor-ligand complex. The receptor-ligand complex is classically referred to as bound, meaning the amount of ligand that is bound to the receptor. The unbound ligand is referred to as free, meaning the amount of ligand that is free and able to interact with the receptor. The parameter measured is the amount of radioactive ligand that is bound to the receptor.

There are two basic types of receptor binding experiments: saturation and competition. When a receptor ligand is not available in a radioactive form, competition binding experiments are used, and the affinity of the unlabeled ligand for the receptor can be measured by measuring its ability to compete with the binding of a radioactive ligand to its receptor.

5.1 Competition binding experiments

In a competition experiment, various concentrations of an unlabeled ligand compete with a fixed concentration of a radiolabeled ligand for binding to the receptor. In these experiments the equilibrium inhibiton constants (K_i) are determined for the unlabelled ligand. This value can be obtained from the IC_{50} value using Cheng-Prusoff equation $K_i = IC_{50} / (1+[L]/K_d)$ where [L] is the concentration of radioactive ligand used and K_d is the affinity of the radioactive ligand for the receptor [41].

Aliquots of frozen $CB_1^{+/+}$ and $CB_1^{-/-}$ mice forebrain and brainstem membranes were centrifuged (40 000 × g, 20 min, 4˚C) to remove sucrose and pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4). Membranes were incubated with gentle shaking at 35˚C for 45 min in a final volume of 1 ml with unlabelled DAMGO, noladin ether or SR144528 (10^{-11} – 10^{-5} M), and ~ 1 nM of [^{3}H]DAMGO. Total binding was measured in the presence of radioligand, non-specific binding was determined in the presence of 10 μM unlabeled naloxone. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester), and

washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/C glass fibers. The radioactivity of the dried filters was detected in UltimaGold^{TM} F scintillation cocktail (Packard) with Packard Tricarb 2300TR liquid scintillation counter. Radioligand binding assays were performed in duplicate and repeated at least three times. Experimental data were analyzed by GraphPad Prism 3.0 software (San Diego, USA, www.graphpad.com) to determine the concentration of the drug that displaced 50% of [^{3}H]DAMGO (IC_{50}).

6. [^{35}S]GTPγS binding experiments

Agonist stimulated [^{35}S]GTPγS binding assay is a widely used functional and biochemical *in vitro* assay used for determination of basic pharmacological characteristics and relative efficacy of ligands. All G-protein-coupled receptors function *via* interaction and activation of G-proteins. It is well established that the key step in this process is induced guanine nucleotide exchange on the G-protein α-subunit. This results in replacement of GDP by GTP followed by conformational rearrangements and dissociation of the G-protein and α-subunit from the βγ complex. The nucleotide exchange process can be monitored by using non-hydrolysable analogue of GTP that contains γ-thiophosphate bond ([^{35}S]GTPγS). Guanine nucleotide exchange is a very early event in the signal transduction cascade thus presents an attractive event to monitor because it is less influenced by other cellular processes. [^{35}S]GTPγS binding assay is mostly feasible using for GPCRs interacting with pertussis-toxin-sensitive G_i family G proteins [151].

Membrane preparations of both CB_1 and CB_2 mice forebrains and brainstems were diluted in 50 mM Tris-HCl buffer (pH 7.4) to get appropriate protein content for the assays (~ 10 μg of protein/sample). The membrane fractions were incubated at 30˚C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-10} – 10^{-5} M) of DAMGO in the presence of excess GDP (30 μM) in a final volume of 1 ml, according to Sim et al. (1995) [209] and Traynor and Nahorski (1995) [195], with slight modifications. Total binding (T) was measured in the absence of test compounds, non-specific binding (NS) was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total

binding. The difference (T–NS) represents basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with Brandel M24R Cell harvester. Filters were washed three times with 5 ml ice-cold buffer (pH 7.4), and the radioactivity of the dried filters was detected in UltimaGold^{TM} F scintillation cocktail (Packard) with Packard Tricarb 2300TR liquid scintillation counter. Stimulation is given as percent of the specific [^{35}S]GTPγS binding observed in the absence of receptor ligands (basal activity). [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times. For the *in vitro* experiments, brainstem membranes were treated with 10^{-10} – 10^{-5} M of DAMGO in the presence or absence of 1 μM NE, 1 μM SR144528 and 1 μM SR144528 + 1 μM NE. LogEC_{50} values and E_{max} values were determined again by GraphPad Prism 3.0.

7. RNA extraction and reverse transcription

Total RNAs were extracted from forebrain and brainstem tissues of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice using Trizol reagent, according to the manufacturer's instructions. RNA was treated with DNaseI in $MgCl_2$ 10 × reaction for 30 min. DNaseI was then heat-inactivated at 65˚C for 15 min, in the presence of 25 mM EDTA. First strand cDNA was synthesized by reverse transcription (5 μg RNA/20 μl reaction volume) using MuLV reverse transcriptase and oligodT primer of Revertaid H-Minus Kit. After an initial denaturation step of 1 min at 95˚C, synthesis of the second strand consisting of 1 h extension at 42˚C and a final extension step of 5 min at 72˚C was done.

8. Qantitative Real-time PCR

Real-time polymerase chain reaction (PCR) is a technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification of a specific sequence in a DNA sample.

This technique is a refinement of the original PCR developed by Kary Mullis and coworkers [201]. By PCR essentially any nucleic acid sequence present in a complex sample can be amplified in a cyclic process to generate a larger number of identical copies that can readily be analyzed. This made it possible, for example, to manipulate DNA for cloning purposes, genetic engineering and sequencing. But as an analytical technique the original PCR method had some serious limitations. By first amplifying the DNA sequence and then analyzing the product, quantification was exceedingly difficult since the PCR gave rise to essentially the same amount of product independently of the initial amount of DNA template molecules that were present. In Real-time PCR the amount of product formed is monitored during the course of reaction by monitoring the fluorescence of dyes or probes introduced into the reaction that is proportional to the amount of product formed. And the number of amplification cycles required to obtain a particular amount of DNA molecules is registered [120].

Quantitative Real-time PCR (QRT-PCR) was performed on a Mini Opticon™ System instrument (BIORAD) with gene-specific primers and SybrGreen dye according to an earlier described protocol [190]. Briefly, the cDNA was diluted 1:5, and 2 μl of this mix was used as template in the QRT-PCR reaction. Primers were designed by using the ArrayExpress software (Applied Biosystems). The following primers were used: MOR forward TCAACTTGTCCCACGTTGATG, reverse AAGCCCCGTGCGGTTAG and β-Actin forward TGACAGGATGCAGAAGGAGA, reverse CGCTCAGGAGGAGCAATG. Reactions were performed in a total reaction volume of 20 μl containing 10 μl of Absolute QPCR mix and 5 mM of each primer. The amplification was carried out with the following cycling parameters: 15 min heat activation at 95˚C, 45 cycles comprising denaturation at 95˚C for 25 s, annealing at 60˚C for 25 s and extension at 72˚C for 20 s. Fluorescent signals were collected after each extension step at 72˚C. Curves were analyzed by the RotorGene software using dynamic tube and slope

correction methods ignoring data from cycles close to baseline. Relative expression ratios were normalized to the endogenous β-Actin gene. Expression ratios were calculated using the Pfaffl method [183]. All the PCRs were performed at least three times in separate runs. Results were expressed as the arithmetical mean for each gene and analyzed using GraphPad InStat 3.06 software. The statistical significance of differences between the groups was determined using one-way ANOVA test.

IV. RESULTS

1. Noladin ether induced effect on μ-opioid receptor in the forebrain of CB_1 cannabinoid knockout mice

In this part of the study we examined whether *in vivo* and *in vitro* administered noladin ether (NE) has any effect on the μ-opioid system in mice forebrain. We used quantitative real-time PCR to measure the changes of μ-opioid receptor (MOR) mRNA levels, [^{35}S]GTPγS functional binding assays to measure the capability of the μ-opioid agonist peptide DAMGO in activating G-proteins via MORs and competition binding assays to directly measure NE binding to MORs. All our experiments were carried out in wide-type ($CB_1^{+/+}$) and CB_1 cannabinoid receptor deficient mice ($CB_1^{-/-}$, knockout) [122].

1.1 QRT-PCR studies after acute NE treatment

Changes of MOR mRNA expression levels after acute NE treatment were measured by QRT-PCR. MOR was detected in both wild-type and CB_1 knockout mice forebrain (Figure 7).

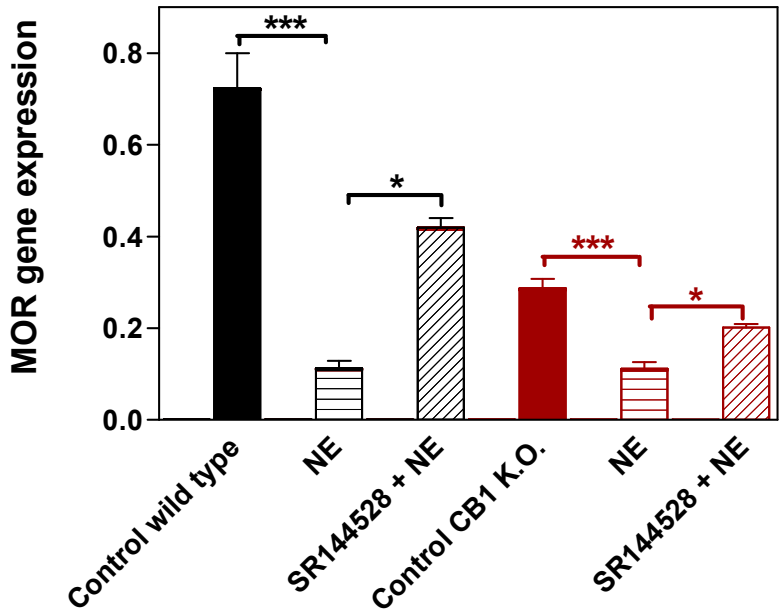

Figure 7: MOR mRNA level in forebrain of wild-type and CB_1knockout mice after acute noladin ether and combined SR144528 + NE treatment.

Treatment of wild-type (nine pooled samples) mice with single intraperitoneal (i.p.) injection of NE at the dose of 1 mg/kg induced a significant decrease (***P<0.001) in MOR mRNA expression in comparison to the vehicle treated controls (black column). Similarly, the same dose of NE caused a significant decrease (***P<0.001) in the level of forebrain MOR mRNAs in CB_1 knockout mice (seven pooled samples) compared to the vehicle-injected control animals (red column). Surprisingly, pretreatment with the CB_2 receptor selective antagonist SR144528 (0.1 mg/kg i.p.) substantially reversed the effect of NE (1 mg/kg i.p.) by significantly increasing the amount of MOR mRNAs in wild-type (*P<0.05) and CB_1 knockout (*P<0.05) mice (Figure 7).

1.2 Acute NE effect on DAMGO-stimulated [^{35}S]GTPγS binding

Coupling efficiency of MOR to the regulatory G-proteins was studied in [^{35}S]GTPγS functional binding assays using the highly MOR specific pure agonist peptide ligand, DAMGO.

MOR mediated G-protein stimulation by DAMGO is characterized by a potency (EC_{50}) value of 39,5 nM and a maximal stimulation (E_{max} or efficacy = 164%) of 64% over the basal (=100%) activity in vehicle-treated CB_1 wild-type control animals (Table 4). Forebrain membranes were used in the [^{35}S]GTPγS binding with 10^{-10} – 10^{-5} M concentration of DAMGO. NE administration (1 mg/kg i.p.) resulted in a decrease in the potency of DAMGO (39.5 nM → 750 nM, n=6, ***P<0.001, one-way ANOVA, Table 4). Pretreatment of the mice with the CB_2 receptor antagonist SR144528 (0.1 mg/kg i.p.) prior to the administration of NE (1 mg/kg i.p.) markedly increased the potency of the μ-opioid agonist DAMGO in activating G-proteins (750 nM → 69.9 nM, n=5, ***P<0.001, Table 4). The efficacy of DAMGO in the $CB_1^{-/-}$ mice was essentially similar to that found in the wild-type animals (E_{max} values of 164% versus 169.5%), although notably different potency values were observed in the $CB_1^{-/-}$ knockout mice (39.5 nM → 250 nM; n=6, **P<0.01, Table 4). Intraperitoneal administration of NE produced about 30% decrease in the efficacy (169.5% → 142%, n=6, ***P<0.001, Table 4) of DAMGO in stimulating the functional coupling of MOR to G-proteins in the $CB_1^{-/-}$ animals. The combined *in vivo* treatments with the CB_2 cannabinoid antagonist SR144528 followed by the agonist NE resulted again in an enhancement of the potency of DAMGO in

stimulating [^{35}S]GTPγS binding to forebrain membrane fractions of $CB_1^{-/-}$ knockout mice (434nM → 82.5nM, n=4, **P<0.01, Table 4).

Table 4 E_{max} and EC_{50} values of the μ-opioid receptor agonist DAMGO in [^{35}S]GTPγS binding experiments in forebrain membrane of wild-type and $CB_1^{-/-}$ mice.

	CB_1 wild-type mice acutely treated with			**CB_1 knockout mice acutely treated with**		
	Vehicle	NE	SR144528 + NE	Vehicle	NE	SR144528 + NE
DAMGO E_{max} ± S.E.M.	164.1 ± 2.6	151.1 ± 4.7 ^{NS}	141.2 ± 2.34 ^{NS}	169.5 ± 4.75	142.0 ± 2.57 ***	151.7 ± 2.3 ^{NS}
DAMGO logEC_{50} ± S.E.M	-7.4 ± 0.11	-6.12 ± 0.18 ***	-7.15 ± 0.16 ***	-6.59 ± 0.17	-6.36 ± 0.14 ^{NS}	-7.08 ± 0.6 **

1.3 In vitro effect of NE on DAMGO stimulated [^{35}S]GTPγS binding

The observed attenuations on MOR signaling after acute *in vivo* administration of NE indicate the presence of interactions between cannabinoid and opioid systems. To reveal the direct interactions, if any, DAMGO-stimulated [^{35}S]GTPγS binding experiments were performed in the presence or absence of *in vitro* added NE.

In vitro administered NE (1 μM) decreased the maximal stimulation of DAMGO in [^{35}S]GTPγS binding assays in CB_1 wild-type forebrain membranes (E_{max} changed from 165% to 139% , n=3, **P<0.01, Figure 8, Table 5). The combined *in vitro* treatments with SR144528 (1 μM) and NE (1 μM) caused an increase in the efficacy (E_{max} changed 139% → 156.6%, n=3, *P<0.05, one-way ANOVA, Figure 8, Table 5) when compared to the NE administration alone. In the CB_1 knockout mice lower potency values of DAMGO were observed in comparison to wild-type (EC_{50} values of 32.7 nM versus 253 nM, n=6, **P<0.01, Figure 8, Table 5). The presence of 1 μM NE induced a robust decrease in the efficacy of DAMGO-stimulated [^{35}S]GTPγS binding in $CB_1^{-/-}$ forebrain (169.5%→ 128.3%, n=6, ***P<0.001, Figure 8, Table 5). Inclusion of 1 μM SR144528 CB_2 antagonist significantly increased the efficacy of DAMGO-stimulated [^{35}S]GTPγS binding when compared to NE administration alone (128.3% → 162.7%, n=3, ***P<0.001, Figure 8, Table 5).

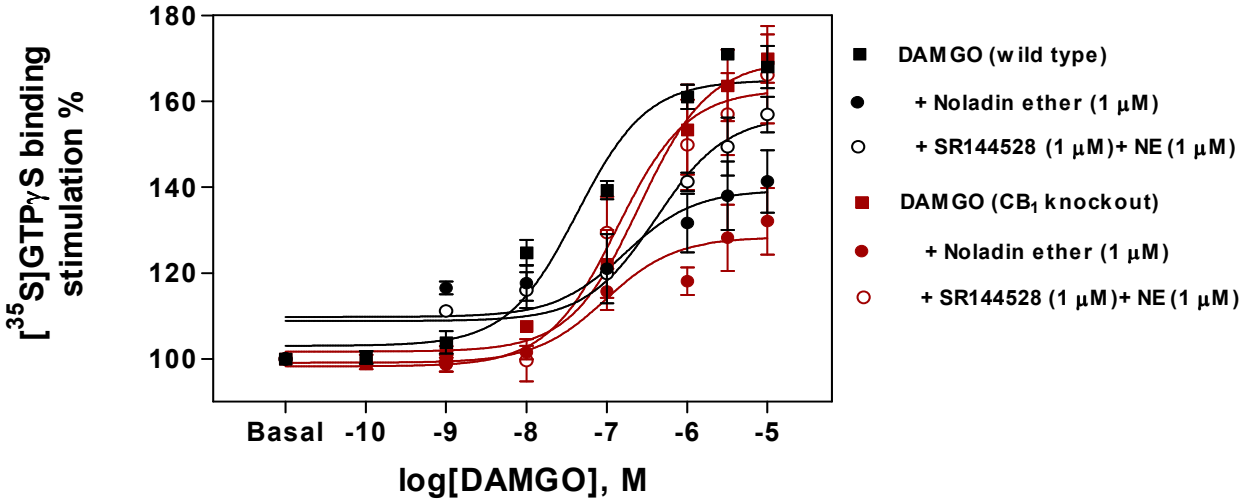

Figure 8: [^{35}S]GTPγS binding assay performed in the forebrain of wild-type and CB_1 knockout mice after *in vitro* administration of noladin ether and combined SR144528 + NE in the presence of μ opioid agonist DAMGO.

Table 5 E_{max} and EC_{50} values of the μ-opioid receptor DAMGO in [^{35}S]GTPγS binding experiments in forebrain membranes of wild-type and $CB_1^{-/-}$ mice.

	Membranes from $CB_1^{+/+}$ mice *in vitro* treated with			**Membranes from $CB_1^{-/-}$ mice *in vitro* treated with**		
	DAMGO	+ NE (1μM)	+ SR144528 (1μM)	DAMGO	+ NE (1μM)	+ SR144528 (1μM)
DAMGO E_{max} ± S.E.M	164.1 ± 2.6	139.0 ± 4.58 **	157.0 ± 4.0 *	169.5 ± 4.75	128.3 ± 3.15 ***	162.7 ± 4.76 ***
DAMGO $logEC_{50}$ ± S.E.M	-7.4 ± 0.11	-6.77 ± 0.5 NS	-6.38 ± 0.2 NS	-6.57 ± 0.17	-7.02 ± 0.3 NS	-6.87 ± 0.22 NS

The observed attenuations caused by NE are similar in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice therefore we can exclude CB_1 receptor contribution in these effects.

1.4 In vitro effect of NE on [^{3}H]DAMGO equilibrium competition binding

To determine the direct binding affinity of the endocannabinoid NE for μ-opioid receptors, competition binding experiments with [^{3}H]DAMGO were conducted

using membranes prepared from forebrain tissues of $CB_1^{+/+}$ (black symbols) or $CB_1^{-/-}$ (red symbols) mice (Figure 9).

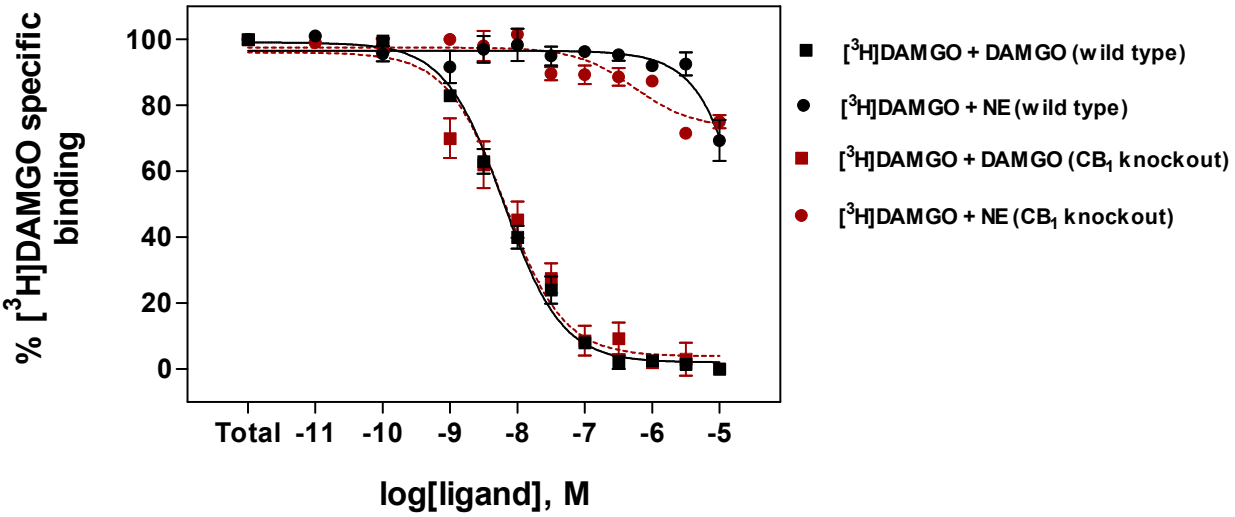

Figure 9: Specific [^{3}H]DAMGO binding (~1nM) in the presence of increasing concentrations of unlabeled DAMGO and noladin ether (NE) in wild-type and CB_1 knockout mice forebrain.

NE failed to displace [^{3}H]DAMGO with high affinity from μ-opioid receptors compared to the affinity of the unlabelled DAMGO, (IC_{50} ≈10 nM) obtained in homologous competition studies in both $CB_1^{+/+}$ and $CB_1^{-/-}$ forebrain membrane fractions (Figure 9).

These results reach the conclusion that under these conditions, the attenuation caused by NE on MOR signaling is mediated *via* CB_2 cannabinoid receptors.

2. Noladin ether and SR144528 induced effect on the μ–opioid system in the brainstem of CB_1 and CB_2 cannabinoid knockout mice

The second part of our study is based on our previous observations that noladin ether produces decrease in the activity of MOR in forebrain and this attenuation can be antagonized by the CB_2 cannabinoid receptor antagonist SR144528, suggesting a CB_2 receptor mediated effect. We decided to continue our work on the brainstem - CB_2 cannabinoid receptors rich area [83, 224] - focusing on the possible interactions between μ and CB_2. We used again quantitative real-time

PCR, [^{35}S]GTPγS binding assay and radioligand binding assay to evaluate the changes on MOR activity after acute noladin ether, combined SR144528+NE and SR144528 treatment alone. For this study we used both CB_1 [122] - and CB_2 [30] cannabinoid receptor knockout mice.

2.1 QRT-PCR studies after acute NE treatment

MOR gene expression was determined in mRNA isolated from mouse brainstem from $CB_1^{+/+}$ (seven to ten pooled samples) and $CB_1^{-/-}$ (seven to ten pooled samples) mice (Figure 10).

No difference was found after treatment with single intraperitoneal (i.p.) injections of NE at the dose of 1 mg/kg in comparison to the vehicle treated $CB_1^{+/+}$ (black column) and $CB_1^{-/-}$ (red column) controls (Figure 10). Statistically significant decrease of MOR expression level in both $CB_1^{+/+}$ (***P < 0.001) and $CB_1^{-/-}$ (***P < 0.001) mice was observed after pretreatment with the CB_2 receptor selective antagonist SR144528 (0.1mg/kg, Figure 10).

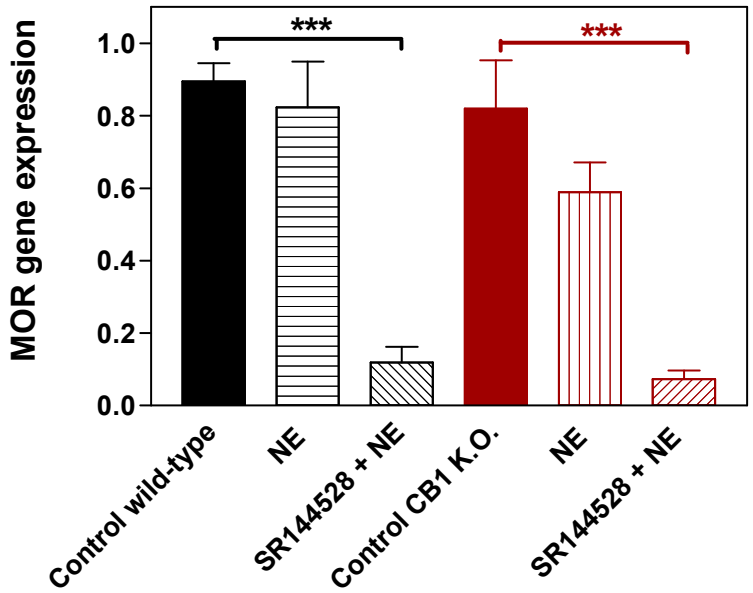

Figure 10: MOR mRNA expression after acute noladin ether and combined SR144528 + NE treatment in CB_1 wild-type and CB_1 knockout mice brainstem.

2.2 Acute NE effect on DAMGO-stimulated [^{35}S]GTPγS binding

Brainstem membranes were used in the [^{35}S]GTPγS binding with 10^{-10} – 10^{-5} M concentration of DAMGO. NE administration (1 mg/kg i.p.) resulted no significant change in the efficacy (170.4% → 164.8%, *n* = 6, Table 6). Pretreatment of mice with the CB_2 receptor antagonist SR144528 (0.1 mg/kg i.p.) prior to the administration of NE (1 mg/kg i.p.) markedly decreased the efficacy of the μ-opioid agonist DAMGO in activating G-proteins (170.4% → 138.4%, *n* = 3, *P < 0.05, Table 6). In the $CB_1^{-/-}$ mice the efficacy of DAMGO was similar to that found in the wild-type animals (E_{max} values of 170.4% versus 172.3%), but lower potency values were observed in the $CB_1^{-/-}$ knockouts (EC_{50} values of 179.2 nM versus 297.9 nM). Intraperitoneal administration of NE caused no change on the brainstem activity of DAMGO in stimulating the functional coupling of MOR to G-proteins in the $CB_1^{-/-}$ animals (172.3% → 162.7%,*n* = 5,Table 6). The combined *in vivo* treatments with SR144528 followed by NE resulted decrease in the efficacy of DAMGO binding in membrane fractions of $CB_1^{-/-}$ mice (172,3% → 134.9%, *n* = 5, *P < 0.05, Table 6).

Table 6 G-protein activation by the μ-opioid receptor agonist DAMGO in brainstem membranes of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice after acute NE and SR144528 + NE treatment.

E_{max} ± S.E.M. (DAMGO)		
***In vivo* treatments**	**CB_1 wild-type mice**	**CB_1 knockout mice**
Vehicle (control)	170.4 ± 4.9	172.3 ± 6.1
NE	164.8 ± 6.9 ^{NS}	162.7 ± 9.9 ^{NS}
SR144528 + NE	138.4 ± 1.9*	134.9 ± 1.7*

2.3 In vitro effect of NE on DAMGO-stimulated [^{35}S]GTPγS binding

Next, we examined if the modulation of MOR receptor activity seen after acute cannabinoid treatments can be observed *in vitro*. For this, we preformed DAMGO-stimulated [^{35}S]GTPγS binding experiments in the presence or absence of *in vitro* added NE.

It was found that *in vitro* administered NE (1 μM) caused no changes in the efficacy of DAMGO in $CB_1^{+/+}$ brainstem membranes (E_{max} values of 165.2% → 160.9%, n = 4, one-way ANOVA, Table 7). The combined *in vitro* treatments with the CB_2 cannabinoid antagonist SR144528 (1 μM) and NE (1 μM) caused a decrease in the efficacy (E_{max} changed 165.2% → 139.5%, n = 4, ***P < 0.001, Table 7). In the CB_1 knockout mice lower potency of DAMGO were observed in comparison to wild-type (EC_{50} values of 172.7 nM versus 249.2 nM). Like in $CB_1^{+/+}$ animals, NE caused no changes in the efficacy of DAMGO-stimulated [^{35}S]GTPγS in $CB_1^{-/-}$ brainstem (E_{max} values of 166.5% → 172.8%, n = 3, Table 7). As we expected, inclusion of 1 μM SR144528 significantly reduced the efficacy of DAMGO when compared to the control (166.5% → 141.9%, n = 3, **P < 0.01, Table 7).

Table 7 *In vitro* effects of cannabinoid compounds on DAMGO-stimulated [^{35}S]GTPγS binding in brainstem membranes of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.

	E_{max} ± S.E.M. (DAMGO)	
***In vitro* treatments**	**$CB_1^{+/+}$ wild-type mice**	**$CB_1^{-/-}$ knock-out mice**
Control	165.2 ± 2.6	166.5 ± 2.6
Noladin ether (NE)	160.9 ± 3.0 ^{NS}	172.8 ± 5.5 ^{NS}
SR144528 + NE	139.5 ± 2.7***	141.9 ± 3.2**

2.4 In vitro effect of NE on [^{3}H]DAMGO competition binding

A possible direct interaction of the endocannabinoid noladin ether with the MOR binding sites were measured by [^{3}H]DAMGO heterologous competition binding assays in brainstem membranes of $CB_1^{+/+}$ (black symbols) and $CB_1^{-/-}$ (red symbols) mice (Figure 11).

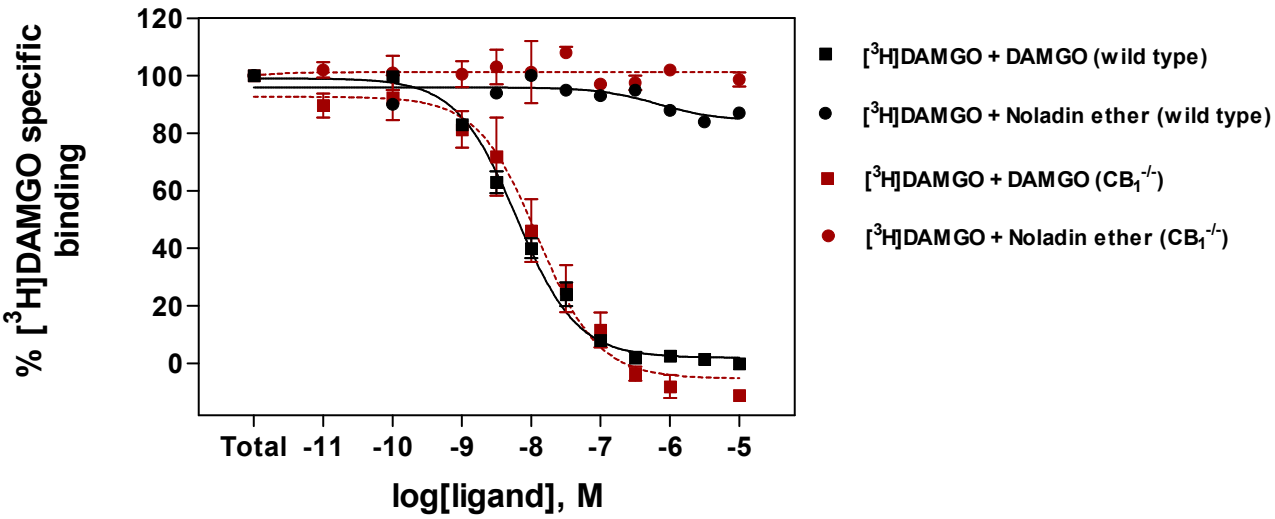

Figure 11: Specific [^{3}H]DAMGO binding (~1nM) in the presence of increasing concentrations of unlabeled DAMGO and noladin ether (NE) in wild-type and CB_1 knockout mice brainstem.

Competition curves demonstrate that NE has practically no effect in displacing [^{3}H]DAMGO, whereas the homologous opioid peptide DAMGO displayed expectedly high affinity (IC_{50}s ≈ 10 nM) in both preparations (Figure 11).

Taken together, our results show that NE failed to have any effect on MOR signaling, both *in vivo* and *in vitro*. However, the observed attenuations on MOR gene expression and MOR signaling after combined SR144528 + NE treatment indicate the presence of interactions between SR144528 and MOR. To reveal this possibility additional animal treatment was done with SR144528 alone.

2.5 QRT-PCR studies after acute SR144528 treatment

We determined MOR gene expression level in $CB_1^{+/+}$ (seven to ten pooled samples) and $CB_1^{-/-}$ (seven to ten pooled samples) mice brainstem after acute SR144528 treatment by quantitative real-time PCR.

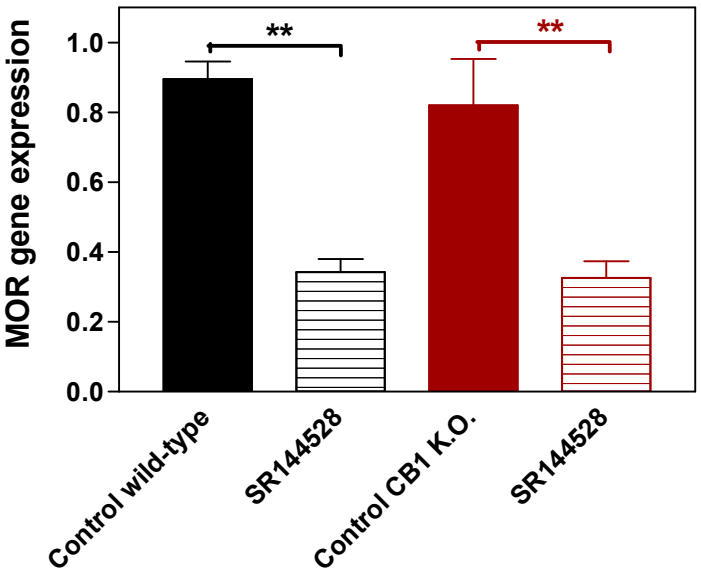

Figure 12: MOR mRNA expression after acute SR144528 treatment in CB_1 wild-type and CB_1 knockout mice brainstem.

Treatment of wild-type mice with single intraperitoneal (i.p.) injections of SR144528 at the dose of 0.1 mg/kg induced significant decrease (**P < 0.01, Figure 12) in MOR mRNA expression when compared to the vehicle treated (black column) controls. A similar decrease in the MOR gene expression was obtained in the brainstem of $CB_1^{-/-}$ after a single treatment with SR144528 (**P < 0.01, Figure 12), when compared to the control (red column).

2.6 Acute SR144528 effect on DAMGO-stimulated [^{35}S]GTPγS binding

We have found a significant decrease in the efficacy of the μ-opioid agonist DAMGO in activating G-proteins after CB_2 receptor antagonist SR144528 (0.1 mg/kg i.p.) treatment (E_{max} values of 199.8% → 171.7%,n = 5, ***P < 0.001, Figure 13, Table 8) in $CB_1^{+/+}$ mice (black symbols). Both, the efficacy and the potency of DAMGO in the $CB_1^{-/-}$ mice was similar to that found in the wild-type animals (E_{max}

values of 199.8% versus 194.5%, Figure 13, Table 8), (EC_{50} values of 145 nM versus 154 nM, data not shown). Intraperitoneal administration of SR144528 caused reduction on the brainstem activity of DAMGO in stimulating the functional coupling of MOR to G-proteins in the $CB_1^{-/-}$ animals (194.5% → 174.6%, n = 4, **P < 0.01, Figure 13, Table 8).

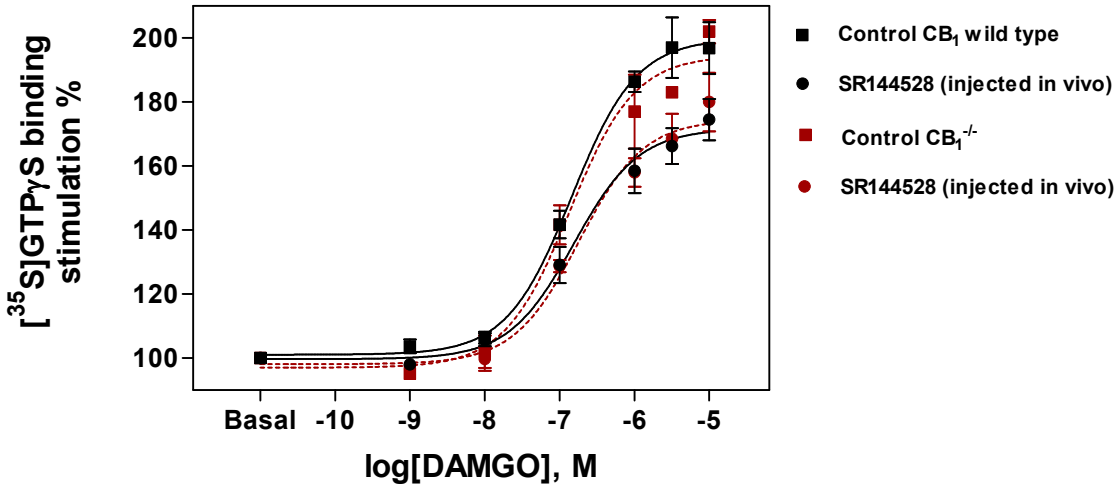

Figure 13: DAMGO-stimulated [^{35}S]GTPγS binding assay performed in the brainstem of wild-type and CB_1 knockout mice after *in vivo* administration of SR144528.

Table 8 DAMGO-stimulated [^{35}S]GTPγS binding in $CB_1^{+/+}$ and $CB_1^{-/-}$ brainstem membranes after acute SR144528 treatment.

	E_{max} ± S.E.M. (DAMGO)	
***In vivo* treatments**	**$CB_1^{+/+}$ wild-type mice**	**$CB_1^{-/-}$ knock-out mice**
Vehicle (control)	199.8 ± 3.7	194.5 ± 4.5
SR144528	171.7 ± 3.2***	174.6 ± 3.7**

2.7 In vitro effect of SR144528 on DAMGO-stimulated [^{35}S]GTPγS binding

To explore whether the previously observed effects of acute SR144528 treatments affects MOR signaling *via* CB_2 receptors, DAMGO-stimulated [^{35}S]GTPγS experiments were performed in the presence or absence of *in vitro* added SR144528 on CB_2 cannabinoid receptor deficient transgenic mice.

It was found that 1 μM SR144528 administered *in vitro* significantly decreased the maximal stimulation of DAMGO in [^{35}S]GTPγS binding assays in $CB_2^{+/+}$ (black symbols) brainstem membranes (E_{max} changed from 217% to 184% in the presence of the CB_2 antagonist, n = 3, ***P < 0.001, Figure 15, Table 9). In the CB_2 knockout mice (red symbols) lower efficacy values of DAMGO were observed in comparison to CB_2 wild-type (E_{max} values of 217.1% versus 196.3% n = 5, **P < 0.01, Figure 15, Table 9). These results are in agreement with the data of the QRT-PCR studies, where we obtained significantly higher MOR mRNA level in $CB_2^{+/+}$ than in $CB_2^{-/-}$ mice brainstem (**P < 0.01, n = 3, Figure 14). The presence of 1 μM SR144528 caused no significant change in the efficacy of DAMGO-stimulated [^{35}S]GTPγS binding in $CB_2^{-/-}$ brainstem (196.3% → 193.6%, n = 5, Figure 15, Table 9).

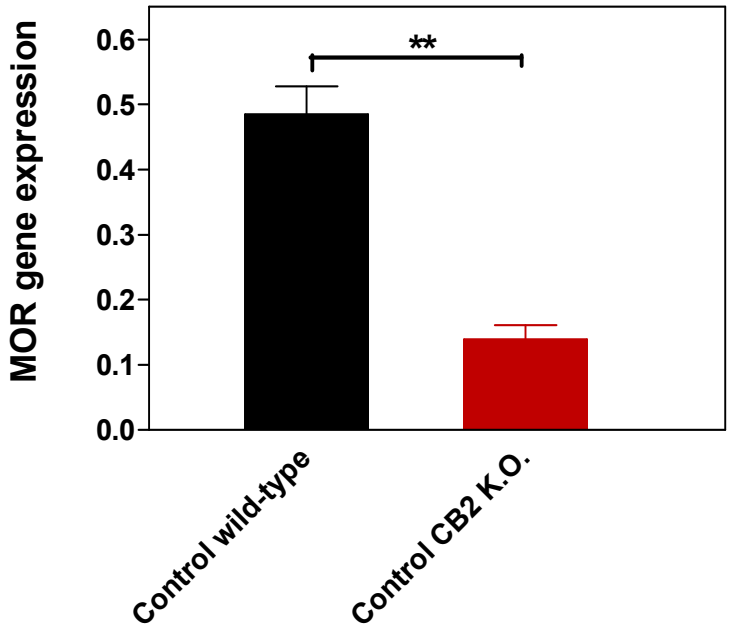

Figure 14: MOR mRNA expression in CB_2 wild-type and CB_2 knockout mice brainstem.

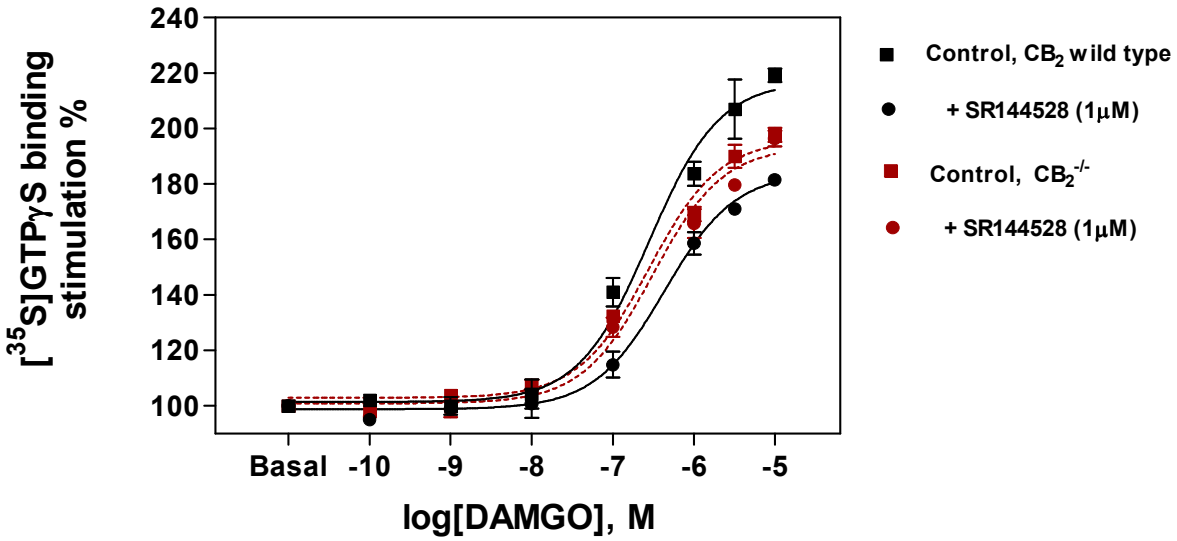

Figure 15: [^{35}S]GTPγS binding assay performed in the brainstem of CB_2 wild-type and CB_2 knockout mice after *in vitro* administration of SR144528 in the presence of μ opioid agonist DAMGO.

Table 9 *In vitro* effects of SR144528 on DAMGO-stimulated [^{35}S]GTPγS binding in brainstem membranes of $CB_2^{+/+}$ and $CB_2^{-/-}$ mice.

	E_{max} ± S.E.M. (DAMGO)	
***In vitro* treatments**	**$CB_2^{+/+}$ wild-type mice**	**$CB_2^{-/-}$ knockout mice**
Control	217.1 ± 4.3	196.3 ± 2.5
SR144528 (1 μM)	184.1 ± 3.8***	193.6 ± 3.0 ^{NS}

The results from QRT-PCR studies clearly show that the deletion of the CB_2 receptor alters the gene expression of the MOR. In [^{35}S]GTPγS binding studies the attenuation caused by SR144528 in $CB_2^{+/+}$ is not observed in $CB_2^{-/-}$. In conclusion, we can strongly suggest that in mouse brainstem the type 2 cannabinoid receptor might be involved in the MOR mediated effects.

2.8 In vitro effect of SR144528 on [^{3}H]DAMGO equilibrium competition binding

Competition binding experiments with [^{3}H]DAMGO were conducted using membranes prepared from brainstem tissues of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice to determine the direct binding affinity of the SR144528 for MOR.

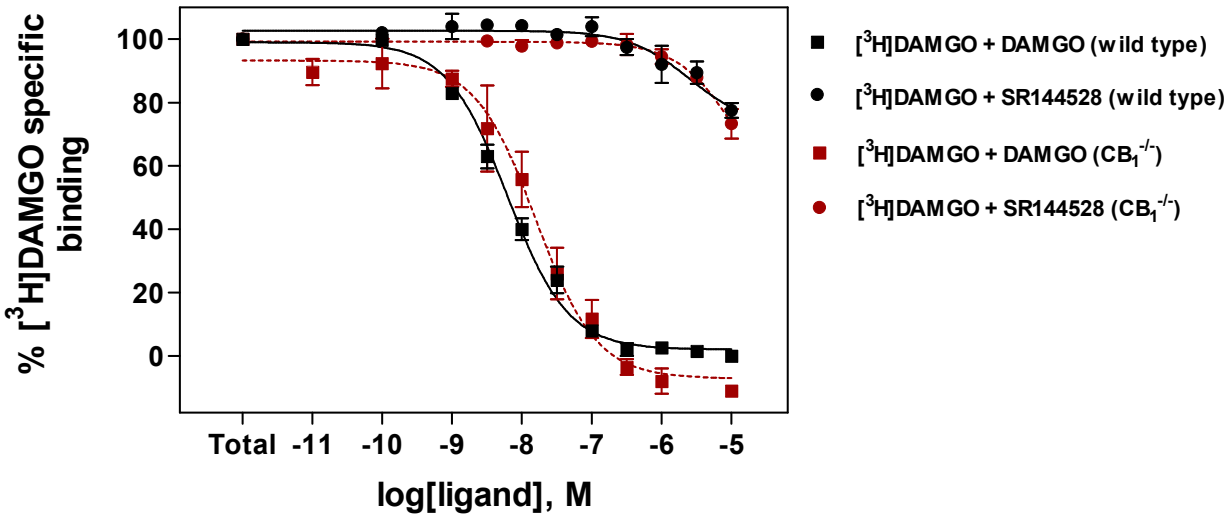

Figure 16: [^{3}H]DAMGO binding (~1nM) in the presence of increasing concentrations of unlabeled DAMGO and SR144528 in wild-type and CB_1 knockout mice brainstem.

Data demonstrate that [^{3}H]DAMGO binding was basically not affected by SR144528 when compared to the affinity of unlabelled DAMGO ($IC_{50} \approx 10$ nM, Figure 16). These results indicate again that under the conditions used, the inhibition caused by SR144528 on MOR signaling is mediated *via* CB_2 cannabinoid receptors.

V. DISCUSSION

Research on the molecular and neurobiological basis of the physiological and neurobehavioral effects of marijuana and cannabinoids lagged behind those of other natural addictive products like opiods because of the lack of specific molecular tools and technology. Later significant and rapid progress was made with the cloning of genes encoding cannabinoid receptors [142, 157], the generation of cannabinoid receptor knockout mice [30, 122, 240], isolation of endocannabinoids [55, 87, 217, 223, 187, 149] and identifications of transporters and enzymes for the biosynthesis and degradation of these endogenous substances [168].

Besides the recognized similarities between the effects of cannabinoids and opioids, progress towards understanding the molecular basis of these similarities and the degree to which the endogenous opioid and endocannabinoid systems interact still has far to go. While CB_1 type cannabinoid receptor attracted interest since its discovery, CB_2 receptor has remained almost overlooked. In the past few years, the established belief that the CNS does not contain CB_2 has been significantly changed. Despite numerous previous studies for over a decade since the cloning of CB_2 cannabinoid receptors have failed to detect CB_2 receptor mRNA or CB_2 ligand binding in the brain [62, 99, 157, 240], more recent data have suggested that CB_2 receptors are present in the normal CNS [81, 163, 224]. Moreover, there is little information about the neuronal function of the CB_2 receptors and its interaction with other systems. In keratinocytes, CB_2 receptor activation stimulates the release of β-endorphin which then acts at opioid receptors to inhibit antinociception suggesting that β-endorphin release is necessary for CB_2 receptor-mediated antinociception [102]. Onaivi and coworkers reported the modification of CB_2 gene expression in different brain regions of animals treated with abused substances like cocaine, morphine and alcohol [170].

In our work we have analyzed the changes of different opioid receptors gene expression levels and activation after acute noladin ether treatment in different parts of wild-type and CB_1 receptor deficient mice brain [122]. Regarding that forebrain and brainstem structures play an important role in reward and pain control as well as counting with the regulated limit of the thesis we have discuss here our results from these brain areas focusing on the possible changes in the μ-opioid receptor system.

In the forebrain study we studied whether the putative endocannabinoid NE causes changes in the expression of opioid receptor mRNAs and/or in the functional coupling of the agonist-activated opioid receptors. Gene expression patterns of MOR was monitored by quantitative real-time RT-PCR, while the primary step in the GPCR signal transduction process of the forebrain μ-opioid receptors [134] was examined in [^{35}S]GTPγS binding assays. It was demonstrated that *in vivo* administration of NE causes attenuations both on μ-opioid receptor gene expression and signaling.

It is known that synthetic as well as endogenous cannabinoid ligands might primarily act on cannabinoid receptors but other cellular targets can also be involved in their widespread effects [for review see ref. 53]. While Hanus et al. [87] showed that NE exhibits high nanomolar affinity for the CB_1 receptors, in the meantime other studies point out that NE acts as a full agonist at CB_2 receptors [208]. This raises the question of the physiological relevance of the action of NE at CB_1 receptors.

Our results clearly show that treating wild-type mice acutely with NE induces a significant decrease in forebrain MOR mRNA expression. Similarly, the same dose of NE caused a substantial reduction in the potency of MOR-selective peptide agonist DAMGO in activating G-proteins in wild-type animals. Surprisingly, an acute single dose of NE given to CB_1 receptor knockout mice led to a similar decrease in MOR mRNA expression and potency in DAMGO-stimulated [^{35}S]GTPγS binding. Pretreatment with the CB_2 receptor selective antagonist SR144528 partially, but significantly reversed the decreasing effect of NE on the level of forebrain MOR mRNA. The same SR144528 treatment also restored the potency-decreasing effect of NE in DAMGO-stimulated [^{35}S]GTPγS binding assays in wild-type and, more importantly, in forebrain membranes of CB_1 receptor knockout mice suggesting the role of CB_2 receptors in this interaction.

The second crucial point concerns the direct interactions among opioids and cannabinoids at the level of their primary and secondary target proteins, i.e., receptors and regulatory G-proteins. Noladin ether, directly added to the reaction mixtures of [^{35}S]GTPγS binding assays, significantly decreased the efficacy of the opioid agonist peptide DAMGO in stimulating G-proteins in forebrain membranes of wild-type mice. The combined *in vitro* treatments with the CB_2 cannabinoid antagonist SR144528 (1μM) and NE (1μM) resulted in an enhancement of the efficacy when compared to the NE administration alone. In other words, the CB_2 receptor antagonist

was able to reverse, at least in part, the inhibitory effect of NE. Similar results were expectedly observed in experiments performed with membrane preparations from CB_1 receptor knockout mice. Thus, CB_1 receptor function can again be excluded to play any role in the *in vitro* action of NE on opioid stimulated G-protein activation.

In the next set of experiments the *in vitro* affinity of NE at MOR was determined. In equilibrium competition assays with [^{3}H]DAMGO NE exhibited fairly week affinity in both $CB_1^{+/+}$ and $CB_1^{-/-}$ forebrain membrane fractions indicating negligible direct interaction with the MOR protein. These data suggest also that attenuation on MOR signaling by NE can not be a direct effect with the receptor, thus it might be either mediated *via* CB_2 cannabinoid receptors, or by an unknown mechanism. Further, additional experiment will be done on CB_2 knockout mice [30]. These studies will give more direct information about the possible involvements of the CB_2 receptors in the observed effects.

Very recently, in forebrain tissues rich in μ-opioid receptors [7] the presence of CB_2 cannabinoid receptors have been shown as well [83]. Our results extend the previous evidence that CB_2 receptors are playing an important role in putative neuronal function and, unexpectedly, NE attenuates μ-opioid receptor function *via* these receptors. The mechanisms involved in these interactions are not clearly understood but possible explanations exist. Recent atomic force microscopy studies show that GPCRs are present in dimeric arrays in native membranes [71]. Opioid receptors are also able to heterodimerize with distantly related family members (CB_1 cannabinoid receptors); these interactions result in attenuation of signaling by both receptors [196]. Based on these facts, CB_2 cannabinoid receptor may serve as an allosteric modulator of the μ-opioid receptor. Coactivation of both receptors can lead to destabilization of receptor-G-protein interactions, resulting in decreased efficacy or potency in G-protein activation and ultimately signal attenuation.

There are data about the involvement of CB_2 cannabinoid receptors in alcohol preference in mice and alcoholism in humans [104], which supports the functional presence of neuronal CB_2 receptors in the mammalian CNS. These studies are consistent with our findings regarding to the presence of functional CB_2 receptors in mouse forebrain [175].

As neuronal CB_2 receptors are present in higher density in the brainstem tissue [224], we investigate the involvement of CB_2 receptors on MORs activation in this brain region as well. We examined whether NE causes changes in the expression of

MOR mRNAs and/or in the functional coupling of DAMGO-activated MOR in brainstem membrane fractions. No significant changes were observed in MORs mRNA expression and signaling after acute NE treatment. We found similar results after *in vitro* administration of NE as well. Interestingly, after pretreatment with CB_2 receptor selective antagonist SR144528, followed by the treatment with NE, the level of brainstem MOR mRNA and the efficacy of DAMGO stimulated [^{35}S]GTPγS binding significantly decreased in $CB_1^{+/+}$ and in $CB_1^{-/-}$ mice. These results are similar to that found *in vitro*, suggesting the involvement of the CB_2 receptor antagonist, SR144528 on MOR signaling. Our observation in distinct parts of the brain suggests that mechanisms involved in NE induced effects on μ-opioid signaling are depending on the investigated area.

To explore the impact of SR144528 on MORs in brainstem, additional animal treatments were done with SR144528 alone. Our results clearly show that acute treating $CB_1^{+/+}$ and $CB_1^{-/-}$ mice with SR144528 induce a significant decrease in brainstem MOR mRNA expression. Similarly, the same dose of SR144528 caused a substantial reduction in the efficacy of MOR selective agonist DAMGO in activating G-proteins in $CB_1^{+/+}$ and $CB_1^{-/-}$ animals, demonstrating that CB_1 receptors are not required for these actions. More direct evidence for the involvement of SR144528 on the observed actions was obtained in CB_2 receptor deficient mice [30]. SR144528, directly added to the reaction mixtures of [^{35}S]GTPγS binding assays, significantly decreased the efficacy of DAMGO in stimulating G-proteins in brainstem membranes of $CB_2^{+/+}$ mice. Importantly, after *in vitro* administration of SR144528 no changes were found in CB_2 knockouts when compared to control. Thus, CB_2 receptor function can be strongly suggested to play a role in the actions of SR144528 on opioid stimulated G-protein activation in brainstem. In equilibrium competition assays with [^{3}H]DAMGO, the cannabinoid antagonist SR144528 exhibited week affinity in both $CB_1^{+/+}$ and $CB_1^{-/-}$ brainstem indicating that there is no direct interaction with the MOR protein. These data shows that decrease on MOR signaling by SR144528 is not a direct effect with the MOR, thus might be mediated *via* CB_2 cannabinoid receptors.

These findings are of importance opening new areas of research in understanding the role of the CB_2 receptor-mediated effects in CNS. Reports by our group [175, 176] and others [83, 8, 15, 104, 170, 224] have identified the functional

presence of CB_2 receptors in CNS contrary to the view that the CB_2 receptors were restricted to the immune cells of the peripheral tissues. In those brain areas, where both MORs and CB_2 receptors are colocalized we can not exclude the heterodimerization of these two receptors. In recent years, cannabinoids have emerged as attractive alternatives or supplements to therapy with opioids for chronic pain states [172, 227]. There is evidence about the involvement of endocannabinoids in the peripheral antinociception induced by activation of μ-opioid receptors [70] and it has been shown that the peripheral endocannabinoid system is an important component of endogenous pain control mechanisms [1].

Although the role of CB_2 on MOR system in brainstem needs to be elucidated, available evidence reported here may yield novel approach for the effects of CB_2 receptors. The nature of the observed effects in forebrain and brainstem needs to be clarified by using a wide range of other techniques, but our results demonstrated functional interactions between brain CB_2 cannabinoid and μ-opioid receptors.

Expanding our understanding of the involvement of CB_2 on MOR function should greatly help to further knowledge of the general mechanisms that underlie the control of the nociceptive responses in the brainstem or in higher organizations of the brain.

VI. SUMMARY

► Intraperitoneal administration of noladin ether to CB_1 wild-type and CB_1 knockout mice decreased MOR gene expression level and MOR G-protein activation in the forebrain. This attenuation can be reversed by pretreatment of mice with the CB_2 cannabinoid antagonist SR144528.

► *In vitro* studies with NE in forebrain membranes of wild-type and CB_1 knockout mice provided similar decrease in the efficacy of DAMGO in [^{35}S]GTPγS binding assays. The combined *in vitro* treatments with SR144528 and NE resulted in an enhancement of the efficacy when compared to the NE administration alone, suggesting a CB_2 receptor mediated effect.

► In mouse brainstem, no significant changes were observed in MORs mRNA expression and signaling after *in vivo* and *in vitro* NE treatments. It seems that the mechanisms involved in NE induced effects on MOR signaling are different depending on the investigated area.

► *In vivo* treatment with the CB_2 antagonist SR144528 caused a signifiacnt decrease in MOR mRNA level and MOR G-protein activation in CB_1 wild-type and CB_1 knockout mice brainstem.

► Ligand stimulated [^{35}S]GTPγS binding assays on CB_2 knockout mice showed that the SR144528 have a significant inhibitory effect on the efficacy of DAMGO in wild-type but not in CB_2 knockout mice brainstem.

► In competition binding studies we have confirmed that neither noladin ether or SR144528 binds directly to the MOR protein in CB_1 wild-type and CB_1 knockout mice forebrain and brainstem.

VII. CONCLUDING REMARKS

The pharmacology of cannabinoid-mediated actions is far from straightforward, indicated by reports of species and tissue differences in actions of ligands [56]. Many of the ligands that are thought to be either CB_1 or CB_2 receptor specific have varying degrees of activity at other receptors, and this must be considered in interpreting experimental responses. Clearly, more research is required to illuminate further the nature of cannabinoid receptors agonist and antagonist action in various conditions. The development of conditional CB_2 receptor knockout mice [31] and of ligands with greater selectivity will serve to further clarify the role of the CB_2 receptor in many physiological and pathological processes in which it appears to be implicated. It is clear that after some years where cannabinoid signaling seemed relatively straightforward, a new phase of research in this field lies ahead.

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