Effects of an F238L point mutation on intracellular trafficking and signaling of the cannabinoid type 1 receptor

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1. INTRODUCTION

1.1 The endocannabinoid system (ECS)

The highly plastic nature of the central nervous system enables animals to quickly sense changes in the environment and react with an adaptation of their behavior. The simplified, canonical way of information flow in the nervous system is considered to be anterograde transmission at chemical synapses of neurons. In anterograde transmission, neurotransmitters are released from presynaptic terminals and subsequently act on postsynaptic cells via various specific receptors. Despite the focus on anterograde signaling, some retrograde signaling systems were found as well, including gases (i.e. nitric oxide), peptides (i.e. dynorphine) and growth factors (i.e. BDNF) (Katona 2012). However, these retrograde messengers only act on specific synapses or cell types (Katona 2012). The first step to discover a more widespread and universal retrograde messenger system which can serve as a feedback mechanism for synaptic transmission, was done almost exactly fifty years ago, when Gaoni and Mechoulam elucidated the structure of the main psychoactive constituent of the plant cannabis sativa, which is Δ^9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964). Around 20 years later, it was suggested that THC could act through a receptor, which was finally cloned in 1990 and termed cannabinoid receptor type 1 (CB1) (Howlett 1985, Matsuda 1990). Another cannabinoid receptor was discovered three years later, which was termed cannabinoid receptor type 2 (CB2) (Mechoulam 2012). The CB1 receptor is one of the most abundant G protein coupled receptors (GPCRs) in the central nervous system (CNS), but it is also expressed in the peripheral nervous system (PNS) and other peripheral tissues. In contrast, the CB2 receptor is predominantly expressed in immune cells (Malfitano 2014). The finding of cannabinoid receptors implicated the existence of endogenous substances binding to these receptors, which were named endocannabinoids (eCB). Indeed, in 1992 and 1995, two lipophilic substances were isolated, which are arachidonoylethanolamide, also called anandamide (AEA), and 2-arachidonoyl glycerol (2-AG) (Mechoulam 2012). Both eCBs are derivatives of the membrane lipid component arachidonic acid (Katona 2012). AEA is a partial agonist for the CB1 and the CB2 receptor and only present in low concentrations in the brain (pmol per gram of tissue). In contrast, 2-AG is a full agonist for the CB1 and the CB2 receptor and found in high levels in the brain (nmol per gram tissue) (Katona 2012). The concept of full and partial agonism will be explained in Section 1.2.2. Furthermore, the endocannabinoid system comprises enzymes to synthesize and degrade eCBs. AEA is synthesized by the N-acylphosphatidyl-ethanolamine-specific phospholipase D (NAPE-PLD) and degraded by the fatty acid amide hydrolase (FAAH) (Kano 2009).

The two isoforms diacylglycerol lipase α and β (DAGLα, -β) synthesize 2-AG, which is degraded by monoacylglycerol lipase (MAGL) (Kano 2009).

1.1.1 The endocannabinoid system and disease

The endocannabinoid system is present in various brain regions in the CNS, in the PNS and in peripheral organs (Pacher 2006). Accordingly, the ECS is involved in an overwhelming amount and diversity of physiological functions and related pathological conditions. For example, CB1 receptor in the cortex, hippocampus and amygdala is implicated in cognitive functions, emotional regulation and learning and memory (Mechoulam 2012). The ECS in the basal ganglia and the cerebellum is involved in the modulation of movement and motivation (Mechoulam 2012). In the sensory nervous system the ECS is an important regulator of pain perception (Piomelli 2014). In the periphery as well as in the nervous system, the ECS is furthermore known to be involved in cell metabolism, proliferation and differentiation and, predominantly through CB2, in inflammatory responses (Pisanti 2013, Malfitano 2014). Accordingly, changes in constituents of the endocannabinoid system have been related with mental disorders such as anxiety, schizophrenia and with neurological conditions such as Alzheimer´s disease, Huntington´s disease and Parkinson´s disease (Pacher 2006). This variety of implications in pathological conditions makes the CB1 receptor an attractive target for medical drugs. However, it also exacerbates the design of drugs aiming at modulating only a defined subset of targets which is necessary to reduce side effects. One example for the problem with side effects is the drug rimonabant, a CB1 receptor blocker, released by Sanofi Aventis to treat obesity. It was removed from the market after it became apparent that it induces depressive symptoms and suicidal behaviors. This example illustrates the importance to understand the mechanisms of functional specificity of the CB1 receptor to narrow down the sides and modes of drugs acting on the ECS.

Despite the function of the CB1 receptor in a variety of cell types and organs, this work mainly focuses on neuronal CB1 receptors in the central nervous system.

1.1.2 The endocannabinoid system in neurons

The ECS in neurons is involved in several forms of synaptic plasticity according to their electrophysiological induction paradigms. One of the first to be described was a form of short term plasticity at GABAergic and glutamatergic synapses called depolarization induced suppression of

inhibition (DSI) or excitation (DSE), respectively (Castillo 2012). In patch clamp experiments, stimulation of a presynaptic cell leads to neurotransmitter release which induces a measurable postsynaptic response. However, if the postsynapse is depolarized artificially through the patch pipette, a subsequent stimulation of the presynaptic cell only causes a reduced transmitter release and hence a reduced postsynaptic response. The explanation for this form of short term plasticity was found to be the depolarization induced production of eCBs by the postsynapse. eCBs can travel retrogradly to inhibit transmitter release in the presynapse by acting on the CB1 receptor. Besides their role in short-term plasticity, eCBs were shown to be involved in homosynaptic as well as heterosynaptic forms of long term depression (LTD) and spike timing dependent plasticity (STDP), a form of synaptic plasticity induced by a precise temporal correlation of presynaptic and postsynaptic activity (Heifets 2010).

eCBS are highly lipophilic substances and are hence not thought to be stored in vesicles, waiting to be released as is the case for many other transmitters. Instead, they are synthesized on demand (Hashimotodani 2013). Two mechanisms have been proposed to induce eCB release in the postsynapse, which are Ca^{2+}-driven eCB release and receptor-driven eCB release (Younts 2014). In the former, Ca^{2+} entry to micromolar levels through postsynaptic voltage gated Ca^{2+} channels or through NMDA receptors, induces 2-AG synthesis (Kano 2009). This mechanism is thought to underlie the electrophysiological phenomena DSI and DSE (Kano 2009). Additionally, the 2-AG synthesizing enzyme DAGL can be found in a complex, called perisynaptic signaling machinery (PSM), with phospholipase Cβ (PLC-β), scaffolding proteins (i.e. homer) and various $G_{q/11}$ coupled GPCRs (Katona 2012). This accounts for the second mechanism of eCB release. Activation of a $G_{q/11}$ coupled receptor stimulates PLC-β to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG serves as a substrate for DAGL to synthesize 2-AG. Interestingly, PLC-β activity can be further stimulated by Ca^{2+}. Hence, both mechanisms can act synergistically (Kano 2009).

Different presynaptic pathways have been described to induce the various forms of eCB dependent plasticity. As a mechanism for short-term depression, the transient inhibition of presynaptic voltage gated calcium channels (VGCC) by the Gβγ subunit of the G protein has been suggested (Castillo 2012). In contrast, long-term synaptic depression might be mediated by the $G\alpha_i$ subunit of the G protein which inhibits adenylyl cyclase (AC) and by that reduces cyclic adenosine monophosphate (cAMP) accumulation. This in turn reduces protein kinase A (PKA) activity, which allows target proteins of this kinase to be dephosphorylated. One target protein of PKA is Rab3A interacting molecule (RIM-1α), which is known to modulate the presynaptic transmitter release machinery and has been proposed to be involved in eCB dependent long-term depression (LTD) (Katona 2008,

Heifets 2009). It has been suggested that eCB mediated LTD could result from a reduction of the excitability of axons of presynaptic neurons by the modulation of K^+ channels (Heifets 2009). Another possible mechanism for eCB mediated LTD is a PKA-dependent reduction in the presynaptic Ca^{2+} response to an arriving action potential by long term modification of presynaptic P/Q type VGCCs (Heifets 2009). Finally, the temporal correlation between presynaptic and postsynaptic activity has been described to be important for eCB mediated spike timing dependent plasticity (Heifets 2009, Min&Nevian 2012). Interestingly, recent evidence suggests that the CB1 receptor on astrocytes could be a key player in eCB mediated spike timing dependent plasticity (Min& Nevian 2012).

Besides its function in synaptic plasticity, the ECS is known to play an important role in brain development and wiring. Cannabis consumption during pregnancy and during adolescence has been shown to have tremendous effects on brain function and psychological health in humans (Schneider 2008, Mulder 2009). This indicates an important function of the ECS during pre- and postnatal development of the nervous system. Indeed, the constituents of the ECS vary in their expression levels during development (Mulder 2009). Furthermore, CB1 receptor activity has been shown to increase neural stem cell proliferation and the migration of newly born neurons (Mulder 2009, Zhou 2014). Finally, during cortical synaptogenesis CB1 receptor and DAGL are implicated in the spatial segregation of glutamatergic and GABAergic synapses (Harkany 2008).

1.2 G protein coupled receptors

Communication between and signal transduction within cells is a crucial prerequisite to orchestrating cellular functions in multicellular organisms. Important players in intercellular communication are membrane proteins which detect extracellular cues and initiate an intracellular response. More than 800 genes of the human genome encode for a special family of membrane proteins, which is the GPCR family (Venkatakrishnan 2013). GPCRs can be activated by a huge variety of physiological mediators, ranging from peptides, lipids, amino acids and photons. Interestingly, a large fraction of GPCRs constitutes olfactory receptors. Importantly, GPCRs are of special interest, as about 45% of current medical drugs act on this class of receptors (Drews 2000). Understanding the structural correlates of GPCR signaling and trafficking is an important goal to improve rational drug design for a large range of applications.

1.2.1 G protein coupled receptor signaling

1.2.1.1 Canonical G protein signaling

Upon activation, the GPCR associates with a heterotrimeric G protein, consisting of a guanosine diphosphate (GDP) binding Gα, a Gβ and a Gγ subunit. There is a variety of Gα subunits classified according to their effector target.

After association of the G protein with the GPCR, GDP is released and exchanged by guanosine-5`-triphosphate (GTP), which is the rate limiting step in GPCR activation (Theresa 2003). The GTP bound Gα and the Gβγ proteins detach from the receptor and can act as effectors on several downstream targets. The bound GTP is hydrolyzed to GDP by the Gα subunits intrinsic GTPase domain. The GDP bound Gα then reassembles with Gβγ and is ready to bind to another GPCR (Harrison 2003) (Fig 1.1). The hydrolysis of GTP can be accelerated by regulator of G protein signaling proteins (RGS). The Gβγ subunit initiates the recruitment of G protein coupled receptor kinases (GRK) to the membrane, which phosphorylate the C-terminus of the GPCR (Kenakin 2011). This leads to the recruitment of β-arrestin to the receptor and hence, a functional uncoupling of G proteins, which desensitizes the receptor and initiates its internalization (Kenakin 2011).

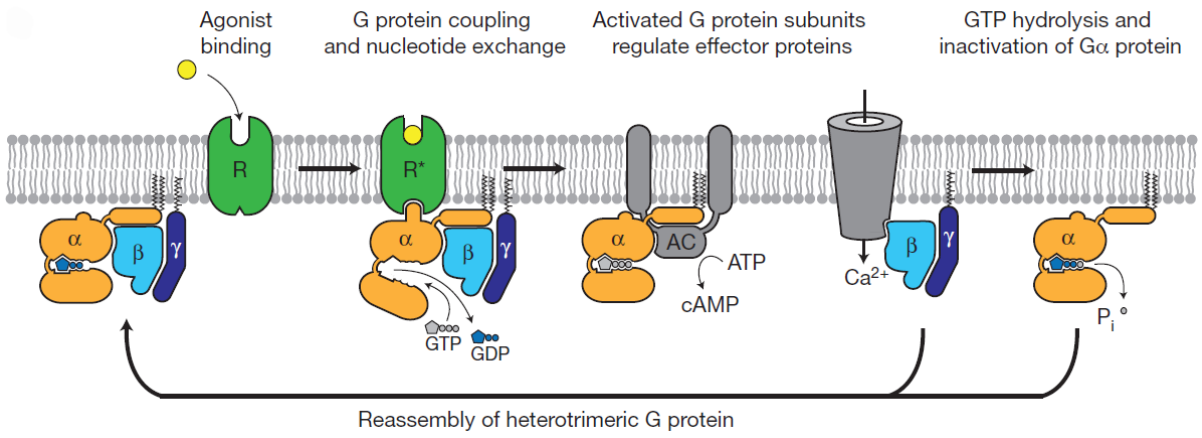


Fig. 1.1 The cycle of G protein signaling at GPCRs

The agonist stabilized active conformation of the GPCR binds the heterotrimeric G protein complex, which leads to an exchange of the Gα bound GDP for GTP. Subsequently, the Gα and Gβγ subunits dissociate from the receptor and can then act on downstream effectors, such as AC or ion channels. The Gα subunit hydrolyses GTP to GDP which leads to the re-association of the heterotrimeric G protein complex, rendering it ready to bind a new receptor. (The figure was taken from Rassmussen 2011)

1.2.1.2 β-arrestin signaling

Additionally to fast and transient signaling via heterotrimeric G proteins, a much slower and prolonged signaling via β-arrestins has been described for some GPCRs (Lefkowitz 2005). Usually, mitogen-activated protein kinases (MAPK) such as ERK1/2, which are activated by the G protein dependent pathway, can enter the nucleus and exert their effect on transcription factors. However, when associated in a signaling complex with GPCRs and β-arrestins, the kinases can no longer enter the nucleus and rather phosphorylate cytosolic targets. It has been concluded that this could constitute one mechanism for subcellular signaling specificity (Lefkowitz 2005). An interesting example for the therapeutic relevance of β-arrestin signaling is the finding that a whole range of antipsychotics, which all act on the dopamine D2 receptor but with various efficacies, share the common property to antagonize the interaction of the D2 receptor with β-arrestin2 (Masri 2004).

1.2.2 Functional selectivity of GPCR signaling and the conformational ensemble model

The efficacy of a molecule at a given receptor has been initially described as the ability of the molecule to induce a response in a given biological system (Stephenson 1956). It depends on the affinity of this ligand to a receptor and the ability of the molecule to activate the receptor and induce a signaling response (Kenakin 2011). In the two-state model of GPCR activity, the receptor is thought to exist in two states, an active or an inactive conformation, and that a pool of receptors exists as a steady state of these two conformations (Seifert 2002). Depending on the proportion of receptors in the active state, receptors are also considered to be constitutively active (Seifert 2002). Based on this model, ligands have been classified as full or partial agonists, neutral antagonists and full or partial inverse agonists (Kenakin 2011) (Fig. 1.2). Agonists are thought to bind to and stabilize the active state of the receptor and hence shift the equilibrium towards active receptors. In the same way, inverse agonists are thought to bind to and stabilize the inactive conformation. “Full” or “partial” refers to the potency of the ligand to stabilize the receptor in the respective conformation and to induce a cellular response. Neutral antagonists can bind to the receptor but do not stabilize any of the above mentioned conformations. However, they can compete with other ligands for binding sites and hence serve as antagonists. Efficacy of a ligand at a given receptor has been considered to be linear for the several biological functions that served as experimental readouts, such as G protein binding, cAMP accumulation, receptor internalization etc. However, ligand efficacy at a given receptor can be different for the many different biological functions it serves (Kenakin 2011). The phenomenon of variating efficacies of ligands to modulate different signaling effectors of a given

GPCR has been named “stimulus trafficking”, “functional selectivity”, “collateral efficacy” or “biased signaling”. In further consequence, efficacies have been attributed not to be linear but “pluridimensional” (Kenakin 2011, Reiter 2012).

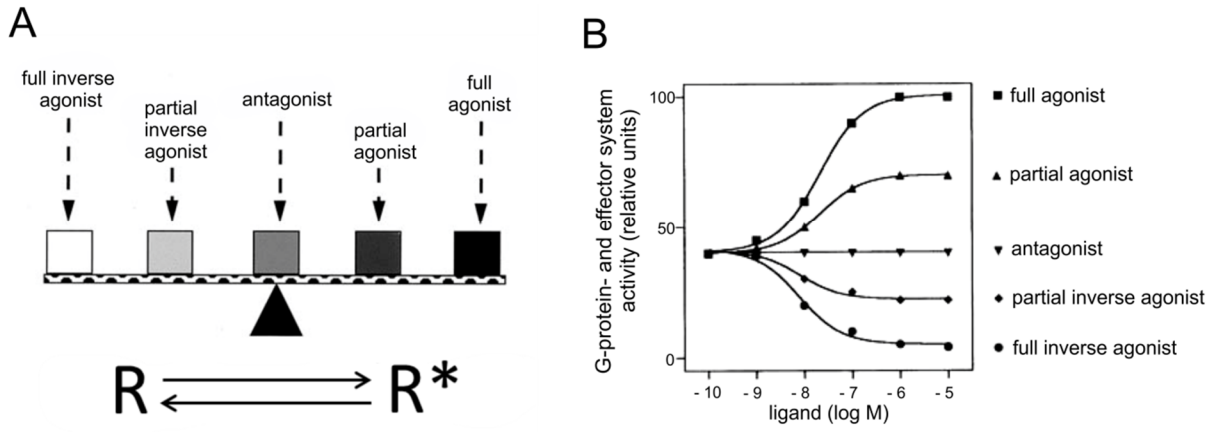


Fig. 1.2 Classification of GPCR ligands

GPCR ligands are traditionally classified according to their potency to activate GPCRs. Agonists stabilize the active conformation (R*), whereas inverse agonists stabilize the inactive conformation (R) of the receptor. Depending on their maximal activation or inactivation of a GPCR, ligands can be classified as full agonist or antagonist (high maximal activation or inactivation) or partial agonist (low maximal activation or inactivation), respectively. Neutral antagonists do not preferentially stabilize the active or inactive conformation of a receptor, but they can compete with agonists for binding sites and can hence serve as antagonists. (The figure was modified from Seifert 2002)

As described in a review by Vaidehi et al., an emerging view on GPCR structures states their existence in “conformational ensembles” rather than just in the two conformations of active and inactive receptors. This view is supported by experimental studies as well as computational molecular dynamics simulation studies, indicating that there are many different active or inactive receptor conformations. Vaidehi et al. described that for every protein a potential energy landscape describes the thermodynamically favorable (minima) or unfavorable (maxima) conformations. GPCRs are conformational flexible proteins, “rolling” between different minima, which facilitates the adaption of several conformational states. However, the minimum for active conformations was suggested to be rugged and to consist of many different minima called “substates” (Vaidehi 2010). These substates could account for signaling diversity of a GPCR and functional selectivity of different ligands (Vaidehi 2010, Kahsai 2011).

1.3 CB1 receptor signaling

1.3.1 Signal transduction pathways and targets modulated by the CB1 receptor

The CB1 receptor is one of the most abundant GPCRs in the brain and exerts its cellular function through different signal transduction pathways. The first pathway described is $G_{i/o}$ mediated inhibition of AC activity. This leads to a reduction in cAMP accumulation and reduced PKA activity. Although $G_{i/o}$ proteins are the best described G proteins to couple to the CB1 receptor, G_s protein coupling has been observed as well (Glass 1997, Jarrahian 2004). Furthermore, CB1 receptor activation has been reported to lead to intracellular Ca^{2+} signals via G_q proteins (Lauckner 2005). This is in particular interesting, because CB1 receptor mediated increase in astrocytic Ca^{2+} has been reported to lead to synaptic strengthening of synapses relatively distant from the cannabinoid releasing synapse (Navarrete 2010). The PKA activity pathway is modulated by the α subunit of the heterotrimeric G protein and can affect gene expression via ERK1/2 phosphorylation and the phosphorylation of a cAMP response element binding protein (CREB), which can act as a transcription factor to enhance the transcription of several proteins (Bosier 2010). In parallel, the βγ subunits of the heterotrimeric G protein modulate the activity of several MAPK pathways, including ERK1/2, JNK and p38MAPK (Bosier 2010). Furthermore, there is evidence that the CB1 receptor signals via PI3K pathways which, via PLC, modulates intracellular Ca^{2+} release and the phosphorylation of AKT (Ozaita 2007) (Fig. 1.3). Very important for the modulation of the synaptic function is the property of the CB1 receptor to modulate ion channels (also see section 1.1.2)

1.3.2 Functional selectivity of CB1 receptor signaling

CB1 receptor signaling has been shown to depend on the cellular environment as well as the nature of the activating ligand. As mentioned above, the CB1 receptor is expressed in a plethora of brain regions, including the cortex, hippocampus, amygdala, basal ganglia and the cerebellum to name a few. However, signaling of CB1 receptor as well as desensitization and down regulation can differ between brain regions (McKinney 2008). For example, it has been reported that the amplification factor for G protein activation induced by the synthetic CB1 receptor agonist WIN55212-2 differs between brain regions in a way that higher receptor occupancy correlates with a lower amplification factor (Breivogel 1997). In agreement with these findings, Steindel et al. recently demonstrated that G protein coupling efficiency further varies between CB1 receptors expressed in different cell types

(Steindel 2013). However, the factors which cause these differences in CB1 receptor signaling remain to be elucidated. Steindel et al. suggest that probably different concentrations of ligands activate the CB1 receptor on different neuronal populations. Furthermore, it has been suggested that different ligand concentrations could differentially activate $G_{i/o}$ or G_s pathways (Bosier 2010). Interestingly in this context, a biphasic effect of cannabinoids has also been observed on the behavioral level (Rey 2012).

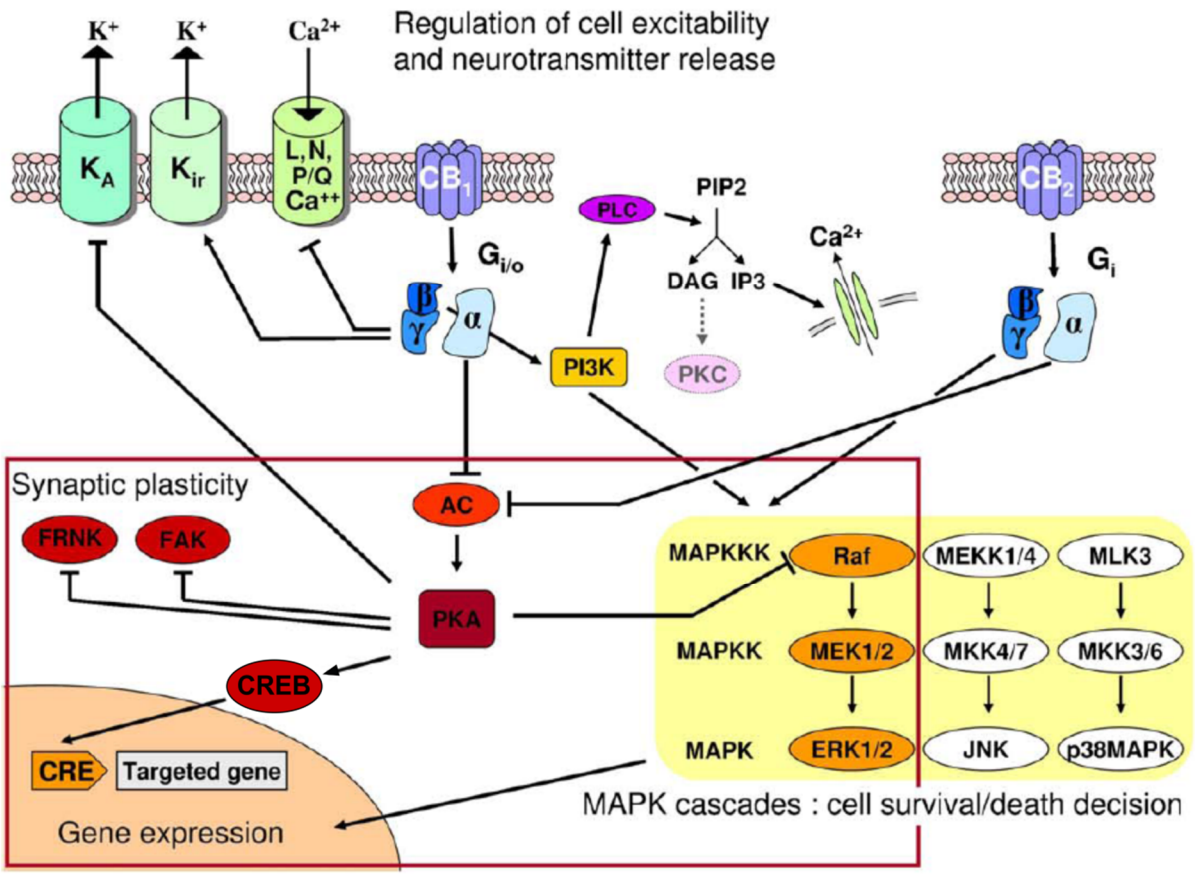


Fig. 1.3 CB1 receptor signaling pathways

Upon activation the CB1 receptor can activate $G_{i/o}$, G_s or G_q proteins. The α subunit can activate (G_s) or inhibit ($G_{i/o}$) the AC, which affects PKA activity and ERK1/2 phosphorylation. The βγ subunits can modulate ion channels. Furthermore, βγ subunits can modulate intracellular Ca^{2+} release and phosphorylation of JNK or p38MAPK. (The figure was modified from Bosier 2010)

Another factor that could account for differences in signaling pathway specificity is the protein expression profile of different cell types. Intuitively, one would assume that the expression of G protein subtypes and their availability within the cell easily affects the signaling pathway chosen by an activated receptor. Furthermore, the expression of other GPCRs can affect G protein selectivity of the CB1 receptor by sequestration and thus competition for a common pool of G proteins (Bosier

2010). Indeed, the CB1 receptor has been shown to share a common pool of G proteins with adrenergic, somatostatin, insulin, IGF-1, and dopamine receptors, but not with purinergic and GABAergic receptors (Jarrahian 2004, Bosier 2010). GPCR expression profiles in a given cell can further affect CB1 receptor signaling, as it has been demonstrated that CB1 receptors can build heteromers with a variety of other GPCRs, including the μ-opioid receptor, the δ-opioid receptor, the orexin 1 receptor, the adenosine2A receptor and the dopamine D2 receptor (Kearn 2005, Rios 2006, Ferre 2010, Ward 2011, Rozenfeld 2012). Aspects of CB1 receptor heteromerization with focus on CB1-D2 receptor interactions will be described in more detail in Section 1.6.

Another protein which interacts with the CB1 receptor and modulates its signaling and probably also its desensitization and internalization is the cannabinoid receptor interacting protein 1a (CRIP1a) (Niehaus 2007, Guggenhuber 2014). Niehaus et al. could show that co-expression of the CB1 receptor and CRIP1a in superior cervical ganglion neurons suppresses CB1 receptor mediated inhibition of VGCC. This might be due to a regulatory effect of CRIP1a on CB1 receptor desensitization and internalization, which will be discussed further in section 1.5.1. The scaffolding protein caveolin-1 has also been reported to interact with the CB1 receptor (Bari 2008), and an effect of caveolin-1 interaction on signaling and trafficking has been shown for other GPCRs (Bhatnagar 2004, Hong 2009). Furthermore, caveolin-1 is enriched in lipid raft domains, which affect eCB signaling in many ways, as it will be described in Section 1.4.1. Finally, posttranslational modifications such as phosphorylation and nitrosylation have also been found to affect CB1 receptor signaling (Bosier 2010).

Additionally to the cellular setting in which the CB1 receptor is embedded, another important factor for the selectivity of the CB1 receptor for different signaling pathways is the nature of the ligand and the presence of allosteric modulators. Various ligands have been found to exert different functions on the CB1 receptor depending on the step within the signaling cascade that is used as readout (Bosier 2010). Interestingly, different cannabinoids have been demonstrated to show different efficacies in inducing typical cannabinoid mediated behavior such as antinociception, hypoactivity, hypothermia and catalepsy (Bosier 2010). For the two orthosteric agonists CP55940 and WIN55212-2, different binding sites within the receptor have been proposed (McAllister 2003). Furthermore, the allosteric modulator ORG27569 differentially affects the binding of CP55940 and WIN55212-2, being a much stronger allosteric modulator of CP55940 as compared with WIN55212-2 (Baillie 2013). This indicates that probably different receptor species exist that bind to and are activated by the two agonists. Another interesting picture emerging from experiments using the allosteric modulator ORG27569 is the biased signaling of CB1 receptors via G proteins and β-arrestin. It has been demonstrated that ORG27569 enhances the maximal binding sites of CP55940, indicating an increase

in active receptor species. However, ORG27569 significantly reduces CP55940 induced G protein binding (Ahn 2012, Baillie 2013). Furthermore, the phosphorylation of ERK1/2 was increased upon co-treatment of the CB1 receptor with ORG27569 and CP55940 (Ahn 2012, Baillie 2013). This indicates an allosteric modulator induced shift from G protein to β-arrestin signaling. ORG27569 treatment alone, and together with CP55940, strongly increased internalization of the CB1 receptor (Ahn 2012). Using an siRNA mediated knockdown approach, Ahn et al. could demonstrate that the ORG27569 mediated increase in ERK1/2 phosphorylation and internalization is mediated by β-arrestin1 and -2, respectively (Ahn 2013). However, it is noteworthy that Baillie et al. did not observe an increased β-arrestin recruitment upon ORG27569 treatment (Baillie 2013).

Negative as well as positive endogenous allosteric modulators of the CB1 receptor have been described recently. One of which are so called "pepcans" (**pep**tide endo**can**nabinoids), which are peptides, derived from α-hemoglobin, with a length of approximately 10 to 20 amino acids (Bauer 2012). Pepcan-12 (12 amino acids long) has been demonstrated to be a negative allosteric modulator of CB1 receptor orthosteric ligand binding and orthosteric agonist induced cAMP modulation, [^{35}S]GTPγS binding and CB1 receptor endocytosis (Bauer 2012). Another endogenous negative allosteric modulator of the CB1 receptor is pregnenolone (Vallée 2014). Pregnenolone is a common precursor of steroids and neurosteroids and hence known to be present in the brain. Vallée et al. could demonstrate that activation of the CB1 receptor by THC stimulated pregnenolone synthesis in the brain. Furthermore, they showed that pregnenolone in turn acts as negative modulator at the CB1 receptor, thus serving as a feedback mechanism for CB1 receptor activity (Vallée 2014). An endogenous positive allosteric modulator for the CB1 receptor, LipoxinA4, has been described by Pamplona et al. (Pamplona 2013). The lipid LipoxinA4 is a known regulator of inflammation in the periphery but it is also present in the brain where it modulates slow wave sleep, neuronal signaling and plasticity (Pamplona 2013). On the molecular level, lipoxinA4 enhances AEA signaling at the CB1 receptor by increasing the affinity of the CB1 receptor to AEA (Pamplona 2013).

Hence, allosteric modulation of the CB1 receptor is a physiological process and a promising target for pharmacological treatment with regards to the reduction of side effects.

1.3.3 Constitutive activity of the CB1 receptor

A constitutive activity has been reported for several GPCRs including the CB1 receptor (Seifert 2002). In the simplified two-state model, a GPCR can switch between active and inactive conformations. Hence, a pool of GPCRs exists in a steady state of active and inactive receptors. Agonists bind to and

stabilize active forms, whereas inverse agonists bind to and stabilize inactive forms of GPCRs, leading to a shift of the equilibrium towards active or inactive receptors, respectively. This means that even in the absence of an agonist a certain proportion of the receptor is in the active state, which results in the observation of a constitutive activity (Seifert 2002). In HEK293 cells and other heterologous expression systems, expression of the CB1 receptor has been shown to result in increased G protein binding compared with cells which do not express the CB1 receptor (Seifert 2002, Ahn 2013). Furthermore, the inverse agonist SR141716 is able to reduce G protein binding in HEK293 cells, which express the CB1 receptor and thereby increasing Ca^{2+} currents in neurons (Nie 2001, Scott 2013). However, in these studies, it has not been excluded that the CB1 receptor might be activated in a cell autonomous manner by 2-AG, which is produced within the same cell. This can be the case if the cells that express the CB1 receptor also express DAGL. Indeed, Turu et al. could show that constitutive activity as well as constitutive endocytosis of the CB1 receptor can be reduced in neurons and heterologous expression systems by treatment of the cells with a DAGL inhibitor (Turu 2007). Additionally, constitutive activity of the CB1 receptor observed in brain tissue might rather be a tonic activity due to tonic eCB production/release by the postsynapse (Katona 2012). In conclusion, it is still under debate whether CB1 receptor constitutive activity is really constitutive, meaning activity in the absence of any ligand, or rather a tonic activity due to cell autonomous or tonic eCB release. Hence, in this work the terms “basal activity” or “basal endocytosis” will be used to describe effects observed without the experimental addition of any agonist.

1.3.4 Signaling of intracellular CB1 receptor

In neurons, it has been suggested that ligand induced endocytosis and subsequent signaling of GPCRs from endosomal compartments is one way to transfer signals across large distances to ensure prolonged signaling (Cosker 2014). Coutts et al. found evidence that CB1 receptors that were endocytosed in the presynaptic site might translocate to the soma (Coutts 2011). In several cell types, a substantial proportion of intracellular CB1 receptor has been observed, which has been suggested not only to constitute a passive store of receptors waiting to be recruited to the membrane but to signal from intracellular compartments (Rozenfeld 2011). Rozenfeld et al. suggested that CB1 receptors can signal from endosomes and even from lysosomes (Rozenfeld 2008). Another interesting function of intracellular CB1 located on mitochondria is the modulation of cellular respiration (Bénard 2012, Morozov 2013, Hebert-Chatelain 2014).

1.3.5 Structural aspects of GPCR and CB1 receptor activity

GPCRs consist of seven transmembrane helices (TMH1-7), which are connected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3). The C-terminus is located on the cytoplasmic side, and in many GPCRs, it contains a helical segment named helix 8 (H8). The N-terminal end faces the extracellular environment. For roughly a decade, high resolution structures of some GPCRs have been resolved (Venkatakrishnan 2013). Venkatakrishnan et al. compared these structures and identified topological consensus residues and interactions within GPCRs that are associated with different activation states as well as ligand and second messenger interactions (Venkatakrishnan 2013). In combination with other spectroscopic methods, biochemical and cell biological studies on point mutations and receptor chimeras, some light was shed on the structural basis of GPCR function. The relationship of GPCR structure and function will be described in this section, based on the publication by Venkatakrishnan et al. and specified for the CB1 receptor, whenever data were available from the literature. Throughout this section, the one letter amino acid code and the Ballesteros & Weinstein numbering system for amino acids within transmembrane helices will be used (Ballesteros & Weinstein 1995). In this system amino acids in a transmembrane helix are described by two numbers (X.Y). X describes the number of the transmembrane helix, whereas Y describes the position relative to the most conserved amino acid of this transmembrane helix which itself is given the number Y=50. Hence, numbers smaller than Y=50 refer to amino acids N-terminal to the most conserved residue, and numbers larger than Y=50 refer to amino acids C-terminal to the most conserved residue. As an example, phenylalanine 238 will be termed “F4.46”. This means it is located in TMH4, and 4 residues N-terminal to the most conserved tryptophane 242 in TMH4, which is termed “W4.50”. This system simplifies the comparison of topologically equivalent amino acid residues between GPCRs with different amino acid sequences.

GPCRs can be roughly divided into three functional parts (Fig. 1.4) which are: (1) The extracellular domains, including the N-terminus and extracellular loops. These domains are involved in the modulation of ligand binding and entry into the ligand binding pocket. (2) The transmembrane domains, consisting of the seven helices. These domains constitute the ligand binding pocket and transduce information to the intracellular side where they are involved in the interaction with G proteins. (3) The intracellular domains contain the C-terminus and intracellular loops. These domains are involved in G protein interaction but also in the interaction with GRKs and β-arrestins.

Furthermore, the C-terminus and ICLs can be posttranslationally modified which can affect receptor activity and subcellular localization (Venkatakrishnan 2013).

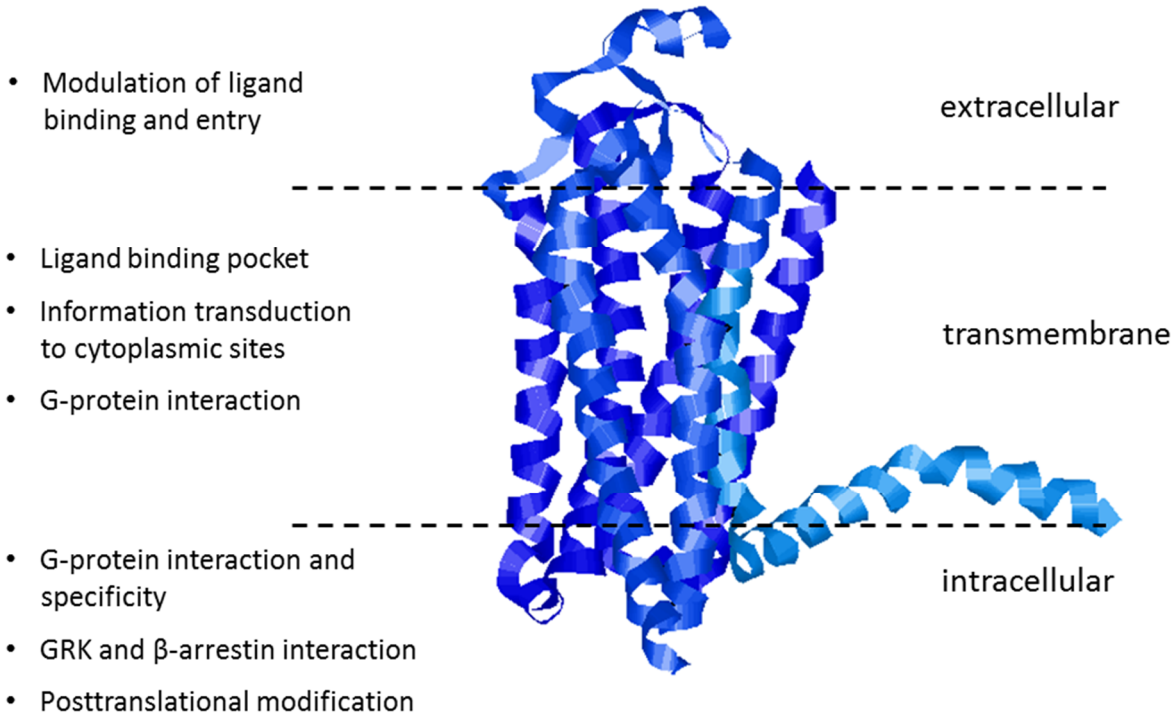


Fig. 1.4 Structure-function relationship of GPCRs

The GPCR structure can be roughly divided into three domains which are the extracellular loops and the N-terminus, the transmembrane domains and the intracellular loops with the C-terminus. The functions of these three domains in GPCR function are depicted (The GPCR structure was generated from the protein structure 2YDO (www.pdb.org) using the RasMol Version 2.6).

1.3.5.1 The extracellular domain

The extracellular domain differs between GPCRs in terms of its function in ligand binding. It can either occlude the ligand binding pocket for hydrophobic ligands (e.g. cannabinoids) or it can leave it water accessible. Common disulfide bridges were found between two cysteins within ECL2 and between ECL2 and TMH3. Although the disulfide bridge between ECL2 and TMH3 is highly conserved among some GPCRs, it is absent in the sphingosine-1-phosphate receptor (S1P1) and the CB1 receptor (Kwang 2009, Venkatakrishnan 2013). It is interesting to note that both, the S1P1 and the CB1 receptor, bind hydrophobic ligands. The X-ray structure of S1P1 shows that all three extracellular loops together with an N-terminal helix build a “hydrophobic lid” on the extracellular surface of the receptor (Hanson 2012). Furthermore, Hanson et al. suggest a lateral entry of the hydrophobic ligand

from the membrane through a gap between TMH1 and TMH7 (Hanson 2012). In the CB1 receptor, K373 in ECL3 has been reported to build a salt bridge with D2.63 in TMH2 which "pulls" ECL3 over the extracellular opening of the receptor and could serve as a lid as well (Marcu 2013). Furthermore, mutations in ECL2 of the CB1 receptor led to a reduction in ligand binding, which is in accordance with the suggested role of ECL domains for ligand binding (Kwang 2009).

1.3.5.2 Transmembrane domains

Within the transmembrane domains, Venkatakrishnan et al. describe 24 non-covalent consensus contacts between 36 topologically equivalent amino acid residues. Most of these interactions are between TMH1 and TMH2, between TMH3 and TMH4, between TMH3 and TMH5 and between TMH3, TMH6 and TMH7. As TMH1 and TMH2 seem not to undergo movements upon activation of the receptor, the authors suggested that these conserved interactions might be important for synthesis and folding of the receptor. Interestingly however, a mutation in TMH2 of the CB1 receptor resulted in abolished agonist induced endocytosis (Roche 1999).

The binding pocket, which is located within the transmembrane domains, contains six topologically equivalent residues that were shown to contact the ligand in a variety of receptors. These are residues 3.32, 3.33, 3.36, 6.48, 6.51 and 7.39. Indeed, for the CB1 receptor, the mutation of aromatic residues at position 3.36 and 6.48 to alanine results in reduced binding of the ligands SR141617 and WIN55212-2 but not CP55940, which led to the suggestion of an aromatic micro-domain being the binding pocket for the two former cannabinoids (McAllister 2003). Halve of the conserved binding site residues described above are located in TMH3. Among the TMH domains, TMH3 serves a unique function because it not only participates in ligand binding but it also builds contacts with all other TMHs except TMH1 and TMH7. The interaction of TM3 with multiple TMH domains is facilitated by its large tilt angle (~35°), which is the angle of the TMH3 helix relative to the perpendicular of the membrane plane (Venkatakrishnan 2013). Furthermore, TMH3 holds contacts with extracellular domains by disulfide bridges with ECL2, and with intracellular domains by salt bridges between the asparagine of the DRY motif in TMH3 and ICL2. Finally, upon activation of the receptor, TMH3 participates in contacting G proteins at the intracellular side. Strikingly, mutations in TMH3 led to constitutive inactivity or constitutive activity in many GPCRs (Venkatakrishnan 2013). In accordance with this, a well-established model to study the effects of CB1 receptor activity states are constitutive active or inactive receptor mutants, where threonine 201 at position 3.46 is mutated to isoleucine or alanine, respectively (Scott 2012, Ahn 2013). Computational simulation of receptor structure recently suggested that these two active or inactive forms of the CB1 receptor differ dramatically in terms of salt bridges between transmembrane helices (Scott 2012, Ahn 2013). One important salt bridge is the

ionic lock between arginine 3.50 of the DRY motif in TMH3 and Asp 6.50 of TMH6. The DRY motif and the ionic lock are conserved in many GPCRs and breakage of this ionic lock is considered to be one step in receptor activation (Stenkamp 2008). In accordance with this, the interaction is lost in the constitutive active CB1 receptor (Scott 2012, Ahn 2013). However, in the CB1 receptor, additionally to the breakage of the ionic lock, an intact D2.63-K3.28 salt bridge seems to be necessary to gain full receptor activity (Ahn 2013). Furthermore, in these CB1 receptor models the active and inactive forms strongly differ in the position of TMH5 and TMH6 (Scott 2012). This is in accordance with findings in the crystal structure of the β-adrenergic receptor in complex with a G protein, where the large outward movement of the cytoplasmic end of TMH6 and the stretching of the cytoplasmic end of TMH5 have been demonstrated to open the cytoplasmic surface and hence the G protein binding site (Rasmussen 2011).

1.3.5.3 The intracellular domain

The intracellular domains are important for interactions of the receptor with G proteins and other proteins. As mentioned above, ICL2 can build salt bridges with the DRY motif in TMH3. This is facilitated by an arginine or tyrosine residue in ICL2, the latter of which can be phosphorylated which modulates receptor activity. In the CB1 receptor, ICL2 has been described to be important for G protein binding and selectivity. In molecular dynamics simulations Shim et al. identified H219, D338, R340, L341 and T334, located in juxtamembrane parts of ICL2 and ICL3, as residues that build h-bonds or hydrophobic interactions with the Gα subunit of inhibitory G proteins (Shim 2013). Interestingly, L222 in ICL2 has been suggested to mediate the selectivity of the CB1 receptor for inhibitory or stimulatory G proteins (Chen 2010).

1.4 Lipid rafts

The canonical view of the plasma membrane is the fluid mosaic model, in which the various membrane lipids merely serve as metabolites, signaling molecules and a solvent for proteins. However about two decades ago, it was stated that the plasma membrane might not exist as an unorganized mixture of various lipids, but that it is rather compartmentalized due to the unique membrane organizing properties of cholesterol and sphingolipids (Simons 1997). These compartmentalized plasma domains where termed “lipid rafts”. Their detergent resistance was one property to identify and prepare lipid rafts and their associated proteins in subsequent publications (Simons 2009). However, the idea of lipid rafts has been controversial ever since. The critic’s strongest and most common argument is that lipid raft preparations exploiting detergent resistance

might only yield preparation artifacts rather than a real biological structure. However, to date, quite some evidence for the existence of lipid rafts comes from findings in model membranes, showing the membrane organizing and lateral segregation properties of cholesterol and sphingolipids (Maccarone 2011). Although detergent resistance is still a property used to biochemically prepare lipid rafts and to identify proteins residing in lipid rafts, other detergent free preparation protocols were established subsequently (Smart 1996, Song 1996). Furthermore, with several imaging methods, dynamic and cholesterol dependent assemblies in the plasma membrane were observed with a size in the nanometer range (10 nm - 50 nm) (Simons 2009). Furthermore, atomic force microscopy experiments detected irregularly shaped, cholesterol dependent plasma membrane compartments with a size of 100 nm - 300 nm (Cai 2012).

Proteins, which are recruited to lipid rafts, primarily include palmitoylated transmembrane proteins, glycosylphosphatidylinositol (GPI) anchored proteins, doubly acylated proteins such as Src kinases, the α subunits of heterotrimeric G proteins and GPCR effector proteins (Simons 2000, Allen 2007). A lot of these proteins are players in signaling pathways, indicating an involvement of lipid rafts in the organization of signaling complexes (Allen 2007). Interestingly, a variety of GPCRs and ionotropic receptors, which act as neurotransmitter receptors, have been described to be recruited to lipid rafts or to depend functionally on cholesterol (Allen 2007). By lateral segregation of signaling molecules and receptors, lipid rafts could contribute to the specificity of different signaling pathways, triggered by the same ligand but acting on different receptor subtypes (Barnett-Norris 2005). Additionally to regulating signaling, lipid rafts have also been suggested to constitute an endocytosis pathway for membrane lipids and proteins (Barnett-Norris 2005).

1.4.1 Lipid rafts and the ECS

Plasma membrane cholesterol has been shown to affect CB1 receptor signaling with regards to G protein binding and downstream effector activation in cell lines. Bari et al. found that cholesterol depletion in C6 glioma cells using methyl-β-cyclodextrin (MβCD) resulted in doubling of the binding efficiency of the CB1 receptor for the agonist CP55940. Furthermore, an increased AEA induced [^{35}S]GTPγS binding and AC signaling was observed (Bari 2005). In accordance with that, cholesterol enrichment had the opposite effect (Bari 2005). Further supporting data comes from experiments in striatal slices (Maccarone 2009).

These findings indicate the possibility that the CB1 receptor in lipid rafts might be less active. Probably, the lipid environment or specific protein interactions within lipid rafts affect the stability of

different receptor activation states (Barnett-Norris 2005). Indeed, the CB1 receptor is able to interact with cholesterol via a cholesterol binding site (CRAC domain) within TMH7 (Epand 2006, Oddi 2011). Mutation of the K402 residue in this CRAC domain results in reduced sensitivity of the receptor to cholesterol enrichment (Oddi 2011). Furthermore, the CB1 receptor can be palmitoylated at residue C415, a posttranslational modification known to promote lipid raft association of proteins (Oddi 2012, Maccarone 2011). Interestingly, the CB1 receptor can directly interact with caveolin-1, which possibly affects CB1 receptor ligand binding site accessibility (Bari 2008). Asimaki et al. found that the formation of an agonist induced signaling complex comprising the CB1 receptor, Src kinases and the fibroblast growth factor receptor depends on cholesterol.

The same study showed that lipid raft recruitment of the CB1 receptor and lateral segregation within the plasma membrane of neurons can be modulated by ligand binding (Asimaki 2011). Indeed, long-term treatment of the CB1 receptor with both agonist (AEA) and inverse agonist (SR141716) for 24 h in human breast cancer cells has been reported to result in reduced lipid raft residence of the CB1 receptor (Sarnataro 2005, Sarnataro 2006). Additionally to the modulation of CB1 receptor signaling, lipid rafts are also involved in CB1 receptor trafficking. This will be described in further detail in section 1.5.2.

1.5 CB1 receptor trafficking

1.5.1 Biosynthesis of GPCRs and the CB1 receptor

Not much is known yet about the biosynthesis and secretion of the CB1 receptor. However, based on data from other GPCRs, some common principals of biosynthesis are concluded, which were described in a review by Achour et al., forming the basis for the following section (Achour 2008). These principals will be specified for the CB1 receptor, whenever data were available from the literature. Synthesis of GPCRs takes place at ribosomes that are associated with the endoplasmic reticulum (ER), which is located in the cell soma. Newly synthesized peptide strands are inserted into the ER through a translocation complex. Some GPCRs contain a cleavable N-terminal signal sequence for ER targeting. However, 90% of GPCRs do not contain such a sequence (Andersson 2003). The CB1 receptor does not contain a targeting sequence and addition of such, or truncation of the long N-terminal tail has been reported to improve surface trafficking of the CB1 receptor (Andersson 2003). The synthesis of GPCRs into the ER is supported by various chaperones belonging to the heat shock protein (HSP) family as well as carbohydrate binding lectin chaperons. During synthesis, the N-

terminal tail becomes glycosylated to ensure recognition by carbohydrate-binding lectin chaperones such as calnexin and calreticulin. However, it is noteworthy that for CB1 receptors N-linked glycosylation of two putative glycosylation sites in the N-terminus seemed not to be important for surface expression (Andersson 2003). Subsequent removal of the glucoses from properly folded proteins allows exiting the ER, whereas miss folded proteins are targeted for degradation via the ER associated degradation pathway (ERAD). After quality control, GPCRs exit the ER via coat protein (COP) II coated buds. Possible ER exit motifs have been suggested to consist of hydrophobic amino acid residues such as phenylalanine and leucine (Ahn 2010). Indeed, hydrophobic residues in helix 8 seem to be important for CB1 receptor surface trafficking (Ahn 2010). In the Golgi apparatus, proteins can be targeted to the plasma membrane, to endosomes/lysosomes or back to the ER and ERAD for degradation. Trafficking from the Golgi to endosomes/lysosomes is mediated by adaptor proteins (AP). It has been reported that in a heterologous expression system the CB1 receptor associates with AP-3, which targets the receptor to lysosomes and prevents its trafficking towards the surface (Rozenfeld 2008). Although not yet much investigated, dimerization of GPCRs has been hypothesized to play a role in GPCR synthesis and secretion as well (Bulenger 2005). For the $GABA_{B1}$ receptor it is very well established that dimerization can mask an ER retention signal which is necessary for receptor exit from the ER (Margeta-Mitrovic 2000). Besides masking ER retention signals, another possible mechanism of hetero- or homodimerization to promote ER exit is to prevent GPCRs to form aggregates due to their highly hydrophobic transmembrane domains. By allowing the ordered interaction between GPCR transmembrane domains, random aggregates could be prevented (Achour 2008). Finally, in homodimers of the same GPCR the symmetry within these dimers could be used as a further quality control mechanism by giving the opportunity to compare the two receptors and by that detect mutations or missfolded domains (Achour 2008). However, further experiments are needed to support these hypotheses.

1.5.2 Endocytosis and postendocytic trafficking of GPCRs and the CB1 receptor

The canonical function of endocytosis and postendocytic trafficking of GPCRs is thought to be inactivation of receptor signaling and the regulation of surface expression and hence availability of receptors to extracellular ligands. However, it is also known that GPCRs can signal from endosomes and that endosomal sorting and trafficking provides a new complexity to GPCR signaling modes (Jean-Alphonse 2012, Cosker 2014). Endocytosis and postendocytic trafficking of GPCRs seem to be

important mechanisms for the targeted distribution of GPCRs as well as prolonged signaling across large distances in polarized cells such as neurons (Winckler 2011, Lasiecka 2011, Cosker 2014). The best studied pathway for GPCR endocytosis is the clathrin coated pit pathway (CCP). Upon activation, GPCRs can be phosphorylated at the C-terminal tail by GRKs, which leads to the recruitment of β-arrestins and adapter proteins. The CB1 receptor has been demonstrated to be phosphorylated by GRK3 which leads to the recruitment of β-arrestin2 (Jin 1999). Indeed, mutation of the phosphorylation sites S426 and S430 of the CB1 receptor in mice has been shown to result in reduced desensitization and tolerance after THC treatment (Morgan 2014). β-arrestin coupling leads to functional uncoupling of G proteins and hence accounts for receptor desensitization. Subsequently, it initiates localization of the receptor to CCP and endocytosis (Jin 1999, Ahn 2013, Hsieh 1999). Clathrin coated pits are invaginations in the plasma membrane which are coated by a scaffold of clathrin polymers. Besides via clathrin coated pit endocytosis, GPCRs have also been described to be internalized via lipid rafts/caveolae. Caveolae are coated plasma membrane invaginations as well and fission of both, CCP and caveolae depends on dynamine (Chini 2004). In 2001 It has been proposed that fission of caveolae results in the formation of a unique endosomal compartment called caveosome, which differs in its pH and other properties from conventional endosomal compartments (Pelkmans 2001). However, the same group questioned the existence of caveosomes nine years later (Hayer 2010). Hence, it is assumed for now that cargo which was endocytosed via lipid rafts/caveolae enters early endosomes (Parton 2010). Both, CCP and lipid raft/caveolae endocytosis have been described for the CB1 receptor (Keren 2003; Wu 2008). These findings are supported by the observations that the CB1 receptor can be found in biochemical lipid raft preparations (Asimaki 2011; Bari 2008; Sarnatarro 2006) and that it interacts and co-localizes with caveolin-1 (Bari 2008).

In a simplified view, after endocytosis the fate of GPCRs can be either recycling or degradation. The postendocytic sorting of GPCRs to these two pathways was reviewed by Hanyaloglu et al., which will be described here and specified for the CB1 receptor, whenever data were available from the literature. In AtT20 cells, treatment of the CB1 receptor with the agonist WIN55212-2 for 20 min led to the reduction in surface receptor, which could be restored after removal of the agonist. The repopulation of the surface was independent from protein synthesis and hence it was attributed to recycling of the receptor (Hsieh 1999). However, after 90 min of agonist treatment, repopulation of the surface depended on protein synthesis (Hsieh 1999). These findings indicate that long-term agonist treatment of the CB1 receptor induces it`s targeting to a pathway distinct from recycling, which is possibly lysosomal degradation. Recycling of the CB1 receptor has further been reported in HEK293 cells (Leterrier 2004, Wu 2008) as well as in primary neurons (Leterrier 2006). In general,

results regarding CB1 receptor postendocytic trafficking are quite contradictory between different groups. In a comparative review by Rozenfeld et al. the authors attributed these variations to differences in experimental settings, i.e. different cell lines, endogenous versus transfected receptors, native versus epitope tagged receptors and C-terminal versus N-terminal tagged receptors (Rozenfeld 2011). According to Hanyaloglu et al., the major postendocytic sorting site is thought to be the early endosomes, but some initial sorting already occurs at the plasma membrane. The phosphorylation of receptors at the plasma membrane can modulate interactions of the receptor with sorting proteins in later endocytic stages, such as postsynaptic density 95/disc large/zonula occludens-1 (PDZ) domain binding proteins, which are important mediators of GPCR recycling. Furthermore, ubiquitination of lysine residues of the receptor initiates endocytosis at the plasma membrane and facilitates lysosomal targeting during endosomal sorting (Fig. 1.5). At the endosomal level, there is a highly conserved sorting machinery called endosomal sorting complex required for transport (ESCRT) -I, -II, -III. A major player initiating the interactions of the receptor with the ESCRT is a protein called hepatocyte-growth factor regulated tyrosine kinase substrate (HRS), which contains an ubiquitin-interacting motif (UIM), and hence binds ubiquitinated proteins. Furthermore, HRS can bind to proteins of the ESCRT-I complex, and hence targets the receptor to the lysosomal pathway. However, not all receptors that are targeted to the lysosome need to be ubiquitinylated. Thus, there are probably other mechanisms to target these receptors for degradation. One such mechanism could be the interactions of GPCRs with GPCR-associated sorting proteins (GASP), which can bind to the C-terminal helix 8 of GPCRs (Hanyaloglu 2008). Indeed, GASP1 has been demonstrated to interact with the C-terminal tail of the CB1 receptor and to mediate it`s down regulation after agonist treatment (Martini 2006). AAV-mediated knockdown of GASP1 in primary neurons led to the attenuation of WIN55212-2 induced down regulation of the CB1 receptor. Furthermore, AAV-mediated overexpression of a dominant negative GASP1 protein *in vivo* resulted in the reduction of analgesic tolerance to WIN55212-2 in mice (Tappe-Theodor 2007). This suggests that GASP1 association is one mechanism to target CB1 receptors to the lysosomal degradation pathway and that this mechanism is important for the development of tolerance to CB1 receptor agonists (Howlett 2010). Another protein implicated in lysosomal targeting of GPCRs is sorting nexin-1 (SNX1), which can bind to the C-terminus of receptors as well as to HRS. However, SNX1 and GASP proteins have also been shown to bind to receptors which are known to recycle upon agonist stimulation, indicating a more complex role for these proteins in postendocytic sorting. Although default GPCR recycling in the absence of lysosomal targeting has been suggested, it is known that recycling of GPCRs can be tightly regulated. A variety of recycling sequences have been identified in GPCRs, most of which correspond to PDZ ligand domains in the distal C-terminus. Interaction with PDZ domain containing proteins seems to be critical for the recycling of many GPCRs (Fig. 1.5)

(Hanyaloglu 2008). The CB1 receptor might indirectly interact with PDZ domain containing proteins via CRIP1a. CRIP1a binds to the C-terminal tail of the CB1 receptor (Niehaus 2007).

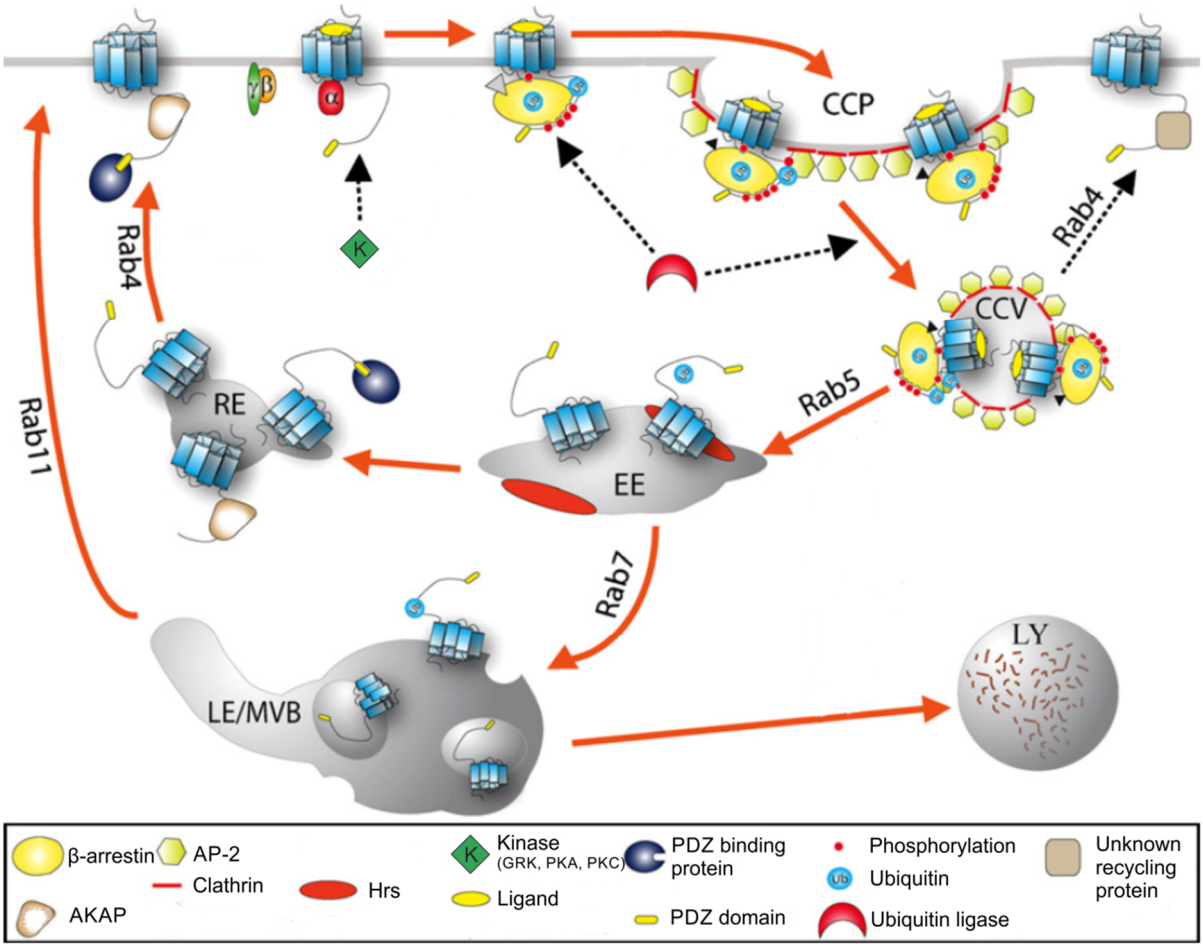


Fig. 1.5 Postendocytic sorting of GPCRs

After endocytosis of GPCRs into early endosomes (EE), receptors can be targeted to lysosomal degradation to lysosomes (LY) via the late endosomal/multi vesicular body compartment (LE/MVB) or recycle back to the membrane via recycling endosomes (RE). Phosphorylation and/or ubiquitination induce internalization of the receptor, i.e via clathrin coated pits (CCP). In the EE, ubiquitinated receptors can interact with HRS which targets it to the LE/MVB compartment for lysosomal degradation. In contrast, interaction of a PDZ ligand domain within the C-terminus of the receptor with a PDZ binding protein targets the GPCR to the recycling pathway. The membrane transport of the various vesicular compartments is regulated by different Ras-related in brain (Rab) proteins. Rab5 mediates early endosome targeting. Rab4 and Rab11 mediate fast and slow recycling, respectively. Rab7 mediates lysosomal targeting. (The figure was modified from Jean-Alphonse 2011).

It further contains a PDZ ligand domain which might mediate a crosslinking with PDZ domain containing proteins (Howlett 2010). Interestingly, CRIP1a overexpression also reduces agonist induced endocytosis and increases G protein binding of the CB1 receptor (Guggenhuber 2014). When

overexpressed in the hippocampus, CRIP1a reduces the severity of chemically induced acute epileptiform seizures (Guggenhuber 2014).

1.5.3 Polarization of the CB1 receptor in neurons

The CB1 receptor in neurons is predominantly targeted to presynaptic sites and growth cones. Considering the highly polarized nature of neurons, an important, unanswered question, is how the CB1 receptor is transported all the way along the axon to its final destination. For the CB1 receptor, as for most GPCRs, no axonal targeting sequence has been identified yet (Lasiecka 2011). Different mechanisms have been described for the trafficking of polarized neuronal proteins. There are cases of mRNA transport in granules and local translation at the target site (Jung 2012). In a review, Lasiecka et al. suggested three other pathways for protein polarization. (1) Proteins, which were translated in the somatic ER, could be sorted into targeting vesicles in the Golgi apparatus and reach their destination via the secretory pathway. (2) Proteins could also be secreted into the plasma membrane in an unspecific manner and gain their polarity by differential endocytosis and by degradation in the somatodendritic membrane and retention in the axonal plasma membrane. (3) The endocytosed protein could be transcytosed to the target site rather than being degraded (Lasiecka 2011).

The CB1 receptor has been reported to undergo differential endocytosis in somatodendritic and axonal compartments as well as transcytosis to gain its polarization towards the axon (Leterrier 2006, McDonald 2007, Simon 2013). In primary hippocampal neurons, which expressed green fluorescent protein (GFP) tagged CB1 receptor, Leterrier et al. found that 4 h after Golgi exit the majority of the CB1 receptor appeared in the somatodendritic surface. However, with proceeding time (8 h and 24 h) the amount of somatodendritic surface CB1 receptor declined and axonal surface receptor increased. Furthermore, they could show that the typical polarization of CB1 receptor towards the axonal surface disappears in the presence of different endocytosis inhibitors. These findings indicate that in case of the CB1 receptor random insertion of the receptor within the plasma membrane and subsequent selective endocytosis from the somatodendritic plasma membrane are one crucial pathway for polarization (Leterrier 2006). With compartment specific antibody labeling of surface CB1 receptor, the same group could recently also demonstrate transcytosis of the endocytosed receptors towards the axon (Simon 2013). They suggested a steady state model for CB1 receptor distribution and polarization on the neuronal surface which highly depends on somatodendritic endocytosis rates (Simon 2013). The somatodendritic endocytosis rate in turn was described to

depend on the receptors activity which can be basal or agonist induced (Turu 2007, Simon 2013) (Fig. 1.6). However, another group did not find an effect of activity on axonal polarization (McDonald 2007).

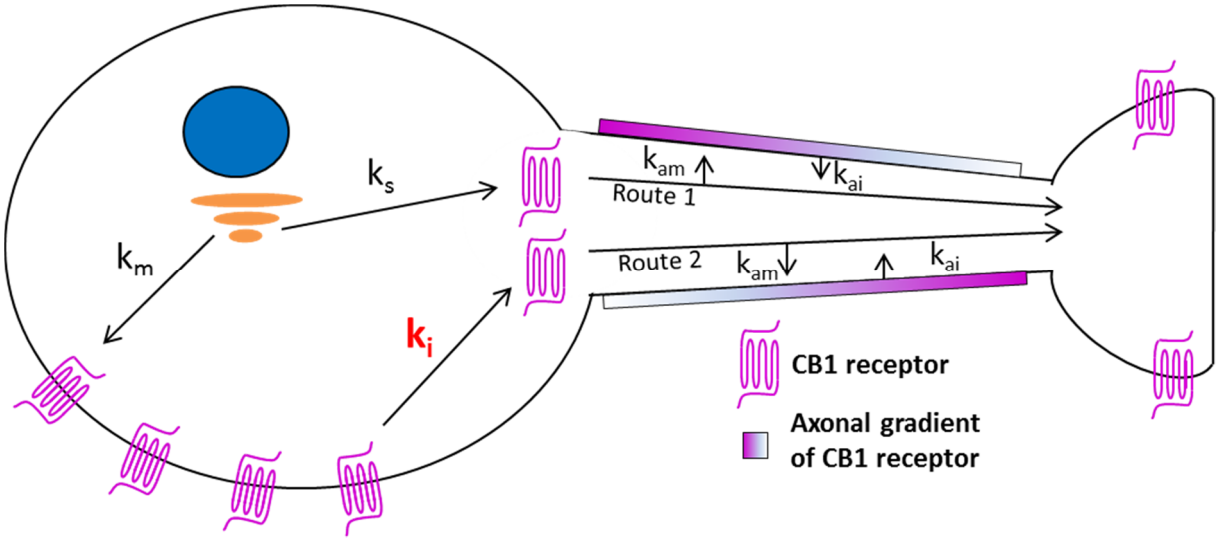


Fig. 1.6 CB1 receptor axonal polarization

The scheme represents a simplified form of a model suggested by Simon et al. for CB1 receptor axonal polarization (Simon 2013). The authors suggest two routes for the CB1 receptor to reach the axon. In route 1, the receptor gets targeted to the axon with a rate constant k_s directly after ER exit. It can fuse anywhere with the axonal membrane. This is more likely to occur in proximal parts of the axon, hence the gradient would decline along the axon. After ER exit the receptor could also be transported to the somatodendritic membrane with a rate constant k_m and be internalized with a rate constant k_i. After endocytosis, the receptor can be transcytosed to the axon by a targeting mechanism which would results in regulated fusion of targeting vesicles at more distal parts of the axon (route 2). Hence, the gradient would increase towards distal parts of the axon. K_{am} and k_{ai} represent fusion rates and internalization rates in the axon, respectively. Using computational and experimental methods Simon et al. revealed that the endocytosis rate k_i of the CB1 receptor is the critical constant that determines polarization and the axonal gradient of the receptor. (The picture was designed according to Simon 2013)

These findings indicate that changes in the activity states of CB1 receptor might not only modulate CB1 receptor signaling but the receptors distribution within the neuron and the neuronal surface (Simon 2013). Although it is currently unknown how these differences might affect neuronal function in particular, it becomes more and more clear that CB1 receptor distribution on the axonal surface can be regulated by intracellular and external cues (such as ligand binding), suggesting a plastic rather than a fixed steady state (Leterrier 2006, Turu 2007, Simon 2013).

1.6 Interactions between the CB1 and the D2 receptor

The CB1 receptor and the D2 receptor are both implicated in physiological functions such as reward, locomotion and motivation as well as cognitive functions. Both receptors are targets for medical drugs but also for drugs of abuse. The popular drug cannabis exerts its psychotropic functions through the CB1 receptor. Furthermore, most of the current addictive drugs of abuse share the common property to modulate dopaminergic transmission (Nestler 2005). Importantly, cannabis consumption during adolescence can entail an increased susceptibility to neuropsychiatric disorders such as schizophrenia or addiction in later life (Schneider 2008). In addition, acute consumption of cannabis can induce schizophrenia-like symptoms (D`Souza 2009). Conventional antipsychotics, which are frequently used to treat schizophrenia symptoms, share the common property of a high affinity for D2 receptors (Miyamoto 2005). However, antipsychotic drugs elicit a range of severe side effects, which still causes problems in patient compliance and long-term treatment (Miyamoto 2005). Understanding the function and the nature of the CB1 and D2 receptor interactions might help to better understand the biochemical correlates of psychosis and addiction and to develop new medical drugs modulating these interactions as a new target for the treatment of these conditions.

1.6.1 GPCR heteromerization

GPCRs do not necessarily exert their function as monomers only, but they can cluster together in homo- or heteromers. Homo- and heteromerization can affect receptor function on multiple levels, including biosynthesis, endocytosis and signaling potency, efficiency as well as selectivity. The resulting combinatorial diversity of GPCR functions further increases the complexity of information processing at the GPCR interface and can contribute to cell type specific signaling (Rozenfeld 2011).

Heteromerization can affect desensitization and endocytosis of GPCRs. The CB1 and Orexin1 (OX1) receptor as well as the adenosine2A (A2A) and D2 receptor have been demonstrated to co-internalize (Torvinen 2004, Ward 2011, Huang 2013). Interestingly, the A2A receptor as well as the D2 receptor co-localizes with caveolin-1. Hence, it could be speculated about an involvement of lipid rafts/caveolae in the co-internalization of the A2A-D2 heteromer (Genedani 2005). Furthermore, it has been concluded from computational studies that heteromerization may regulate lipid raft recruitment of GPCRs (Fallahi-Sichani 2009). Internalization of GPCRs can be initiated by phosphorylation of the C-terminus and subsequent recruitment of β-arrestin. The heteromerization

of the CB1 receptor with the δ-opioid receptor (DOR) has been demonstrated to increase β-arrestin recruitment and hence CB1 receptor desensitization (Rozenfeld 2011).

These examples illustrate that drugs targeting a GPCR might also modulate the availability of off-target GPCRs, which is an important finding with regards to side effects of medical drugs. As desensitization and endocytosis of GPCRs are thought to be one mechanism for drug tolerance, the effect of heteromerization on β-arrestin recruitment, lipid raft association and endocytosis of GPCRs might be an interesting phenomenon to understand cross-tolerance and cross-desensitization of medical drugs.

1.6.2 The CB1-D2 receptor heteromer

The ECS and the dopaminergic system can interact on several levels. Although there are quite some studies addressing the question how D2 receptor or CB1 receptor activity modulate each other's expression, the data are quite contradictory and do not generate a clear picture yet (see table 9.1 in appendix). This might be due to the fact that different genetic or pharmacological models were used which differ in the time period in which the receptors activity is modulated (knockout animals vs. AAV mediated knockdown and pharmacological treatment). Furthermore, different methods were used to measure receptor protein levels (immunohistochemistry vs. radioligand binding).

However, regarding the function, it has been found that the D2 receptor and the CB1 receptor couple to and hence compete for a common pool of G_i proteins (Jarrahian 2004). Furthermore, the co-activation and even the co-expression of both receptors cause a switch from G_i to G_s signaling, which was attributed to heterodimerization of the two receptors (Glass 1997, Jarrahian 2004). The physical interaction of the CB1 receptor and the long splice variant of the D2 receptor (D2L) has been demonstrated by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) (Kearn 2005, Marcellino 2008, Przybyla 2010). Kearn et al. and Przybyla et al., but not Marcellino et al., agree in the finding that co-activation of CB1 receptor and D2 receptor increases heteromerization. Interestingly, Przybyla et al. further found that activation of the CB1 or D2 receptor increases intracellular heteromers, which could lead to speculations about co-trafficking of these receptors. However, further experimental evidence is needed to support this idea. In addition to dimerization with one another, both CB1 and D2 receptors are known to dimerize with the A2A receptor, and hence all three receptors might exist as a trimeric or multimeric receptor mosaic (Marcellino 2008). Biochemical as well as behavioral evidence for the CB1-D2-A2A interaction comes from a study by Marcellino et al.. The authors found that activation of the CB1 receptor in striatal membrane

preparations reduces the affinity of the D2 receptor for dopamine (Marcellino 2008). Accordingly, in behavioral experiments they showed that pharmacological activation of the CB1 receptor could inhibit the hyperlocomotor effect of a D2 receptor agonist. Importantly, this effect occurred with a concentration of CB1 receptor agonist which did not show any effect on locomotion when applied alone. The effect of CB1 receptor activation on D2 receptor-induced hyperlocomotion was blocked by an A2A receptor antagonist (Marcellino 2008). In contrast to D2 receptor agonist mediated hyperlocomotion, pharmacological activation of the CB1 receptor induces hypolocomotion and catalepsy in rodents (Martin 1987, Monory 2007). Current publications implicate that this effect might at least partially depend on CB1-D2-A2A interactions (Andersson 2005, Borgkvist 2008). This indicates that the CB1-D2-A2A interaction could be an important modulator of striatal function and related behavior. Although these findings could be explained by the interactions of CB1, D2 and A2A receptors, they do not directly tell whether it is a consequence of heteromerization of the three receptors or a crosstalk in downstream signaling events. However, although further studies are needed, probably using less complex models such as cultured cells, the behavioral studies give valuable support for the relevance to study the heteromerization of these receptors.

When thinking about heteromerization and signaling interactions of two GPCRs, the obvious prerequisite to do so is co-expression of these two receptors in the same cell type, in the same brain area and, in case of heteromerization, the same structure within a cell. The CB1 receptor and the D2 receptor are both expressed in high abundance in the striatum (Jackson 1994; Breivogel 1998). Furthermore, co-expression on the mRNA level was found in the olfactory tubercle, the piriform cortex, the enthorhinal/perirhinal cortex area and the dentate gyrus of the hippocampus (Hermann 2002). D2 receptor protein can be located either at presynapses as autoreceptors on dopaminergic neurons (D2 short isoform (D2S)) or postsynaptically (D2L) (Mei 2009). However, CB1 receptor protein is predominantly present in the presynaptic area, and the CB1 receptor is not expressed in dopaminergic neurons of the substantia nigra (SN) or ventral tegmental area (VTA) (Fitzgerald 2012). This means that interaction of the CB1 receptor with D2S autoreceptors on dopaminergic neurons is unlikely. Furthermore, postsynaptic D2 receptors in medium spiny neurons (MSN) of the striatum are located in the dendrites whereas CB1 receptors in MSN are predominantly found presynaptically in the axon (Fitzgerald 2012). However, CB1 and D2 receptors could interact extra synaptically in MSNs in the somatodendritic area. Probably one of the most interesting interaction sites of CB1 and D2 receptors is the corticostriatal synapse. Long-term depression at the corticostriatal synapse has been demonstrated to depend on the D2 receptor as well as the CB1 receptor (Lovinger 2010). Furthermore, activation of the D2 receptor together with mGluR1 receptors on the corticostriatal postsynapse has been shown to stimulate cannabinoid release (Lovinger 2010). The CB1 receptor is

present on the presynaptic site of the corticostriatal glutamatergic synapse (Lovinger 2010). Dopaminergic projections from the SN and VTA contact the postsynapse of the corticostriatal synapse to modulate glutamate transmission. Interestingly, however, it might be that the D2 receptor is also present at the corticostriatal presynapse (Bamford 2004). Hence, an axo-axonal synapse or heterosynaptic volume transmission between dopaminergic neurons and the cortical axon of the corticostriatal synapse are possible (Bamford 2004). The D2 receptor on the presynaptic site of the corticostriatal synapse has been suggested to select highly active synapses and, in turn, to weaken less active synapses, which would lead to a low-pass filter effect (Bamford 2004). It needs to be mentioned however, that the existence of the presynaptic D2 receptor at the corticostriatal synapse is still controversial and its responsibility for low-pass filtering has been challenged by Yin et al., who propose a different mechanism (Yin 2006).

Finally, the D2 receptor has also been shown to be expressed in cortical astrocytes, where their activation affects intracellular Ca^{2+} levels (Khan 2000). The CB1 receptor in cortical astrocytes has also been reported to modulate intracellular Ca^{2+} dynamics and thereby modulate spike timing dependent plasticity (Min & Nevian 2012). Hence, it could also be possible that CB1 and D2 receptors build heteromers in astrocytes.

1.7 The CB1F238L receptor mutant rat

As for all proteins, the structure of a GPCR in a cellular environment is predominantly determined by it`s amino acid sequence. Point mutations in the amino acid sequence of a GPCR are a common and usefull tool to investigate the role of this amino acid in a GPCR structure and functions. A point mutation can affect GPCR folding, a variety of receptor functions such as ligand binding, conformational flexibility and hence activity, or protein interactions which might also modulate the receptors activity or it`s subcellular localization and trafficking. Thus, the characterization of point mutations in GPCRs helps to shed light on the relation between structure and functions of these receptors.

The point mutation characterized in this work has been recently published by Schneider et al. (Schneider 2014). A single missense point mutation has been introduced in the *cnr1* gene of a Fischer rat by N-ethyl-N-nitrosourea (ENU) mutagenesis. In the amino acid sequence of the CB1 receptor the mutation leads to the exchange of phenylalanine 238 in TMH4 to a leucine residue (Fig. 1.7). The authors state a gain of function for the mutant receptor as concluded from computational,

biochemical, electrophysiological and pharmacological experiments. Furthermore, mutant rats display a behavioral phenotype similar to that observed in adolescent rats when compared to adult rats (Schneider 2014). Additionally, mutant rats respond stronger to CB1 receptor agonist and inverse agonist treatment with regards to food intake and locomotor activity, respectively (Schneider 2014). In biochemical experiments, the mutant receptor shows increased agonist stimulated G protein binding. In electrophysiological experiments, there is a reduction in presynaptic transmitter release probability, which can be explained by hyperactivity of the mutant CB1 receptor (Schneider 2014). Many of the affected behavioral paradigms such as locomotor activity, reward sensitivity and risk affinity are also known to be modulated by the dopaminergic system. Interestingly, positron emission tomography (PET) experiments with a D2 receptor radiotracer showed an increased binding potential in the striatum (Schreckenberger, unpublished). Hence, the point mutation in the CB1 receptor might also affect dopaminergic signaling.

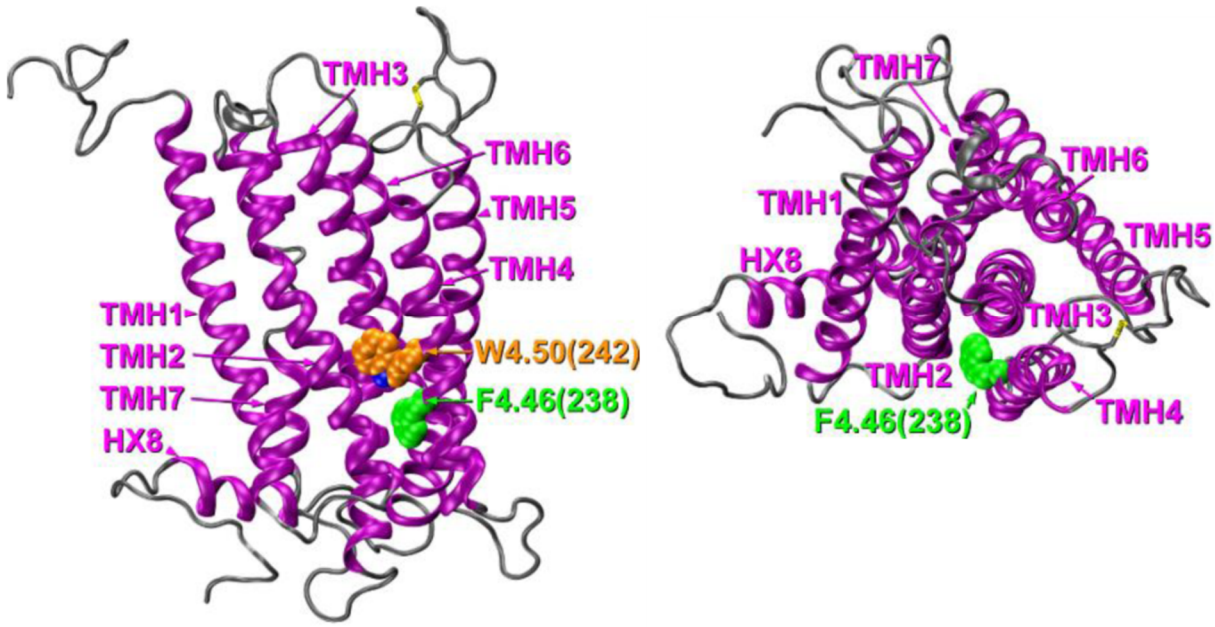


Fig. 1.7 *In silico* modelling of the CB1F238L receptor mutant

In the CB1F238L receptor mutant the phenylalanine 238 (green; 4.46 in the Ballesteros & Weinstein system) is exchanged by a leucine residue. Phenylalanine 238 is located in the TMH4 at the lipid facing side between a TMH2-3-4 bundle and directly below the highly conserved tryptophan 242 (orange; 4.50 in the Ballesteros & Weinstein system) (Schneider 2014). (The Figure was taken from Schneider 2014).

1.8 Aim of the study

It has previously been demonstrated that the F238L mutation in the fourth transmembrane domain of the CB1 receptor affects the behavior of Fischer rats in several behavioral paradigms. In this study, it was aimed at investigating the effect of the F238L mutation at level of the cell biological properties of this mutant CB1 receptor. To this end, cell surface expression, endocytic trafficking and intracellular signaling cascades of the CB1F238L receptor were investigated in transiently and stably transfected HEK293 cells, and compared to the wild-type CB1 receptor. Furthermore, it was aimed at testing whether the F238L mutation affects the polarization of the CB1 receptor towards the axon of primary hippocampal neurons.

Many of the behavioral prototypes, which were affected by the CB1F238L mutation in rats, are known to be modulated by the dopaminergic system. Previous positron emission tomography experiments revealed an increased binding potential of a D2 receptor radiotracer in the striatum of CB1F238L receptor mutant rats. However, the D2 receptor mRNA level was unaltered in the mutant rat. Due to these contradictory results on D2 receptor expression, an additional aim of this study was to examine the D2 receptor protein level in the mutant rat striatum by means of receptor autoradiography and western blot analysis. Furthermore, experiments in transiently transfected HEK293 cells were performed aiming at characterizing the heteromerization of the CB1 receptor with the D2 receptor. To further study the effect of the F238L mutation in the CB1 receptor on heteromerization with the D2 receptor, HEK293 cells were generated, which stably express the D2 receptor either alone, or together with the wild-type or F238L mutant CB1 receptor.

2. MATERIAL AND METHODS

2.1. Material

2.1.1 Tissue

Brain tissue from CB1F238L mutant fisher rats was generously provided by Dr. Miriam Schneider from the Central Institute of Mental Health in Mannheim.

Striatal tissue from D2 receptor knockout mice was generously provided by Dr. Marcelo Rubinstein from the University of Buenos Aires.

2.1.2 Devices

Autoradiography

Cryostate	Microtom Cryostat Cryo-Star HM 560 MV, Microm International
Phosphoimager	Storage Phosphor System Cyclone®Plus, Perkin Elmer
Trithium sensitive screen	TR storage phosphor screen, Perkin Elmer

Table 2.1

Cell culture

Cell counter	Neubauer cell counter, Superior Marienfeld Germany
Incubator	HERACELL 150i CO2 Incubator, Thermo Scientific
Sterile Hood	HERAsafe, Heraeus Instruments

Table 2.2

Centrifuges

Centrifuge 5417R, Eppendorf
Heraeus MegaFuge 16R, Thermo Scientific
L8-60M Ultracentrifuge, Beckman
Megafuge 1.OR, Heraeus Instruments

Table 2.3

Microscopes

Leica AF 6000
Zeiss LSM510 UV META Axiovert 200M laser scanning microscope
Zeiss LSM-710 confocal microscope

Table 2.4

Molecular Biology

Gel documentation System	UVsolo TS Imaging system, Biometra
Gel Electrophoresis chamber	Compact M Horizontal Gel Electrophoresis Apparatus, Biometra
Nanodrop	NanoDrop 2000c Spectrophotometer, Thermo Scientific
Thermocycler	T3000 Thermocycler, Biometra

Table 2.5

Proteinbiochemistry

Spectrophotometer	BioPhotometer, Eppendorf
Sonicator	Bandelin Sonopuls HD60
Western blot chemiluminescence imaging system	Fusion-SL advance™ system, Peqlab
Western blot system	Mini-PROTEAN 3, Biorad

Table 2.6

Radioligand binding assay

Harvester	Brandel Cell Harvester
Scintillation counter	Tri-Carb 2800 TR, Perkin-Elmer,

Table 2.7

2.1.3 Drugs

Drugs

(+)-butaclamole hydrochloride	Sigma Aldrich
Dopamine hydrochloride	Sigma Aldrich
SR141716A	Generously provided by the NIMH Chemical Synthesis and Drug Supply Program
Tetrahydrolipstatin	Cayman Chemicals
WIN55212-2	BioTrend Chemicals AG

Radioactively labeled substances	Perkin Elmer

Table 2.8

2.1.4 Plasmids

Nr	Plasmid name	Protein expression	Generated by
1	pAM/CBA-pL-WPRE-bGHpA	/	Stephan Guggenhuber
2	pcDNA3.1	/	Invitrogen
3	pcDNA3.1_Zeo	/	Invitrogen
4	pcDNA3_rCB1wt	rCB1wt	Frauke Steindel
5	pcDNA3_rCB1F238L	rCB1F238L	Frauke Steindel
6	pcDNA3_HA-rCB1wt	HA-rCB1wt	Generated in this work
7	pcDNA3_HA-rCB1F238L	HA-rCB1F238L	Generated in this work
8	pcDNA3.1-rCB1-WT-EGFP	rCB1wt-EGFP	Frauke Steindel
9	pcDNA3.1-rCB1-F238L-EGFP	rCB1F238L-EGFP	Frauke Steindel
10	pBS II SK(+) -Flag-Drd2L	Flag-rD2L	MWG Eurofins Operon
11	pAM/CBA-pL-Flag-D2L-WPRE-bGHpA	Flag-rD2L	Generated in this work
12	pcDNA3_Zeo_Flag-D2L	Flag-rD2L	Generated in this work
13	pcDNA3_rD2L	rD2L	Frauke Steindel
14	pAM/CBA-pL-HA-rCB1-WPRE-bGHpA	HA-rCB1wt	Stephan Guggenhuber

Table 2.9

2.1.5 Primary antibodies

Epitope	Clonality/Species	Application	Company
HA	Monoclonal/ mouse	WB: 1:1000 ICC: 1:1000	Covance
HA	Monoclonal/ mouse	ICC: 1:200 /1:400	Sigma
Tubulin	Monoclonal/ mouse	WB: 1:200,000	Sigma
Actin	Rabbit/ polyclonal	WB: 1:1000	Millipore
Caveolin-1	Rabbit/ polyclonal	WB: 1:1000	Santa Cruz
Map2	Rabbit/ polyclonal	ICC: 1:600	Sigma
D2R	Rabbit/ polyclonal	WB: 1:1000 IP: 1:250	Millipore
p44/42 Phospho-ERK 1/2 (THR202/Tyr204)	Monoclonal/ mouse	WB: 1:1000	Cell Signaling
p44/42 ERK 1/2	Rabbit/ polyclonal	WB: 1:1000	Cell Signaling
Lamp-2	Monoclonal/ mouse	ICC: 1:100	Abcam

Table 2.10 ICC: Immunocytochemistry; IP: Immunoprecipitation; WB: Western Blot

2.1.6 Secondary antibodies

Antibody	Application	Company
Alexa488-goat anti mouse	ICC: 1:1000	Life technologies
Alexa546-goat anti mouse	ICC: 1:1000	Life technologies
Cy2-donkey anti mouse	ICC: 1:600	Jackson Immuno
Cy3-donkey anti mouse	ICC: 1:600	Jackson Immuno
Cy5-donkey anti rabbit	ICC: 1:600	Jackson Immuno
HRP-goat anti mouse	WB: 1:5000	Dianova
HRP-goat anti rabbit	WB: 1:5000	Dianova

Table 2.11 ICC: Immunocytochemistry; WB: Western Blot

2.1.7 Materials

Consumables	Company
Chemicals	AppliChem, Biochrom AG, Calbiochem, Fluka Chemika, Merck, Roth, Sigma Aldrich
Glass ware	Schott, VWR
Plastic ware	BD-Falcon, CytoOne, Greiner bio-one, Nunc

Table 2.12

2.1.8 Buffers and solutions

Standard buffer

PBS	136,9 mM NaCl, 2,7 mM KCl, 8 mM Na2HPO4, 1,47 mM KH2PO4; pH7.4
TBS	50 mM Tris/HCl, 150 mM NaCl; pH7.4

Table 2.13

Molecular Biology

Ampicilin solution	100 mg/ml stock of ampiclillin Na-salt in 75% ethanol. Use 1:1000
Ethidiumbromide solution	Ethidiumbromidlösung 0,025 % (250 µg/ml), Roth
LB agar	1.5 mg/ml agar in LB medium
LB medium	in 1L: 10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl; pH 7.0 (autoclaved)
Phenol-Chloroform-Isoamylalcohol-Mix	Roti®-Phenol /C/I (25:24:1), Roth
10 x TBE	108 g Trisbase, 55 g boric acid, 0.74 g EDT, to 1 L with dest H2O

Table 2.14

[^{35}S]GTPγS binding

Assay buffer	50 mM Tris/HCl, 1 mM EGTA, 3mM MgCl2, 100mM NaCl; pH 7.4
Membrane buffer	50 mM Tris/HCl, 3 mM MgCl2, 0.2 mM EGTA, Roche EDTA free complete protease inhibitor; pH 7.4
Scintillation fluid	Scintillation cocktail Aquasafe 300 plus, Zinsser Analytic
Washing buffer	50 mM Tris/HCl; pH 7.4

Table 2.15

CB1 receptor equilibrium binding

Cell buffer	50 mM Tris/HCL, 1 mM EDTA and 3 mM $MgCl_2$; pH 7.4

Table 2.16

D2 receptor equilibrium binding

Binding buffer	20 mM Tris/HCl, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, Roche EDTA free complete protease inhibitor; pH 7.4
Membrane buffer	20 mM Tris/HCl, 100 mM NaCl, 7 mM $MgCl_2$, 1 mM EDTA, Roche EDTA free complete protease inhibitor; pH 7.4

Table 2.17

Cell culture

HBS buffer	25 mM HEPES, 137 mM NaCl, 5 mM KCl, 15 mM glucose, 1.5 mM $CaCl_2$, 1.5mM $MgCl_2$; pH7.4, osmolarity adjusted to within ± 10 mOsM of the culture medium
HEBS buffer	50 mM HEPES, 28 mM NaCl, 1.5 mM Na2HPO4; pH 7.05
Lipofectamine	Lipofectamine ®2000 Life technologies
Poly-L-lysine	1mg/ml Poly-L-lysine (Sigma) in dH_2O

Table 2.18

Primary neuron culture

B27	B27® Supplement (50x), serum free, gibco® life technologies™
Feeding medium	Neurobasal medium supplemented with B27®, glutaMAX™ and penicillin/streptamycin

Glutamax	GlutaMAX™ Supplement, gibco® life technologies™
Neurobasal	Neurobasal® Medium, gibco® life technologies™
Plating medium	Neurobasal medium supplemented with horse serum (10%), B27®, glutaMAX™ and penicillin/streptamycin
Transfection medium	Neurobasal medium supplemented with glutaMAX™ and B27®

Table 2.19

HEK293 cell culture

DMEM	Dulbecco`s Modified Eagle Medium (+) 4.5 g/L D-Glucose, (+) L-Glutamine, (-)Pyruvate, gibco® life technologies™
IMDM	Iscove`s Modified Dulbecco`s Medium, gibco® life technologies™
Non-essential amino acids	MEM NEAA 100 x, gibco® life technologies™
Penicilin/ Streptamycin	PenStrep, 5000 U/ml Penicilin, 5000 µg/ml Streptamycin, gibco® life technologies™
Plating medium	DMEM containing 10% fetal calf serum, 1% (v/v) Penicilin/Streptamycin, 1% (v/v) sodium pyruvate and 1% (v/v) non-essential amino acids
Serum free medium	DMEM containing 1% (v/v) Penicilin/Streptamycin, 1% (v/v) sodium butyrate and 1% (v/v) non-essential amino acids
Sodium pyruvate	Sodium pyruvate 100 mM, gibco® life technologies™
Transfection Medium	IMDM containing 5% FCS
Trypsin solution	0.05% Trypsin-EDTA, gibco® life technologies™
Versene solution	Versene solution, 0.48 mM EDTA in PBS, gibco® life technologies™
Zeocin stock solution	Zeocin™, Zeocin 100 mg/ml in sterile water, gibco® life technologies™

Table 2.20

Protein biochemistry

Acrylamide solution	Rotiphorese®Gel 40 (37,5:1), Roth
Autoradiograph buffer	120 mM NaCl, 2 mM CaCl, 1 mM MgCl2, 50 mM Tris/HCl; pH 7.4
Bradford solution	Biorad Quick Start™ Bradford 1x Dye Reagent
ECL solution	Amersham™ ECL Prime™ Western Blotting Detection Reagent, GE Healthecare
Extraction buffer	20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% TritonX100, protease inhibitor; pH7.4
IP-buffer	150mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris, protease- and phosphatase inhibitor cocktail; pH 7.5, 0,5 % CHAPS
10x Laemmli buffer	0,25 M Tris/HCl, 1,92 M Glycin
Lysisbuffer	150mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris, protease- and phosphatase inhibitor cocktail; pH 7.5, 1 % CHAPS
Protease inhibitor	cOmplete Tablets EDTA-free, EASYpack, Roche
Protease-Phosphatase Inhibitor cocktail	Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X), Thermo Scientific
5 x sample buffer	100 ml: 15 g SDS, 15,6 ml Tris/HCl 2 M; pH 6, 57.5 g Glycerol 87%, 16.6 ml β mercaptoethanol, to 100 ml with dest H2O
TBST	50 mM Tris/HCl, 150 mM NaCl; 0.1% Tween® 20; pH7.4
Western blot running buffer	100 ml 10x Laemmli-buffer, 5 ml SDS 20%, 895 ml H_2O
Western blot transfer buffer	100 ml 10x Laemmli-buffer, 200 ml Ethanol, 700 ml H_2O

Table 2.21

2.1.9 Kits

Molecular Biology

Gel purification	PureLink™ PCR Quick Gel Extraction Kit, Invitrogen
Maxi preparation	PureLink™ HiPure Plasmid Miniprep Kit, Invitrogen
Mini preparation	PureLink™ Quick Plasmid Miniprep Kit, Invitrogen
PCR purification	PureLink™ PCR Purification Kit, Invitrogen

Table 2.22

2.1.10 Enzymes and molecular markers

Enzymes

BamHI, EcoRI, HindIII, NotI, PvuI, Phusion® High-Fidelity DNA Polymerase, T4-DNA-Ligase and Calf intestine phosphatase (CIP) were purchased from New England Biolabs

Molecular markers

DNA ladder	MassRuler DNA Ladder Mix, Ready-to-Use, 80-10,000 bp, Thermo Scientific
Protein ladder	PageRuler Prestained Protein Ladder, Thermo Scientific

Table 2.23

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using the Phusion^{R} High-Fidelity DNA Polymerase in a total volume of 50 µl of buffer supplied with the polymerase and the following components:

Component	Concentration
Buffer	1 x
dNTPs	200 µM
Primer forward	0.5 µM
Primer reverse	0.5 µM
Template DNA	10 ng
Phusion polymerase	1 Unit
dH_20	Fill up to 50 µl

The PCR was performed with the following thermocycler parameters:

Step	Temperature	Time	Cycle number
Initial Denaturation	98°C	3 min	1 x
Denaturation	98°C	15 sec	
Annealing and Elongation	72°C	30 sec	25 x
Final Elongation	72°C	10 min	1 x

2.2.1.2 Analytical and preparative agarose gel electrophoresis

PCR products or digested DNA fragments were analyzed and separated on an agarose gel (0.6 % agarose in TBE) containing ethidiumbromide to visualize DNA. A molecular marker was run in one lane to determine the size of the fragments which were to be analyzed or prepared. In case of a preparative gel electrophoresis the band was cut out of the gel under UV light and the DNA was purified using the Gel purification kit following the manufacturer's instructions.

2.2.1.3 Determination of DNA concentration

The concentration of DNA in solution was determined by spectrophotometry exploiting the fact that DNA can absorb light of a wavelength of 260 nm. The measurements were performed using the Nanodrop device.

2.2.1.4 Restriction of DNA

Enzymatic restriction of DNA was performed using restriction enzymes from NEB in the buffer recommended by the manufacturer. The following mixture incubated for 2 h at 37°C.

Component	Amount
dH_2O	Fill up to 20 µl
10 x buffer	2 µl
DNA	10 µg
Restriction enzyme	2 µl

The restriction product was analyzed via agarose gel electrophoresis. The band corresponding to the restricted vector, insert or PCR product were cut out under UV light and purified using the Gel purification kit following the manufacturer`s instructions.

2.2.1.5 Dephosphorylation of 5` and 3` ends of DNA

To avoid the re-ligation of empty vector, the 5` and 3`ends of the restricted vector where dephosphorylated. Hence, in the ligation step the vector can only ligate with the phosphorylated insert. 1.5 µl CIP phosphatase was added to 8.5 µl of purified restricted vector and incubated for 1 h at 37°C. The enzyme was removed by gel electrophoresis and purification of the dephosphorylated vector.

2.2.1.6 Ligation of DNA

Ligation of vector and insert was performed with a vector to insert ratio of 1 : 8 using the T4-Ligase and the buffer supplied by the manufacturer. The following reaction incubated at 4°C over-night.

Component	Amount
dH_2O	Fill up to 20 µl
10x buffer	2 µl
Vector	35 ng
Insert	$Insert\ ng = 8x \frac{Insert\ bp\ x\ Vector\ ng}{Vector\ bp}$
Ligase	1 µl

2.2.1.7 Transformation

To transform chemocompetent DH5α bacteria, 6 µl of the ligation reaction was added to 100 µl of bacteria and swirled gently. The reaction incubated for 30 min on ice and was followed by a heat shock of 90 sec at 42°C in a water bath. Subsequently, the reaction was put on ice for 2 min and 250 µl of LB medium at room temperature was added. The reaction incubated at 37°C for 30 – 60 min in a shaking incubator. 100 µl of the reaction was spread on LB agar plates containing 100 µg/ml ampicillin, to select for transformed bacteria, and incubated for 16 h at 37°C.

2.2.1.8 Minipreparation of plasmids

Colonies of transformed bacteria were picked and amplified in 2 ml LB medium per colony at 37°C over-night to gain minicultures. The LB medium contained 100 µg/ml ampicillin to select for transformed bacteria. The plasmid was prepared using the minipreparation kit following the manufacturer's instructions.

2.2.1.9 Maxipreparation of plasmids

200µl of miniculture was amplified in 200 ml LB medium at 37°C over-night. The LB medium contained 100 µg/ml ampicillin to select for transformed bacteria. The plasmid was prepared using the maxipreparation kit following the manufacturer's instructions.

2.2.1.10 Cloning strategy for the HA tagged CB1wt and CB1F238L receptor constructs

BamHI and NotI restriction sites, a 5` Kozak sequence as well as an N-terminal human influenza hemagglutinin (HA) tag were added to the rat CB1wt or rat CB1F238L receptor sequence by PCR using the following primers:

Forward: GCGGATCCACCATGGCATACCCATATGATGTCCCCGACTACGCGAAGTCGATCCTAGATG GCCTTG

Reverse: GGCGCGGCCGCTCACAGAGCCTCGGCGGA

As template DNA the pcDNA3_rCB1wt and pcDNA3_rCB1F238L constructs were used (Nr 4 and Nr 5 in table 2.9). The resulting PCR product was cloned into the target vector pcDNA3.1 (Nr 2 in table 2.9) using BamHI and NotI restriction sites.

2.2.1.11 Cloning strategy for constructs of Flag tagged D2L receptor in two different target vectors

The following sequence was synthesized and cloned into a pBS II SK(+) vector by Eurofins MWG Operon (Nr 10 in table 2.9):

gaattc*atggattacaaagacgatgacgataag***gatccactgaacctgtcctggtacgatgacgatctggagaggcagaactggagccggc ccttcaatgggtcagaagggaaggcagacaggccccactacaactactatgccatgctgctcaccctcctcatctttatcatcgtctttggcaat gtgctggtgtgcatggctgtatcccgagagaaggctttgcagaccaccaccaactacttgatagtcagccttgctgtggctgatcttctggtggcc acactggtaatgccgtgggttgtctacctggaggtggtgggtgagtggaaattcagcaggattcactgtgacatctttgtcactctggatgtcat

gatgtgcacagcaagcatcctgaacctgtgtgccatcagcattgacaggtacacagctgtggcaatgcccatgctgtataacacacgctacagc
tccaagcgccgagttactgtcatgattgccattgtctgggtcctgtccttcaccatctcctgcccactgctcttcggactcaacaatacagaccaga
atgagtgtatcattgccaaccctgcctttgtggtctactcctccattgtctcattctacgtgcccttcatcgtcactctgctggtctatatcaaaatct
acatcgtcctccggaagcgccggaagcgggtcaacaccaagcgcagcagtcgagctttcagagccaacctgaagacaccactcaagggcaac
tgtacccaccctgaggacatgaaactctgcaccgttatcatgaagtctaatgggagtttcccagtgaacaggcggagaatggatgctgcccgcc
gagctcaggagctggaaatggagatgctgtcaagcaccagtcccccagagaggacccggtatagccccatccctcccagtcaccaccagctca
ctctccctgatccatcccaccacggcctacatagcaaccctgacagtcctgccaaaccagagaagaatgggcacgccaagattgtcaatccca
ggattgccaagttctttgagatccagaccatgcccaatggcaaaacccggacctcccttaagacgatgagccgcagaaagctctcccagcaga
aggagaagaaagccactcagatgcttgccattgttctcggtgtgttcatcatctgctggctgcccttcttcatcacgcacatcctgaatatacact
gtgattgcaacatcccaccagtcctctacagcgccttcacatggctgggctatgtcaacagtgccgtcaaccccatcatctacaccaccttcaac
atcgagttccgcaaggccttcatgaagatcttgcactgctgaaagctt

The sequence contains the restriction sites EcoRI and HindIII (underscored), a Flag tag sequence (*italic*) and the coding sequence for the long isoform of the rat D2 receptor (D2L) (**bold**).

The Flag-D2L sequence was cut out and cloned into the target vector pAM/CBA-pL-WPRE-bGHpA (Nr 1 in table 2.9) and pcDNA3.1 Zeo (+) (Nr 3 in table 2.9) using EcoRI and HindIII restriction sites.

2.2.2 Cell biological methods

2.2.2.1 Culture of human embryonic kidney 293 cells

Human embryonic kidney cells (HEK293) were grown in DMEM plating medium. Twice a week cells were split in a 1:4 to 1:6 dilution for experiments and to maintain the culture. For this cells were washed two times with warm PBS and treated with trypsin-EDTA solution for approximately 1 min until the cells visibly detached from the dish. Subsequently 10 ml of warm plating medium was added to stop the enzymatic reaction and cells were centrifuged at 100 g and room temperature for 5 min. The cell pellet was re-suspended in plating medium and an appropriate amount was plated in a flask containing pre-warmed medium.

2.2.2.2 Primary hippocampal neuron culture

Primary hippocampal neurons were prepared by Keri Hildick at the University of Bristol as described (Martin 2004).

Hippocampi from E18 Wistar rats were removed. Cells were dissociated by 15 min of trypsin digestion and by mechanical dissociation. 500,000 cells per 35 mm or 50,000 cells per 22 mm poly-L-lysine covered coverslips were plated in pre-warmed plating medium. Two days after plating the medium was changed to feeding medium. Cells were kept in feeding medium until use.

2.2.2.3 Transient transfection of HEK293 cells

HEK293 cells were transiently transfected using the calcium precipitation method. 24 h after plating HEK293 cells on poly-L-Lysin coated coverslips in a 6 well plate, the medium was replaced by transfection medium for 2 h. A transfection mix was prepared by dissolving the DNA in sterile water and the addition of the $CaCl_2$ solution. Subsequently, HEBS buffer was added slowly and drop wise. The volumes of the constituents are listed below for the different well sizes. The final transfection mixture was allowed to stand at room temperature for 5 min. It was then vortexed and added drop wise to the cells. 24 h later, the medium was changed to plating medium. Another 24 h later, cells were used for further experiments.

	6 well plate	**10 ⌀cm dish**
H2O	145 µl	800 µl
CaCl2 (2.5 M)	20 µl	125 µl
Plasmid DNA	5 µg	15 µg
HEBS buffer	170 µl	1100 µl

2.2.2.4 Transient transfection of primary hippocampal neurons

Primary hippocampal neurons at seven days in vitro (DIV) were transfected with 0.75 µg of HA-rCB1wt (Nr 6 in table 2.9) or HA-rCB1F238L (Nr 7 in table 2.9) constructs using Lipofectamin®2000. Prior to transfection coverslips with cells were transferred to wells containing 1 ml of transfection medium. 1.5 µg plasmid DNA was mixed with 100 µl Neurobasal medium and vortexed briefly. 1.125 µl of Lipofectamin®2000 were mixed with 100 µl Neurobasal medium, vortexed briefly and

incubated at room temperature. After 5 min the Lipofectamin®2000 dilution and the plasmid dilution were mixed and incubated at room temperature for 20 min. Subsequently the mixture was added to the coverslips drop wise and coverslips were placed into the incubator at 37°C. After 1.5 h coverslips were placed back into their conditioned medium and kept in the incubator. After 48 h the cells were used for immunocytochemistry.

2.2.2.5 Stable transfection of HEK293 cells

20 µg of the plasmid DNA were linearized by restriction with PvuI-HF in NEB buffer 4 and 100 µg/ml bovine serum albumin (BSA). The digestion incubated at 37°C over-night and linearization was validated by analyzing 1 µl of digestion product on an agarose gel containing ethidiumbromide. As a control the same amount of circular plasmid was loaded. Linearized plasmid DNA was purified via phenol-chloroform extraction. 1 volume of Phenol-Chloroform-Isoamylalcohol-Mix was added to 1 volume of linearized DNA and vortexed for 15 sec. After centrifugation for 15 sec with 1000 g at room temperature, 1 volume of the upper phase was mixed with 1 volume of chloroform and vortexed. After centrifugation for 15 sec with 1000 g at room temperature, 0.1 volume of the upper phase were mixed with 3 M Na-Acetate pH 5.2 and 2 volumes of 100 % (v/v) ethanol and the mixture incubated at -20°C for 1 h. After centrifugation with 18,000 g at 4°C for 5 min the pellet was washed in 70 % (v/v) ethanol in H_2O and centrifuged again with 18,000 g at 4°C for 5 min. The supernatant was discarded, the pellet air-dried under a sterile hood and re-suspended in sterile PBS. The DNA concentration was measured by spectrophotometry using a Nanodrop device. HEK293 cells were transfected with 24 µg of linearized DNA (table 2.23) in a 10 cm cell culture dish using Lipofectamine®2000 following the manufacturer`s instructions. 48 h after transfection, the medium was changed to plating medium containing the appropriate selection agent as listed in table 2.23. HEK293 cells were transfected with 24 µg of linearized DNA (table 2.23) in a 10 cm cell culture dish using Lipofectamine®2000 following the manufacturer`s instructions. 48 h after transfection, the medium was changed to plating medium containing the appropriate selection agent as listed in table 2.23.

Cell line	Transfected cells	Transfected plasmids	Selection agent in culture medium
HEK^{CB1wt}	HEK293	pcDNA3_HA-rCB1wt	1 mg/ml G418
$HEK^{CB1F238L}$	HEK293	pcDNA3_HA-rCB1F238L	1 mg/ml G418
HEK^{D2L}	HEK293	pcDNA3_Zeo_Flag-D2L	40 µg/ml zeocin
$HEK^{D2L+CB1wt}$	HEK^{CB1wt}	pcDNA3_Zeo_Flag-D2L	1 mg/ml G418 + 40 µg/ml zeocin
$HEK^{D2L+CB1F238L}$	$HEK^{CB1F238L}$	pcDNA3_Zeo_Flag-D2L	1 mg/ml G418 + 40 µg/ml zeocin

Table 2.23

Cells were allowed to grow and passaged when necessary. After two passages, cells were detached from the plate by trypsin treatment, pelleted and re-suspended in plating medium.

Cells were counted using a Neubauer cell counter and the suspension was adjusted to 4 cells per ml. 250 µl of cell suspension was added per well of a 96 well plate. After 8 days colonies were visible in the single wells. Clones were picked only from those wells that contained only a single colony. Receptor expression in the picked colonies was examined and quantified by immunocytochemistry and western blot analysis.

The cell lines were cultured as described in section 2.2.2.1 except that the selection agent was present in the culture medium at all times (table 2.23).

2.2.2.6 Immunocytochemistry with HEK293 cells

HEK293, HEK^{CB1wt}, $HEK^{CB1F238L}$, HEK^{D2L}, $HEK^{D2L+CB1wt}$ or $HEK^{D2L+CB1F238L}$ cells were seeded on poly-L-Lysin coated coverslips. After 48 h cells were washed in PBS and fixed in 4 % PFA in PBS for 10 min. After washing with PBS, cells were permeabilized with 0.05 % (v/v) Triton-X100 in PBS for 5 min. After blocking with 4 % (v/v) goat serum in PBS for 15 min, primary antibody in 4% goat serum was added for 2 h. After two 5 min washes with PBS, fluorophore conjugated secondary antibody in 4 % goat serum was added for 30 min. Subsequently, cells were washed once for 5 min in PBS and nuclei were

stained with 4',6-Diamidin-2-phenylindol (DAPI) for 5 min. Cells were washed once in PBS and mounted on moviol. All steps were carried out at room temperature.

2.2.2.7 Co-localization assay

Assay principle

To test postendocytic trafficking of the CB1F238L receptor, co-localization experiments with markers for two different endocytic compartments, lysosomes and early endosomes/recycling endosomes, were performed. An enhanced green fluorescent protein (EGFP) tagged form of the CB1wt or CB1F238L receptor was transiently transfected into HEK293 cells. Immunocytochemistry in these cells was performed using an antibody against Lamp2 to visualize lysosomes. To mark recycling endosomes transferrin receptors (TrfR) were stained with fluorescently labeled transferrin (Invitrogen). Subsequently, images were taken to visualize CB1 receptors and the respective markers. Quantitative co-localization analysis was performed gaining two measures of correlation between the green (CB1 receptor) and red (Lamp2 or TrfR) channels which are the Pearsons correlation coefficient (PCC) and one of the two Manders coefficients (M1 and M2) (Manders 1993).

The Pearsons correlation coefficient describes how well the pixel intensity patterns correlate between two channels. It is described by equation 2.1 where R_i is the intensity of the red channel in pixel i, R_{aver} is the average intensity of the red channel over all pixels, G_i is the intensity of the green channel in pixel I and G_{aver} is the average intensity of the green channel over all pixels. The Pearsons correlation coefficient can range from -1 (which indicates maximal anti-correlation) over 0 (which indicates no correlation) to 1 (which indicates maximal correlation). As for each pixel the average intensity is subtracted, this coefficient is insensitive to constant background. However, PCC is sensitive to the intensity of the two channels in each pixel and to the number of objects in the respective channels.

$$PCC = \frac{\sum_i (R_i - R_{aver}) \times (G_i - G_{aver})}{\sqrt{\left[\sum_i (R_i - R_{aver})^2 \times \sum_i (G_i - G_{aver})^2\right]}}$$

Equation 2.1 Calculation of the Pearsons correlation coefficient PCC

If the number of red objects, for instance, is much higher than the number of green objects, than the Pearsons correlation coefficient will be very low, even if all green pixels were also red (Fig. 2.1). In many biological samples this is the case. Furthermore, in biological samples it is not only interesting to know how well two channels correlate in a picture, but one would rather like to know the proportion of one channel that correlates with the other (i.e. how much CB1 receptor correlates with Lamp2). For this purpose the Manders coefficients M1 and M2 can be determined, which are more meaningful with respect to biological questions (Manders 1993). The Manders coefficients are calculated by equation 2.2 where R_i is the intensity of the red channel in pixel i and G_i is the intensity of the green channel in pixel i. For M1, $R_{i,\,coloc}$ either equals the value of R_i if the intensity of the green channel in pixel i is bigger than 0 or it equals 0 if the intensity of the green channel in pixel i is 0. The same principle applies for M2.

$$M_1 = \frac{\sum_i R_{i,coloc}}{\sum_i R_i}, \qquad M_2 = \frac{\sum_i G_{i,coloc}}{\sum_i G_i}$$

$$R_{i,coloc} = R_i \;\; if \;\; G_i \; > 0 \; and \; R_{i,coloc} = 0 \;\; if \;\; G_i = 0$$

$$G_{i,coloc} = G_i \;\; if \;\; R_i \; > 0 \; and \;\; G_{i,coloc} = 0 \;\; if \;\; R_i = 0$$

Equation 2.2 Calculation of the two Manders coefficients M1 and M2

This makes the Manders coefficients independent of the intensity values of overlapping pixels. However, it also makes M1 and M2 sensitive to background which is why a threshold was set manually for each picture in the experiments of this work. M1 indicates the fraction of the red channel that co-localizes with green. M2 indicates the fraction of the green channel that co-localizes with red.

To distinguish significant from random co-localization, the costes randomization algorithm was applied using 200 randomizations. This algorithm, according to Costes et al. (Costes 2004), generates 200 pictures in which it scrambles the pixels of the original picture randomly. For each of these pictures it generates a Pearsons correlation coefficient and compares it with the correlation coefficient of the original picture. If more than 95% of the scrambled pictures yield a correlation coefficient smaller than the one obtained for the original picture, the correlation coefficient for the original picture is considered statistically significant.

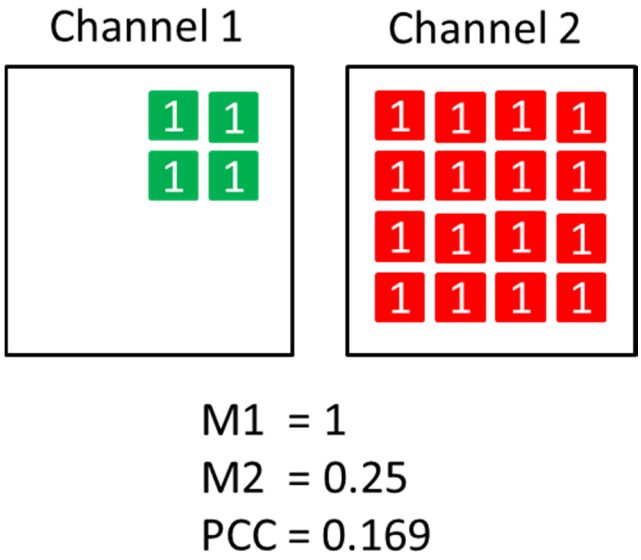


Fig. 2.1 The Manders and Pearsons correlation coefficient

All green pixels in channel 1 co-localize with red pixels in channel 2. However, not all red pixels in channel 2 co-localize with green pixels in channel 1. The Pearsons correlation coefficient only describes the correlation between the two channels and is hence very low in this example. However, the Manders coefficients M1 and M2 can distinguish between green pixels that co-localize with red pixels (M1) and vice versa (M2). Hence, M1 is high whereas M2 is low. The Manders coefficient and especially M1 are more meaningful with regards to the biological question addressed in this work.

Due to the point spread function of the microscope, the intensity of one pixel correlates with the intensity of its neighboring pixels. This is why pixels were divided into blocks with the approximate size of the calculated point spread function for costes randomization. Instead of pixels, these blocks were scrambled during costes randomization.

Assay protocol

HEK293 cells plated on poly-L-Lysine coated coverslips were transiently transfected with CB1wt-EGFP (Nr 8 in table 2.9) or CB1F238L-EGFP (Nr 9 in table 2.9).

Transferrin receptor immunocytochemistry

Cells were kept in serum free medium for 1 h prior to the experiment. Transferrin-Alexa546 conjugate solved in PBS was added to the cells in a final concentration of 5 µg/ml for 10 min at 37°C. Cells were washed with PBS 2 times and fixed in 4 % PFA in PBS for 10 min. After washing the cells with PBS, coverslips were mounted on moviol. If not indicated otherwise, all steps were carried out at room temperature.

Lamp 2 immunocytochemistry

Cells were kept in serum free medium for 1 h prior to the experiment. Lamp2 was labeled as described in section 2.2.2.6 using a monoclonal antibody against Lamp2.

Image acquisition

Z-stack pictures were taken using the 100 x oil immersion objective (oil refractive index n=1.518) of a Zeiss LSM-710 confocal microscope and the Zen 2011 SP2 software. DAPI was excited with 405 nm and emission was filtered by a 410 nm - 585 nm filter. GFP was excited with 488 nm and emission was filtered by a 495 nm – 556 nm filter. Alexa546 was excited with 543 nm and emission was filtered by a 548 nm – 680 nm filter. To improve the signal to noise ratio, pictures were taken with 4 x averaging of each channel in a frame scanning mode. Picture properties were 8 bit depth, 1024 x 1024 pixel resolution and a voxel size of (X) 80 nm x (Y) 80 nm x (Z) 38 nm for Lamp2 co-localization and (X) 60 nm x (Y) 60 nm x (Z) 17 nm for TrfR co-localization.

Quantitative analysis of co-localization

Co-localization of the CB1 receptor with Lamp2 or TrfR in Z-Stack images was analyzed using the JACOP plugin of the ImageJ Fiji software. A region of interest (ROI) containing only one cell was chosen and pixels outside the ROI were deleted. Images were cropped to fit the ROI. Pixel based analysis algorithms were used, yielding the Pearsons correlation coefficient and the two Manders coefficients (Manders 1993). To distinguish between significant and random co-localization the costes randomization method was applied (Costes 2004) using a xy block size of 2 pixels (120 nm) and a z block size of 1 pixel (170 nm). In the experiments of this work all Pearsons correlation coefficients or Manders coefficients yielded a costes significance of 100 %.

2.2.2.8 Polarization assay

Assay principle

In neurons the CB1 receptor is predominantly targeted to the axonal plasma membrane. The extend of this polarization can be described by the polarization index A/D were A is the amount of surface CB1 receptor in axonal membranes and D is the amount of surface CB1 receptor in dendritic membranes (Leterrier 2006). A polarization index smaller than 1 indicates polarization towards dendrites. Accordingly, a polarization index larger than 1 indicates polarization towards the axon (Leterrier 2006). To test the polarization behavior of the CB1F238L receptor, primary hippocampal

neurons were transfected with HA-CB1wt or HA-CB1F238L receptor and immunohistochemistry was performed. First, surface CB1 receptor was labeled by incubating the living neurons with a monoclonal HA antibody. After fixation and permeabilization, total CB1 receptor (intracellular and surface) was labeled using the same antibody. In parallel MAP2 was stained to label the somatodendritic compartment. Images were taken from cells which displayed similar CB1 receptor expression levels (visually estimated from total CB1 receptor staining) and surface CB1 receptor expression in dendrites and different parts of the axon was quantified.

Assay protocol

Immunocytochemistry

DIV 5 primary hippocampal neurons were transfected with HA-CB1wt or HA-CB1F238L receptor. After 48 h they were placed in pre-warmed HBS buffer and were allowed to adjust for 5 min at room temperature. Primary HA antibody (Sigma 1:200) in HBS buffer was added to the coverslips for 20 min. After washing the coverslip 3 times in HBS, cells were fixed with pre-warmed PFA solution (4 % (v/v) paraformaldehyde, 5 % (w/v) sucrose in PBS). After 15 min the coverslips were washed 3 times in PBS and residual PFA was quenched by adding 50 mM ammonium chloride in PBS for 5 min. Subsequently, coverslips were washed 3 times with PBS and blocked with 10 % (v/v) horse serum in PBS for 10 min. Cy3 conjugated secondary antibody was applied in 5 % (v/v) horse serum in PBS for 45 min in the dark. After 3 washes with PBS, cells were permeabilized with 0.1 % (v/v) TritonX100 in PBS for 10 min, washed 2 times with PBS and blocked with 10 % (v/v) horse serum for 20 min. Primary antibodies against HA (Sigma 1:400) and MAP2 in 5 % (v/v) horse serum were added to the coverslips for 1 h. After washing 3 times with PBS, Cy2 conjugated anti mouse secondary antibody and Cy5 conjugated anti rabbit secondary antibody were added for 1.5 h. Subsequently, coverslips were washed 3 times in PBS and mounted in fluoromount. All steps were performed at room temperature.

Image acquisition

Cells were imaged using the 40 x oil objective of a Zeiss LSM510 UV META Axiovert 200M laser scanning microscope and the Zeiss LSM Data Server software. Cy2 was excited with an Argon/2 laser at 488 nm and emission was detected using a 505-530 band pass filter. Cy3 was excited with a helium neon laser at 543 nm and emission was detected using a 560-615 band pass filter. Cy5 was excited with a helium neon laser at 633 nm and emission was detected using a 650 long pass filter. Z-stacks of four optical slices per cell were recorded with 4 x averaging, 1024 x 1024 pixels and 8 bit depth. If necessary, multiple pictures per cell were collected to capture the entire neuron.

Quantitative analysis of fluorescence intensity

Quantification of fluorescence was performed using the ImageJ software. Maximum projections were used to quantify surface CB1 receptor fluorescence. 20 µm x 1 µm ROIs from dendrites (Map2 positive) and proximal, intermediate and distal parts of the axon (Map2 negative) were selected and fluorescence intensity was measured for the red channel (surface CB1 receptor). To avoid any bias, ROIs where selected in merged pictures showing only the green (total CB1 receptor) and blue (Map2) channel. Average axonal surface CB1 receptor was calculated by taking the average of surface CB1 receptor in proximal, intermediate and distal parts of the axon. Average dendritic surface CB1 receptor was calculated by taking the average of surface CB1 receptor in two to three different dendrites of one cell. The surface CB1 receptor polarization index (A/D ratio) for each cell was determined by dividing average axonal surface CB1 receptor (A) by average dendritic surface CB1 receptor (D). To measure the gradient of surface CB1 receptor expression from the proximal to the distal axon, values for the intermediate and distal axon were expressed as percent of the value for the proximal axon. The experimenter was blind to the genotype throughout the imaging and analysis procedure to avoid any bias.

2.2.3 Protein biochemical methods

2.2.3.1 SDS-PAGE and Western blotting

Assay principle

Sodiumdodecylsulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) is a method to separate proteins according to their size. Proteins were mixed with sample buffer containing reducing and denaturing agents (β-mercaptoethanol, SDS). Additionally to denaturing the protein, the negatively charged SDS covers the charge of the proteins and allows their electrophoretical separation according to their size. After separation, the proteins are electrophoretically transferred to a nitrocellulose membrane were they can be detected by antibodies and enhanced chemiluminescence.

Assay protocol

Cell lysate preparation

Cells were washed two times with ice cold PBS and scraped into lysisbuffer. For the ERK1/2 phosphorylation experiment, cells were directly scraped into lysisbuffer without the PBS washing steps. Cells in lysisbuffer either lysed on a rotor at 4°C for 1 h (ERK1/2 phosphorylation experiment) or overnight when receptors were to be analyzed in the subsequent western blot. Nuclei and debris were removed by centrifugation at 1000 g and 4°C for 10 min. The supernatant was collected and the protein concentration was determined by Bradford assay. 10 - 20 µg of protein was mixed with 5 x sample buffer. If receptors were to be analyzed, the samples were not heated to avoid aggregation of the hydrophobic proteins. For the ERK1/2 phosphorylation assay, samples were heated to 95 °C for 5 min.

Brain lysate preparation

The striata of hemispheres of 2 wild-type and 2 mutant rats were separately homogenized in a glass dounce homogenizer in 500 µl of D2 binding membrane buffer. The protein concentration was determined by Bradford assay. 10 µg of protein were mixed with extraction buffer and rotated for 1 h at 4°C. 5 x sample buffer was added.

SDS-PAGE and Western blot

Proteins were separated on a 10 % polyacrylamide gel in running buffer for 2 h with a constant voltage of 80 V. The SDS-PAGE gel was composed of a resolving gel and an overlaid stacking gel with the following composition:

Resolving gel

H_2O	5.7 ml
1.5 M Tris-HCl; pH 8.8	3.0 ml
SDS 20%	60 µl
Acrylamid/ Bisacrylamid 40%	3.0 ml
TEMED	6 µl
APS	60 µl

Stacking gel

H_2O	4.4 ml
1.5 M Tris-HCl; pH 8.8	0.76 ml
SDS 20%	30 µl
Acrylamid/ Bisacrylamid 40%	0.76 ml
TEMED	6 µl
APS	60 µl

Subsequently proteins were transferred to a nitrocellulose membrane by wet blotting in transfer buffer for 75 min with constant 300 mA. Membranes were stained with ponceau for 5 min to control for successful transfer of proteins to the membrane. After that, the membranes were blocked with 5 % (w/v) nonfat dry milk in TBST for 1 h at room temperature and primary antibody was added in 5 % (w/v) nonfat dry milk over-night at 4°C. Primary antibody was removed and after 3 x 10 min washes with TBST, horseradish peroxidase coupled secondary antibody was added in 5 % (w/v) nonfat dry milk and incubated for 30 min at room temperature. After 2x 10 min washes with TBST and a 10 min wash with PBS, membranes were incubated with ECL solution according to the manufacturer's instructions and imaged with the Peqlab Fusion-SL system using the fusion software version 15.16 by Vilber lourmat. Quantification of western blot pictures was performed using the bio1D software version 15.02 by Vilber lourmat. Calculation of molecular weight bands was performed using the fusion software 15.16 by Vilber lourmat.

2.2.3.2 Basal [^{35}S]GTPγS binding assay

Assay principle

The [^{35}S]GTPγS binding assay is used to measure the coupling of heterotrimeric G proteins to a GPCR. Coupling to G proteins can be induced by agonist stimulation but it can also be caused by basal activity of the receptor. When an active receptor binds a heterotrimeric G protein, the GDP which is bound to the Gα subunit is exchanged by GTP. Under physiological conditions the GTPase domain of

the Gα subunit hydrolyses GTP to GDP which allows the heterotrimeric G proteins to reassemble. However, under the conditions in this assay [^{35}S]GTPγS substitutes endogenous GTP (Harrison 2003). The γ-triphosphate bond in [^{35}S]GTPγS is resistant to hydrolysis which leads to the accumulation of [^{35}S]GTPγS bound Gα proteins (Harrison 2003). Using a filter, [^{35}S]GTPγS bound Gα proteins can be harvested whereas unbound [^{35}S]GTPγS is washed away. As more active receptor species are in the assay or as higher the ability of the receptor to activate G proteins as more [^{35}S]GTPγS bound Gα proteins are generated and as more radioactivity remains in the filter. Hence, by measuring the amount of radioactivity remaining in the filter one can draw conclusions about the activity state of the receptor (Harrison 2003).

Assay protocol

HEK^{CB1wt} and HEK^{CB1F238L} cells were washed two times with PBS and scraped into membrane buffer. Cells were homogenized by 20 strokes in a glass dounce homogenizer and nuclei and debris was removed by centrifugation at 1000 g and 4°C for 10 min. Crude membranes were pelleted by centrifugation of the supernatant at 100,000 g and 4°C for 1 h. The pellet was resuspended in 1 ml of membrane buffer with 10 strokes in a glass dounce homogenizer. The protein concentration of homogenates was determined by Bradford assay and adjusted with membrane buffer. Protein, GDP, cold GTP, BSA and [^{35}S]GTPγS were mixed in a final volume of 1 ml as indicated below. Per experiment quadruplicates were measured for each genotype. All constituents were dissolved in membrane buffer.

	Binding	**Nonspecific binding**
GDP	1 µM	1 µM
Cold GTP	/	10 µM
BSA	1 % (w/v)	1 % (w/v)
Protein	5 µg	5 µg
[^{35}S]GTPγS	1 nM	1 nM

The reaction incubated for 1 h at 30°C under agitation and the reaction was stopped by addition of ice cold washing buffer and rapid filtration. 2.5 ml of scintillation fluid was added to the filter and scintillation was counted. Specific binding was determined by subtraction of nonspecific binding.

The CB1 receptor content for each membrane homogenate was determined by western blot analysis. 1 µl of 20 % CHAPS (w/v) in dH_2O was added to 19 µl of membrane homogenate to gain a final concentration of 1 % CHAPS (w/v) and incubated over-night under rotation. The resulting 20 µl of protein lysate was separated by SDS-PAGE and analyzed by western blot. Specific binding was normalized against CB1 expression.

2.2.3.3 ERK1/2 phosphorylation assay

Assay principle

As described above, activation of the CB1 receptor can lead to various cellular responses one of which is the phosphorylation of ERK1/2. Hence, in this experiment the effect of the CB1F238L receptor mutation on basal and agonist induced ERK1/2 phosphorylation was to be examined. HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated either with vehicle, with the inverse agonist SR141716, or the agonist WIN55212-2. Subsequently, the phosphorylation of ERK1/2 was determined by western blot using antibodies specific for phosphorylated or total ERK1/2. To describe the degree of ERK1/2 phosphorylation phosphoERK1/2 was divided by total ERK1/2.

Assay protocol

1h prior to the experiments HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were kept in serum free medium. Subsequently, serum free medium containing vehicle (DMSO), 100 nM WIN55212-2 or 100 nM SR141716 was added to the cells and incubated at 37°C. After 5 min cells were lysed and processed for western blotting. Phosphorylated and total ERK1/2 were detected on the western blot using a monoclonal antibody against phosphoERK1/2 and a polyclonal antibody against total ERK1/2, respectively.

To describe the degree of ERK1/2 phosphorylation phosphoERK1/2 was divided by total ERK1/2. Relative values of ERK1/2 phosphorylation were gained by normalizing agonist or inverse agonist treated samples to vehicle treated samples of the same genotype. Absolute values of ERK1/2 phosphorylation were gained by first normalizing all values against CB1 receptor expression. Secondly, all groups were normalized against CB1wt receptor vehicle treated samples. CB1 receptor

expression levels were determined for each experiment by probing the same membrane with antibodies against HA and tubulin. HA expression levels were determined by dividing HA by tubulin.

2.2.3.4 Equilibrium binding assay

Assay principle

In this assay the affinity of a receptor for a given ligand can be concluded from its ability to replace another ligand which is radioactively labeled. A constant concentration of the radiolabeled ligand ([^{3}H]CP55940 for the CB1 receptor and [^{3}H]raclopride for the D2 receptor) and various concentrations of the cold ligand to be examined (SR141716, WIN55212-2, CP55940 for the CB1 receptor and dopamine for the D2 receptor) were added to membrane preparations containing the receptor of interest. The cold ligand competes with the labeled ligand for binding sites at the receptor. Hence, increasing concentrations of the cold ligand lead to a reduction of receptor bound radioligand. After filtration only receptor bound radioligand will be harvested, unbound radioligand will be washed away. By plotting remaining radioligand against the concentration of cold ligand an IC_{50} (**i**nhibitory **c**onstant) can be determined, which is the concentration of cold ligand that replaces 50 % of the labeled ligand. As higher the IC_{50} as higher concentrations of cold ligand are needed to replace 50 % of the labeled ligand. The pIC_{50} is the negative logarithm of IC_{50}. Hence, from an increased IC_{50} (or a decreased pIC_{50}) a reduction in the affinity of the cold ligand for the receptor binding site can be concluded and vice versa.

In contrast to the before mentioned ligands, the allosteric modulator ORG27569 does not compete with the radioligand for CB1 receptor binding sites but has been shown to increase [^{3}H]CP55940 binding to the receptor (Ahn 2012). Hence, plotting ORG27569 concentrations against receptor bound [^{3}H]CP55940 shows an increase in total [^{3}H]CP55940 bound which yields the maximum occupancy of the receptor (E_{max}).

Assay protocol for CB1 receptor equilibrium binding

HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were scraped into cold PBS and pelleted with 2000 g at 4°C for 4 min. After the cells where re-suspended in cell buffer, the protein content was measured by Bradford assay. Binding experiments were performed by Dr. Gemma Baillie at the University of Toronto. Equilibrium binding assays were performed using the cannabinoid receptor agonist [^{3}H]CP55940, at a concentration of 0.7 nM. 1 mg/ml BSA and 50 mM Tris/HCl buffer was used in a total assay volume of 500 µl containing 0.01 % DMSO. Binding was initiated by adding 1 mg/ml of cell suspension. Assays

were incubated at 37°C for 60 min. The reaction was stopped by the addition of ice cold wash buffer that contained 1 mg/ml BSA and vacuum filtration using a 24-well sampling manifold Brandel cell harvester. Each reaction tube was washed 6x with a 500 µl aliquot of buffer. The filters were then oven dried for 60 min, placed scintillation vials with 4 ml of scintillation fluid and radioactivity was quantified by liquid scintillation spectrometry. Specific binding is defined as the difference between the binding that occurred in the presence and absence of 1 µM CP55940 and varied between 70% and 90% of the total binding.

Assay protocol for D2 receptor equilibrium binding

HEK293 cells were transiently transfected either with Flag-rD2L receptor (Nr 11 in Table table 2.9) alone or with Flag-rD2L receptor and HA-rCB1wt receptor (Nr 4 in Table table 2.9). Cells were washed two times with PBS and scraped into membrane buffer. After homogenization with 20 strokes in a glass dounce homogenizer, nuclei and debris were pelleted by centrifugation at 1000 g and 4°C for 10 min. Crude membranes were pelleted by centrifugation of the supernatant at 50,000 g and 4°C for 2 h. The membrane pellet was re-suspended in binding buffer. The protein content of the suspension was determined by Bradford assay. Binding reactions were performed in a total volume of 300 µl. First, buffer was added to the reaction tube, than various concentrations of dopamine were added followed by (+)-butaclamol for determination of non-specific binding. After that, protein and subsequently [^{3}H]raclopride were added. Concentrations were as depicted below.

	Data points	**Maximum binding**	**Nonspecific binding**
Binding buffer	Fill up to 300 µl	Fill up to 300 µl	Fill up to 300 µl
Dopamine	10 nM – 1 mM	/	/
(+) - butaclamol	/	/	10 µM
Protein	80 µg/reaction	80 µg/reaction	80 µg/reaction
[^{3}H]raclopride	2 nM	2 nM	2 nM

The reaction incubated at 30°C for 45 min to equilibrium. The reaction was stopped by addition of ice cold washing buffer and rapid filtration. After 4 washes with washing buffer filters were added to scintillation fluid, vortexed for 20 sec and allowed to stand over-night. The next day scintillation was counted. Experiments were performed in duplicates. The mean for each data point as well as maximum binding (binding of [^{3}H]raclopride in the absence of any dopamine) and non-specific binding (binding of [^{3}H]raclopride in the presence of (+)-butaclamol) was calculated. Non-specific binding was subtracted from all other values and data points were expressed as percent of maximum binding.

2.2.3.5 Trypsin protection assay

Assay principle

To quantitatively determine the surface expression of tagged receptors a modification of the protocol published by Grimsey et al. was used (Grimsey 2010). This protocol exploits the property of trypsin to enzymatically cleave the N-terminal tag of the receptor which is located at the extracellular side. However, intracellular receptor is protected from trypsin. This means that in a western blot using an antibody against the N-terminal tag, the band in trypsin treated samples represents intracellular receptor whereas the band in the control treated samples represents total receptor. Hence, surface CB1 receptor can be calculated by equation 2.3.

$$\%\, Surface\ CB1 = 100 - \left(\frac{Intracellular\ CB1}{Total\ CB1} \times 100\right)$$

Equation 2.3 Calculation of the percentage of surface CB1 receptor in the trypsin protection assay

Assay protocol

HEK^{CB1wt} and HEK^{CB1F238L} cells were placed into serum free medium 1 h prior to the experiment. For endocytosis experiments cells were either treated with 100 µM WIN55212-2 or 100 µM SR141716 in serum free medium for 45 min, with 450 mM sucrose or 5 mM MβCD in serum free medium for 30 min or with tetrahydrolipstatin (THL) for 2 h. Afterwards, the medium was removed and either 2 ml of versene (control) or trypsin solution were added. After 4 min 10 ml of cold plating medium was added to stop the enzymatic reaction. Cells were transferred to a 15 ml tube, pelleted at 4°C and 100 g for 5 min and washed two times with cold PBS to remove any residual trypsin. The pellet was re-suspended in lysis buffer and the lysate was processed for western blotting. Signals for HA were

normalized against tubulin or actin. Percent intracellular CB1 receptor was calculated by dividing normalized HA signal in trypsin treated cells by normalized HA signal in versene treated cells and multiplied by a hundred. In conclusion percent surface CB1 receptor was calculated by subtracting percent intracellular CB1 receptor from a hundred (equation 2.3).

2.2.3.6 Detergent free lipid raft preparation

To prepare lipid rafts from HEK293 cells a detergent free sucrose gradient protocol was used. HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were placed into serum free medium 1 h prior to the experiment. When the effect of SR141716 on lipid raft allocation of the CB1 receptor was tested, cells were treated with 100 µM SR141716 in serum free medium for 45 min. Medium was removed and cells were scraped into cold PBS and pelleted with 100 g and 4°C for 5 min. The supernatant was removed, the pellet was re-suspended in 900 µl ice cold 45 % (v/v) sucrose in TBS and sonicated with 3 strokes for 10 sec. The homogenates were passed through a 25 G cannula for 20 times. 800 µl of the homogenate was placed into an ultracentrifuge tube and overlayed with 2700 µl of 30 % (v/v) sucrose in TBS and 500 µl of 5 % (v/v) sucrose in TBS. The sucrose gradient was centrifuged in a Beckmann ultracentrifuge using a SW40TI rotor at 4°C and 100,000 g for 26 h. Subsequently, 8 x 400 µl fractions and one 800 µl fraction were collected from the top (fraction 1) to the bottom (fraction 8). The 800 µl bottom fraction included the re-suspended pellet. 8 µl of each fraction were mixed with 5 x sample buffer and analyzed by western blot. The lipid raft marker caveolin-1 was used to validate enrichment of lipid rafts in low density fractions. Western blot signals for HA where normalized against caveolin-1 signals. For each fraction the relative amount of normalized HA content was expressed as percentage of total normalized HA signal of all fractions. The sum of values for fractions 2-4 were taken as lipid raft content. The sum of values for all other fractions was taken as non-raft content. Values for lipid raft associated CB1F238L receptor were expressed as percentage of values for lipid raft associated CB1wt receptor.

2.2.3.7 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) of the D2 and CB1 receptor was performed using a protocol modified from Kearn et al. (Kearn 2005).

HEK293 cells were transiently transfected with HA-rCB1wt receptor (Nr 14 in table 2.9) alone or together with rD2L receptor (Nr 13 in table 2.9). Cells were placed on ice and washed 2 times with

cold PBS. The cells were scraped into ice cold PBS and pelleted at 100 g and 4°C for 10 min. The cell pellet was re-suspended in IP buffer and lysed under rotation at 4°C for 45 min. Nuclei and debris were removed by centrifugation at 14,000 g and 4°C for 10 min. The protein concentration of the supernatant was determined by Bradford assay and adjusted to 200 µg/ml with IP buffer. 40 µl containing 16 µg of protein were kept as "input" for western blot analysis.

A preclear was performed to determine whether the target protein binds un-specifically to the beads. 50 µl sepharose-A bead slurry was washed 2 times with IP buffer. All bead washing steps throughout the protocol were carried out by centrifugation at 16,000 g and 4°C for 20 sec. 200 µg of protein in 1 ml were added to the beads and incubated under rotation at 4°C for 30 min. The supernatant was removed and used for IP. The preclear-beads were kept overnight at 4°C. For IP, 4 µl (4 µg; 1:250) of polyclonal primary antibody against the D2 receptor was added to the precleared lysate and incubated on a rotor over night at 4°C. 50 µl sepharose-A beads were washed 2 times with IP buffer and the protein-antibody mix was added. After incubation under rotation at 4°C for 1 h the supernatant was removed.

The preclear- as well as the IP-beads were washed with cold IP buffer for 4 times and 1 time with cold PBS. Protein from preclear- and IP-beads as well as from the input was denatured by addition of 1x sample buffer and analyzed by western blot analysis using a monoclonal antibody against the HA tag of the CB1 receptor.

2.2.3.8 Receptor autoradiography

Coronal cryosections of CB1F238L mutant rat brains were produced. Slides carrying the cryosections were kept at -80°C for long term storage. D2 receptor autoradiography was performed as described by Zavitsanou et al. (Zavitsanou 2010). Slides containing the striatum were pre-incubated in autoradiography buffer for 20 min at room temperature. The slides then incubated in the same buffer containing 4 nM [^{3}H]raclopride at room temperature in the presence (control for non-specific binding) or absence of 10 µM (+) – butaclamol. After 60 min the slides were washed 2 times for 5 min in ice cold buffer at 4°C. Buffer was removed from slides by dipping in dH_2O and slides were air dried over-night. Subsequently a tritium sensitive screen was exposed to the slides for 5 days and measured in a phosphoimager. Grey values of three ROIs were quantified using the OptiQuant™ software. The ROIs were the entire striatum, the lateral striatum and the nucleus accumbens. Background was determined by measuring grey values of an area outside the brain slice and subtracted from grey values of the ROIs. Grey values of non-specific binding were subtracted

manually before statistical analysis. A standard curve was determined using a tritium standard (American Radiolabeled Chemicals) to estimate nCi/mg from grey values. Grey values were measured from 18 hemispheres of 3 mutant and 2 wild-type rats. Each hemisphere was considered as one n.

3. RESULTS

The CB1F238L mutant rat showed behavioral as well as electrophysiological and biochemical changes that indicate differences in CB1 receptor function (Schneider 2014). However, how the point mutation affects CB1 receptor signaling and its subcellular distribution and trafficking is difficult to examine in a complex organism such as a rat. Hence, in this work, a simpler model system was generated to address possible effects of the point mutation on CB1 receptor cell biology. The sequence for an HA epitope was added N-terminally to the wild-type (CB1wt) and F238L mutant rat CB1 receptor (CB1F238L) cDNA and cloned in a eukaryotic expression vector. HEK293 cells, a commonly used model to study GPCR functions (Atwood 2011), were generated which stably express the HA tagged forms of CB1wt (HEK^{CB1wt}) and CB1F238L ($HEK^{CB1F238L}$). Using these cells, the subcellular distribution, trafficking and signaling of the receptor were examined. Axonal polarization of the mutant receptor in primary neurons was assessed by ectopically expressing CB1wt or CB1F238L receptors in rat primary hippocampal neurons.

There is evidence pointing to an effect of the point mutation in the CB1 receptor on dopaminergic signaling. To address this, the interaction of the CB1wt receptor with the D2 receptor was assessed by co-immunoprecipitation and radioligand binding. Furthermore, the expression of D2 receptors in the striatum of CB1F238L mutant rats was measured by means of receptor autoradiography and western blot. To further analyze the effect of the CB1 receptor point mutation on D2 receptor function, HEK293 cell lines were generated which express the D2 receptor alone or together with the wild-type or mutant CB1 receptor.

3.1 Cloning of HA tagged forms of the CB1wt or CB1F238L receptor

An HA tag was added to the sequence of the CB1wt and CB1F238L receptor, respectively, by PCR and was subsequently cloned into the pcDNA3 expression vector. To improve protein expression, a Kozak sequence was added upstream to the HA-receptor sequence. Transgene expression is controlled by a cytomegalovirus promotor (P CMV). Furthermore, the vector contains a neomycin gene for the expression of an aminoglycoside phosphotransferase. Cells expressing this phosphotransferase gain resistance against the antibiotic geneticin (G418), which allows the selection of eukaryotic cells that were stably transfected with this vector by addition of geneticin to the culture medium. The vector contains an ampicillin resistance gene to select transformed bacteria (Fig. 3.1).

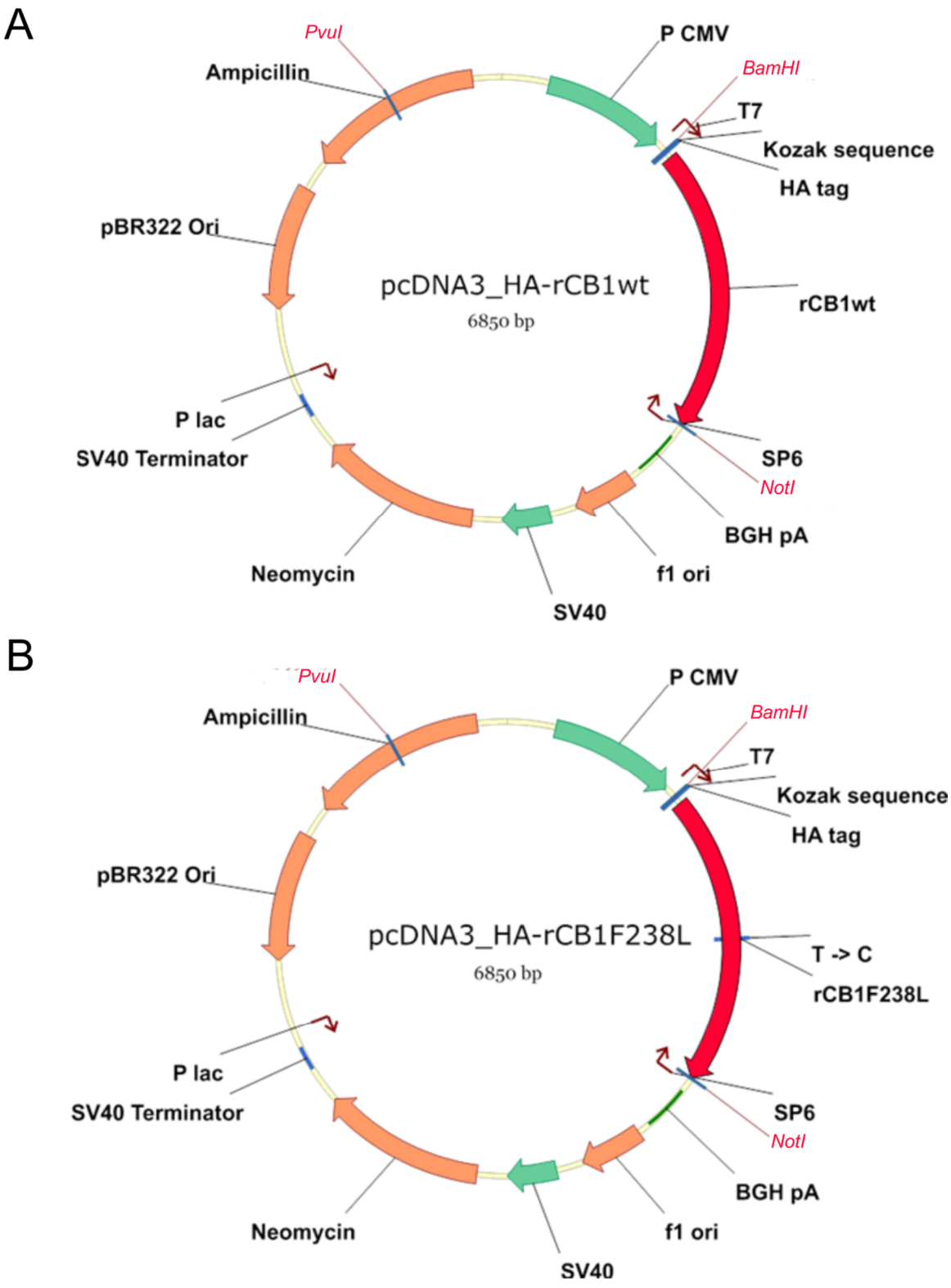


Fig. 3.1 pcDNA3_HA-rCB1wt and pcDNA3_HA-rCB1F238L vector maps

HA-tagged CB1wt (A) or CB1F238L (B) sequences were cloned into the pcDNA3 expression vector under the control of a CMV promotor (P CMV). The neomycin gene enables the selection of stably transfected cells with geneticin (G418). The vector contains an ampicillin resistance gene to select transformed bacteria. (Vector maps were generated using Vector NTI Advance™ 11.0).

3.2 Generation of HEK293 cells stably expressing the HA-CB1wt or the HA-CB1F238L receptor

HEK293 cells were stably transfected with HA-CB1wt or HA-CB1F238L constructs and positive clones were selected by addition of geneticin to the culture medium. Transgene expression was validated by immunocytochemistry and western blot experiments (Fig. 3.2). For experiments with $HEK^{CB1F238L}$ and HEK^{CB1wt} cells, those clones were picked which showed moderate and similar expression levels between genotypes. CB1 receptor expression levels were determined by western blot analysis using a monoclonal antibody against the HA epitope. Four bands of 78 kD, 68 kD, 51 kD and 47 kD were observed in the western blot for HA-CB1 (Fig. 3.2A). All bands were demonstrated to be specific as compared with untransfected HEK293 cells that do not express HA-CB1 (Fig. 3.2B).

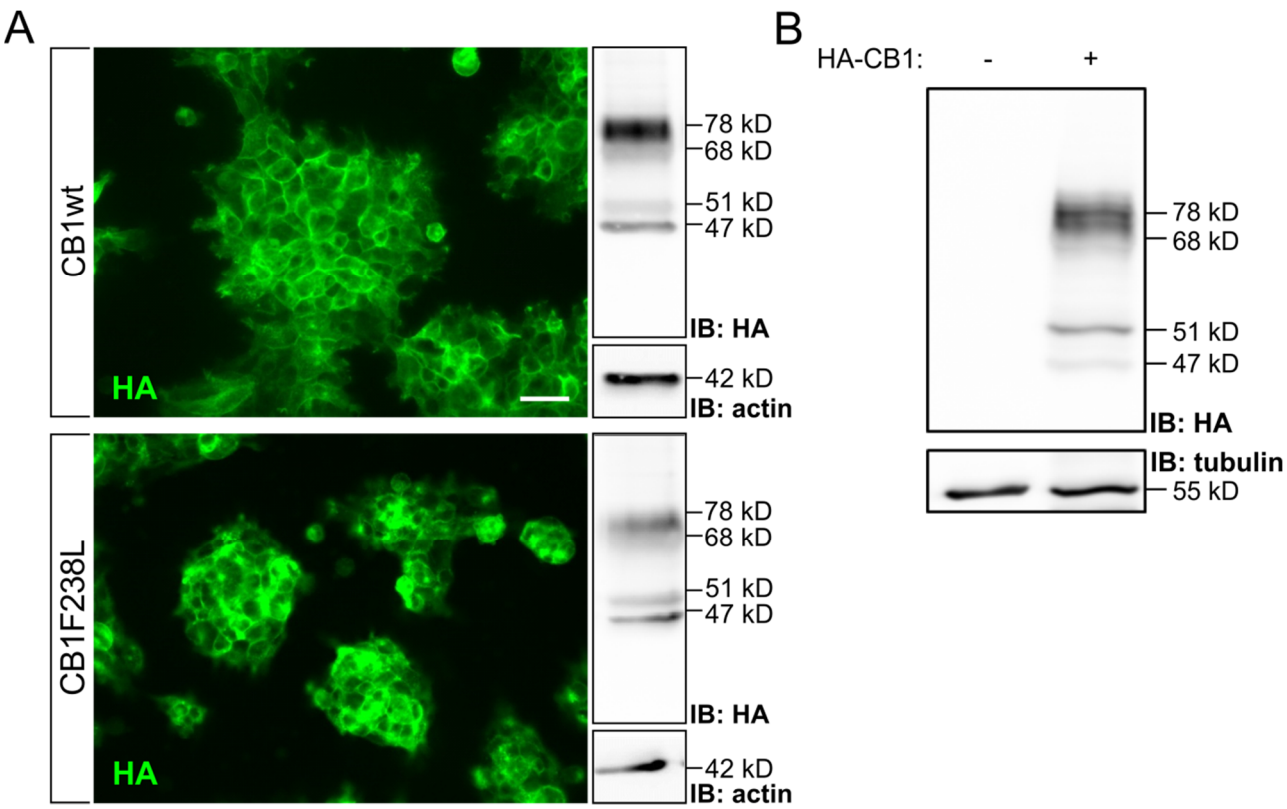


Fig. 3.2 Characterization of HEK^{CB1wt} and $HEK^{CB1F238L}$ cells

HEK293 cells were stably transfected with pcDNA3-HA-CB1wt or pcDNA3-HA-CB1F238L. Both constructs include a geneticin resistance cassette to select positive clones by keeping the cells in medium containing geneticin. (A) In order to evaluate the expression of HA-CB1, immunocytochemistry was performed using a monoclonal antibody against the HA-tag of the receptor. Scalebar: 20µm. (B) Relative expression levels of HA-CB1 receptor were quantified by western blot analysis. Four bands were detected at 78 kD, 68 kD, 51 kD and 47 kD. Two clones with moderate and similar CB1 receptor expression were chosen. All four bands detected in the western blot are specific as compared with un-transfected HEK293 cells. IB: Immuno blot

3.3 The F238L mutation affects basal endocytosis of the CB1 receptor

3.3.1 Reduced surface expression of the CB1F238L receptor

During immunocytochemical characterization of $HEK^{CB1F238L}$ and HEK^{CB1wt} cells, a difference in surface expression of the two receptors was observed in several clones. The CB1wt receptor showed intracellular as well as surface distribution, whereas the CB1F238L receptor showed reduced surface expression compared to the wild-type receptor. Two quantitatively determine the surface expression of the receptor, a trypsin protection assay was applied, exploiting the ability of trypsin to cleave the extracellular HA tag from the receptor (Fig. 3.3A).

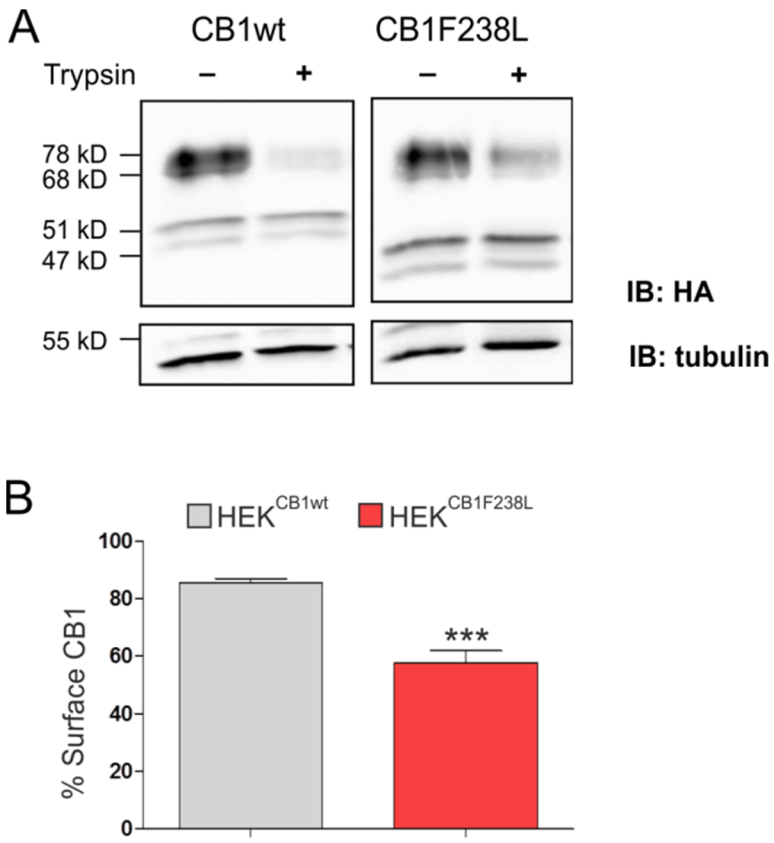


Fig. 3.3 Reduced surface expression of the CB1F238L receptor

(A) HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated with trypsin or versene as control. Trypsin is able to cleave the extracellular HA-tag. Thus, bands in control treated samples represent total CB1 receptor, whereas bands in trypsin treated samples represent intracellular CB1 receptor. (B) Surface HA-CB1 was calculated and expressed as percent of total HA-CB1 as described in Methods. 85.49% (±1.44 SEM) of HA-CB1wt, but only 57.68% (±4.26 SEM) of HA-CB1F238L were found to be located to the plasma membrane. (Student`s t-test. Data are presented as the mean ± SEM of n=7 independent experiments. ***, $p<0.001$). IB: Immuno blot.

In contrast, intracellular receptor is protected from trypsin. Using this assay and western blot analysis, the surface expression of the wild-type and mutant CB1 receptors was quantified. The band in control treated samples represents total CB1 receptor, whereas the band in trypsin treated samples represents intracellular CB1 receptor. Hence, surface CB1 receptor can be calculated as described in methods.A significant difference in plasma membrane localization was observed, with 85.49% (±1.44SEM) of HA-CB1wt, but only 57.68% (±4.26SEM) of HA-CB1F238L being expressed on the cell surface (Fig. 3.3B).

3.3.2 Inverse agonist treatment rescues surface expression of the CB1F238L receptor

As for many other GPCRs, agonist treatment can induce CB1 receptor endocytosis, whereas inverse agonist treatment can reduce endocytosis (Hsieh 1999, Grimsey 2010). To test if the differences in surface expression are due to differences in the internalization characteristic of the mutant receptor, endocytosis of the CB1F238L receptor was examined. HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated with 100 nM of the CB1 receptor agonist WIN55212-2 for 45 min, which is known to induce strong endocytosis of the CB1 receptor (Hsieh 1999) (Fig. 3.4A). WIN55212-2 treatment induced strong internalization of HA-CB1wt as well as HA-CB1F238L (Fig. 3.4B). Furthermore, there was no significant difference in the effect of WIN55212-2 on internalization between the two receptors (Fig. 3.4C). In contrast to agonists, inverse agonist treatment is known to stabilize the CB1 receptor at the cell surface (Grimsey 2010). Hence, it was tested whether the inverse agonist SR141716 was able to increase HA-CB1F238L surface levels. Indeed, treating the cells with 100 nM SR141716 for 45 min rescued surface expression of the mutant receptor to wild-type levels (Fig. 3.4B).

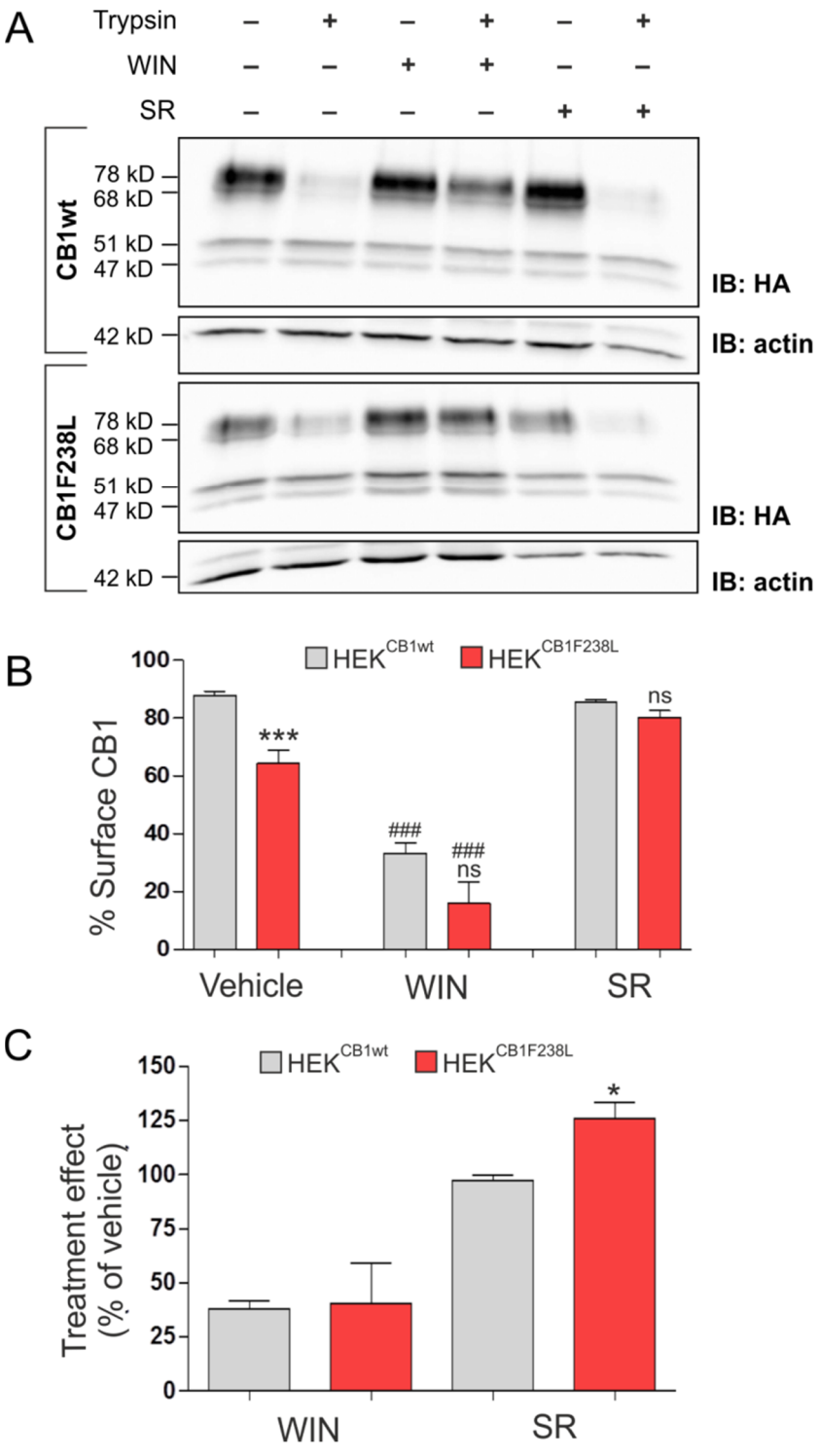


Fig. 3.4 Inverse agonist treatment rescues surface expression of the CB1F238L receptor

(A) HEK^{CB1wt} and HEK^{CB1F238L} cells were treated either with vehicle, 100 nM WIN (agonist) or 100 nM SR (inverse agonist) for 45 minutes. Surface expression was analyzed by a trypsin protection assay. (B) Strong internalization after WIN treatment was observed for both receptors. SR treatment rescued surface expression of CB1F238L receptor to wild-type levels. (Two way ANOVA and Bonferroni`s post-hoc test. Data are presented as the mean ± SEM of n=4 independent experiments. *, $p<0.05$; ***, $p<0.001$; ns, $p>0.05$; *, wild-type vs. mutant; #, WIN or SR vs. vehicle). (C) The effect of SR on surface expression is significantly bigger for CB1F238L receptor. (Student`s t-test between genotypes. Data are presented as the mean ± SEM of n=4 independent experiments). IB: Immuno blot

3.4 Enhanced basal endocytosis of the CB1F238L receptor is mediated by lipid rafts/caveolae

3.4.1 Cholesterol depletion rescues surface expression of the CB1F238L receptor

The finding that inverse agonist treatment rescues surface expression of CB1F238L indicates a possible involvement of basal endocytosis in this phenotype. To test this hypothesis, two described pathways of CB1 receptor internalization, clathrin coated pit and lipid raft/caveolae mediated endocytosis, were inhibited. HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated for 30 min either with hypertonic sucrose, to inhibit CCP endocytosis (Heuser 1989), or with 5 mM MβCD, a cholesterol depleting agent that disrupts lipid rafts/caveolae (Fig. 3.5A). Sucrose treatment did not affect surface expression of the CB1F238L receptor. In contrast, MβCD treatment led to an increase in surface expression of the CB1F238L receptor (Fig. 3.5B).

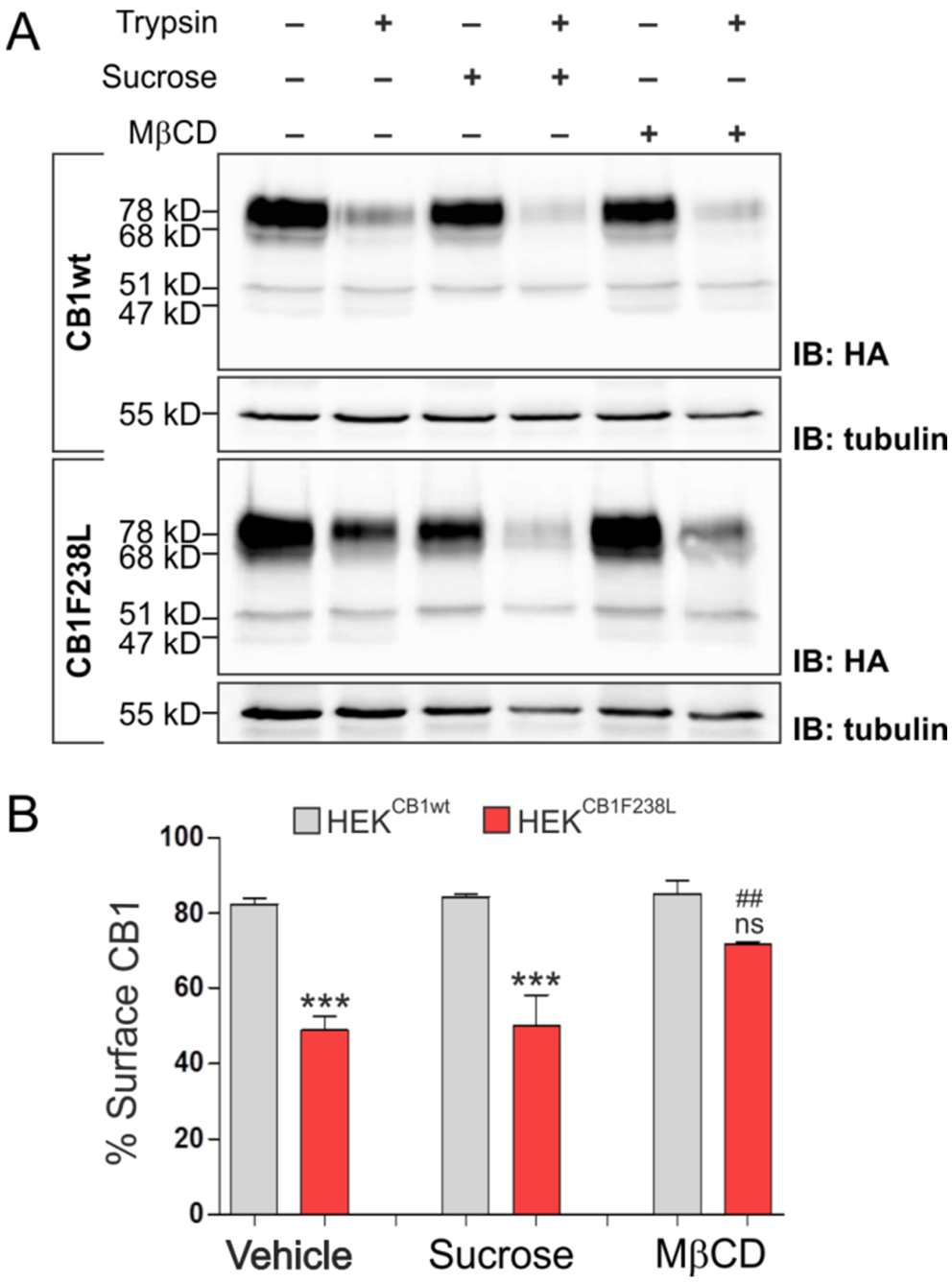


Fig. 3.5 Enhanced basal endocytosis of the CB1F238L receptor via lipid rafts

(A) HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated for 45 min either with 450 mM sucrose to inhibit clathrin coated pit endocytosis or 5 mM MβCD to inhibit caveolae mediated endocytosis. Surface expression was analyzed by a trypsin protection assay. (B) Only MβCD treatment significantly increased surface expression of CB1F238L receptor. (Two way ANOVA and Bonferroni`s posthoc test. Data are presented as the mean ± SEM of n=3 independent experiments. **, $p<0.01$; ***, $p<0.001$; n.s., $p>0.05$; ***,** wild-type vs. mutant; #, Sucrose or MβCD vs. vehicle). IB: Immuno blot

3.4.2 Increased recruitment of the CB1F238L receptor into lipid rafts/caveolae

The CB1 receptor has been reported to be present in biochemical lipid raft preparations (Sarnataro 2005, Asimaki 2011). As disruption of lipid rafts/caveolae rescued surface expression of the CB1F238L receptor, the association of the mutant receptor with biochemical lipid raft preparations was tested. Lipid rafts from HEK^{CB1wt} and HEK^{CB1F238L} cells were prepared using a detergent free protocol in a discontinuous sucrose gradient (Fig. 3.6A).

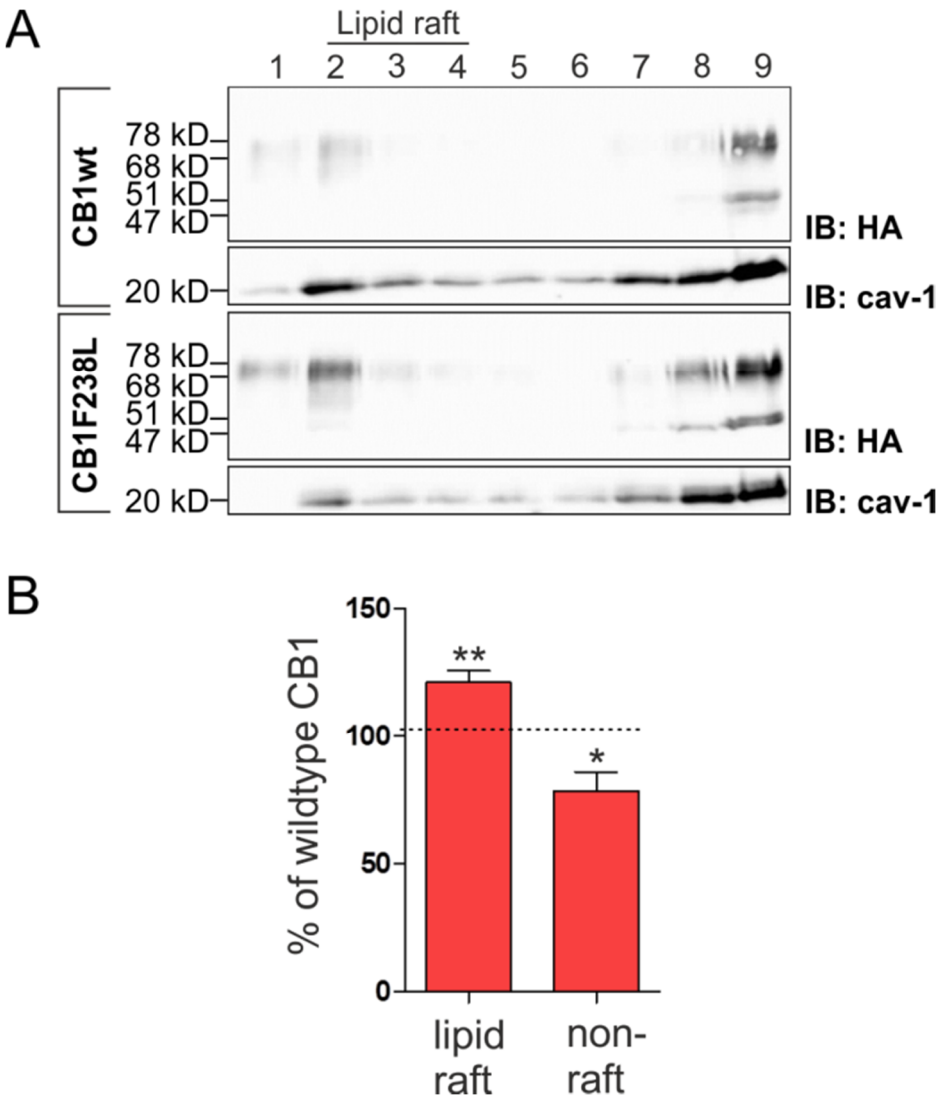


Fig. 3.6 Increased recruitment of the CB1F238L receptor into lipid rafts

(A) Lipid rafts were prepared from HEK^{CB1wt} and HEK^{CB1F238L} cells and analyzed by western blot. HA signals were normalized against caveolin, and values for caveolin rich fractions 2-4 were chosen as lipid raft fractions, all others as non-raft fractions. (B) Values for CB1F238L receptor were expressed as percentage of CB1wt receptor. (One sample t-test. Data are presented as the mean ± SEM of n=7 independent experiments. *, $p<0.05$; **, $p<0.01$). IB: Immuno blot

The HA-CB1 receptor content in different fractions of the gradient was quantified by western blot. The scaffolding protein caveolin-1 was used as a marker for lipid raft fractions, and the HA signal was normalized against caveolin-1 for each fraction. Fractions 2-4 were chosen as lipid raft fractions, all others as non-raft fractions. The amount of CB1F238L in lipid raft or non-raft fractions was expressed as percent of CB1wt receptor. There was significantly more CB1F238L receptor in the lipid raft fractions, and accordingly less in the non-raft fraction, as compared with the CB1wt receptor (Fig. 3.6B).

3.4.3 Inverse agonist treatment increases lipid raft/caveolae recruitment of the CB1F238L receptor

SR141716 treatment as well as lipid raft disruption by MβCD was able to increase surface expression of the CB1F238L receptor. It has been suggested by others that SR141716 as well as AEA binding can reduce lipid raft recruitment of the CB1 receptor (Sarnataro 2005, Sarnataro 2006). To test whether SR141716 treatment has an effect on lipid raft recruitment of the CB1wt and the CB1F238L receptor under the conditions used in the previous experiments of this work, HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were treated with SR141716, and lipid rafts were prepared (Fig. 3.7A). Quantification of the HA-CB1 content in lipid raft fractions 2-4 showed a significant increase in lipid raft associated CB1F238L receptor after treating the cell with SR141716. The increase was significant when comparing SR141716 treated lipid raft associated CB1F238L to vehicle treated CB1F238L receptor as well as when compared to SR141716 treated CB1wt receptor. A tendency of an increase in lipid raft associated CB1wt receptor upon SR141716 treatment was observed, too. However, this effect was smaller compared with the mutant receptor and did not reach statistical significance (Fig. 3.7B).

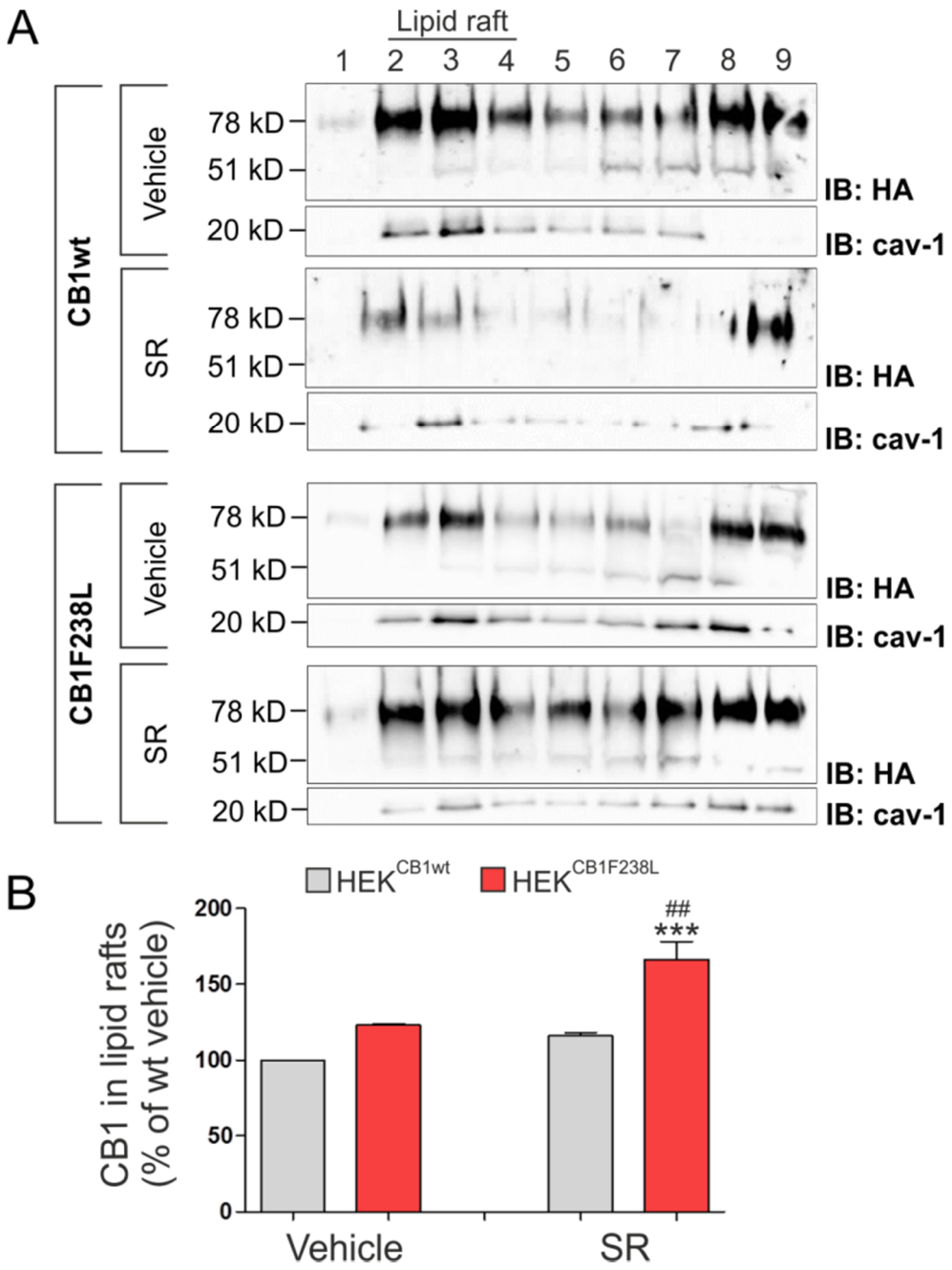


Fig. 3.7 Inverse agonist treatment increases lipid raft association of the CB1F238L receptor

(A) HEK^{CB1wt} and HEK^{CB1F238L} cells were treated with 100 nM SR for 45 min. Subsequently, lipid rafts were prepared and analyzed (B) CB1 receptor in lipid rafts was expressed as percent of vehicle treated CB1wt receptor. There is a significant increase in lipid raft associated CB1F238L receptor after inverse agonist treatment as compared with inverse agonist treated CB1wt receptor and vehicle treated CB1F238L receptor. (Two way ANOVA and Bonferroni`s posthoc test. Data are presented as the mean ± SEM of n=3 independent experiments. ##, p<0.01; ***, p<0.001; ***,** wild-type vs. mutant; #, vehicle vs. SR141716). IB: Immuno blot

3.5 The DAGL inhibitor THL does not affect surface expression of the CB1F238L receptor

The CB1 receptor is considered to be a constitutive active GPCR (Bosier 2010, Kwang 2012). However, it remains to be clarified whether it is a true constitutive activity in the absence of any ligand or if the CB1 receptor is activated in an autocrine manner by cell intrinsic DAGL activity. Turu et al. showed in Chinese Hamster Ovary cells, HEK293 and primary hippocampal neurons that basal activity as well as basal endocytosis of the CB1 receptor can be reduced by inhibiting DAGL activity with tetrahydrolipstatin (THL) (Turu 2007). Hence, with the trypsin protection assay, it was tested whether surface expression of the mutant CB1 receptor can be rescued by THL treatment (Fig. 3.8A), using the same conditions as Turu et al.. However, THL treatment did neither affect surface expression of the CB1wt receptor nor of the CB1F238L receptor (Fig. 3.8B).

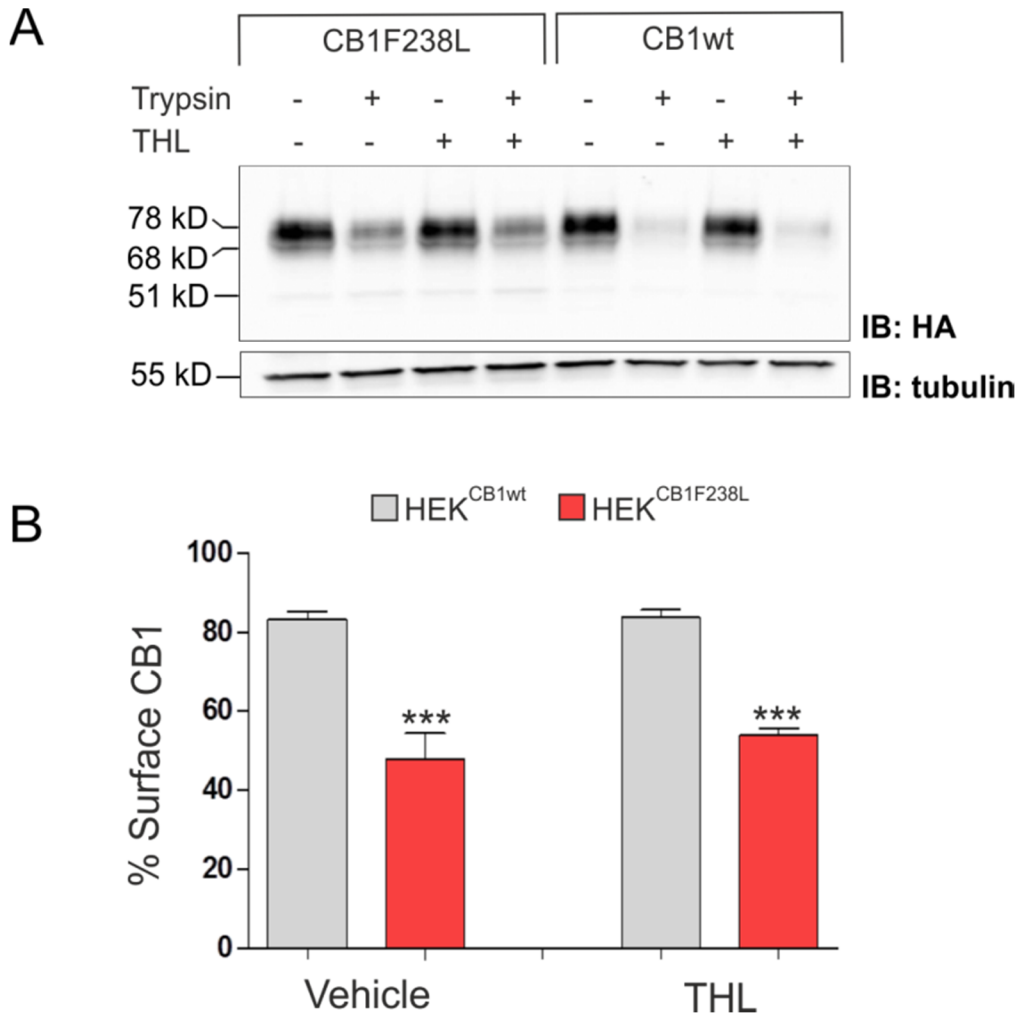
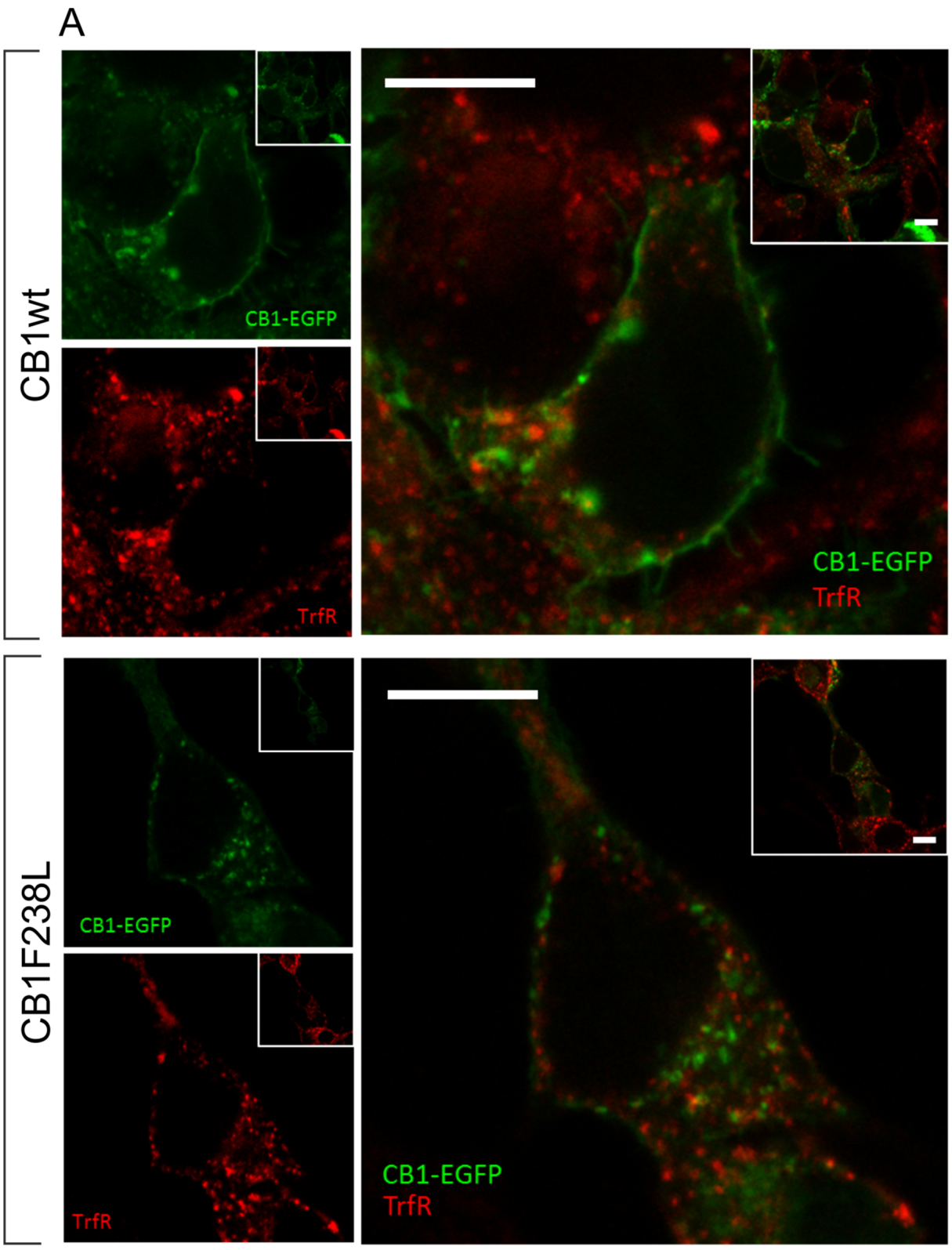


Fig. 3.8 The DAGL inhibitor THL does not affect surface expression of the CB1F238L receptor

(A) HEK^{CB1wt} and HEK^{CB1F238L} cells were treated for 2 h with the DAGL inhibitor THL. Surface expression was analyzed by a trypsin protection assay. (B) THL treatment did not affect surface expression of CB1F238L. (Two way ANOVA and Bonferroni`s posthoc test. Data are presented as the mean ± SEM of n=4 independent experiments. ***, p<0.001; ***,** wild-type vs. mutant). IB: Immuno blot

3.6 Increased targeting of the CB1F238L receptor into lysosomes

It has been reported that after endocytosis, the CB1 receptor can enter the lysosomal degradation pathway or recycle back to the plasma membrane (Rozenfeld 2011). To test, which of these routes is used by the endocytosed CB1F238L receptor, co-localization experiments where performed using C-terminally enhanced green fluorescent protein (EGFP)-tagged CB1wt or CB1F238L receptor constructs, which were transiently transfected into HEK293 cells. Co-staining with TrfR or Lamp2 was performed, as markers for early /recycling endosomes or lysosomes, respectively.


A
CB1wt
CB1-EGFP
TrfR
CB1-EGFP
TrfR
CB1F238L
CB1-EGFP
TrfR
CB1-EGFP
TrfR

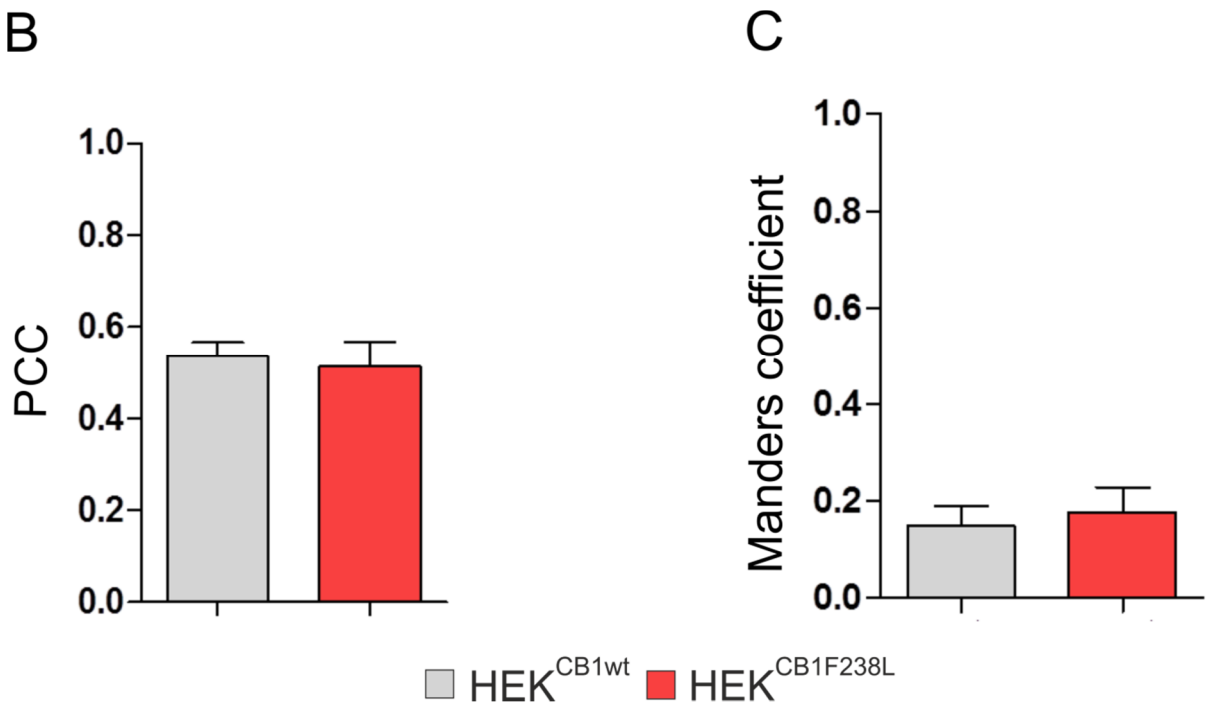
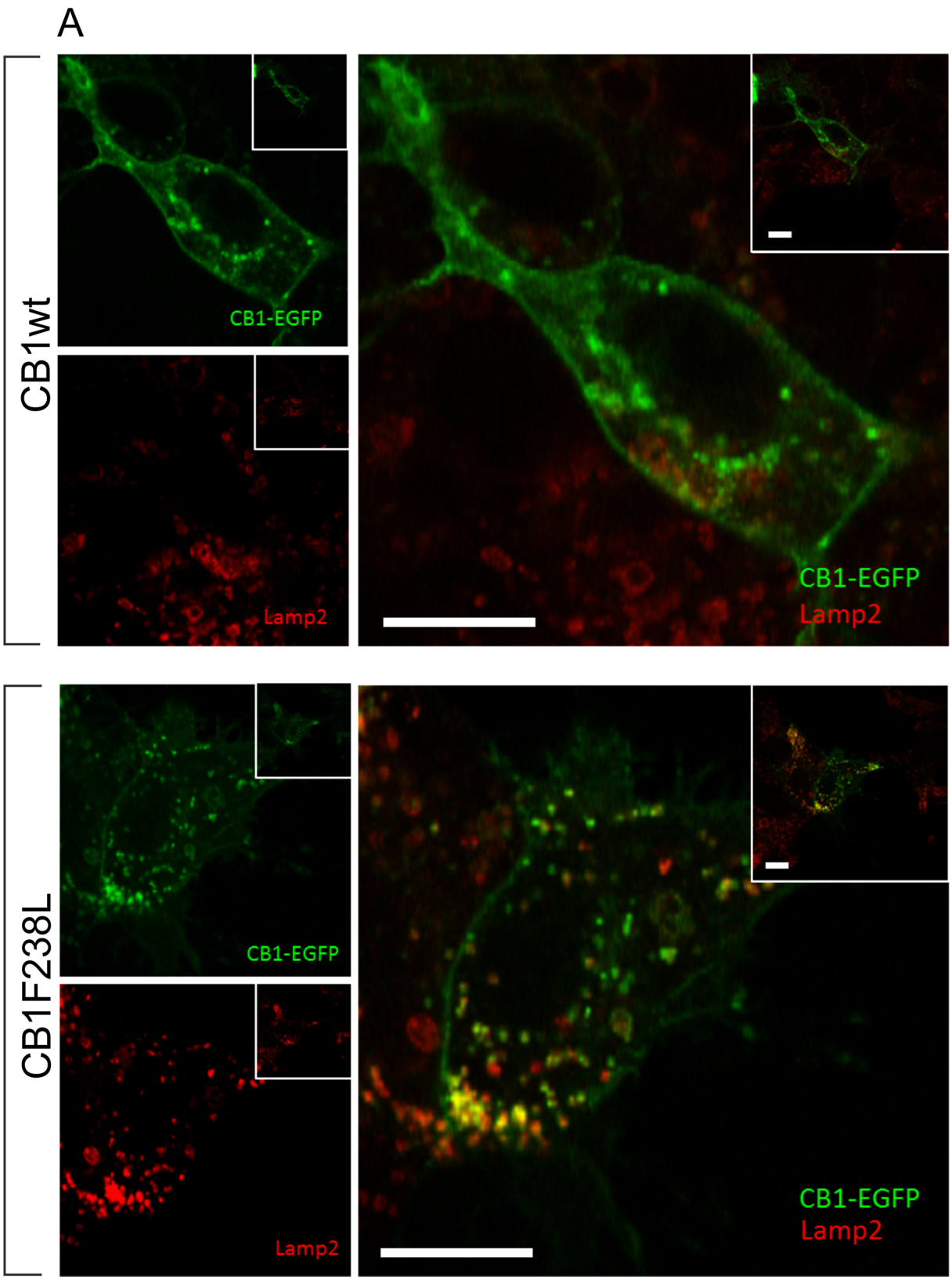


Fig.3.9 Co-localization of the CB1F238L receptor with the transferrin receptor

(A) HEK293 cells were transiently transfected with either CB1wt-EGFP or CB1F238L-EGFP. The transferrin receptor was labeled by addition of Alexa546 conjugated transferrin to the culture medium for 10 minutes and z-stacks were recorded. (B, C) Quantitative co-localization analysis was performed, yielding a Manders coefficient describing the fraction of CB1 that co-localizes with the transferrin receptor and a Pearsons correlation coefficient (PCC) describing the correlation between the red and green channel. No co-localization with the transferrin receptor was observed for CB1wt or CB1F238L. (Student`s t-test; Data are presented as the mean ± SEM of n = 6 cells expressing CB1wt and n = 5 cells expressing CB1F238L. Scale bar 10µm).

Co-localization was quantified gaining the Pearsons correlation coefficient (PCC) as a measure of the correlation between the pixels in the red (Lamp2 or TrfR) and green (CB1 receptor) channel. Furthermore, the Manders coefficient was determined, which described the fraction of CB1 receptor that co-localizes with Lamp2 or TrfR (Fig. 2.1). Neither the CB1wt receptor nor the CB1F238L receptor co-localized to a substantial proportion with the transferrin receptor, and no difference in Pearsons correlation coefficient or Manders coefficient was observed between the two genotypes (Fig. 3.9). In contrast, there is a significant increase in the Manders coefficient M1 as well as the Pearsons correlation coefficient for the co-localization of the CB1F238L receptor with the lysosomal marker Lamp2, as compared with the CB1wt receptor (Fig. 3.10).


A
CB1wt
CB1-EGFP
Lamp2
CB1-EGFP
Lamp2
CB1F238L
CB1-EGFP
Lamp2
CB1-EGFP
Lamp2

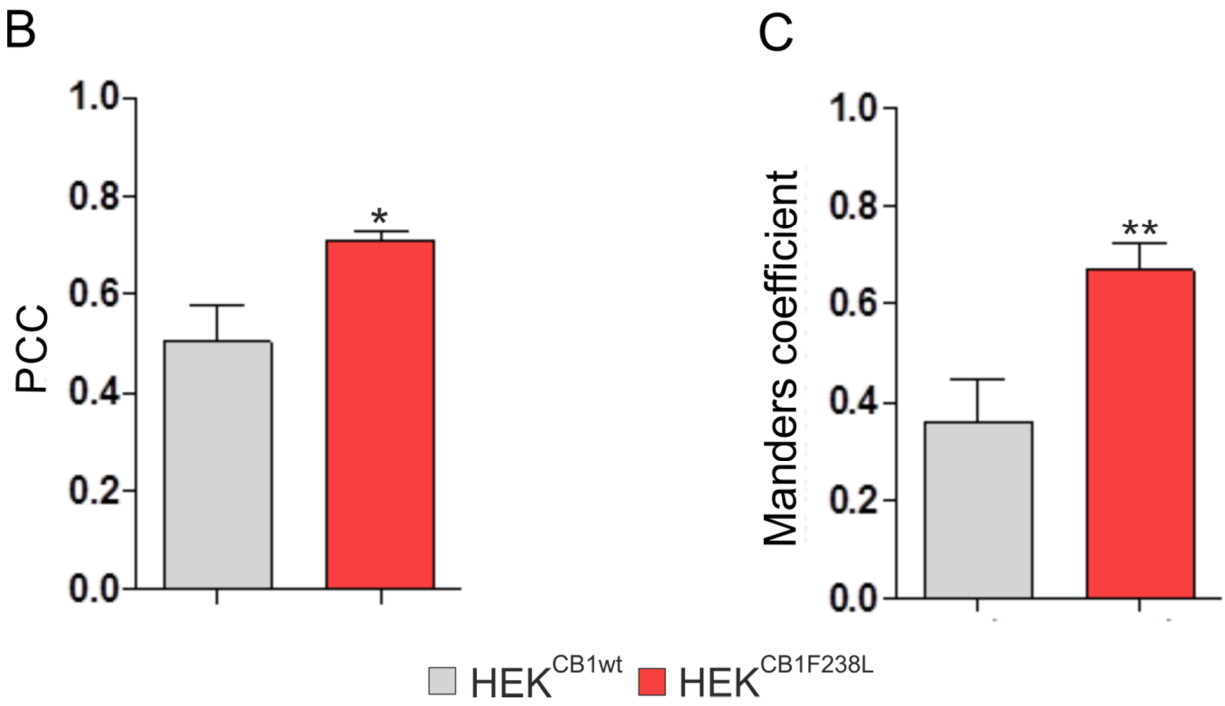
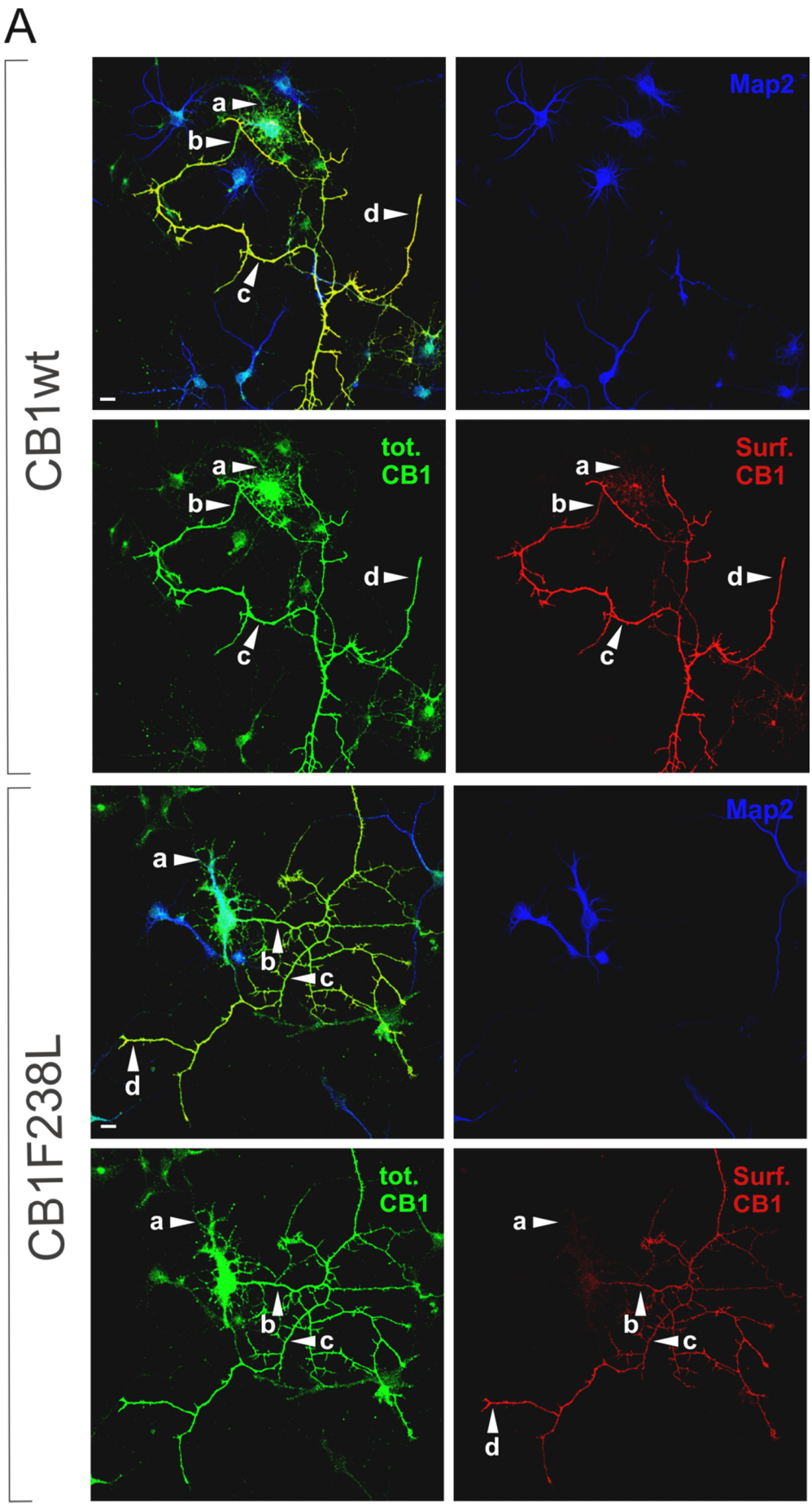


Fig. 3.10 Co-localization of the CB1F238L receptor with lysosomes

(A) HEK293 cells were transiently transfected with either CB1wt-EGFP or CB1F238L-EGFP. Immunocytochemistry against the lysosomal marker Lamp2 was performed and z-stacks were recorded. (B, C) Quantitative co-localization analysis was performed, yielding a Manders coefficient describing the fraction of CB1 that co-localizes with lamp2 and a Pearsons correlation coefficient (PCC) describing the correlation between the red and green channel. With both coefficients a significant increased co-localization of CB1F238L with lamp2 was observed as compared with CB1wt. (Student`s t-test; Data are presented as the mean ± SEM of n = 8 cells per genotype. *, $p<0.05$; **, $p<0.01$. Scale bar 10µm).

3.7 Increased axonal polarization of the CB1F238L receptor

Somatodendritic endocytosis has been shown to be one crucial step for axonal polarization of the CB1 receptor (McDonald 2007, Simon 2013). As the CB1F238L receptor shows increased endocytosis in HEK293 cells, the effect of the point mutation on axonal polarization of the CB1 receptor was examined. Therefore, primary hippocampal neurons were transfected with either HA-CB1wt or HA-CB1F238L, and surface receptor expression in dendrites and axons was measured by quantitative immunofluorescence (Fig. 3.11A).


A
CB1wt
CB1F238L
Map2
tot. CB1
Surf. CB1
a
b
c
d

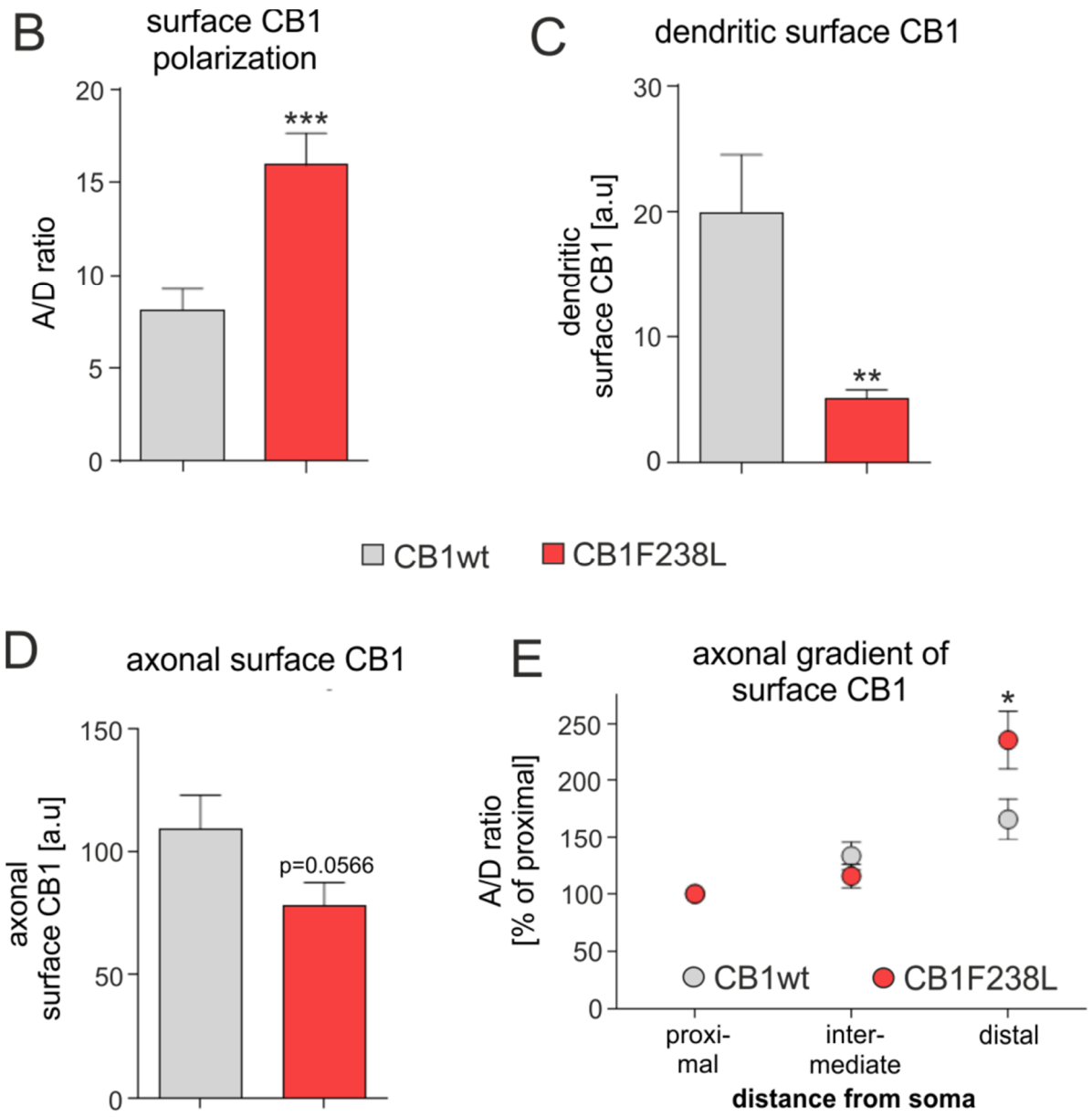


Fig. 3.11 Increased axonal polarization of the CB1F238L receptor in primary hippocampal neurons

Immunocytochemistry was performed using primary rat hippocampal neurons which were transfected with either HA-CB1wt or HA-CB1F238L. (A) Surface HA-CB1 receptor is shown in red, total HA-CB1 receptor in green and Map2 in blue. Surface HA-CB1 receptor was quantified in dendrites (Map2+) (a), proximal axon (Map2-) (b), intermediate axon (Map2-) (c) and distal axon (Map2-) (d) (Scale bar: 20 µm). (B) The polarization index (A/D ratio) for surface CB1 receptor was determined by dividing average axonal surface fluorescence (*A*) by average dendritic surface fluorescence (*D*). The polarization index for CB1F238L is significantly increased. (C) There is a significant reduction in dendritic surface CB1F238L receptor. (D) In the axon there is a small reduction in surface CB1F238L, which did not reach statistical significance. (Student`s t-test between genotypes. Data are presented as the mean ± SEM of 21 HA-CB1wt cells and 30 HA-CB1F238L cells of two independent experiments. **, $p<0.01$; ***, $p<0.001$). (E) Intermediate and distal axonal surface CB1 receptor is expressed as percent of surface CB1 receptor in the proximal axon. (Repeated measures ANOVA and Bonferroni`s post hoc test. Data are presented as the mean ± SEM of 19 HA-CB1wt cells and 26 HA-CB1F238L cells of two independent experiments. *, $p<0.05$).

To examine the polarization of the receptor within the neuron, average surface CB1 receptor in the axon (A) was divided by average surface CB1 receptor in the dendrites (D) to gain a polarization index (A/D ratio) (Leterrier 2006). A polarization index of 1 indicates no polarization, values smaller than 1 indicate polarization towards the dendrites and values larger than 1 indicate polarization towards the axon. For both the CB1wt receptor as well as for the CB1F238L receptor, polarization indices were larger than 1, indicating axonal polarization. The axonal polarization index of the CB1F238L receptor was almost double as high as for the CB1wt receptor (Fig. 3.11B). The large and significant difference between the polarization indices is paralleled by a significant and strong reduction of CB1F238L receptor in the dendritic surface (Fig. 3.11C). Furthermore, a smaller reduction of CB1F238L receptor in the axonal surface was observed. However, this difference did not reach statistical significance (Fig. 3.11D). The model by Simon et al. predicts that increased somatodendritic endocytosis leads to an increased gradient of surface receptor expression towards the distal part of the axon (Simon 2013). When expressing surface CB1 receptor values in the intermediate and distal axon as percent of surface CB1 receptor values in the proximal axon, a measure of the gradient of surface CB1 receptor along the axon was gained. A significantly increased axonal gradient towards the distal end of the axon was observed for the CB1F238L receptor as compared with the wild-type receptor (Fig. 3.11E).

3.8 Effect of the F238L mutation on CB1 receptor signaling

Previous data suggest a role of receptor activity in endocytosis and polarization of the CB1 receptor (Simon 2013). As the effect of the point mutation on CB1 receptor trafficking was predominantly observed under basal conditions, the basal activity of the receptor was tested. Basal activity of the CB1 receptor in HEK^{CB1wt} and $HEK^{CB1F238L}$ cells was tested by measuring [^{35}S]GTPγS binding and ERK1/2 phosphorylation. Furthermore, agonist or inverse agonist effects on ERK1/2 phosphorylation were examined in the two cell lines.

3.8.1 Effect of the F238L mutation in the CB1 receptor on basal [^{35}S]GTPγS binding

[^{35}S]GTPγS binding is a method to quantify the binding of G proteins to GPCRs in the presence or absence of ligands. Agonist stimulated [^{35}S]GTPγS binding with brain homogenates of the CB1F238L mutant rat revealed an increase in G protein binding to the agonist stimulated mutant CB1 receptor

(Schneider 2014). However, the CB1 receptor has been reported to show basal activity as well. To test the effect of the point mutation on basal activity, [^{35}S]GTPγS binding was performed using homogenates of HEK^{CB1wt} and HEK^{CB1F238L} cells in the absence of any ligand (Fig. 3.12A). Untransfected HEK293 where used as a negative control to determine CB1 independent [^{35}S]GTPγS binding in HEK293 cells. CB1 receptor contents of the homogenates were determined by western blot, and the [^{35}S]GTPγS binding was normalized against CB1 receptor content (Fig. 3.12B). Both CB1wt and CB1F238L receptors showed significant basal [^{35}S]GTPγS binding as compared with untransfected HEK293 cells (Fig. 3.12A). A significant reduction in basal [^{35}S]GTPγS binding of the CB1F238L receptor was observed as compared with the wild-type receptor (Fig. 3.12B).

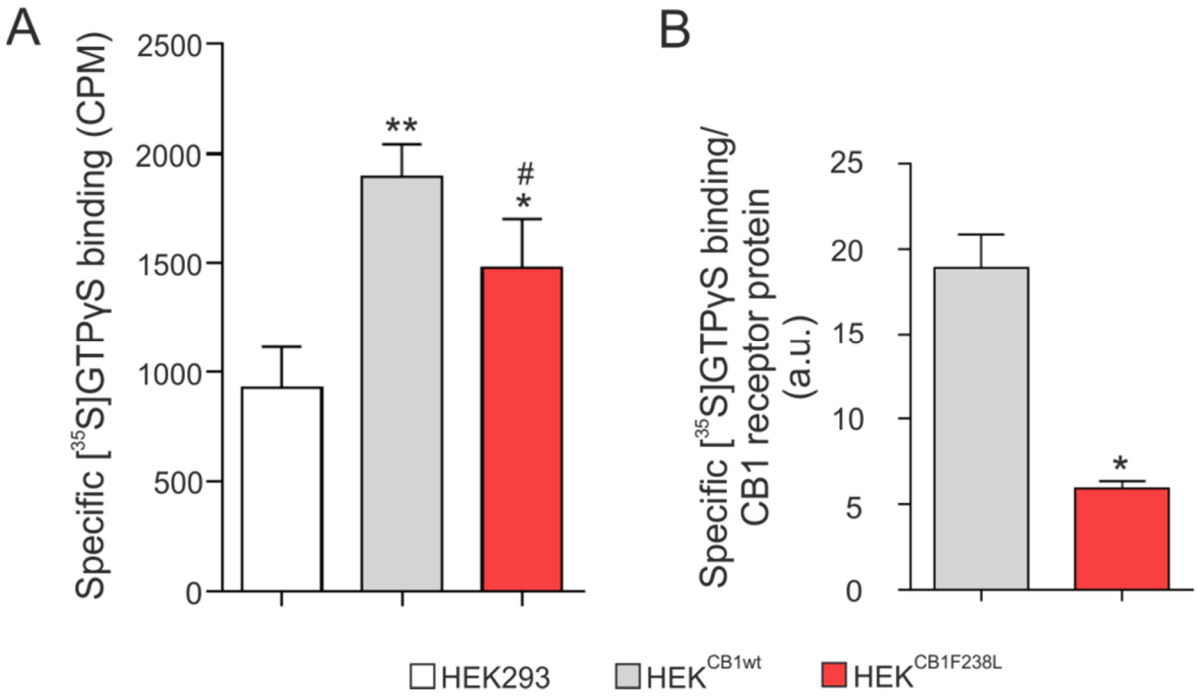


Fig. 3.12 Decreased basal [^{35}S]GTPγS binding of the CB1F238L receptor

Basal [^{35}S]GTPγS binding was measured using homogenates of HEK^{CB1wt} and HEK^{CB1F238L} cells. (A) Both CB1wt and CB1F238L showed basal activity as compared with untransfected HEK293 cells. The basal activity of CB1F238L is significantly reduced as compared with CB1wt (One way ANOVA and Bonferroni`s Multiple Comparison posthoc test. Data are presented as the mean ± SEM of n=3 independent experiments. *, p<0.05; **, p<0.01; ***,** transfected vs. untransfected; #, wild-type vs. mutant). (B) The amount of CB1 receptor in the homogenates used for [^{35}S]GTPγS binding was quantified by western blot analysis and specific [^{35}S]GTPγS binding was normalized against CB1 levels. A significant and strong decrease in basal activity of the CB1F238L receptor was observed as compared with the CB1wt receptor. (Two tailed student`s t-test and Welch`s correction. Data are presented as the mean ± SEM of n=3 independent experiments. *, p<0.05).

3.8.2 Effect of the F238L mutation in the CB1 receptor on basal and stimulated ERK1/2 phosphorylation

ERK1/2 is a downstream target of the mitogen activated protein kinase pathway activated by the CB1 receptor. Stimulation of the CB1 receptor leads to an increase in ERK1/2 phosphorylation (Bosier 2010). To support the results found with the [^{35}S]GTPγS binding experiments, ERK1/2 phosphorylation was analyzed via western blot in HEK^{CB1wt} and HEK^{CB1F238L} cells. There are no data on agonist induced ERK1/2 phosphorylation in the CB1F238L rat. Hence, in this work ERK1/2 phosphorylation was determined not only under basal conditions but also under agonist or inverse agonist stimulated conditions (Fig. 3.13A). Under basal conditions, a reduced phosphorylation of ERK1/2 in HEK^{CB1F238L} cells was observed as compared with HEK^{CB1wt} (Fig. 3.13B). When compared to vehicle treated cells of the same cell line, the effect of WIN55212-2 on ERK1/2 phosphorylation was slightly increased for the CB1F238L receptor as compared to the wild-type receptor (Fig. 3.13C). In contrast, the effect of SR141716 was reduced for the CB1F238L receptor as compared to the CB1wt receptor (Fig. 3.13C). However, these effects did not reach statistical significance. By comparing ERK1/2 phosphorylation of all conditions with ERK1/2 phosphorylation in vehicle treated HEK^{CB1wt} cells a measure of a total ERK1/2 phosphorylation was gained. In this case, ERK1/2 phosphorylation was normalized against HA-CB1 expression to exclude any effect of a variation in receptor expression. Again, a non-significant tendency of a reduced effect of SR141716 on the mutant receptor was observed as compared to the wild-type receptor (Fig. 3.13D). However, the total ERK1/2 phosphorylation was significantly reduced in WIN55212-2 treated HEK^{CB1F238L} cells as compared with HEK^{CB1wt} cells (Fig. 3.13D).

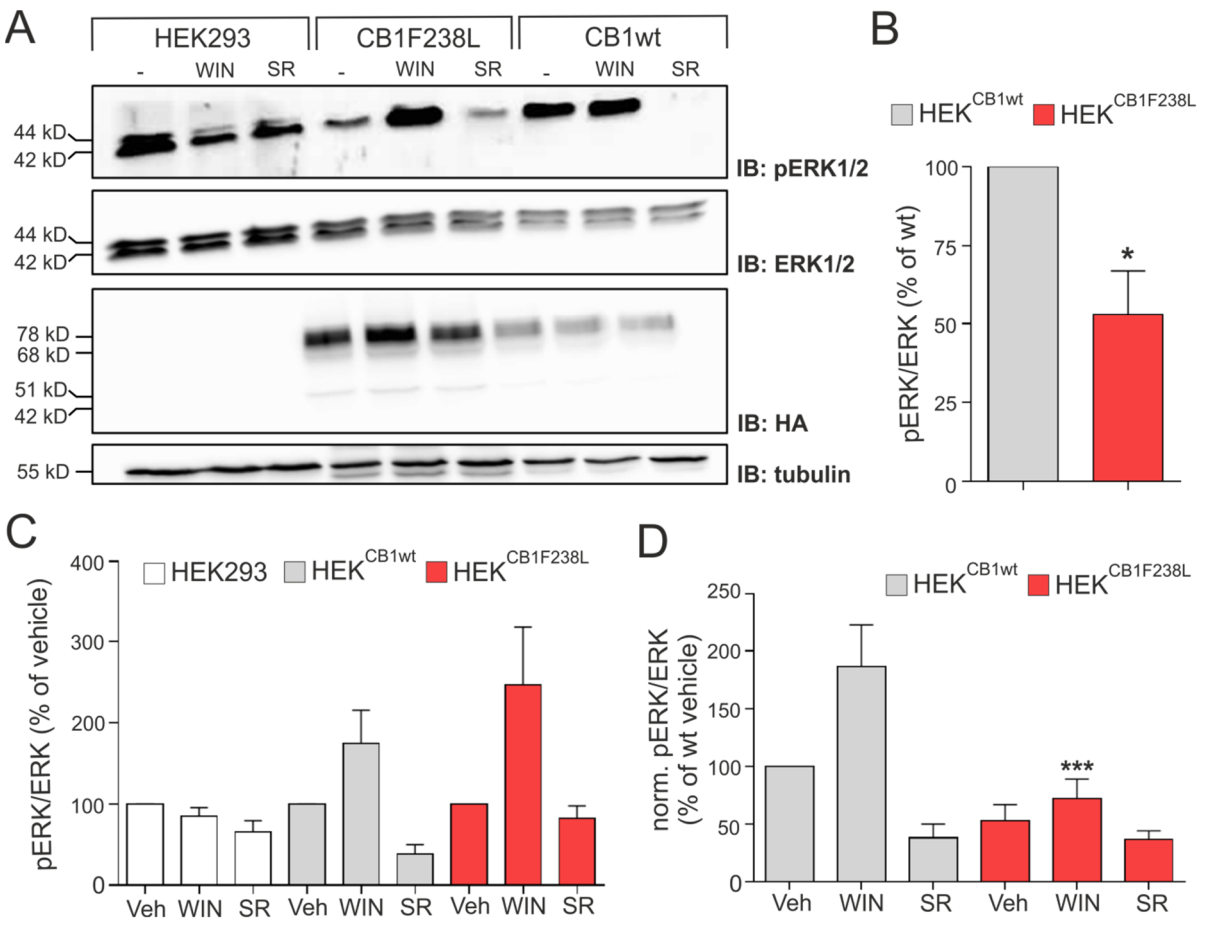


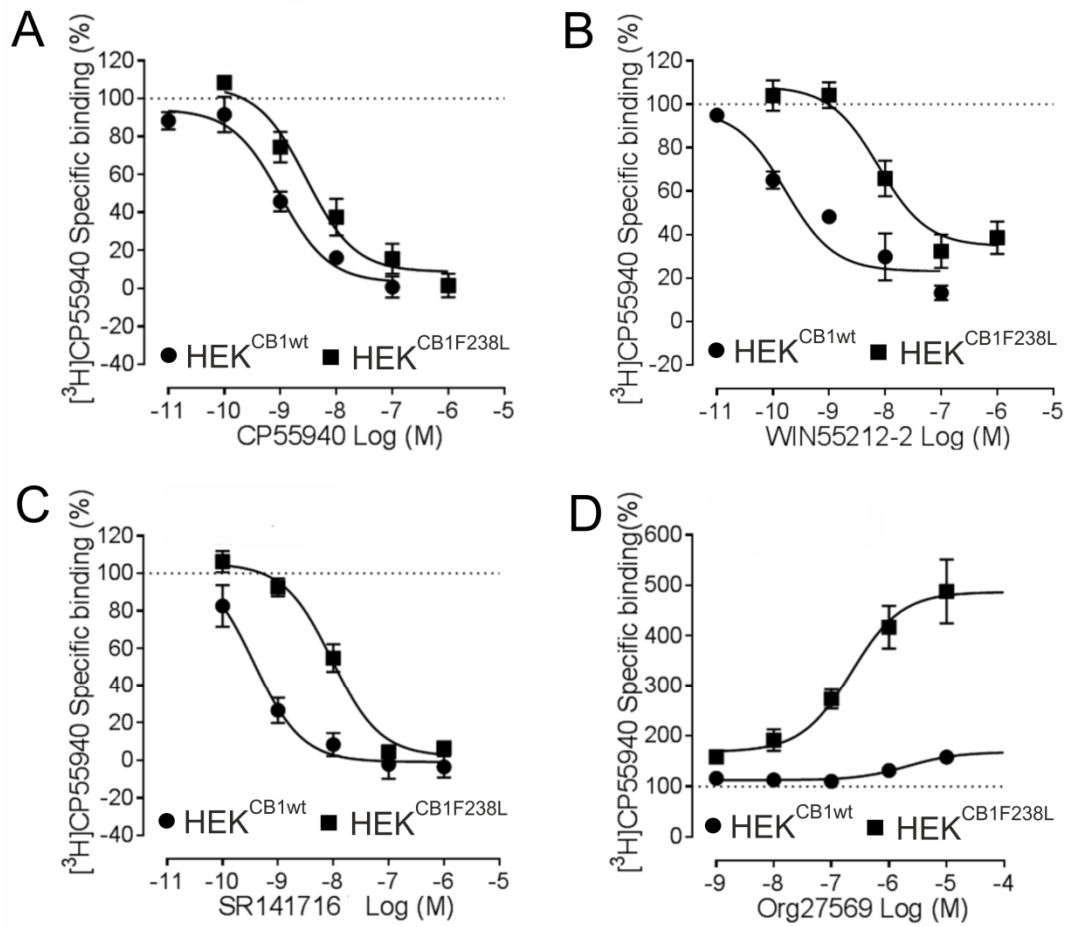
Fig. 3.13 Basal and ligand induced ERK1/2 phosphorylation

(A) HEK293, HEK^{CB1wt} and HEK^{CB1F238L} cells were treated either with vehicle, with 100 nM WINor with 100 nM SR for 45 min. ERK1/2 phosphorylation was analysed by western blot as described in methods. (B) ERK1/2 phosphorylation in vehicle treated HA-CB1F238L cells was expressed as percent of vehicle treated HA-CB1wt cells. Basal ERK1/2 phosphorylation was significantly lower with vehicle treated HA-CB1F238L as compared with HA-CB1wt. (One sample t-test. Data are presented as the mean ± SEM of n=5 independent experiments. *, $p<0.05$). (C) Relative ERK1/2 phosphorylation levels were expressed as percent of vehicle treated samples from the same cell line. No significant effect was observed. However, a trend of a reduced effect of SR on CB1F238L as compared with CB1wt was observed ($p=0.0567$). (Two way ANOVA and Bonferroni`s posthoc test. Two tailed t-test. Data are presented as the mean ± SEM of n=5 independent experiments.). (D) To determine absolute values, ERK1/2 phosphorylation was normalized against HA-CB1 expression and expressed as percent of normalized wild-type vehicle treated samples. There was a significantly smaller effect of WIN on CB1F238L as compared with CB1wt. Although not significant, a trend of a smaller effect of SR on CB1F238L as compared with CB1wt was also observed. (Two way ANOVA and Bonferroni`s posthoc test. Data are presented as the mean ± SEM of n=5 independent experiments. ***, $p<0.001$; ***,** wild-type vs. mutant). IB: Immuno blot

3.8.3 Reduced ligand binding potency but increased allosteric modulation of the CB1F238L receptor

There were significant differences in the effect of CB1 receptor agonists and inverse agonists on the behavior of the CB1F238L receptor mutant rat (Schneider 2014). Furthermore, there were significant differences in the effect of SR141716 on surface expression and lipid raft association of the CB1F238L receptor in HEK293 cells. However, the efficacy and potency of a ligand at a GPCR depends on the affinity of the receptor for this ligand. Hence, to assess the effect of the point mutation on ligand affinities, the affinity of the mutant receptor for the agonists CP55940 and WIN55212-2 and for the inverse agonist SR141716 was measured using HEK^{CB1wt} and $HEK^{CB1F238L}$ cells.

The allosteric modulator ORG27569 has been reported to increase the CB1 receptors affinity to CP55940 (Ahn 2012). It might also be that the point mutation affects allosteric modulation of the CB1 receptor. Hence, the effect of ORG27569 on CP55940 binding was also measured for the CB1F238L receptor. Ligand binding experiments were performed by Dr. Gemma Baillie from the University of Toronto. In the competition binding experiments, the CB1F238L receptor showed a right shift in the binding curve and a significantly reduced pIC_{50} for the competition of both, WIN55212-2 and SR141716 with [^{3}H]CP55940 (Fig. 3.14B,C,E). This indicates a reduction in the affinity of the mutant receptor for these two ligands because more unlabeled ligand is needed to replace half of the radiolabeled ligand (IC_{50}). pIC_{50} represents the negative logarithm of IC_{50}. In contrast, pIC_{50} for CP55940 was not significantly different, indicating no changes in the CB1F238L receptors affinity for CP55940 (Fig. 3.14A,E). ORG27569 induced allosteric modulation of CP55490 binding was found to be significantly stronger at the CB1F238L receptor as compared with the wild-type receptor (Fig. 3.14D,E).



E

Ligand	CB1wt		CB1F238L	
	pIC_{50} ± SEM (M)	E_{max} (95%CL) (M)	pIC_{50} ± SEM (M)	E_{max} (95%CL) (M)
CP55940	9.0±0.15	n.d	8.5±0.20	n.d
SR141716	9.4±0.29	n.d	8.1±0.20 *	n.d
WIN55212-2	9.8±0.29	n.d	8.0±0.11*	n.d
ORG27569	5.7±0.50	168 (127-209)	6.7±0.26	486 (416-558) *

Fig. 3.14 Changes in ligand binding properties of the CB1F238L receptor

Using HEK^{CB1wt} and $HEK^{CB1F238L}$ cells competition binding experiments were performed. The radiolabeled CB1 receptor agonist [^{3}H]CP55940 was replaced by CP55940, WIN55212-2 or SR141716. (A) No effect of the F238L mutation on CP55940 affinity was observed. (B) The F238L mutation causes a decrease in the affinity of CB1 receptor for the agonist WIN55212-2. (C) The F238L mutation causes a decrease in the affinity of CB1 for the inverse agonist SR141716. (D) The positive allosteric effect of ORG27569 on [^{3}H]CP55940 binding was increased for CB1F238L as compared with CB1wt. (E) pIC_{50} for competition binding experiments and E_{max} for allosteric modulation experiment show the significantly reduced affinity of CB1F238L for WIN55212-2 and SR141716 but not CP55940. Furthermore the E_{max} of [^{3}H]CP55940 binding in the presence of ORG27569 was significantly increased for CB1F238L as compared with CB1wt. (Student`s t-test. Data are presented as the mean ± SEM of n=3-8 independent experiments. *, $p<0.05$). (Competition binding experiments were performed by Dr. Gemma Baillie at the University of Toronto)

3.9 Interactions of the CB1 receptor with the D2 receptor

CB1 and D2 receptor signaling interferes at several levels of the signaling cascade. Amongst others, the two receptors can directly interact by building heteromers. Heteromerization of CB1 and D2 receptor affects their ligand affinity and signaling selectivity (Glass 1997, Marcellino 2008). To address questions of general heteromerization, co-immunoprecipitation was performed to reproduce and confirm the interaction of CB1 and D2 receptors in the present work. Furthermore, radioligand binding experiments were performed to test, whether the mere co-expression of the CB1 receptor affects D2 receptor affinity for dopamine. Additionally, the D2 receptor expression in the CB1F238L mutant rat was measured by means of receptor autoradiography and western blot analysis. Finally, HEK293 cells were generated that stably express the Flag-D2L receptor alone or together with HA-CB1wt or HA-CB1F238L. These cells serve as a tool to examine possible effects of the point mutation in the CB1 receptor D2 receptor functions.

3.9.1 Cloning of a FLAG tagged form of the D2 receptor into two different target vectors

The sequence for a C-terminally Flag tagged rat D2L receptor was synthesized by MWG Eurofins Operon and cloned into an pBS II - SK(+) vector. For transient transfection of HEK293 cells, the sequence was clones into a pAM/CBA vector, where the transgene is expressed under the control of a CMV early enhancer/chicken β actin (CAG) promotor (Fig. 3.15A). For stable transfection of HEK293 cells, the Flag-D2L sequence was cloned into a pcDNA3_Zeo vector, where the transgene is expressed under the control of a CMV promotor. The vector contains a zeocin resistance gene, which allows the selection of stably transfected cells by the addition of zeocin to the culture medium (Fig. 3.15B).

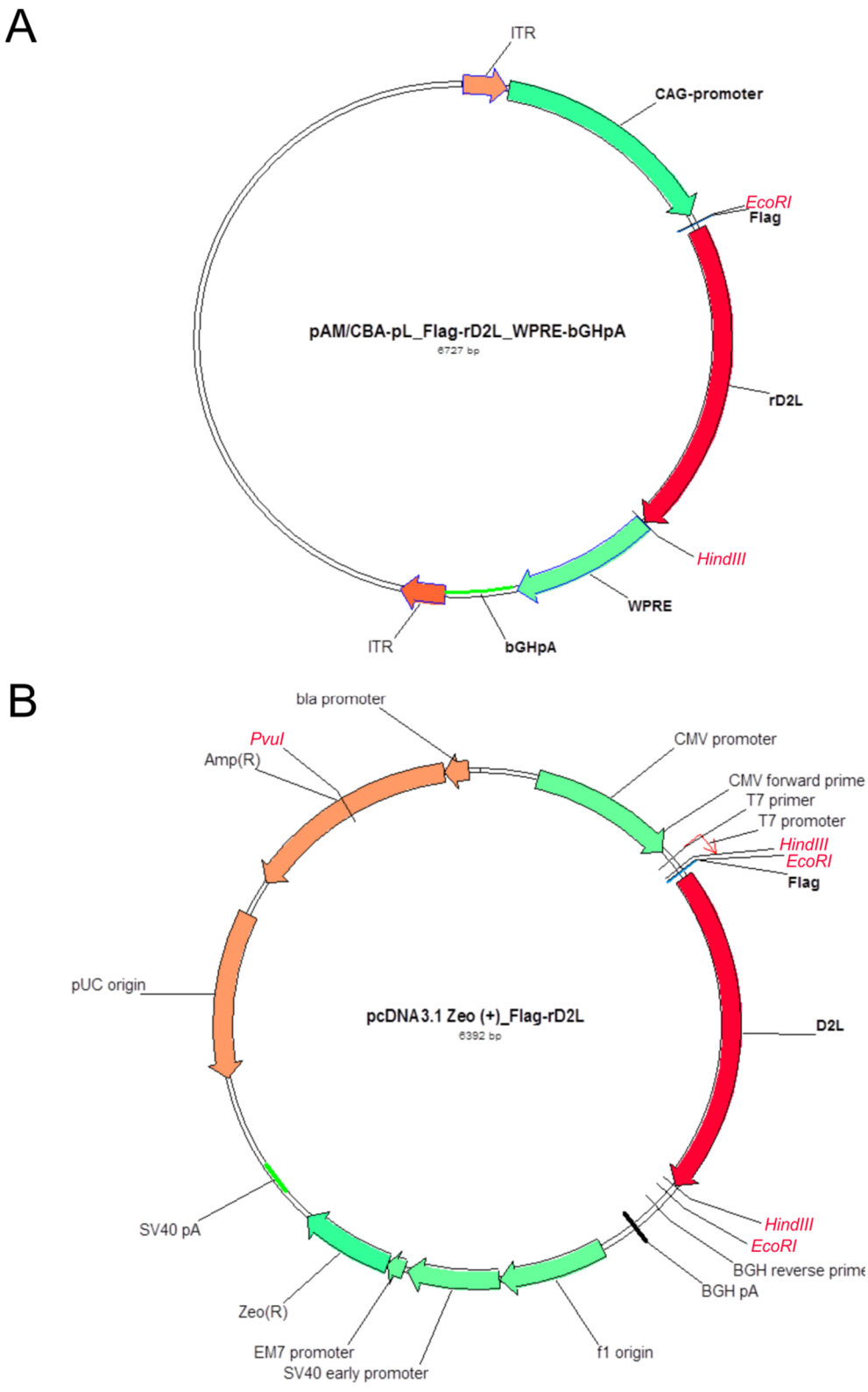


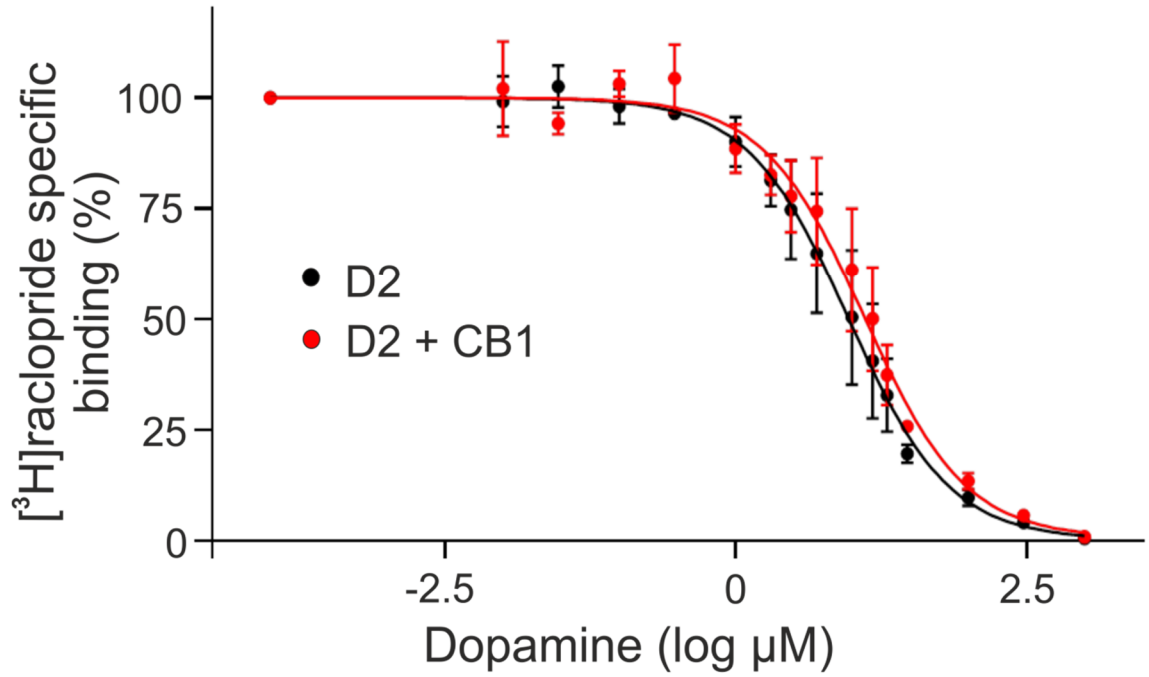
Fig. 3.15 pAM/CBA_Flag-D2L and pcDNA3_Zeo_Flag-D2L vector maps

Flag-D2L sequences were cloned into a pAM/CBA (A) or a pcDNA3_Zeo (B) expression vector. (A) The transgene is expressed under control of a CMV early enhancer/chicken β actin (CAG) promotor. (B) The transgene is expressed under control of a CMV promotor. The zeocin resistance gene allows the selection of stably transfected cells with zeocin. (Vector maps were generated using Vector NTI Advance™ 11.0).

3.9.2 Co-expression of the CB1 receptor causes a reduction in the affinity of the D2 receptor for dopamine

Heterodimerization and pharmacological co-activation of CB1 and D2 has been demonstrated to change G protein binding specificity in the CB1 receptor (Glass 1997). Jarrahian et al. found that the mere presence of the D2 receptor, without any agonist treatment, sufficiently modulates G protein binding to the CB1 receptor (Jarrahian 2004). In turn, agonist binding of the CB1 receptor in striatal membrane preparations has been demonstrated to reduce the affinity of the D2 receptor for dopamine (Marcellino 2008). However, it remains open whether the mere presence of the CB1 receptor affects the affinity of the D2 receptor for dopamine. To address this question, HEK293 cells where transiently transfected with either the D2 receptor alone or together with the CB1 receptor. Competition binding experiments where performed to assess the ability of dopamine to displace [^{3}H]raclopride from the D2 receptor. IC_{50} values were determined, which describe the concentration of dopamine that is needed to displace 50% of [^{3}H]raclopride from the D2 receptor and can hence be used as a measure for the affinity of the receptor for dopamine. Co-expression of the D2 receptor with the CB1 receptor led to a small shift of the dopamine competition curve towards higher dopamine concentrations (Fig. 3.16A). Accordingly, the IC_{50} is significantly higher for the D2 receptor in the presence of the CB1 receptor (Fig. 3.16B).

A



B

	D2	D2 + CB1
IC_{50} ± SEM (µM)	5.976 ± 0.9092	8.506 ± 1.168*

Fig. 3.16 Changes in the affinity of the D2 receptor for dopamine in the presence of the CB1 receptor

Using HEK293 cells transiently transfected with Flag-rD2L alone or together with HA-rCB1wt competition binding experiments were performed. The radiolabeled D2 receptor agonist [^{3}H]raclopride was replaced by varying concentration of the endogenous ligand dopamine. (A) A small right shift in the binding curve was observed indicating a slight reduction in D2 receptor affinity for dopamine in the presence of the CB1 receptor (B) IC_{50} for competition binding experiments were determined showing the significantly reduced affinity of D2 for dopamine in the presence of the CB1 receptor. (Paired Student`s t-test. Data are presented as the mean ± SEM of n=3 independent experiments. *, $p<0.05$).

3.9.3 The CB1 receptor co-immunoprecipitates with the D2 receptor

The physical interaction of CB1 and D2 receptors has been previously demonstrated by co-immunoprecipitation from co-transfected HEK293 cells (Kearn 2005). Using a protocol modified from Kearn et al., the co-immunoprecipitation experiments were reproduced. In immunoprecipitates with an antibody against the D2 receptor, the 78 kD and the 47 kD Band of HA-CB1 were detected by

western blot. Additionally, a 92 kD band was observed (Fig. 3.17). As a negative control, the same experiment was performed using cells that do not express the D2 receptor but only the CB1 receptor. Even after long exposure, only the 92 kD band was detectable, indicating that the 92 kD band is unspecific, and the 78 kD and 47 kD bands specifically represent the CB1 receptor.

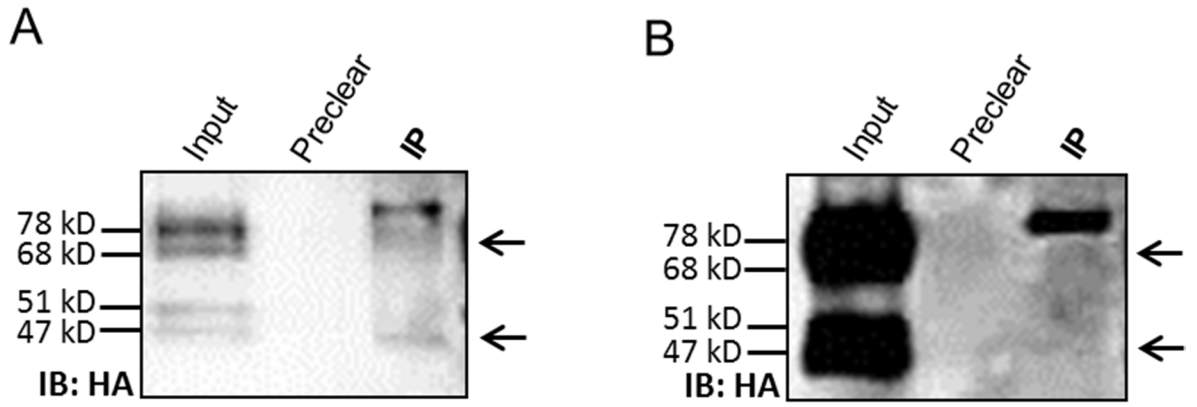


Fig. 3.17 The CB1 receptor co-immunoprecipitates with the D2 receptor

(A) Using HEK293 cells transiently transfected with the D2 receptor and the HA-CB1wt receptor, co-immunoprecipitation experiments were performed with a polyclonal antibody against the D2 receptor and subsequent western blot analysis with an antibody against the HA tag of the CB1 receptor. In the western blot, three bands of 92 kD, 78 kD and 47 kD were detectable in the IP fraction. (The picture represents results of n=3 independent experiments). (B) As a negative control, the same co-immunoprecipitation was performed with HEK293 cells that were transiently transfected only with the HA-CB1wt receptor. Even after overexposition of the western blot, only the 92 kD band was detectable, indicating that the bands at 78 kD and at 47 kD in A (arrows) are specific. (The picture shows results of 1 experiment). IB: Immuno blot; IP: Immunoprecipitation

3.9.4 D2 receptor expression in the striatum of the CB1F238L mutant rat

PET experiments detected an increased binding potential of D2 receptors in the striatum of CB1F238L mutant rats (I. Miederer; M. Schreckenberger, unpublished data). This could point to an increased amount of D2 receptor protein in the mutant rat striatum. However, in situ hybridization did not reveal any difference in D2 receptor mRNA levels (F. Steindel, unpublished data). The binding potential of the radiotracer used for PET experiments not only depends on available D2 receptor protein but also on the affinity of the receptor for dopamine and the dopamine level in the respective brain area, because dopamine can compete with the radiotracer for binding sites (Mukherjee 2005, Buchsbaum 2006, Vrieze 2011). This is why additional experiments where

performed to determine the D2 receptor protein amount in striata of CB1F238L mutant rats using autoradiography and western blot analysis.

3.9.4.1 Autoradiography of striatal D2 receptor in the CB1F238L mutant rat

Striatal D2 receptor protein levels were measured in brain slices of CB1F238L rats by labeling with the D2 receptor ligand [^{3}H]raclopride (Fig. 3.18A). Grey values in autoradiograms were quantified in the entire striatum, in the lateral striatum and in the nucleus accumbens (Fig. 3.18B). A very small but significant increase in specific [^{3}H]raclopride binding was observed in the entire striatum and the lateral striatum, but not in the nucleus accumbens (Fig. 3.18C).

3.9.4.2 Western Blot of striatal D2 receptor of the CB1F238L mutant rat

Western blot experiments were performed to further asses the amount of D2 receptor protein in the striata of CB1F238L mutant rats (Fig. 3.19A). A polyclonal antibody raised against a 28 AA peptide within the third intracellular loop of the receptor was used, thus detecting the long as well as the short isoform of D2. Hippocampus lysate was used as a negative control, because this brain area expresses very low levels of D2 receptor as compared with the striatum (Jackson 1994). Antibody specificity was further demonstrated by western blot using lysates of D2 receptor knockout mice (Fig. 3.19C). As compared with the two negative controls, a specific band at about 100 kD was observed in lysates from wild-type and mutant rats (Fig. 3.19A). There was significantly less D2 receptor protein detectable in lysates from CB1F238L mutant rat striata compared with the wild-type (Fig. 3.19B).

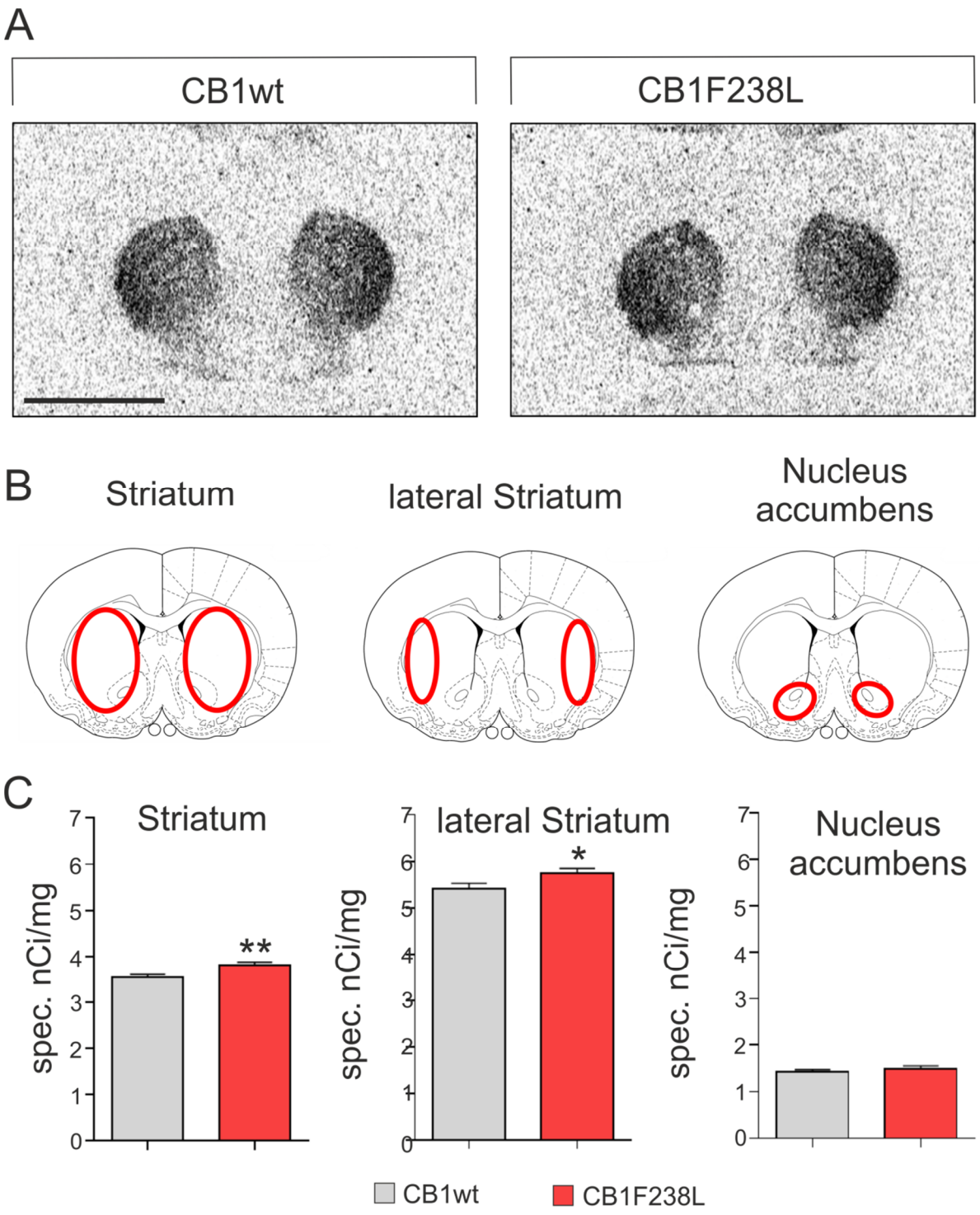


Fig. 3.18 Autoradiography of the D2 receptor in the striatum of CB1F238L mutant rats

(A) Autoradiography with striatal cryosections of the CB1F238L rat brain was performed using the radiolabeled D2 ligand [^{3}H]raclopride. (B) Grey values were quantified in the entire striatum, lateral striatum and nucleus accumbens as depicted in the schematic brain section. (The picture of the schematic brain section was modified from Paxinos 1997). (C) A very small but significant increase in [^{3}H]raclopride binding in the striatum and lateral striatum but not the nucleus accumbens of the CB1F238L mutant was observed as compared with wild-type rats. (Student`s t-test. Data are presented as the mean ± SEM of n= 18 striatal hemisphere slices per genotype of 3 mutant and 2 wild-type animals. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., $p>0.05$).

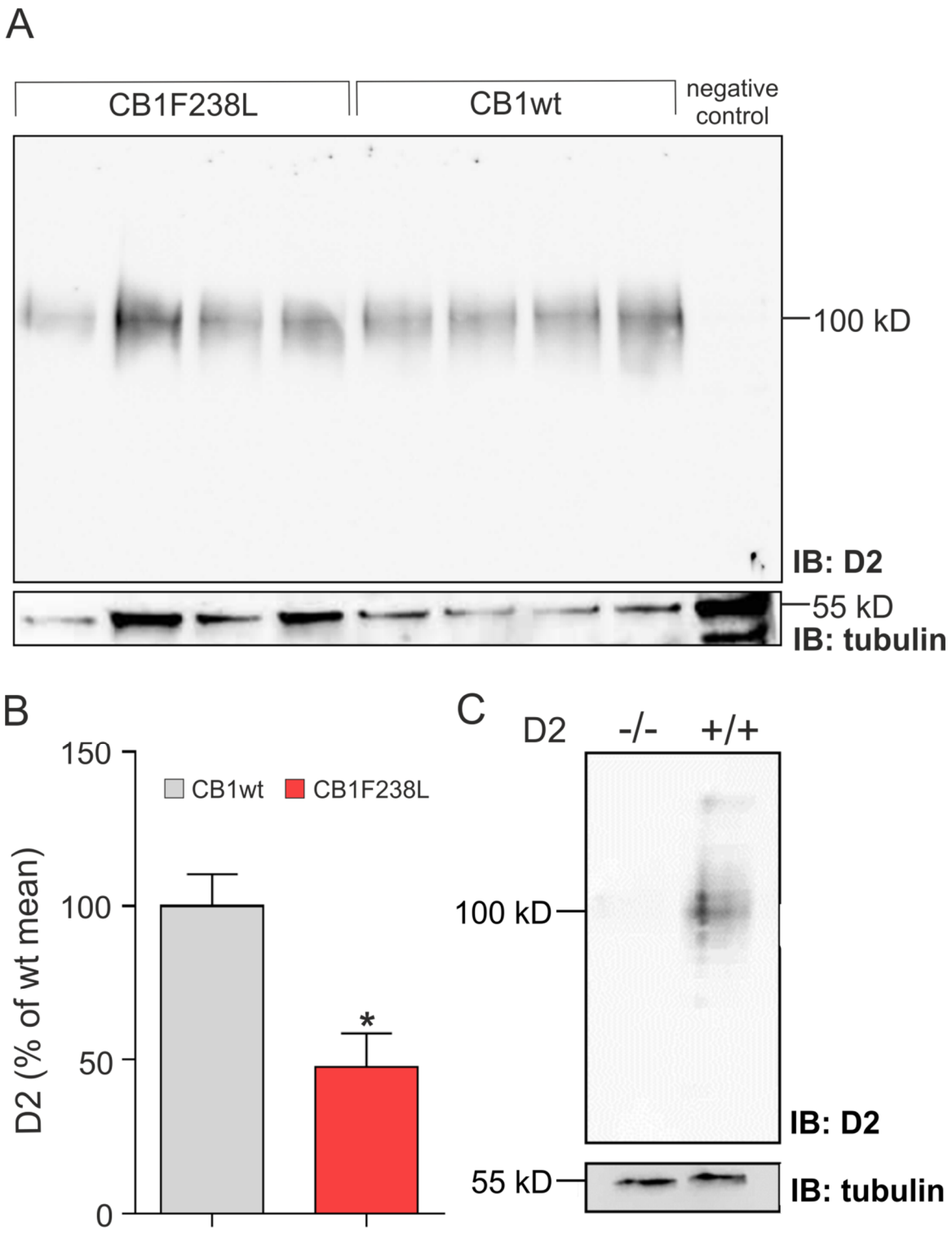


Fig. 3.19 Western blot analysis of the D2 receptor in the striatum of CB1F238L mutant rats

(A) The relative amount of D2 receptor protein in the striatum of CB1F238L mutant rats was analyzed by western blot using a polyclonal antibody against the D2 receptor. One band at 100 kD was observed which was specific as compared with hippocampus lysate as a negative control. (B) There was significantly less D2 receptor protein in the striata of CB1F238L mutant rats as compared with wild-type rats. (C) The western blot of wild-type (+/+) and D2 receptor knockout (-/-) mice further demonstrates the specificity of the 100 kD band observed with the polyclonal D2 antibody. (Student`s t-test. Data are presented as the mean ± SEM of n= 4 animals per genotype. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., $p>0.05$). IB: Immuno blot

3.9.5 Generation of HEK293 cells stably expressing the D2 receptor alone or together with the wild-type and F238L mutant CB1 receptor

HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were stably transfected with Flag-D2L, and stable clones were selected by addition of zeocin to the culture medium. D2 receptor expression was analyzed by western blot using the polyclonal antibody against the D2 receptor. Four bands were observed for $HEK^{D2L\text{-}CB1wt}$ and $HEK^{D2L\text{-}CB1F238L}$ cells.

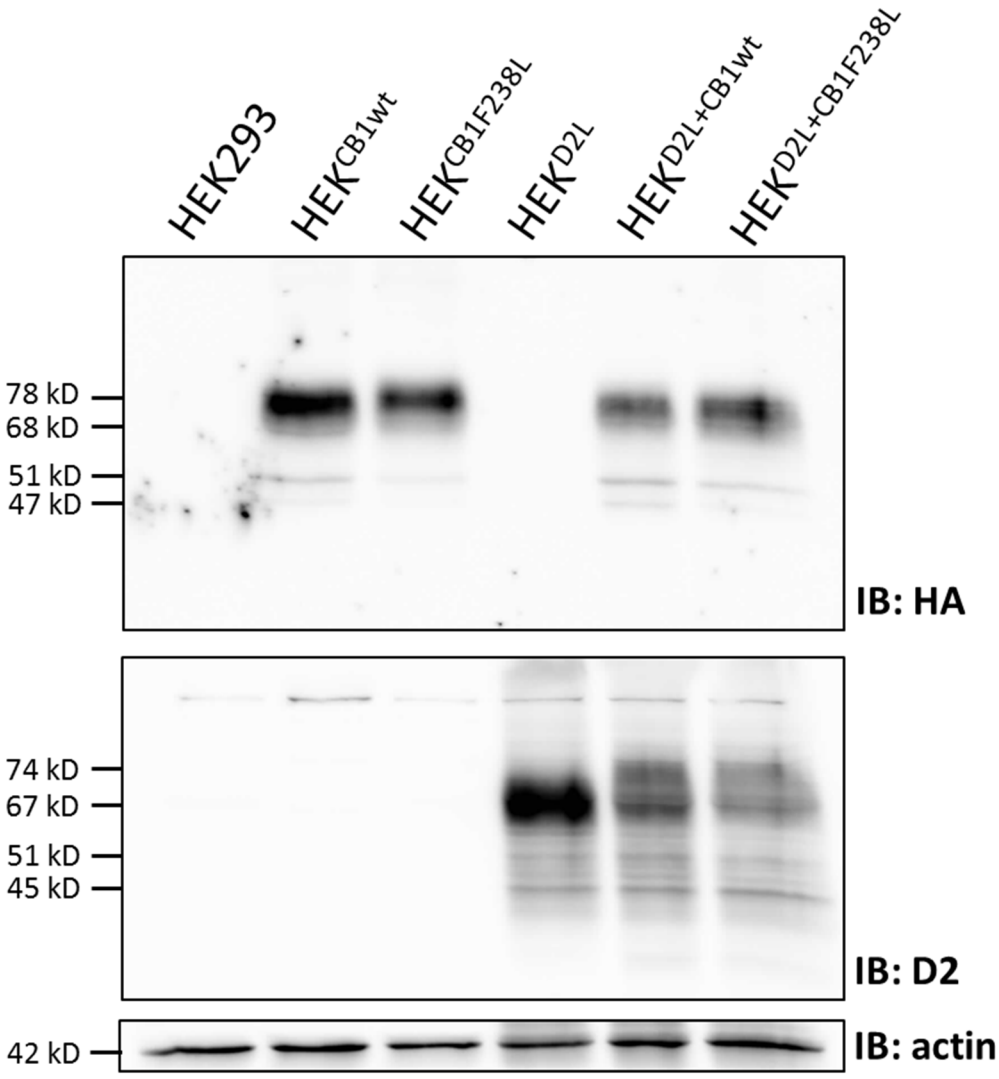


Fig. 3.20 Characterization of HEK^{D2}, $HEK^{D2\text{-}CB1wt}$ and $HEK^{D2\text{-}CB1F238L}$ cells

HEK293, HEK^{CB1wt} and $HEK^{CB1F238L}$ cells were stably transfected with pcDNA3-Flag-D2L constructs, which include a zeocin resistance cassette to select positive clones by keeping the cells in medium containing zeocin. In order to evaluate and quantify the expression of Flag-D2, western blot analysis was performed using the polyclonal antibody against the D2 receptor. In all three cell lines, three bands were detected at 67 kD, 51 kD and 45 kD. In cells which co-express Flag-D2 and HA-CB1 receptors, an additional band at 74 kD was observed. All the bands were specific as compared to HEK293, HEK^{CB1wt} and $HEK^{CB1F238L}$ cells. IB: Immuno blot

The bands were at 74 kD, 67 kD, 51 kD and 45 kD, and all of them were shown to be specific as compared to untransfected HEK293 cells, HEK^{CB1wt} and $HEK^{CB1F238L}$ cells as negative controls. However, in the HEK^{D2L} cell line, only three bands were detectable at 67 kD, 51 kD and 45 kD. Furthermore, the 67 kD band had a higher intensity as compared to $HEK^{D2L\text{-}CB1wt}$ and $HEK^{D2L\text{-}CB1F238L}$ cells (Fig. 3.20).

4. DISCUSSION

The CB1 receptor is one of the most abundant GPCRs in the brain (Mechoulam 2012). It is involved in a huge variety of physiological functions and pathological conditions mediated by various brain regions, the peripheral nervous system and peripheral organs (Pacher 2006). Due to this diversity, the CB1 receptor constitutes a promising target for medical treatment. However, this diversity also entails the risk of side effects of potential drugs. Hence, understanding the mechanisms contributing to the functional selectivity of the CB1 receptor constitutes an important goal for the improvement of rational drug design. Furthermore, evidence is emerging that GPCR trafficking affects receptor signaling and, in neurons, regulates their polarization towards the axon. However, not much is known yet about the trafficking and polarization of the CB1 receptor. In this work, the role of a phenylalanine residue in TMH4 in CB1 receptor trafficking and signaling was examined by characterizing a F238L point mutated CB1 receptor in cell culture experiments.

4.1 The CB1F238L receptor undergoes enhanced basal endocytosis through lipid rafts/caveolae

Surface expression of the CB1wt and the CB1F238L receptor was examined in HEK^{CB1wt} and $HEK^{CB1F238L}$ cells using a trypsin protection assay. The trypsin protection assay exploits the fact that trypsin can cleave the extracellular, N-terminal HA tag, which was added to the CB1 receptor. This renders only intracellular receptor detectable by an antibody against the HA tag in western blot analysis. This allows the quantification of surface receptor (Grimsey 2010). However, it could be that protein interactions protect trypsin cleavage sites from trypsin access and hence inhibit proteolytic cleavage. If the F238L mutation affects protein interactions of the CB1 receptor, it might influence trypsin cleavage. What speaks against this possible caveat is that trypsin cleaves peptides at lysine or arginine residues. The HA-tag is located at the N-terminus of the receptor which contains 10 potential trypsin cleavage sites. For the trypsin protection assay used in this work, it is not important where in the N-terminus the cleavage occurs, and it should be unlikely that all of the 10 cleavage sides are occluded due to differential protein interactions.

A significant reduction in surface expression of the CB1F238L receptor was observed as compared with the wild-type receptor. The reduced surface expression could be explained by retention of the CB1 receptor in the ER or Golgi apparatus or by enhanced endocytosis. Endocytosis of the CB1

receptor is known to be induced by agonist treatment and to be inhibited by inverse agonist treatment (Hsieh 1999, Grimsey 2010). Indeed, WIN55212-2 treatment induced a strong endocytosis of the CB1F238L receptor, which did not differ in its extent from the CB1wt receptor. Hence, agonist induced endocytosis does not seem to be affected by the point mutation. However, the experimental setup used in this work cannot resolve the endocytosis kinetics, which could differ between the two receptors. As the point mutation significantly reduces the affinity of the CB1 receptor for the agonist WIN55212-2, it might well be that there are differences in the endocytosis kinetics induced by a defined WIN55212-2 concentration. An experiment showing the time course of agonist induced receptor endocytosis could give further interesting insights.

As mentioned above, inverse agonist treatment can prevent endocytosis of the CB1 receptor. SR141716 treatment was able to fully rescue surface expression of the CB1F238L receptor to wild-type levels. Interestingly, although the CB1F238L receptor showed decreased affinity for SR141716, the effect of this inverse agonist on surface expression was significantly higher for the CB1F238L receptor than for the CB1wt receptor. These findings are a first indicator that it might be basal endocytosis and not the biosynthesis and transport to the plasma membrane of the CB1 receptor that is affected by the F238L mutation. To find further support for this idea, the two described CB1 receptor internalization pathways, which are clathrin coated pit and lipid raft/caveolae endocytosis, were inhibited. Clathrin coated pit endocytosis was inhibited by hypertonic sucrose treatment, which induces an abnormal polymerization of clathrin and hence inhibits pit formation (Heuser 1989). Lipid raft/caveolae endocytosis was inhibited by MβCD-mediated depletion of membrane cholesterol, a lipid essential for lipid raft formation. MβCD, but not hypertonic sucrose treatment, rescued surface expression of CB1F238L receptor, indicating that it is enhanced lipid raft/caveolae mediated basal endocytosis accounting for the reduced surface expression of CB1F238L receptor. It needs to be mentioned that MβCD treatment has been reported also to affect clathrin coated pit budding under certain conditions (Rodal 1999, Subtil 1999). However, the finding that sucrose treatment did not alter surface expression of the CB1F238L receptor indicates that with the conditions used in this work the CB1F238L receptor undergoes increased caveolae/lipid raft mediated rather than clathrin coated pit endocytosis. As the CB1 receptor has been shown to co-immunoprecipitate and co-localize with caveolin-1 (Bari 2008), it would be interesting, however, to see whether an siRNA-mediated knockdown of caveolin-1 would have the same effect as MβCD treatment as compared with a knockdown of clathrin heavy chain or overexpression of the C-terminus of AP180, an inhibitor of clathrin coated pit endocytosis.

If the CB1F238L receptor undergoes increased lipid raft/caveolae mediated endocytosis, it might also have an increased preference to reside in lipid rafts/caveolae. To test this hypothesis, lipid

rafts/caveolae were prepared from HEK^{CB1wt} and HEK^{CB1F238L} cells and analyzed by western blot. Indeed, there was an increase in lipid raft/caveolae associated CB1F238L receptor as compared with the wild-type receptor. It is known that ligand binding can modulate lipid raft recruitment of the CB1 receptor (Sarnataro 2005, Sarnataro 2006). In the two studies by Sarnataro et al., 24 h treatment with AEA or SR141716 in a human breast cancer cell line led to a reduction in lipid raft associated CB1 receptor. Taking together the finding that SR141716 treatment rescues surface expression of CB1F238L receptor and that SR141716 treatment has been reported to reduce CB1 receptor lipid raft association nurtures the idea that SR141716 induces the movement of the CB1F238L receptor out of lipid rafts/caveolae and hence protects it from being internalized via this pathway. To test if SR141716 treatment removes CB1F238L receptor from lipid rafts/caveolae, the same lipid raft/caveolae preparation experiment as before was conducted. However, this time in the presence or absence of SR141716. Surprisingly, SR141716 treatment significantly increased the amount of CB1F238L receptor in lipid rafts/caveolae as compared with vehicle treated mutant and wild-type receptor. There is also a tendency of increased lipid raft/caveolae associated CB1wt receptor upon SR141716 treatment. However, this effect did not reach statistical significance. In conclusion, SR141716 treatment does not seem to protect the CB1F238L receptor from basal endocytosis by keeping it out of lipid rafts/caveolae. It might rather inhibit the endocytosis through lipid rafts. Hence, inhibition of lipid raft/caveolae mediated endocytosis of CB1F238L receptor by SR141716 treatment could lead to an accumulation of CB1F238L receptors in lipid rafts.

The contradictory effects of SR141716 treatment on lipid raft association of the CB1 receptor observed in this work as compared to the findings by Sarnataro et al. could be due to different durations of SR141716 treatment. In this work, CB1 receptor expressing HEK cells were treated only for 45 min, whereas Sarnataro et al. treated the CB1 receptor, which was furthermore expressed in a different cell line, for 24 h. It has previously been shown that 2 min of agonist treatment increases, whereas 15 min treatment decreases, lipid raft/caveolae association of the CB1 receptor (Asimaki 2011). Although different cell lines were used in this work and the study by Sarnataro et al., which renders the results hardly comparable, it is tempting to speculate that it might not solely be the conformational and activity states of the CB1 receptor that mediate lipid raft association, because it should be assumed that SR141716 stabilizes the inactive conformation regardless of the duration of treatment. It might rather be that short-term and long-term inverse agonist treatment induce different protein interactions or posttranslational modifications of the receptor, which could affect its lipid raft allocation.

Indeed, there is evidence that the phosphorylation of a receptor could regulate its trafficking fate. It has been proposed that the pattern of phosphorylation of the GPCR C-terminus can determine

different functions of the recruited β-arrestin (Kenakin 2011). Depending on the conformation induced or stabilized by the ligand, different GRKs, which can compete with one another for receptor binding sites (i.e. GRK2/3 vs GRK5/6), can phosphorylate the receptor`s C-terminus at varying residues (Kenakin 2011). This implicates that the phosphorylation pattern of a GPCRs C-terminus can be regulated and determines the trafficking fate of the receptor. Furthermore, it has been demonstrated that phosphorylation of the β-1 adrenergic receptor (β-1AR) by protein kinase A (PKA) or GRK directs the receptor to different endocytic pathways (Rapacciuolo 2003). PKA phosphorylation induces lipid raft/caveolae mediated endocytosis of β-1AR, whereas GRK phosphorylation at residues different from PKA phosphorylation sites induces clathrin coated pit endocytosis (Rapacciuolo 2003). The CB1 receptor is known to be phosphorylated by GRK3 and PKC, but no phosphorylation by PKA was reported yet (Garcia 1998). However, it is not known whether the PKC mediated phosphorylation of a serine in ICL3 affects endocytosis of the CB1 receptor. Interestingly, phosphorylation of a serine in ICL3 of the β-1AR by PKA has been shown to promote recycling of the receptor after endocytosis (Gardner 2007).

In conclusion, as the CB1F238L receptor shows stronger basal lipid raft/caveolae mediated endocytosis, it would be interesting to test if there are differences in the phosphorylation pattern of the CB1F238L receptor C-terminus or ICL3 as compared with the CB1wt receptor, for example using an LC-MS screening approach. This might help to understand the role of phosphorylation of GPCRs and especially the CB1 receptor in endocytic sorting.

4.2 DAGL inhibition does not affect surface expression of the CB1F238L receptor

Basal endocytosis as well as basal activity of GPCRs is usually considered to be constitutive, which means in the absence of any ligand. However, in case of the CB1 receptor it is possible that the receptor is activated in a cell autonomous way if the cell that expresses the CB1 receptor also expresses DAGL. The CB1 receptor could then be activated by 2-AG, which was produced from the very same cell. Indeed, Turu et al. demonstrated that THL mediated inhibition of DAGL in cultured primary hippocampal neurons, in Chinese hamster ovary cells (CHO) and in HEK293 cells increases surface expression of heterologous CB1 receptor (Turu 2007). However, in this work, the treatment of HEK^{CB1wt} and $HEK^{CB1F238L}$ cells with THL under the same conditions as used by Turu et al. did not alter surface expression of CB1wt or CB1F238L receptor. At least for the CB1wt receptor, a positive effect on surface expression would be expected to reproduce the findings by Turu et al.. One could conclude that cell autonomous 2-AG activation of the CB1 receptor is not the cause of the basal

endocytosis of CB1F238L receptor observed in this work. However, additional experiments are needed to support this idea. Such experiments could be siRNA mediated knockdown of DAGL and evaluation of 2-AG contents in the cells and the cell culture medium by mass spectrometry to control for the functional inactivation of DAGL. Furthermore, binding experiments could be performed to determine the affinity of CB1F238L receptor for 2-AG.

4.3 The F238L mutation affects axonal polarization of the CB1 receptor in primary hippocampal neurons

There is experimental evidence that somatodendritic endocytosis of the CB1 receptor is one crucial step for the polarization of the receptor towards the axon (Leterrier 2006, McDonald 2007, Simon 2013). Furthermore, it has been suggested that the rate of somatodendritic endocytosis is the key parameter that determines the extent of receptor polarization (Simon 2013). The endocytosis rate in turn has been suggested to be determined by the receptor`s activity (Simon 2013). However, only clathrin coated pit endocytosis has been suggested to play a role in this mechanism. Nothing is known about the role of lipid raft/caveolae mediated endocytosis in polarization of the CB1 receptor.

Since the F238L mutation causes increased endocytosis of the CB1 receptor, it was hypothesized that the mutant receptor would show a stronger polarization towards the axon. To test this hypothesis, surface polarization of transfected CB1wt or CB1F238L receptor was measured in primary hippocampal neurons. Surface expression was quantified for three dendrites and for the axonal areas proximal, intermediate or distal to the soma. To determine a polarization index, average surface CB1 receptor in dendrites was divided by average surface expression on the entire axon. Furthermore, a surface CB1 receptor gradient was determined by plotting surface CB1 receptor against the three distances relative to the soma. The polarization indices for both CB1wt and CB1F238L receptor were larger than 1, indicating a polarization towards the axon. This is in accordance with findings from previous studies (Leterrier 2006, Simon 2013). Furthermore, in support of the above mentioned hypothesis, the polarization index for the CB1F238L receptor was double as high as for the CB1wt receptor. This was predominantly due to the strong reduction in dendritic surface CB1F238L receptor. Furthermore, there was a significantly stronger gradient of surface CB1F238L receptor along the axon as compared with the CB1wt receptor. This is in accordance with the predictions from Simon et al., which state that the stronger the endocytosis of a receptor is, the steeper is its surface expression gradient towards the axon tip (Simon 2013). From these findings the emerging picture is that the CB1F238L receptor undergoes increased basal endocytosis in the somatodendritic area,

probably by lipid raft/caveolae mediated endocytosis. The subsequent transcytosis of endocytosed CB1 receptor towards the axon could account for the increased polarization of the CB1F238L receptor.

However, although this hypothesis is plausible in the light of what is already known about CB1 receptor polarization, some other scenarios could be possible too, and additional experiments are needed for clarification of this issue. The mutant receptor could also be selectively targeted to the axon via a route distinct from transcytosis, directly after synthesis without ever occurring at the somatodendritic surface (route 1 in Fig. 4.1). One mechanism for this could be differential sorting of the mutant receptor in the Golgi apparatus. However, there is no experimental evidence so far of such a route for the CB1 receptor. As the CB1F238L mutation in the rat does not affect untranslated regions of the CB1 gene, an effect on a possible mRNA transport towards the axon is unlikely. In addition, so far there is no experimental evidence for such CB1 receptor mRNA transport towards the axon. The most likely alternative mechanism is increased basal endocytosis of the CB1F238L receptor in the somatodendritic area and subsequent sorting into lysosomes where the receptor is degraded. As the endocytosis rate in the axon is 4 times lower than in the somatodendritic area (Simon 2013), this would lead to a selective removal of CB1 receptor from the somatodendritic membrane and hence a polarization towards the axon. However, in this case, the CB1 receptor in the axon needs to be transported there by another targeting mechanism. This scenario is supported by the finding that significantly more CB1F238L receptor co-localizes with the lysosomal marker Lamp2 in HEK293 cells as compared with the wild-type receptor. However, it might be that the transcytosis machinery is not fully expressed in HEK293 cells, as these are non-polarized cells and hence, in the absence of these endosomes, the receptor becomes targeted to lysosomes. Furthermore, lysosomal CB1 receptor has previously been reported to associate with AP-3 in HEK293 cells (Rozenfeld 2008). A neuronal form of AP-3 is known to target vesicles towards the axon (Danglot 2007), and Rozenfeld et al. speculate that neuronal AP-3 might be involved in the axonal polarization of the CB1 receptor (Rozenfeld 2008).

To test conclusively if it is somatodendritic endocytosis and transcytosis that mediate the increased axonal polarization of CB1F238L receptor, compartment specific antibody feeding of surface CB1 receptor in the somatodendritic area and subsequent detection of the labeling antibody in the axonal compartment needs to be performed, as it has been done by Simon et al. (Simon 2013). To prove that the increased axonal polarization of the CB1F238L receptor in neurons is lipid raft/caveolae mediated, the same experiment could be performed after knockdown of caveolin-1.

The CB1 receptor in growth cones is known to be an important player in the axonal guidance and synaptogenesis of glutamatergic and GABAergic synapses (Harkany 2008). As the CB1F238L receptor

shows a stronger gradient towards distal parts of the axon, it might be enriched in growth cones. If this is the case, it would be interesting to examine neuronal migration and growth cone guidance in CB1F238L or CB1wt receptor expressing neurons as differences in these processes could affect the hardwiring of the mutant rat brain.

4.4 Diverse effects of the F238L mutation on CB1 receptor activity

As mentioned before, the CB1 receptor shows basal as well as agonist stimulated activity. With samples from the CB1F238L receptor mutant rat, Schneider et al showed that stimulation with various agonists (WIN55212-2, CP55940, HU-210) results in increased [^{35}S]GTPγS binding to the receptor as compared with wild-type rats. This, together with computational simulation, led to the conclusion that the CB1F238L receptor is more active than the wild-type receptor. This was supported by electrophysiological experiments showing a decreased presynaptic transmitter release probability in mutant rats (Schneider 2014). Although not significant, the finding of this work that WIN55212-2 has a slightly stronger effect on CB1F238L receptor induced ERK1/2 phosphorylation supports these findings. However, although the relative agonist induced activation of the CB1F238L receptor seems to be higher, the absolute agonist induced activation seems to be lower than in the wild-type receptor. Therefore, the mutant receptor probably has reduced basal activity but is more easily activated by an agonist. This is further supported by the finding that the allosteric modulator ORG27569 has a much stronger effect on CP55940 binding to the CB1F238L receptor than the wild-type receptor. This would mean that although the relative activation via agonists is increased for the CB1F238L receptor, the total agonist induced activity cannot reach the level of the wild-type receptor. This is probably only true for the agonist concentration used in these experiments. A dose response curve of agonist induced total ERK1/2 phosphorylation could determine whether the mutant receptor can reach the same plateau as the wild-type receptor. The idea that the mutant receptor shows reduced basal activity is supported by the finding that basal [^{35}S]GTPγS binding as well as basal ERK1/2 phosphorylation in HEK^{CB1F238L} was significantly decreased as compared with HEK^{CB1wt}. Furthermore, although not statistically significant, the effect of inverse agonist treatment on ERK1/2 phosphorylation seemed to be smaller for the CB1F238L receptor as compared with the wild-type receptor, a finding that can also be explained by reduced basal activity of the CB1F238L receptor.

The idea of reduced basal activity of the mutant receptor might seem contradictory to the electrophysiological results found in the rat. These results indicate a reduced presynaptic release

probability due to increased CB1 receptor signaling (Schneider 2014). However, although these measurements (paired pulse ratio, spontaneous excitatory postsynaptic currents) were performed in the absence of any pharmacological agonist or electrophysiologically stimulated eCB release, it could still be that there is a tonic activation of the CB1 receptor due to tonic eCB release. Hence, they would not reflect constitutive activity of the receptor.

Although the allosteric modulator ORG27569 increases CP55940 binding, it reduces CP55940 induced G protein binding but not ERK1/2 phosphorylation (Ahn 2012, Ahn 2013, Baillie 2013). This has been explained by an ORG27569 mediated shift from G protein signaling to β-arrestin signaling (Ahn 2012, Ahn 2013). The strongly increased effect of ORG27569 on the CB1F238L receptor and the clear endocytosis phenotype of the mutant receptor shown in this work could tempt to speculate that the mutation somehow affects interaction of the CB1 receptor with β-arrestins. This could be tested using β-arrestin recruitment assays and knockdown approaches for example. The ERK1/2 phosphorylation experiments and the endocytosis experiments in this work were performed using the agonist WIN55212-2. Although ORG27569 also affects WIN55212-2 pharmacology, the effect is much stronger for CP55940 (Baillie 2013). Hence, it would be interesting to examine the effect of CP55940 on endocytosis of the mutant receptor. Furthermore, the binding sites for WIN55212-2 and CP55940 have been suggested to be different. An aromatic microdomain between TMH 3-4-5-6 has been suggested to constitute the binding pocket for WIN55212-2 and SR141716 but not for CP55940 (McAllister 2003). Together with the finding that the F238L mutation in TMH4 reduces the affinity of the WIN55212-2 and SR141716 but not for CP55940, this could indicate that F238 participates in WIN55212-2 and SR141716 binding. However, the finding that the allosteric modulator ORG27569 has a much stronger effect on CP55940 binding to the CB1F238L receptor as compared with the wild-type receptor, could also implicate that F238 in TMH4 participates in the transmission of information from the allosteric modulator binding site to the CP55940 binding site. It could also be that F238 is a part of the ORG27569 binding site. This should be tested by saturation binding of radiolabeled ORG27569.

There is evidence from D2 receptors and calcitonin receptors that TMH4 is involved in the homomerization of these receptors (Lee 2003, Harikumar 2010). Furthermore, there is evidence for cooperativity in D2 receptor homo-oligomers with regard to agonist binding (Kara 2010). It has been reported that the CB1 receptor might also exist as homomers (Wager-Miller 2002). In conclusion, it is feasable that TMH4 could also be involved in homomerization of the CB1 receptor. If homomerization leads to cooperativity with regard to agonist binding and if this cooperativity is due to allosteric modulation of the monomers by each other, this could underlie the strong difference in allosteric modulation between the wild-type and F238L mutant receptor. Hence, it would be very

interesting to examine the homomerization of the F238L mutant receptor as compared with the wild-type receptor. This could be addressed by transfecting Flag-tagged CB1wt or CB1F238L constructs into the HEK^{CB1wt} and $HEK^{CB1F238L}$ cell lines generated in this work.

4.5 Contradictory relationship between activity and endocytosis of the CB1F238L receptor

As mentioned above, it is believed that basal endocytosis is due to basal activity of the CB1 receptor (Simon 2013). Furthermore, it has been suggested that the axonal polarization index is positively proportional to receptor activity as evaluated by means of constitutively active or inactive CB1 receptor mutants and pharmacological treatment (Simon 2013). However, as discussed above, it seems that the CB1F238L receptor shows increased agonist induced activity but reduced basal activity. In this work, the polarization in primary neurons was measured in the absence of any exogenous ligand. Hence, it is not increased ligand induced activity that could account for increased polarization. Although cell autonomous 2-AG could be present in this setup, DAGL inhibition in $HEK^{CB1F238L}$ cells did not affect CB1F238L receptor endocytosis. In conclusion, it seems that, although the CB1F238L receptor is less active, it still shows increased polarization which is contradictory to what has been stated in the literature so far.

The mechanism thought to link receptor activity to endocytosis is G protein mediated recruitment of GRKs (and probably other kinases like PKA or PKC) which phosphorylate the receptor`s C-terminus and hence initiates β-arrestin binding to the receptor and subsequent clathrin coated pit endocytosis. However, the F238L mutation in the CB1 receptor does not seem to affect clathrin coated pit endocytosis but lipid raft/caveolae mediated endocytosis instead. Probably, lipid raft/caveolae mediated endocytosis and polarization do not depend on CB1 receptor activity as much as clathrin coated pit endocytosis. Additionally, the point mutation might affect protein interactions that regulate CB1 receptor endocytosis and trafficking, which could further mask the regulatory role of receptor activity. To test this hypothesis, pull down experiments with the mutant and wild-type receptor and subsequent proteomic screening for differences in trafficking related protein interactions could be performed.

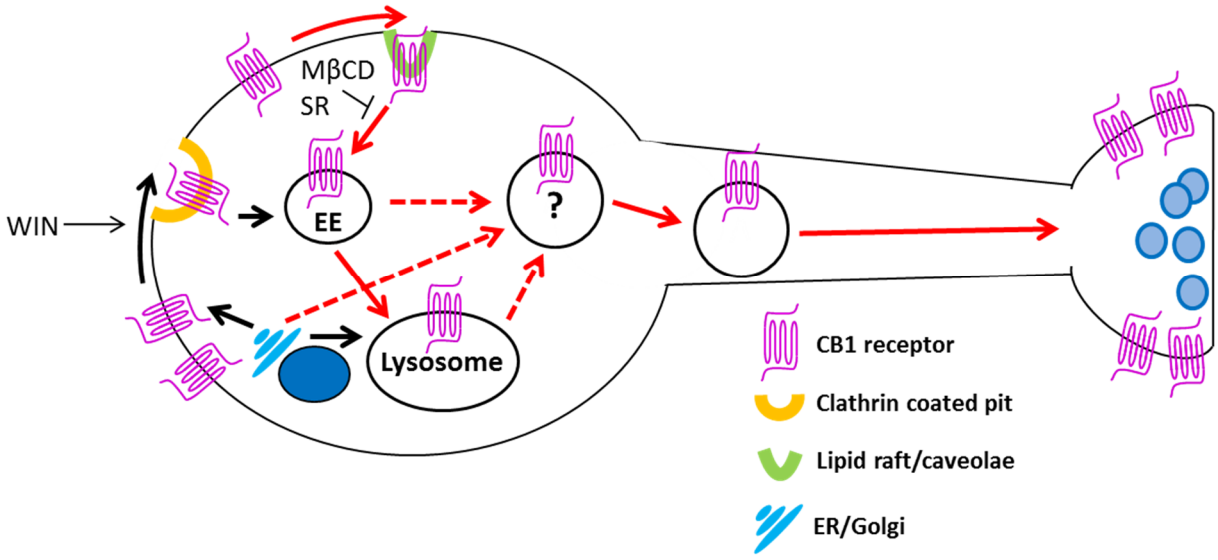


Fig. 4.1 Trafficking of the CB1F238L receptor

The proposed trafficking of the CB1F238L receptor is depicted. Red arrows indicate pathways that were demonstrated in this work to be increased for the CB1F238L receptor compared with the wild-type receptor. Dashed arrows indicate pathways that are hypothesized to be changed for the CB1F238L receptor, but that need further experimental verification. The CB1F238L receptor undergoes increased lipid raft mediated basal endocytosis and is targeted to lysosomes in HEK293 cells. In primary neurons the CB1F238L receptor shows an increased polarization towards the axon. Although it is likely that the increased endocytosis causes increased transcytosis of the CB1F238L receptor, this hypothesis needs further experimental proof. Furthermore, the nature of the vesicles that transport the CB1 receptor to the axon is not yet determined. EE: early endosomes; ER: endoplasmic reticulum

4.6 The CB1 and the D2 receptor physically and functionally interact in HEK293 cells

Activation of the CB1 receptor has been reported to reduce the affinity of the D2 receptor for dopamine in striatal membrane preparations (Marcellino 2008). However, it is not known whether already the co-expression of CB1 and D2 receptors, without any pharmacological activation, modulates D2 receptor affinity. To address this question, competition binding experiments were performed with HEK293 cells transiently expressing the D2 receptor alone or together with the CB1 receptor. The mere co-expression of the CB1 receptor reduced the affinity of the D2 receptor for dopamine as demonstrated by a significantly increased IC_{50} for dopamine. The effect of the CB1 receptor on D2 receptor affinity has been attributed to heteromerization of these two receptors (Marcellino 2008). Indeed, the physical interaction of CB1 and D2 receptors has been demonstrated among others by co-immunoprecipitation from HEK293 cells (Kearn 2005). In western blots of D2 receptor immunoprecipitates, Kearn et al. found the high molecular CB1 receptor band of about 66 kD. This finding could be reproduced in this work. However, in this work an additional 47 kD CB1

receptor band was detected in co-immunoprecipitates. The calculated molecular weight of the HA tagged CB1 receptor is around 54 kD (S. Ruehle, unpublished data). In rat brain samples as well as in HEK293 cells, the high molecular band (around 70 kD) of the CB1 receptor has been reported to represent the mature N-linked glycosylated form of the receptor whereas the low molecular weight bands represent unglycosylated receptor (Song 1995, Andersson 2003, S. Ruehle, unpublished data). However, the role of these glycosylations for CB1 receptor function is not yet known. The finding that the D2 receptor co-immunoprecipitates with unglycosylated forms of the CB1 receptor might however lead to the speculation that the heteromerization of the two receptors could take place already in early stages of receptor synthesis.

Although statistically significant, the effect of CB1 receptor co-expression on D2 receptor affinity was quite small and the standard deviation between experiments was relatively high. This might be due to the fact that transient transfection of HEK293 cells with the two receptors does not necessarily mean that both receptors are co-expressed in the same cells. It might be that, even if expression of both receptors is high, they are expressed in separate cells in the culture, and hence, never meet. This means that, although for every experiment the interaction between CB1 and D2 receptors is necessarily higher in co-transfected cells than in single transfected cells, the total amount of CB1 and D2 receptor interaction might vary between experiments. Therefore, in this work HEK293 cells were generated which stably express CB1 and D2 receptors to ensure co-expression and similar expression levels of both receptors in each cell of a culture. These cells show similar expression levels of CB1 and D2 receptors and hence a similar ratio between these two receptors which is important for quantitative experiments on heteromerization. Western blots of the D2 receptor stably expressed in HEK293 cells showed three bands with molecular weights of 67 kD, 51 kD and 45 kD. Interestingly, when co-expressed with the CB1 receptor, a fourth band appeared at 74 kD. All four bands where specific as compared with several negative controls. In contrast, no difference in band size was observed for the CB1 receptor when co-expressed with the D2 receptor. It could be speculated that co-expression of the two receptors and/or heteromerization affects posttranslational modification of the D2 receptor causing this band shift. This might be an interesting subject for future studies using the cell lines generated in this work.

4.7 Controversial effects of the CB1F238L mutation on D2 receptor expression in the rat striatum

PET experiments with anesthetized CB1F238L mutant rats revealed an increased binding potential of the D2 receptor radioligand [^{18}F]fallypride in the striatum of the mutant rats (I. Miederer, M. Schreckenberger, unpublished data). The first interpretation of these results was an increase in D2 receptor protein. However, D2 receptor mRNA levels where not changed as assessed by in situ hybridization (Steindel, unpublished data). Nevertheless, the PET binding potential of a radioligand does not solely depend on the amount of receptor protein. Endogenous dopamine can compete with the radioligand for binding sites (Mukherjee 2005, Buchsbaum 2006, Vrieze 2011). Hence, a decrease in dopamine release or a decrease in the D2 receptor`s affinity for dopamine could also lead to increased radioligand binding. D2 receptor affinity for dopamine is known to be modulated by CB1 receptor activity and could hence be affected by the mutation (Marcellino 2008). However, dopamine levels seemed not to be changed in the striatum of mutant rats (M. Schneider, R. Spanagel, unpublished data).

In this work, D2 receptor protein expression in the striatum of mutant rats was measured by additional methods such as receptor autoradiography and western blot. Autoradiography with the D2 receptor radioligand [^{3}H]raclopride revealed a significant but still vanishingly small increase in D2 receptor protein. In contrast, western blot analysis indicated a significant decrease in D2 receptor protein. It needs to be mentioned here, that the sample size of these experiments was small and further experiments might be needed. However, the recent findings that D2 receptor trafficking affects radioligand binding (Guo 2010, Quelch 2014) led to a speculative hypothesis that might help to integrate the contradictory results gained so far.

Guo et al. used chemical inactivation of surface D2 receptors to differentially measure the affinity of several D2 receptor radioligands and endogenous ligands at intracellular or surface D2 receptors (Guo 2010). They found that for [^{3}H]raclopride and [^{18}F]fallypride the affinity to surface receptors was two fold and three fold increased, respectively, than for intracellular receptors. For dopamine, the affinity to surface receptors was twenty times higher than to intracellular receptors. One of the authors` explanations for the large difference in affinity change between the radioligands and dopamine is that the membrane is less permeable to dopamine than to the radioligands. This is probably due to dopamine`s reduced lipophilicity (Guo 2010). Importantly, this would mean that radioligands strongly compete with dopamine at surface D2 receptors but compete less at intracellular receptors, because dopamine hardly crosses the plasma membrane (Fig. 4.2).

In conclusion, an increase in intracellular D2 receptor paralleled by a decrease in surface receptor could cause an increased binding potential of [^{3}H]raclopride and [^{18}F]fallypride because of a reduced competition of the radioligands with endogenous dopamine (Fig. 4.2). Accordingly, if the CB1F238L receptor affects D2 receptor trafficking this could explain the increased binding potential of [^{18}F]fallypride in the striatum of the mutant rat even if there is no increase in D2 receptor protein.

There are some arguments supporting the idea that the F238L mutation in the CB1 receptor could affect D2 receptor trafficking. The F238L mutation is located at the lipid facing side of TMH4 (Schneider 2014). Lipid facing residues in TMH4 have been shown to mediate homodimerization of the calcitonin receptor (Harikumar 2010). Furthermore, TMH4 has been shown to be involved in D2 receptor dimerization (Lee 2003). In addition, it has been demonstrated that CB1 and D2 receptors can heteromerize (Kearn 2005, Marcellino 2008). Hence, it might well be that a mutation in the lipid facing side of TMH4 of the CB1 receptor could affect heteromerization of CB1 and D2 receptors. Furthermore, co-internalization of GPCRs has been shown for several GPCR heteromers including some involving the CB1 receptor (Torvinen 2004, Ward 2011, Huang 2013). Hence, co-trafficking of CB1 and D2 receptors is not unlikely. Strikingly, in this work an increased basal endocytosis of the CB1F238L receptor has been demonstrated as compared with the wild-type receptor. In conclusion, it could be that the increased basal endocytosis of the CB1F238L receptors causes an increased basal endocytosis of the D2 receptor as well and hence an increased radioligand binding potential. Although there are quite some theoretical arguments for this hypothesis it is highly speculative and needs experimental clarification. To test if the F238L mutation in the CB1 receptor affects D2 receptor heteromerization and co-trafficking HEK^{D2L}, HEK$^{D2L\text{-}CB1wt}$ and HEK$^{D2L\text{-}CB1F238L}$ cells were generated in this work, which constitute a useful tool for future experiments.

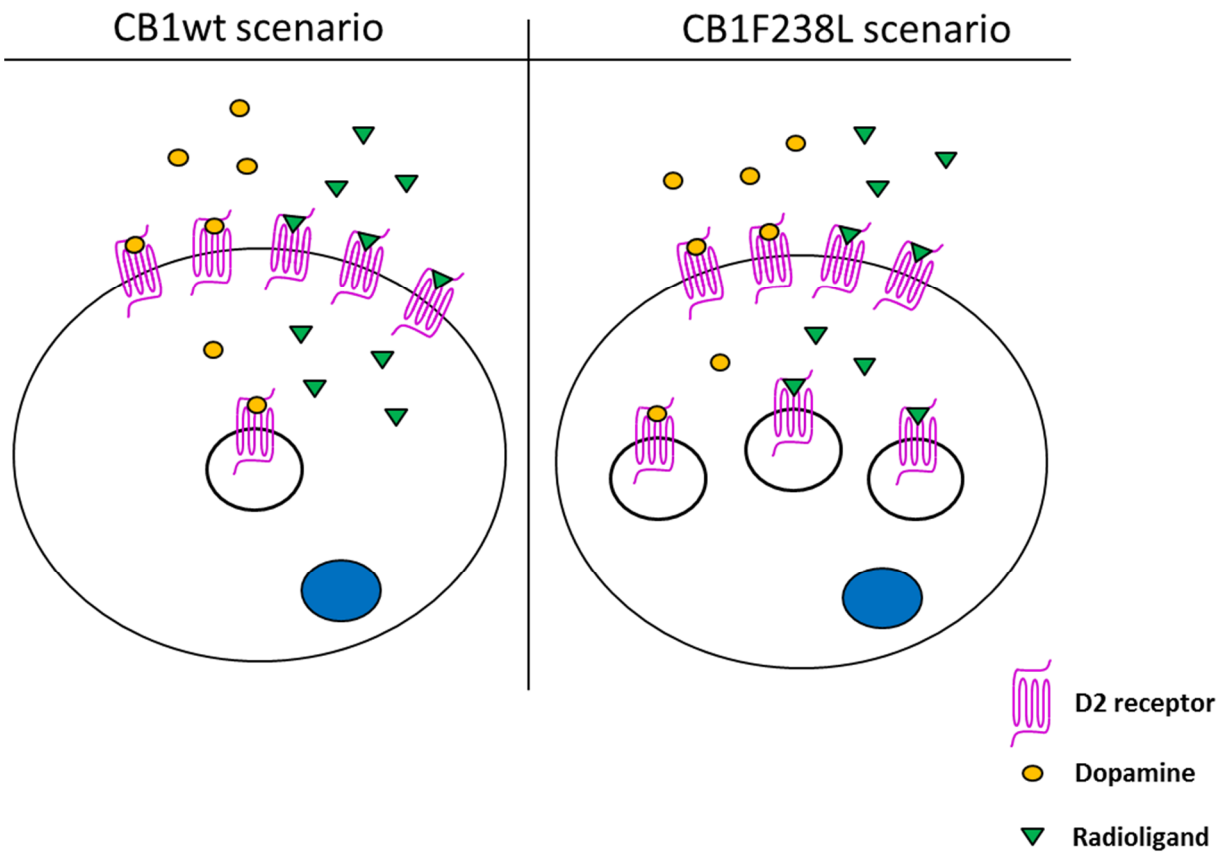


Fig. 4.2 Working hypothesis for the effect of CB1 and D2 receptor co-trafficking on D2 receptor radioligand binding potentials. The plasma membrane has been suggested to be less permeable for dopamine as compared with the radioligands [^{3}H]raclopride and [^{18}F]fallypride (Guo 2010). This might cause less competition between dopamine and the radioligands at intracellular receptors than at surface receptors. Hence, if the D2 receptor is predominantly located intracellularly it would result in an increased binding potential of the radiotracer although the amount of D2 receptor protein is unchanged. This could be the case, if the D2 receptor undergoes increased basal endocytosis together with the CB1F238L receptor.

5. SUMMARY

The CB1 receptor is involved in many physiological functions, such as learning, memory and regulation of emotion. Furthermore, it plays a role in various pathological conditions, e.g. psychiatric and neurodegenerative. The CB1 receptor`s availability and it`s polarization towards the axon and the presynapse is amongst others regulated by endocytosis. It has been further suggested that endocytosis and polarization of the CB1 receptor positively correlate with the receptor`s activity. In this work, the effect of a F238L mutation in the fourth transmembrane domain of the CB1 receptor on its trafficking and signaling was examined. The CB1F238L receptor showed a reduced surface expression in HEK293 cells, which could be rescued by inverse agonist treatment and was found to be caused by an increased basal endocytosis via lipid rafts/caveolae. In line with this finding, an increased lipid raft allocation of the mutant CB1 receptor was observed. In accordance with the role of endocytosis in receptor polarization, the CB1F238L receptor showed increased axonal polarization, when ectopically expressed in primary hippocampal neurons. However, although the CB1F238L receptor showed increased endocytosis and polarization, its basal activity was found to be reduced. It was concluded that there might be other mechanisms for the regulation of CB1 receptor polarization, additionally to the receptor`s activity.

The CB1 receptor is known to form heteromers with other GPCRs. Previous work by others showed the heteromerization of the CB1 receptor and the D2 receptor by co-immunoprecipitation and FRET. Additionally, activation of the CB1 receptor led to a reduction in the affinity of the D2 receptor for dopamine. In the present work, the co-immunoprecipitation of the two receptors was reproduced. Furthermore, it was shown that the mere co-expression of the CB1 receptor affects dopamine binding to the D2 receptor. In previous experiments, the CB1F238L mutation in rats was shown to lead to an increased binding potential of a D2 receptor PET radiotracer. In general, the binding potential of D2 radiotracers has been proposed to depend on D2 receptor expression and the subcellular localization of the receptor. However, previous experiments did not reveal changes in D2 receptor mRNA levels in the CB1F238L receptor mutant rat. Thus, further experiments were performed in order to determine the protein level of the D2 receptor in the striatum of the CB1F238L mutant rat. However, receptor autoradiography and western blot experiments yielded contradictory data. As the F238L mutation in the CB1 receptor affects the receptor`s trafficking, and as receptors can co-traffick in heteromers, it was then hypothesized that differential co-trafficking of the D2 receptor with the CB1F238L receptor could account for the differences observed in the PET ratiotracer binding potential. To test this hypothesis, HEK293 cells were generated which express the D2 receptor either alone or together with the CB1wt receptor or the CB1F238L receptor.

5.1 Zusammenfassung

Der CB1 Rezeptor ist an vielen physiologischen Prozessen beteiligt, wie beispielweise beim Lernen und Erinnern oder bei der Regulation von Emotionen. Dementsprechend ist dieser Rezeptor ebenfalls in pathologische Prozesse bei psychiatrischen oder neurodegenerativen Erkrankungen involviert. Die Verfügbarkeit des CB1 Rezeptors und dessen Transport zur Präsynapse wird unter anderem durch Endozytose reguliert. Es gibt Hinweise darauf, dass die Endozytose des CB1 Rezeptors wiederum in einem positiven Zusammenhang mit seiner Aktivität steht. In dieser Arbeit wurde der Effekt der F238L Mutation in der vierten Transmembrandomäne des CB1 Rezeptors auf dessen subzellulären Transport und dessen Signaltransduktion untersucht. Es wurde gezeigt, dass der CB1F238L Rezeptor eine verringerte Oberflächenexpression aufweist, was auf verstärkte „lipid raft/caveolae“ vermittelte Endozytose zurückgeführt werden konnte. Dementsprechend wurde eine verstärkte Anreicherung des CB1F238L Rezeptors in „lipid raft“ Präparationen beobachtet. Auch konnte die Oberflächenexpression durch die Stimulation mit einem inversen Agonisten wieder erhöht werden. In Übereinstimmung mit dem Zusammenhang zwischen Endozytose und axonalem Transport des CB1 Rezeptors wurde eine verstärkte axonale Polarisation des CB1F238L Rezeptors in transfizierten, primären Neuronen beobachtet. Obwohl die Endozytose und axonale Polarization des CB1F238L Rezeptors verstärkt ist, wurde eine verringerte basale Aktivität dieses Rezeptors gemessen. Eine Schlussfolgerung aus diesem Widerspruch ist, dass es neben der Rezeptoraktivität noch andere Mechanismen zur Regulation der axonalen Polarisation des Rezeptors gibt.

Es ist bekannt, dass der CB1 Rezeptor mit dem Dopamin D2 Rezeptor heteromerisiert, wie in früheren Arbeiten mit Hilfe von Ko-Immunpräzipitationen und FRET gezeigt wurde. Die Aktivierung des CB1 Rezeptors reduziert die Affinität des D2 Rezeptors für Dopamin. In dieser Arbeit wurde die Ko-Immunpräzipitation des CB1 Rezeptors und des D2 Rezeptors reproduziert. Außerdem wurde gezeigt, dass bereits die Ko-Expression des CB1 Rezeptors die Affinität des D2 Rezeptors für Dopamin reduziert. In früheren Experimenten wurde ein verringertes Bindungspotential für einen D2 Rezeptor Radioliganden im Striatum der CB1F238L mutanten Ratte beobachtet, obwohl die D2 Rezeptor mRNA Menge unverändert war. Die durchgeführten Experimente ergaben ebenfalls widersprüchliche Ergebnisse hinsichtlich der D2 Rezeptor Proteinmenge im Striatum der CB1F238L mutanten Ratte. Da der CB1F238L Rezeptor veränderte Transporteigenschaften aufweist und da GPCRs in heteromeren ko-transportiert werden können, wurde eine Hypothese aufgestellt, die eine Veränderte subzelluläre Lokalisation des D2 Rezeptors mit einem veränderten Bindungspotential des D2 Rezeptor Radioliganden in Verbindung bringt. Für zukünftige Experimente zur Überprüfung dieser Hypothese wurden HEK293 Zellen hergestellt, die den D2 Rezeptor entweder allein, oder zusammen mit dem CB1wt Rezeptor oder dem CB1F238L Rezeptor exprimieren.

6. ABBREVIATIONS

A	ampere
aa	amino acid
AAV	Adeno-associated virus
A2A	adenosine 2A receptor
AC	adenylate cyclase
AEA	arachidonoylethanolamide, anandamide
2-AG	2-arachidonoyl glycerol
AP	adapter protein
APS	ammonium persulphate
β-1AR	β1 adrenergic receptor
bp	base pair
°C	degree celcius
Ca	calcium
CAG	CMV early enhancer/chicken β actin
cAMP	cyclic adenosine monophosphate
cav-1	caveolin 1
CCP	clathrin coated pit
Ci	Curie
CMV	cytomegalovirus
CNS	central nervous system
COP	coat protein
CP55940	2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol
CRAC	cholesterol recognition amino acid consensus
CRE	cAMP response element
CRIP1a	cannabinoid receptor interacting protein 1a
C-terminal	carboxy-terminal

Cy	indocarbocyanin
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DMSO	dimethylsulfoxide
dH_2O	distilled water
dNTP	desoxynucleosid triphosphate
DOR	δ-opioid receptor
DSE	depolarization induced suppression of excitation
DSI	depolarization induced suppression of inhibition
EC	extracellular loop
eCB	endocannabinoid
ECS	endocannabinoid system
EE	early endosome
ENU	N-ethyl-N-nitrosourea
ESCRT	endosomal sorting complex required for transport
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation pathway
ERK	extracellular regulated kinase
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
Fig.	Figure
g	gram
GASP1	GPCR-associated sorting protein 1
GDP	guanosine diphosphate
GRK	G protein coupled receptor kinases
GTP	guanosine-5`-triphosphate
h	hour
HA	hemagglutinin
HRP	horseraddish peroxidase

HRS	hepatocyte-growth factor regulated tyrosine kinase substrate
IC	intracellular loop
ICC	immunocytochemistry
IP	immunoprecipitation
IP3	inositol 1,4,5-trisphosphate
K	potassium
k	kilo
K_A	voltage gated potassium channel
K_{ir}	inwardly rectifying potassium channel
kD	kilo Dalton
L	liter
Lamp2	lysosome associated membrane protein 2
LC-MS	liquit chromatography - mass spectrometry
LE/MVB	late endosome/multi vesicular body
Log	logarithm
LTD	long term depression
LTP	long term potentiation
µ	micro
m	milli
M	molar
min	minutes
MAGL	monoacylglycerol lipase
MAP	microtubuli associated protein
MAPK	mitogen activated protein kinase
MβCD	methyl-β-cyclodextrine
MSN	medium spiny neuron
n	nano
Na	sodium
NAPE-PLD	N-acylphosphatidyl-ethanolamine-specific phospholipase D

N-terminal	amino-terminal
ORG27569	5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide
OX1	orexin 1 receptor
PC	preclear
PCC	Pearsons correlation coefficient
PDZ	postsynaptic density 95/disc large/zonula occludens-1
PET	positron emission tomography
PIP2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
PSM	perisynaptic signaling machinery
Rab	ras-related in brain
RE	recycling endosome
RGS	regulator of G protein signaling proteins
RIM-1α	rab3A interacting molecule 1α
S1P1	sphingosine-1-phosphate receptor
SDS	sodium dodecyl sulphate
sec	second
SEM	standard error of the mean
siRNA	small interfering RNA
SN	substantia nigra
SNX1	sorting nexin-1
SR141716	5-(4-Chlorphenyl)-1- (2,4-dichlorphenyl)-4- methyl-N-(piperidin-1- yl)-1H-pyrazol-3-carboxamid
STDP	spike timing dependent plasticity
TEMED	N,N,N‘,N‘-tetramethylendiamine
THC	Δ^9-tetrahydrocannabinol

THL	tetrahydrolipstatine
TMH	transmembrane helix
TrfR	transferrine receptor
V	volt
VGCC	voltage gated calcium channels
VTA	ventral tegmental area
v/v	volume per volume
WB	western blot
WIN55212-2	(R)-(+)-[2,3-Dihydro-5-methyl- 3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-enzoxazin-6-yl]-1-napthalenylmethanone
w/v	weight per volume
wt	wild-type
UIM	ubiquitin-interacting motif

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9. APPENDIX

9.1 Table of Figures

9.2 Plasmid sequences

9.2.1 pcDNA3_rCB1wt (no. 4 in table 2.9)

ggccgctcgagcatgcatctagagggccctattctatagtgtcacctaaatgctagagctcgctgatcagcctcgactgtgccttctagttgccagc
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9.2.2 pcDNA3_rCB1F238L (no. 5 in table 2.9)

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9.2.3 pcDNA3_HA-rCB1wt (no. 6 in table 2.9)

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9.2.4 pcDNA3_HA-rCB1F238L (no. 7 in table 2.9)

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9.2.5 pcDNA3.1-rCB1-WT-EGFP (no. 8 in table 2.9)

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9.2.6 pcDNA3.1-rCB1-F238L-EGFP (no. 9 in table 2.9)

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9.2.7 pAM/CBA-pL-Flag-D2L-WPRE-bGHpA (no. 11 in table 2.9)

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9.2.8 pcDNA3_Zeo_Flag-D2L (no. 12 in table 2.9)

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9.3 Table 9.1

Experimental model	mRNA	Protein	Function	Reference
Reduced CB1 signaling				
CB1 knockdown (AAV + shRNA)	D2 ↓	D2 ↓	n.d	Blume 2012
CB1 knockout	D2 –	D2 ↑	n.d	Gerald 2006, Houchi 2005
SR141716 treatment 3 days	D2 ↓	n.d	n.d	Gerald 2006
SR141716 treatment 13 days	n.d	D2 ↑	n.d	Crunelle 2011
Increased CB1 signaling				
CP55940 treatment	n.d	n.d	- D2 affinity for DA ↓ - Membrane and intracellular heterodimers ↑	Marcellino 2008 Przybyla 2011
THC treatment	D2 –	D2 ↑	n.d	Ginovart 2012
Constitutive active CB1 mutant	n.d	n.d	- intracellular heterodimers ↑	Przybyla 2012
Reduced D2 signaling				
D2 knockdown (AAV + shRNA)	CB1 ↓	CB1 ↓	n.d	Blume 2012
D2 knockout	n.d	CB1 ↑	n.d	Thanos 2010
Antipsychotic treatment	CB1 –	CB1 ↓	n.d	Urigüen 2009
Increased D2 signaling				
Quinpirole treatment	n.d	n.d	- intracellular heterodimers ↑	Przybyla 2010
Co-activation of CB1 and D2				
HU210 + Quinpirole treatment	n.d	n.d	- G_i to G_s switch	Glas 1997
CP55940 + Quinpirole treatment	n.d	n.d	- heterodimers ↑	Kearn 2005

– : Unchanged; ↓ : Decreased; ↑ : Increased; n.d : Not determined