universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Pharmacology of $GABA_A$ receptor subtypes: Phytocannabinoids act on $GABA_A$ receptors "

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna, 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie UG200
Betreut von / Supervisor:	Ass. Prof. Priv.-Doz. Dr. Margot Ernst, PhD

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Acknowledgements

This thesis was performed in the laboratory of Margot Ernst at the Center of Brain Research at the Medical University of Vienna. Margot supported many aspects of the work in the laboratory. Driven by her own curiosity she gave me the opportunity encouraging my inquiring mind to explore a topic of my own interest. Additionally, I want to thank Margot`s team for the assistance in the laboratory. Furthermore, I want to thank Tibor Harkany for his inspiring discussions and technical advices. I am grateful for the help of David Siebert and Michael Schnürch from the Technical University of Vienna in providing analysis and quantification methods. In addition, I want to thank Lukas Weigl, a great electro physiologist from the Department of Anesthesia at the AKH in Vienna and his team for teaching me patch clamp techniques in HEK and CHO cells.

Introduction

$GABA_A$ receptor structure and receptor arrangement determine pharmacology

$GABA_A$ receptors are ligand gated ionotropic receptors, found in different cell types. They are members of the Cys-loop receptor superfamily. Five $GABA_A$ receptor subunits, form pentameric trans-membrane ion channel proteins. In their open state, they are permeable for chloride and hydrogen carbonate ions. In mammalian cells, nineteen different subunits were identified: six α, three β, three γ, three ρ, one δ, one ε, one θ and one π subunit. Moreover, the genes of human $GABA_A$ receptor subunits are located on seven different chromosomes and illustrated in a phylogenetic tree shown in Figure 1. The amino acid sequence shows the highest similarities between α_1 and α_2, α_4 and α_6, γ_1 and γ_2, ρ_1 and ρ_2 and β_2 and β_3 subunits. Furthermore, the ε subunit is more related to γ subunits and the θ subunit is more related to the β subunits. Additionally, the sequences of β_2 and β_3 subunits are more similar to each other compared to β_1 (Sigel and Steinmann 2012).

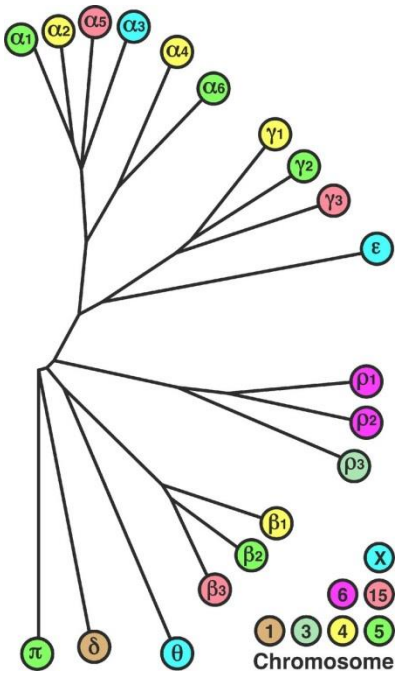

Figure 1: Phylogenic Tree. The phylogenic tree shows the sequence similarities and the chromosomal loci of the 19 human $GABA_A$ receptor subunits (Sigel and Steinmann 2012).

Furthermore, the genes form clusters on four different chromosomes (4, 5, 15, X), except for δ and ρ subunits on chromosomes 1, 3 and 6. The subunits of the postulated most common receptor subtypes in the rat brain $\alpha_1\beta_2\gamma_2$ are on the same chromosome which is 5.

Each subunit consists of one large extracellular domain (ECD) including the N-terminal end. Below the ECD lies the transmembrane domain (TMD). The TMD consists of four trans-membrane helices (TM1, TM2, TM3 and TM4). TM4 includes the C-terminal end. TM1 and TM2 as well as TM3 and TM4 are connected by an intracellular domain (ICD) whereas only a fragment is shown in the crystal structure. Each subunit has two sides, termed principal or (+) side and complementary or (-) side which form interfaces when the receptor is assembled. These interfaces play an important role in formation of allosteric or agonist binding sites for compounds (Sieghart 2015).

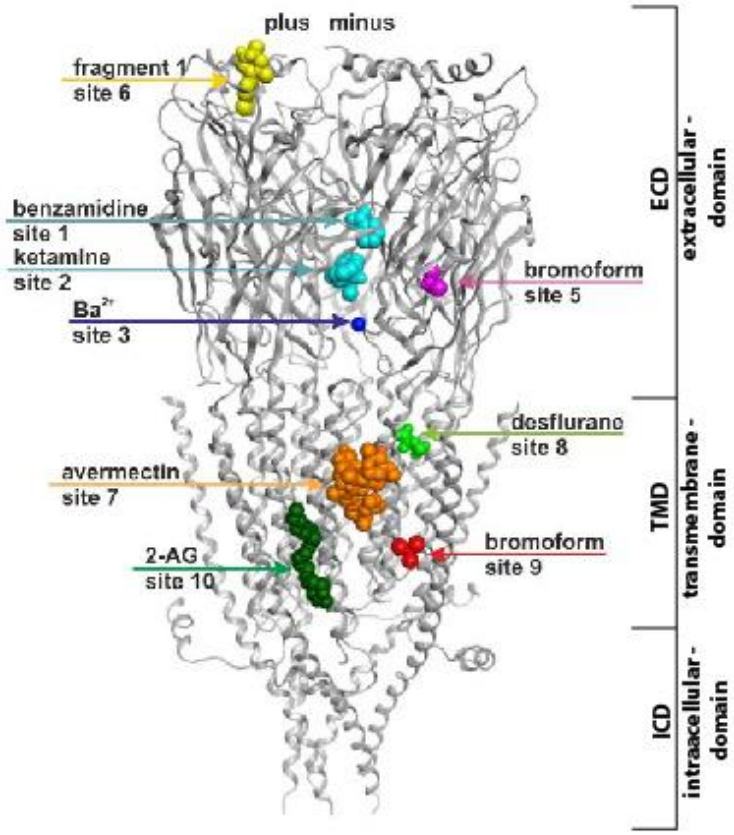

Figure 2: Binding sites on the $GABA_AR$. A homology model based on 4COF (ECD, TMD) and 4PIR (ICD) including one representative ligand per representative site. Each of the sites could occur in five subunits or at five interfaces respectively (Puthenkalam, Hieckel et al. 2016).

In biochemistry, a ligand defines any molecule which binds to a protein or another molecule at a specific site, by non-covalent binding. This binding occurs through intermolecular forces such as Van der Waals forces or ionic and hydrogen bonds. These non-covalent bonds are found to be relatively weak, because no electrons are shared in the bond. Nevertheless, working together, they are considered to be strong and specific (Alberts, Bray et al. 2013).

Expression of subunits in diverse patterns among cell types and regions in the brain, peripheral tissues, glands and organs appear. Thus, a complex variety of receptors, capable of individual pharmacology occur. A common way to estimate expression of $GABA_A$ receptors, is the detection of subunit mRNA in a cell and tissue dependent manner by RT-PCR. Occasionally, this method can be combined with immuno-histochemical methods and western blotting to prove the existence of translated proteins. Through in situ hybridisation and immunohistochemical analysis, the expression of $GABA_A$ receptors was studied more detailed in the mouse brain (Hortnagl, Tasan et al. 2013).

The most abundant $GABA_A$ receptor subtypes in the mammalian brain contain two α, two β and one γ subunits. A large fraction of $GABA_A$ receptors contains extracellular orthosteric agonist binding sites for GABA (gamma amino butyric acid) at β(+) α(-) interfaces (Smith and Olsen 1995). Another binding site for GABA was identified in αβδ receptors at the β(+)δ(-) interface (Lee, Absalom et al. 2016) and at ρ/ρ interfaces (Lukasiewicz 1996).

In the CNS, $GABA_A$ receptors are mostly located in and around synapses (synaptic, perisynaptic, extrasynaptic and glia receptors) to produce inhibition of neurons. In physiological conditions, activation of $GABA_A$ receptors in neurons, leads to an opening of ion channels and to chloride influx causing hyperpolarization of the cell. GABA thus seems to be the major inhibitory neurotransmitter in the nervous system. Based on their location, the GABA sensitivity is regulated by subunit composition. Synaptic receptors contain α, β and γ subunits while extra synaptic receptors often contain α_4, α_6 and δ subunits or the α_5 subunit and produce tonic inhibition (Wang 2011).

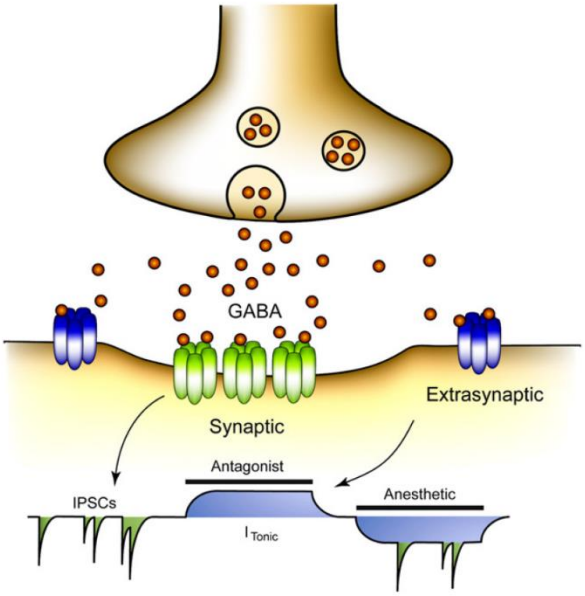

Figure 3: GABAergic synaptic transmission including synaptic and extrasynaptic receptors and their function (Bonin and Orser 2008).

Thus, the GABA sensitivity is higher in extrasynaptic receptors than in synaptic receptors.

In the mouse brain, the distribution of subunits is shown in **Figure 4**. Of note for the results, the immune reactivity of α_6 is restricted to the cerebellum and the spinal trigeminal tract (STT).The α_4 and δ subunits are co-localized in the striatum and thalamus but not in cerebellum and hippocampus (Hortnagl, Tasan et al. 2013).

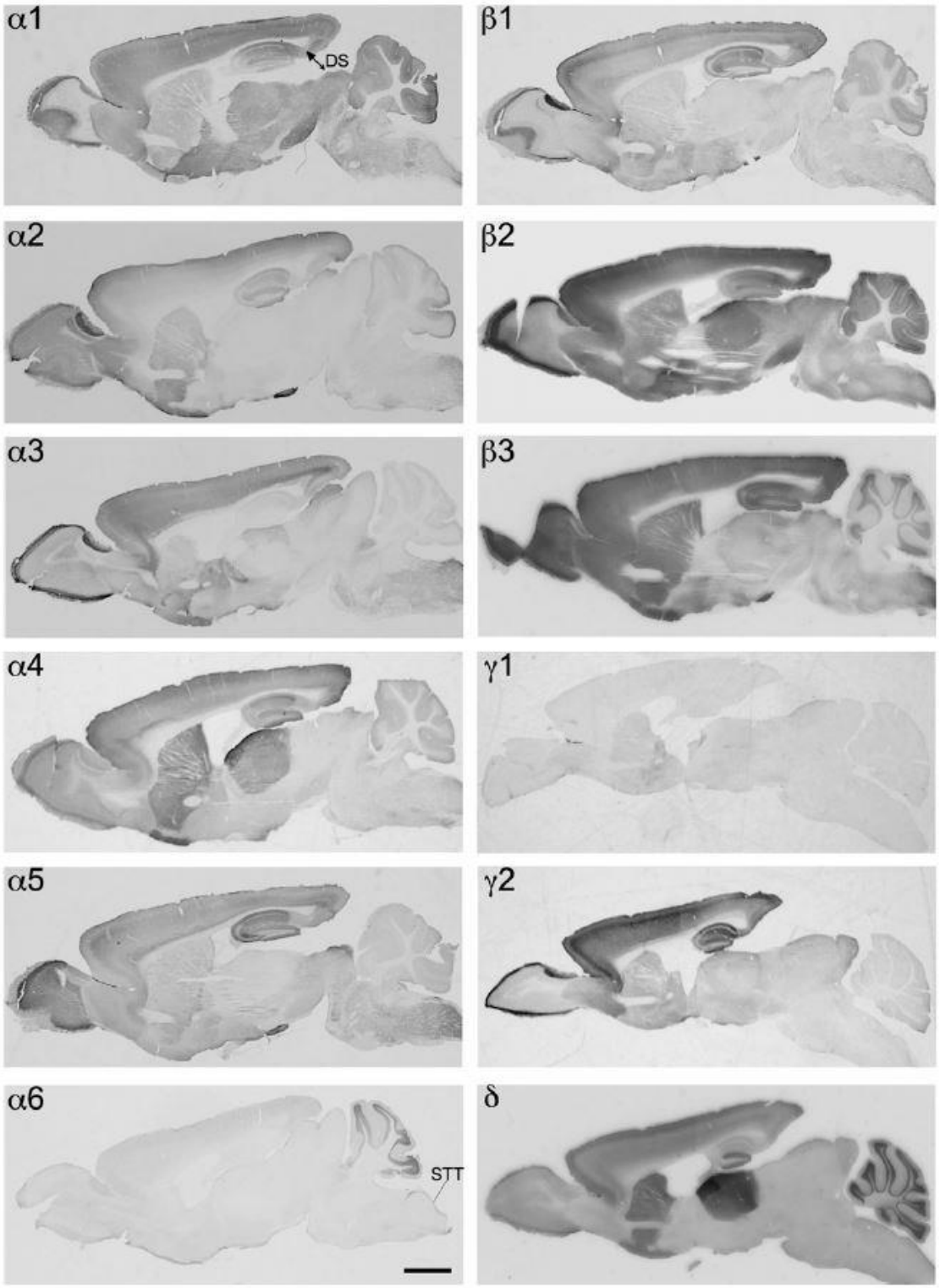

Figure 4: $GABA_A$ subunits among the brain. Differences in distribution of $GABA_A$ subunit immunoreactivities in saggital sections of the mouse brain at lateral level 1.2 mm (Hortnagl, Tasan et al. 2013).

To this end, the expression pattern of $GABA_A$ receptor subunits in the rat brain was evaluated by immunohistochemistry. In contrast, the α_6 subunit is mainly expressed in the cerebellum and often forms receptors including β and δ subunits or β and γ subunits. Evidence suggest that receptors including different α subunits exists. Interestingly, α_6 and α_4 subunits are more often co-localized with β and δ subunits than with γ subunits. Moreover, the α_1 subunit was found in all brain regions while other α subunits were more selective to distinct regions (Pirker, Schwarzer et al. 2000).

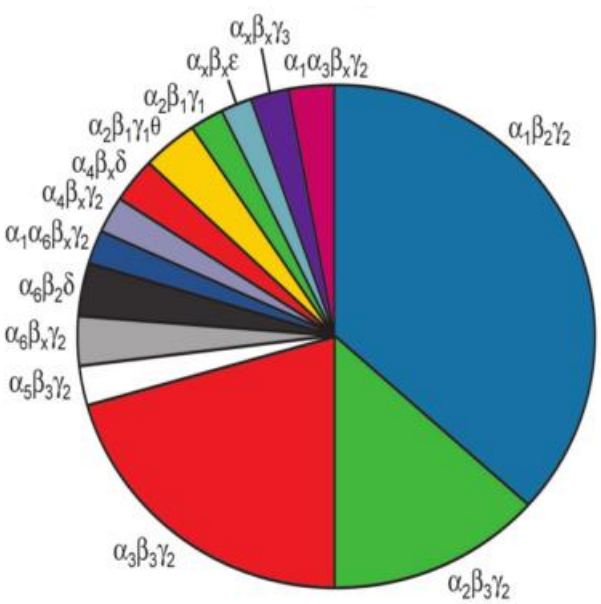

Figure 5: $GABA_A$ receptor pie chart. A broad but incomplete representation of abundances of $GABA_A$ receptor compositions in the rat brain whereas other compositions exists and not all of the shown compositions have been confirmed (Whiting 2003).

$GABA_A$ receptor list 2008

A. Identified			
α1β2γ2	α4βγ2	α5βγ2	α6β3δ
α2βγ2	α4β2δ	α6βγ2	ρ
α3βγ2	α4β3δ	α6β2δ	
B. Existence with high probability			
α1β3γ2	α5β3γ2	αβ	
α1βδ	αβ1γ/αβ1δ	α1α6βγ/α1α6βδ	
C. Tentative			
ρ1	αβγ1	αβθ	
ρ2	αβγ3	αβπ	
ρ3	αβε	αxαyβγ2	

Table 1: $GABA_A$ receptor table. Olsen and Sieghart propose a list of identified compositions of subtypes and those which exist tentatively or with high probability (Olsen and Sieghart 2009).

Beside the broad range of $GABA_A$ receptors expression patterns in the CNS, peripheral tissues contain GABA $_A$ receptors too.

In isolated human airway smooth muscle cells (ASM), proteins of α_4, α_5, β_3, γ_2 and δ subunits had been detected. Their role is associated with muscle relaxation and suggests pharmacological relevance in bronchial asthma or COPD (Mizuta, Xu et al. 2008).

Moreover, the appearance of ε subunits in the human heart has been concluded (Davies, McCartney et al. 2002). Nevertheless, ε subunits alone are not able to form receptors, responding to GABA, nor including α or β subunits. Otherwise, in recombinant cells ε containing $GABA_A$ receptors need to include both α and β or α, β and γ subunits respectively, to be responsive to GABA (Bollan, Baur et al. 2008). However, α_1 and ε subunits can establish picrotoxin insensitive receptors which evoke chloride currents (Bollan, Baur et al. 2008). Neither the function nor the composition of ε subunit containing receptors has been consistently studied.

In the human liver α_3 and ε subunits have been identified. In addition, different human liver carcinoma cell lines e.g. Huh7 express mRNA of α_1, α_2, α_3, α_4, β_1 and ε. Furthermore, quantitative variation of subunit expression in human liver carcinoma and non-tumor tissue was evaluated. Interestingly, pre-treatment with

GABA remarkably reduced the amount of intrahepatic liver metastasis and primary tumor formation in vivo, using an orthotopic model of liver carcinoma in mice (Chen, Bao et al. 2012).

Leydig cells of mice contain $GABA_A$ receptors, assembled by a subset of subunits (α_1, α_2, β_1, β_3, γ_1), which seemingly interact with the proliferation of those in vitro. Moreover, evidence for a regulatory role in testosterone production is given. Interestingly, the presence of α_2, β_1, β_2, β_3 and γ_3 subunits was reported in postnatal mouse testicles (Geigerseder, Doepner et al. 2004).

In STC-1 cells, a cholecystokinin (CCK) secreting gastrointestinal neuroendocrine (NE) cell line, mRNA of α_2, α_3, α_5, β_1, β_3, and δ subunits was found. Furthermore, electrophysiological patch clamp experiments identified GABA evoked currents and indicated the presence of $GABA_A$ receptors. In addition, the existence of functional receptors was confirmed through interaction of muscimol. Consequently, blocking of the currents by picrotoxin or bicuculline support the claim (Glassmeier, Herzig et al. 1998). Moreover, ingestion of probiotics such as L. rhamnosus regulate emotional behaviour and influenced $GABA_A$ receptor α_2 subunit expression in mice in basolateral amygdala, frontal cortex and hippocampus. To this end, the involvement of the vagus nerve as possible part of gut-brain axis can be considered (Bravo, Forsythe et al. 2011).

$GABA_A$ receptors exist to a certain extent on cells of the immune system. In CD4+ cells of a type 1 diabetes mouse lineage α_1, α_2, β_1, β_2, γ_3 and δ subunits had been identified (Tian et al. 2004). In T-cells of an EAE (encephalitogenic) mice line α_1, α_4, β_2, β_3, γ_1 and δ subunits were found (Bjurstöm, Wang et al. 2008). In Wistar rats α_1, α_2, α_3, α_4, α_6, β_2, β_3, γ_1, ρ_1, ρ_2, ρ_3, π and θ subunits had been identified in cells isolated from mesenteric lymph nodes. In comparison, in CD4+ and CD8+ cells isolated from human pancreatic lymph nodes only α_1, α_5, β_1, ρ_2 and π subunits had been found (Mendu, Bhandage et al. 2012). The discrepancy among data demonstrates the variation in expression of T-cell types and species. Interestingly, differences in expression were reported among donors of the same species (Barragan, Weidner et al. 2015). In mouse and human dendritic cells (DC), mRNA of α_3, α_5, β_1, β_3 and ρ_1 subunits had been identified. Moreover,

GABA evoked currents in DC had been reported. In addition, interfering in GABAergic signalling of DC infected by *T. gondii* had effects on DC migratory speed and chemotactic response. In astrocytes, subunit mRNA of α_1, α_2, α_3, α_5, β_1, β_2, β_3, γ_3, θ, π, ρ_1 and ρ_3 was found (Fuks, Arrighi et al. 2012).

Mainly ρ subunit containing receptors were found inside the retina besides other locations in the CNS (Lukasiewicz 1996).

Ligands and pharmacology of $GABA_A$ receptors

As $GABA_A$ receptor subtypes differ in structure, a variety of allosteric binding sites arise, causing distinct functionality.

Agonists, antagonists and channel blocker

Muscimol, a toxin of the mushroom *amanita muscaria* is an agonist of $GABA_A$ receptors, while bicuculline a component of the flower (*Dicentra cucullaria*) selectively inhibits $GABA_A$ receptors as an antagonist (Curtis, Phillis et al. 1959). Furthermore, one of several channel blockers, picrotoxin is a non-competitive antagonist of $GABA_A$ receptors but also effective on other Cys-loop receptor members and derives from asian plant *anamirta cocculus* (Johnston and Kennedy 1978).

Tranquilizer

Based on an allosteric binding site at the extracellular α(+) γ(-) interface, those receptors are sensitive to benzodiazepines such as chlordiazepoxide, diazepam, flunitrazepam, lorazepam and many others, exclusively in the presence of GABA. Benzodiazepines increase the binding affinity for GABA at the orthosteric binding site and extend the GABA-induced chloride current (Sieghart 2015). Moreover, they increase the frequency of channel opening (Bianchi, Botzolakis et al. 2009). Furthermore, members of the benzodiazepine class can interact with additional binding sites, such as diazepam. Indeed, micromole concentrations of diazepam had been reported to potentiate GABA-induced currents in receptors without a γ_2 subunit, like other anaesthetics, such as etomidate, which suggests a different binding site on αβ subunit complexes, (Walters, Hadley et al. 2000). Additionally, some benzodiazepines like flurazepam possibly have a third site of action in

some of the α(+)β(-) interfaces, which interestingly decrease the GABA-evoked currents (Baur, Tan et al. 2008). Flunitrazepam, a positive allosteric modulator (PAM) of $\alpha_{1, 2, 3, 5}$/ β_3/ γ_2 containing receptors has negative modulatory effects in $\alpha_6\beta_3\gamma_2$ receptors (Sieghart 2015).

Anaesthetics

Some anaesthetics can potentiate GABA-evoked currents at lower concentrations and additionally evoke chloride currents in absence of GABA at higher concentrations. Seemingly, inhalative anaesthetics such as isoflurane, enflurane, halothane, chlorophorm and ethanol interact with a binding pocket on the α subunit (Sieghart 2015). Furthermore, Imidazoles such as etomidate can interact with subunit interfaces β(+)α(-) and β(+)β(-) but not simultaneously. (Chiara, Dostalova et al. 2012). It was demonstrated that β_3 homopentamers form functional receptors in HEK293 cells where currents can be evoked by general anaesthetic such as propofol (Davies, Kirkness et al. 1997). It had been the first crystallized $GABA_A$ receptor (Miller and Aricescu 2014). They were affected by millimolar concentrations of GABA. A binding site for propofol was identified between TM1 and TM2 of a single β_3 subunit (Yip, Chen et al. 2013). Moreover, propofol is able to bind to trans-membrane interfaces α(+)β(-), γ(+)β(-) and β(+)α(-) (Sieghart 2015). Alanine mutations experiments on the intracellular loop of $\alpha_1\beta_2$ receptors discovered a possible binding site for propofol on this intracellular loop (Moraga-Cid, Yevenes et al. 2011).

Barbiturates such as phenobarbital, are agonists of $GABA_A$ receptors and elicit chloride currents in the absence of GABA, by a proposed binding site at the trans-membrane interfaces α(+)β(-) and γ(+)β(-) (Chiara, Jayakar et al. 2013). In summary, barbiturates increase the opening duration of $GABA_A$ receptors ion channel pore (Bianchi, Botzolakis et al. 2009).

Other synthetic compounds

Some β-carbolines as DMCM are capable of biphasic activity on $GABA_A$ receptors. A high affinity binding to the benzodiazepine site leads to negative

allosteric modulation (NAM). Otherwise, the response is reversed at higher concentrations and DMCM acts as a PAM (Thomet, Baur et al. 1999).

Pyrazoloquinolinones were identified to potentiate GABA evoked chloride currents by an allosteric binding site at the extracellular α(+)β(-) interface. 10 µM LAU177 enhanced the maximum GABA currents twelve times in $\alpha_1\beta_3\delta$ $GABA_A$ receptor subtypes and preferably potentiated currents at low GABA (EC_{3-5}) approximately 3000 %. In contrast, $\alpha_1\beta_3\gamma_2$ and $\alpha_1\beta_3$ (app. 1000 %). In summary, it can be used as a test compound to confirm the existence of subunit expression in recombinant $\alpha_1\beta_3\delta$ transfected cells (Mirheydari, Ramerstorfer et al. 2014).

Endogenous compounds

Furthermore, it was confirmed that histamine has agonistic properties in β_3 homopentamers and can modulated by other compounds (Hoerbelt, Ramerstorfer et al. 2016). Moreover, histamine is capable of potentiation of GABA evoked currents in α_4 containing synaptic and extrasynaptic receptors (Bianchi, Clark et al. 2011), (Saras, Gisselmann et al. 2008).

GABA evoked currents are pH dependent. $GABA_A$ receptors seemingly have distinct allosteric binding sites for H^+ and Zn^{2+} (Huang, Chen et al. 2004). Therefore, it is important to re-adjust pH when high concentrations of compounds such as histamine-HCl are used for measurements (Hoerbelt, Ramerstorfer et al. 2016).

Cholesterol derived neurosteroids such as pregnenolone, progesterone, DHP, allopregnanolone and in addition adiol, androstane and THDOC were found to enhance GABA evoked chloride currents. In addition, metabolites of progesterone like deoxycorticosterone and testosterone were suggested as positive modulators of $GABA_A$ receptors (Wang 2011). Allopregnanolone and THDOC produced biphasic enhancement of GABA currents in granule cells in the cerebellum (Maksay and Fodor 2011).

Endocannabinoids such as 2-AG, were found to selectively modulate β_2 containing $GABA_A$ receptors (Sigel, Baur et al. 2011), due to a binding pocket in the TMD of a β_2 subunit (Baur, Kielar et al. 2013).

Natural compounds

Flavenoids like apigenin and luteolin had been shown to target and modulate $GABA_A$ receptors (Johnston 2015). Quercetin and its glycosides quercetin-3-O-rhamnoside, Rutin and quercetin-3-2-rhamnosylrutinoside inhibit GABA evoked currents in ρ_1 containing receptors (Kim, Lee et al. 2015). An IC_{50} value 4.4 µM of quercetin for GABA (1µM) was found and use-independency was concluded (Goutman and Calvo 2004). The flavan-3-ol derivative Fa131 acts as a positive modulator on both $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_2$ wild-type $GABA_A$ receptors expressed in X. oocytes (Fernandez, Karim et al. 2012). The flavonoid viscosin, isolated from linn, was identified as positive modulator of $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_2\beta_2\gamma_{2L}$ $GABA_A$ receptors in X. oocytes, in a flumazenil insensitive manner (Karim, Irshad et al. 2015).

Many terpenes were found to modulate $GABA_A$ receptors, listed in **Table 6**.

Valerenic acid a sesquiterpenoid compound occuring in valerian is a PAM of $GABA_A$ receptors. The binding site was identified to coincide with the propofol and etomidate binding site on the β_3 subunit (Luger, Poli et al. 2015).

Cannabinoids and the endocannabinoid system

The endocannabinoid system is a retrograde neuronal signalling system consisting of receptors, enzymes, ligands and other proteins (Alger 2002). Additionally, endocannabinoid receptors were found on immune cells (Jean-Gilles, Braitch et al. 2015). The major endocannabinoid receptors are G_I coupled receptors as CB_1, CB_2 and orphan receptors as GPR55, GRP18 and GPR119 (Brown 2007). Enzymes as fatty acid amide hydrolase (FAAH) for hydrolysis of AEA (Cravatt, Demarest et al. 2001) or monoacylglycerol lipase (MAGL) for hydrolysis of 2-AG contribute to the endocannabinoid system (Dinh, Carpenter et al. 2002). Moreover, the endocannabinoid system modulates synaptic plasticity, neuronal excitability, reward and motivation, emotions, learning and memory, locomotion, appetite and thermoregulation via CNS abundant CB_1 receptors. Activation of cannabinoid receptors outside the CNS are involved in immune response, regulation of blood pressure, gastric and intestinal motility, lipogenesis,

bone development, fertility and acute or inflammatory nociception (Nicolussi and Gertsch 2015).

Apparently, cannabinoids in the broader sense are ligands of receptors of the endocannabinoid system and are classified in endo- phyto- and synthetic cannabinoids. The class of endogenously produced cannabinoids is termed endocannabinoids (e.g. 2-AG, AEA). Moreover, the chemical structure of endogenous and synthetic cannabinoids can differ from classical phytocannabinoids such as non-classical cannabinoids, eicosanoids and aminoalkylindoles (Znaleziona, Ginterova et al. 2015). Synthetic cannabinoids such as HU-211, WIN55212-2, JWH-073, AM-630 and CP47.497) in general act on the cannabinoid system and their affinity to cannabinoid receptors is shown in **Table 2**. Phytocannabinoids are plant originated cannabinoids and refers to a group of C_{21} terpenophenolic compounds found in *Cannabis sativa* L (Brenneisen 2007). In this work, only phytocannabinoids were investigated.

In the cannabis plant, phytocannabinoids are produced biosynthetically from precursors of plant terpenes such as isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Those derive via the deoxyxyllulose phosphate pathway (Fellermeier, Eisenreich et al. 2001). Next, geranyl diphosphate and olivetolic acids are converted into cannabigerolic acid (CBGA). The phytocannabinoid CBGA is enzymatically catalysed into tetrahydrocannabinolic acid (THCA) by THCA synthase and cannabidiolic acid (CBDA) by CBDA synthase, the most abundant phytocannabinoids in their acid form (Taura, Morimoto et al. 1996). Other phytocannabinoids such as cannabichromenic acid (CBCA) are catalysed from CBGA by CBCA synthase or from cannabinerolic acid (CBNA) with a lower V_{max} (Morimoto, Komatsu et al. 1998). The loss of the acidic group is called decarboxylation and the products were Δ9-THC (later referred to as THC), CBD and CBG. More than 60 different phytocannabinoids have been identified (Brenneisen 2007).

Pharmacological effects of phytocannabinoids are widespread and not exclusively targeting the endocannabinoid system (Izzo, Borrelli et al. 2009). Interestingly, other plant compounds were identified to target the

endocannabinoid system. Alkylamides from *Echinacea* bind to CB_2 receptors with a K_i of 60nM a higher affinity than some endocannabinoids (Raduner, Majewska et al. 2006). Furthermore, micromolar concentrations of tea catechins had been identified to bind cannabinoid receptors (Korte, Dreiseitel et al. 2010). Kavalactones especially yangonin targets cannabinoid receptors (Ligresti, Villano et al. 2012). Interestingly, the kavalactone kavain, the major constituent of the anxiolytic kava extract, was identified to potentiate $GABA_A$ receptors (Chua 2016).

Pharmacological targets of cannabinoids

Seemingly, the phytocannabinoid cannabidiol (CBD) is the most active phytocannabinoid with more than 65 so far identified molecular targets, including ion channels, receptors, enzymes and transporter (Ibeas Bih, Chen et al. 2015). Beside the actions of CBD, several phytocannabinoids also act on targets outside the endocannabinoid system.

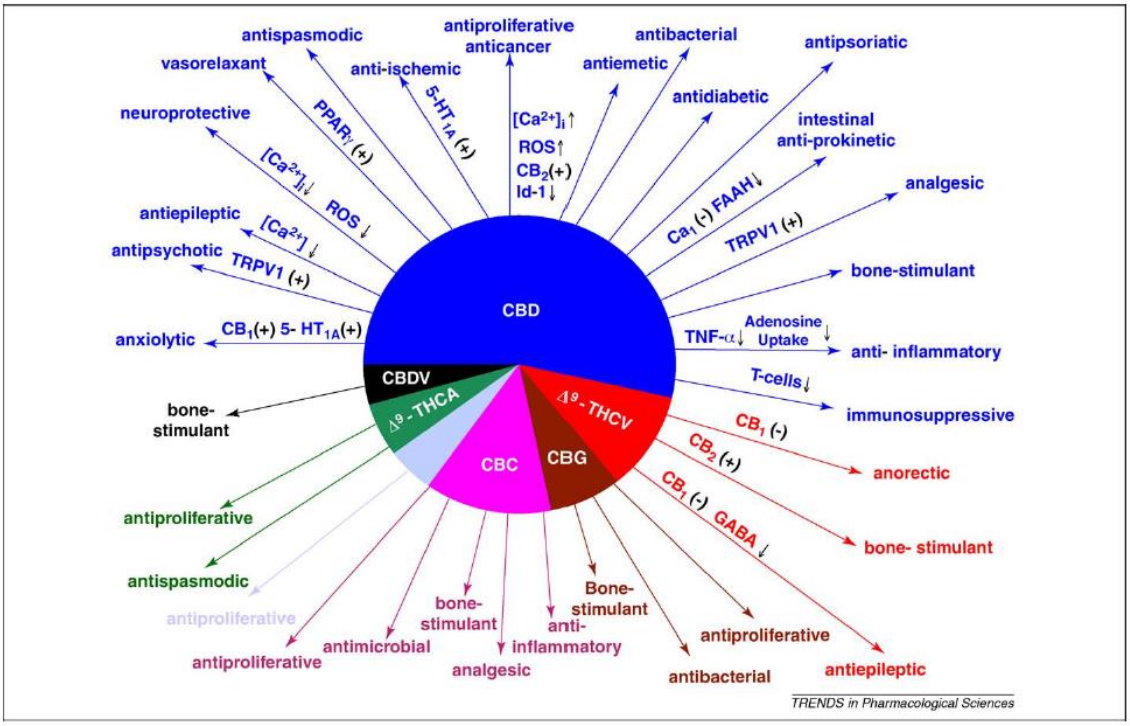

Figure 6: Phytocannabinoid actions. Physiological effects of phytocannabinoids, molecular targets of known or pharmacologically suspected targets, are indicated (Izzo, Borrelli et al. 2009).

Cys-loop receptors

Cannabinoids have been shown to act also on members of the Cys-loop receptor family. It was demonstrated that the endocannabinoids 2-AG, 1-AG, noladine ether (NE) and AEA target and modulate the most abundant $GABA_A$ receptor subtype $\alpha_1\beta_2\gamma_2$ and other subtypes. Furthermore, weak positive modulation of THC at 3 µM was demonstrated. Moreover, synthetic cannabinoids and the CB_1 receptor antagonists rimonabant (SR141716) and AM251 are positive modulators of $GABA_A$ receptor subtype $\alpha_1\beta_2\gamma_2$ (Baur, Gertsch et al. 2012).

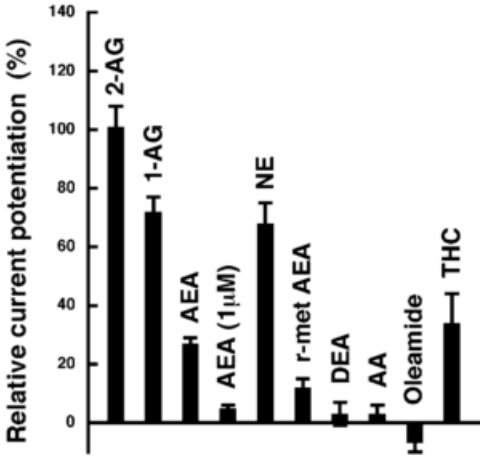

Figure 2: Relative current potentiation of different endocannabinoids, except THC, relative to the endocannabinoid 2-AG in recombinant $\alpha_1\beta_2\gamma_2$ receptors expressed in Xenopus laevis oocytes (Sigel, Baur et al. 2011).

Endocannabinoids such as 2-AG and AEA target α_7-nACh receptors and show reversible inhibition in the presence of acetylcholine at nanomolar concentrations while the synthetic cannabinoid WIN55212-2 acts only at micromolar concentrations (Oz, Zhang et al. 2004). CBD shows full inhibition of α_7-nACh receptors in the micromole range (IC_{50}: 11.3µM) while maximum effect of Δ9-THC (later referred to as THC) show approximately 20 % inhibition (Oz et al. 2004). AEA and WIN55212-2 inhibit human 5-HT_{3A} receptor activity in nanomolar range (Barann, Molderings et al. 2002). AEA is a positive modulator of α1 and α1β glycine receptors in oocytes and isolated VTA neurons (Hejazi, Zhou et al. 2006). CBD and THC inhibit human 5-HT_{3A} receptor activity due to a desensitization dependent mechanism (Xiong, Koo et al. 2011). THC inhibits human 5-HT_{3A} receptor activity possibly through a modulatory binding site in nanomolar (IC_{50}: 38.4nM) range (Barann, Molderings et al. 2002). CBD reversible inhibits human

5-HT_{3A} receptor activity (IC_{50}: 600nM) in a concentration dependent manner (Yang, Galadari et al. 2010), (Mahgoub et al. 2013). CBD directly activates and additionally acts as a positive modulator on α_1 and $\alpha_1\beta$ glycine receptors (Ahrens, Demir et al. 2009). In animal experiments, different derivatives of cannabidiol alleviate neuropathic pain in rats, which is attributed to the potentiation of currents in α_3-GlyR in lumbar spinal cord slices. The site of action was identified at position S296, a serine residue in the α_3-TM domain, by mutagenesis (Xiong, Cui et al. 2012).

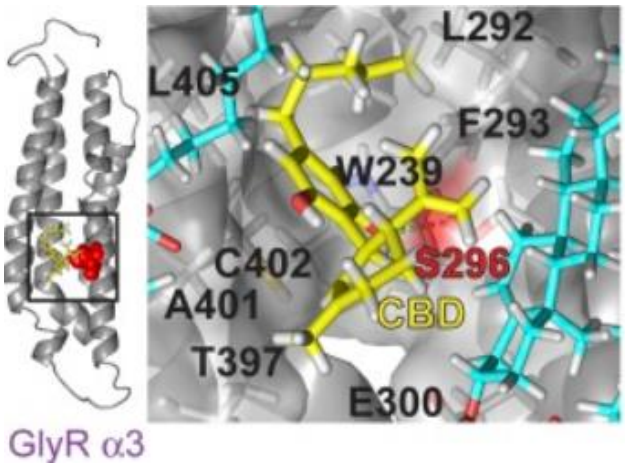

Figure 8: Docking of CBD into GlyR. Docking of CBD into the proposed binding pocket of the GlyR α_3-TMD (Xiong, Cui et al. 2012).

The proposed binding site is conserved and a homologous site in the $GABA_AR$ β_2 subunit was identified as 2-AG binding site by Sigel et al. 2011. Furthermore, THC is a positive modulator of α_1 and $\alpha_1\beta$ glycine receptors in oocytes and isolated VTA neurons (Hejazi, Zhou et al. 2006). Long-term treatment of AβPP/PS1 mice is associated with reduced GluR2/3 and increased levels of $GABA_A$ receptor subunit α_1 in cannabinoid-treated animals treated with botanical drug substance (BDS), including high amount of CBD and THC but also low amounts of CBG, CBC and other cannabinoids, THC 0.75mg/kg + CBD 0.75mg/kg (Aso, Andrés-Benito et al. 2016).

G-protein coupled receptors

THC is an partial agonist of CB_1 and CB_2 receptors with K_i values in the low nanomolar range (Pertwee 2008). CBD seems to antagonize agonists of the CB1

receptor (K_B: 79nM) and CB2 receptor (K_B: 138nM) in binding studies in mouse brains. Furthermore, CBD displays inverse agonist properties (EC50: 503nM) on human CB2 receptors transfected into CHO cells (Thomas, Baillie et al. 2007).

Interestingly only CBD can antagonize GPR55 (IC_{50}: 445nM) an orphan G-protein coupled receptor also called novel cannabinoid receptor, which is found across the brain and in different cell types. Other cannabinoids as THC, 2-AG, AEA and noladine ether (NE) activate the receptor (Ryberg, Larsson et al. 2007) (Izzo, Borrelli et al. 2009).

Table 1 Profile of agonist activities of ligands at GPR55, CB_1 and CB_2 receptors

Ligand	*GPR55 EC_{50} (nM) $GTP\gamma S$ binding*	*GPR55 E_{max} (%)*	*CB_1 EC_{50} (nM) $GTP\gamma S$ binding*	*CB_1 E_{max}(%)*	*CB_2 EC_{50} (nM) $GTP\gamma S$ binding*	*CB_2 E_{max}(%)*
Anandamide	18±3	73±5	31±6	66±4	27±6	58±5
Noladin ether	10±1	95±7	37±5	89±5	>30000	
2-Arachidonoylglycerol	3±1	99±2	519±48	92±6	618±45	87±5
Virodhamine	12±3	160±10	2920±325	75±9	381±34	91±10
Palmitoylethanolamide	4±1	92±1	>30000		19800±2821	93±12
Oleoylethanolamide	440±145	92±3	>30000		>30000	
Δ^9-THC	8±1	92±5	6±1	61±5	0.4±0.1	67±3
Cannabidiol	Antagonist		>30000		>30000	
Cannabinol	>30000		>30000		>30000	
Abnormal cannabidiol	2523±579	76±17	>30000		>30000	
AM281	>30000		Antagonist		Antagonist	
AM251	39±3	88±4	Antagonist		Antagonist	
WIN55,212-2	>30000		18±3	101±14	1±0.2	97±8
HU210	26±7	78±3	0.2±0.03	91±2	0.5±0.1	99±6
O1602	13±2	99±4	>30000		>30000	
CP55940	5±1	100±2	0.2±0.01	100±2	0.3±0.01	100±4

Values shown are the means±s.e.m. derived from five independent experiments.

Table 2: Ligand effects on endocannabinoid receptors. This table lists the activities of cannabinoid ligands on GPR55, CB1 and CB2 receptor (Ryberg, Larsson et al. 2007).

CBD acts on $5HT_{1A}$ receptors, G_i coupled serotonin receptor in the brain and possible target for anxiolytic drugs. CBD displayed agonistic properties in binding assays on $5HT_{1A}$ transfected CHO cells (Russo, Burnett et al. 2005). Accordingly, a part of the neuroprotective effect of CBD may be caused by this effect. It`s controversial if CBD is an agonist or a positive modulator of $5HT_{1A}$ receptors (Campos, Fogaça et al. 2016). According to results of bioluminescence resonance energy transfer (BRET) experiment, the formation of CB_2/$5HT_{1A}$ heterodimers in HEK293 seems reasonable (Pazos, Mohammed et al. 2013).

CBD was found to be a partial agonist at D_2 receptors. Indeed, the demonstrated effect was considered to be biphasic, concluded from a binding study with radio-domperidone (K_D: 11nM) and seemed comparable to the effects of aripiprazole (an antipsychotic drug) (Seeman 2016).

CBD was shown to bind to μ-opioid and δ-opioid receptors in rat cerebral cortex membrane homogenates. A possible modulation had been concluded but was not shown (Kathmann, Flau et al. 2006).

Other ion channels

CBD and CBDV (Cannabidivarin) were shown to dose-dependently activate and rapidly desensitize recombinant rat TRPV1, TRPV2 and TRPA1 channels in HEK293 cells. Furthermore, it was shown that CBD and CBG (Cannabigerol) block voltage-gated Na^+ channels (Iannotti, Hill et al. 2014).

Enzymes

CBD shows inhibition of following enzymes:

ACAT (<10μM), AANAT (Arylalkylamine N-acetyltransferase), Catalase, Complex I (8.2 μM), Complex II (19.1 μM), Complex IV (18.8 μM), COX1 (Cyclooxygenase 1), COX2 (Cyclooxygenase 2), CYP2C19 (1.55 μM), CYP2D6 (6.52 μM), CYP3A4 (11.7 μM), CYP3A5 (1.65 μM), CYP3A7 (24.7 μM), CYP2C9 (2.7 μM), CYP1A1 (0.537 μM), CYP1A2 (~3.5 μM), CYP1B1 (~5 μM), DAGL-α (Diacylglycerol lipase), FAAH (Fatty acid amide hydrolase) (15.2μM), IDO, LOX-5 (Arachidonat-5-Lipoxygenase) (73.73 μM), ALOX-15 (arachidonate 15-lipoxygenase) (2.56 μM), MAGL (Monoacylglycerol lipase), N-acylethanolamine acid amide hydrolase (> 100 μM), NAD(P)H quinone reductase, Phospholipase A2 (134 μM), Progesterone 17α-hydroxylase, SOD (Superoxiddismutase), Testosterone 6α-hydroxylase, Testosterone 16β-hydroxylase

And stimulation in:

Sphingomyelinase, Phospholipase A2 (6.4 μM), Glutathione reductase, GSH peroxidase (Glutathionperoxidase), HMG-CoA Reductase, FAAH, Complex I.

Methods

Two electrode voltage clamp

Oocytes were isolated from mature female *Xenopus laevis* (Nasco, WI, USA). After anaesthetizing in ice-cold 0.17% Tricain (Ethyl-m-aminobenzoat, Sigma, MO, USA) decapitation was performed. Stage 5-6 oocytes were obtained from the ovary lobe, dissected into smaller parts and incubated in calcium free xenopus ringer (XR/NDE) and 1mg/ml collagenase (type IA, Sigma, MO, USA) for 60 to 90 minutes. Then the oocytes were placed in XR/NDE which was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (XR+/NDE+). The follicular layer was detached by pipetting trough a sterile glass Pasteur pipette. A day later after resting at 18°C, the cells were placed in XR for mRNA injection. 40nl (2.5 - 4 ng) mRNA was injected with a *Nanoject 2* (Drummond Scientific Company, USA) into each oocyte. The subunit ratio was 1:1:5 for αβγ (exception: 3:1:5 for $\alpha_4\beta\gamma/\alpha_6\beta\gamma$) and 1:1 for αβ receptors (exception: 2:1 for $\alpha_4\beta/\alpha_6\beta$) and 1:3:5 for αβδ receptors according (Mirheydari, Ramerstorfer et al. 2014), (Varagic, Ramerstorfer et al. 2013).

For parallel two electrode voltage clamp (TEV) recordings a Dagan CA-1B Oocyte Clamp and a Dagan TEV-200A two-electrode voltage clamp (Dagan Corporation, Mineapolis, MN, USA) was used. Two electrodes were placed in a bath of XR/NDE solution containing 90mM NaCl, 1mM $MgCl_2$, 1mM KCl, 1mM $CaCl_2$ and 5mM HEPES-NaOH (pH 7.4). The oocytes were impaled by two microelectrodes containing 2M KCl with a serial resistance of 1-3MΩ. A on top gravity flow was placed over the oocytes providing constant flow of XR. The perfusion could be switched to different solutions containing GABA or GABA and compound. The compounds were pre-dissolved in DMSO to 30mM stocks and diluted in XR/NDE containing an amount of GABA referenced to EC_{3-5}.

Two different experiments were carried out.

Compound effects were determined at low GABA EC_{3-5}

The EC_{3-5} was estimated by titration of different concentrations of GABA. The current was normalized to the current obtained from 1mM GABA. After maximum

concentration of GABA (1mM) 15 min washing time were provided to ensure full recovery from desensitization before further measurement occurred. Before compound was applied, two controls of GABA EC_{3-5} conc. were recorded. Washing time between measurements was at least 3 min. Different concentrations of substance were co-applied with a GABA EC_{3-5} control.

10µM of substance were co-applied with different GABA concentrations

First, a full GABA dose response curve was measured. The valves were flushed with NDE/XR in the 3 min washing time between the measurements. After a washing time after the highest GABA concentration (1mM), 10µM substance was co-applied with the same GABA concentrations used before including washing periods of 3 min.

All recordings were performed at a holding potential of -60 mV and RT. The data was digitized by a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA, USA). The data was recorded by Axon pClamp Software (Molecular Devices, USA) and analysed by Prism6 (GraphPad Software, USA).

Noteworthy is that the assembly of $GABA_A$ receptors in oocytes can be influenced by the ratio of subunit RNA, the expression time and other factors. Furthermore, the configuration can vary between oocytes and batches. A mixture of populations occurs including homopentameric and binary receptors with different stoichiometry of α and β subtypes. Therefore, variation of response to GABA as well to substance can occur. The use of test compounds is necessary to test the configuration of the assembled receptors to gain comparable data. For tertiary receptors Zn^{2+} is used to test for presence of binary (α β) receptors. For γ_2 containing receptors, diazepam was used except for incorporation of a_4 and a_6 subunits. For a_6 containing receptors, Compound 6 was the appropriate test substance. For δ containing receptors, DS2 was used to test the incorporation of δ subunits (Mirheydari, Ramerstorfer et al. 2014). LAU177 a synthetic compound was used to potentiate maximum GABA currents in $\alpha_1\beta_3$ receptors. The substances were co-applied together with a GABA control. Only in HEK293 cells an additional pre-application of substance was used.

Patch clamp technique

HEK293 cells were cultivated in growth media: DMEM, high glucose, GlutaMAX™ Supplement (Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific) and 1 % Penicillin-Streptomycin 10,000 U/mL (Thermo Fisher Scientific) at 37°C and 5% CO_2.

Cells were cultivated in T-75 flasks and passaged at ~90% confluency. Cells were passaged to small petri dish (10 cm^2) and transfected at ~75% confluency. Transfection media was prepared from 100µl DMEM, high glucose, GlutaMAX™ Supplement (Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific) and 0.5µl – 1µl (1µg/µL) linearized plasmid DNA for each subtype. After 2min 4µl TurboFect™ Transfection Reagent (Thermo Fisher Scientific) was added and vortexed. After 15min 700µL of DMEM, high glucose, GlutaMAX™, 10% FBS were placed on the cells and 100µl of the transfection media was dropped above. The cells were incubated for 4-6h at 37°C and 5% CO_2 before the media was changed to growth media.

After 48h the cells were used for patch clamp techniques.

Bath solution for whole cell recordings contains 140mM NaCl, 2mM MgCl2, 2.5mM CaCl2, 10mM HEPES and 20mM glucose. The pH of 7.4 of 1l of solutions was adjusted by 2.2ml of 2M KOH and the measured osmolality was 330mosmol/kg.

Pipette solution for whole cell recordings contained 140mM CsCl, 10mM EGTA, 0.7mM MgCl2, 2mM CaCl2, 10mM HEPES, 5mM glucose and 4mM ATP/Mg. pH of 7.3 was adjusted with CsOH and measured osmolality was 320mosmol/kg.

Cells were grown on small petri dish and placed at the head stage of a fluorescence microscope. A silver wire bath electrode was placed in the dish. A tube for continuous medium exchange was placed at the edge of the dish. The seal was accomplished by microelectrodes with a series resistance of 2-5 MΩ. Compounds were applied by a gravity flow perfusion system to a manifold tube and digitally regulated by an electromagnetic valve.

Docking

Docking was performed with GOLD. An existing α_6 homology model (Puthenkalam, Hieckel et al. 2016) was used. Only one α_6 subunit was used for the docking. The simplified molecular-input line-entry system (SMILES) data of THC and CBD was downloaded from Wikipedia and implemented into MOE. SER287 was declared as center (10 Å) of a binding site. A subset of 10 amino acids in the region was defined for flexible sidechain amino acids. 100 different docking poses had been calculated and sorted according high GOLD scores and CHEM scores.

Statistics

Data was analysed and monitored with prism 6 (graph pad software). Data points of GABA dose response curves were fitted for each cell individually to notice the variation of EC50 (**Figure 9 B**). After elimination of outliers (CI95%) of GABA and substance data (whole datasets were removed **Figure 9 A**), fits were produced for the GABA dose response curve (**Figure 9 C**): (log(agonist) vs. response -- Variable slope (four parameters)); and for each substance respectively (**Figure 9 D-G**).

$$Y = Bottom + (Top - Bottom)/(1 + 10^{((logEC50 - X) * Hill\ Slope)})$$

Compounds were labelled individually (THC: blue, CBD: green, CBG: orange and THCV: purple) through whole results part for clarity. Error bars display SEM (Standard Error of Mean). Statistical significance was tested with unpaired t-test and 95% confidence interval.

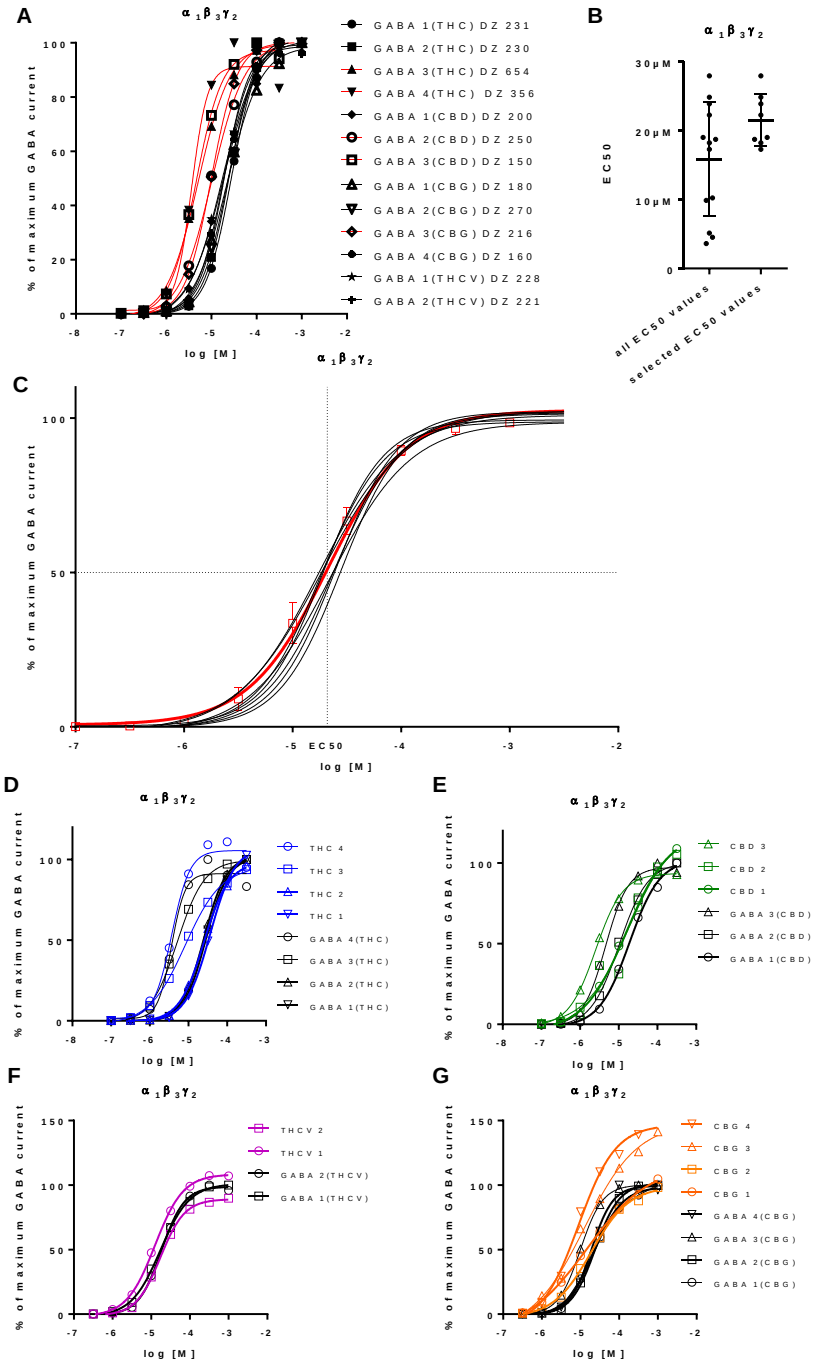

Figure 3: Statistics example. ***[A]*** *All GABA dose response curves of* $\alpha_1\beta_3\gamma_2$ *experiments taken together to eliminate outliers (red).* ***[B]*** *EC50 values of selected compared to all.* ***[C]*** *Curve fit (red) of pooled and selected GABA dose response curves.* ***[D]*** *Detection and elimination of outliers and respective THC measurements (thin).* ***[E]*** *Detection and elimination of outliers and respective CBD measurements (thin).* ***[F]*** *Detection and elimination of outliers and respective THCV measurements (thin).* ***[G]*** *Detection and elimination of outliers and respective CBG measurements (thin).*

Results

Screening of major phytocannabinoids in different $GABA_A$ receptor subtypes.

A screening with CBD and THC in different $GABA_A$ receptors at GABA EC_{3-5} was carried out, illustrating an overview of substance effects. In addition, effects of CBD and THC on $GABA_A$ receptor subtype combinations were compared at 10 µM substance concentration, shown in **Figure 10** (dose dependent effects were not shown).

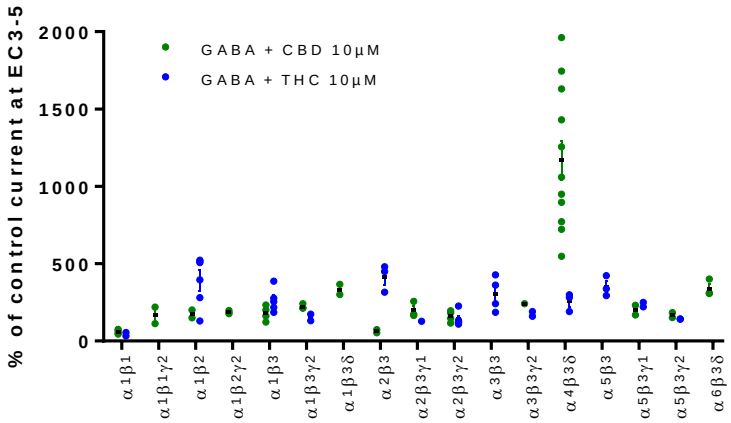

Figure 10: EC3-5 overview. An overview of 10µM compound effects of THC and CBD coapplied with EC3-5 GABA concentrations in individual GABAAR subtypes.

EC 3-5 GABAA Receptor	n	10µM CBD Mean ± SEM	n	10µM THC Mean ± SEM
$\alpha_1\beta_1$	4	60 ± 7	2	44 ± 11
$\alpha_1\beta_1\gamma_2$	2	167 ± 54		
$\alpha_1\beta_2$	2	175 ± 25	5	444 ± 47
$\alpha_1\beta_2\gamma_2$			2	198 ± 9
$\alpha_1\beta_3$	4	179 ± 24	6	267 ± 28
$\alpha_1\beta_3\gamma_2$	6	223 ± 6	2	152 ± 22
$\alpha_1\beta_3\delta$	2	333 ± 33		
$\alpha_2\beta_3$	2	64 ± 10	3	415 ± 51
$\alpha_2\beta_3\gamma_1$	3	198 ± 29	2	126 ± 2
$\alpha_2\beta_3\gamma_2$	4	160 ± 18	6	142 ± 17
$\alpha_3\beta_3$			4	304 ± 50
$\alpha_3\beta_3\gamma_2$	1	242	2	175 ± 15
$\alpha_4\beta_3\delta$	12	1170 ± 127	3	257 ± 34
$\alpha_5\beta_3$			3	351 ± 38
$\alpha_5\beta_3\gamma_1$	2	200 ± 31	2	236 ± 15
$\alpha_5\beta_3\gamma_2$	2	168 ± 17	2	142 ± 1
$\alpha_6\beta_3\delta$	3	339 ± 31		

Table 3: Table of EC_{3-5} potentiation. Table of corresponding modulation of GABA currents at EC_{3-5} including the standard error of mean (SEM).

CBD (green) and THC (blue) showed modulatory effects in most GABA receptor combinations, compared at 10µM substance concentration at GABA EC_{3-5}, shown in **Figure 10**. The modulatory effects were quantified and normalized to a GABA control listed in **Table 3**. Negative modulatory effects of CBD and THC in $\alpha_1\beta_1$ receptors were displayed (60% and 44 % respectively). In $\alpha_2\beta_3$ receptors, CBD showed negative modulatory effects (64%) (n=2) while THC showed moderate positive modulation (415%) (n=3). In all other tested combinations, CBD and THC showed positive modulatory effects. THC showed moderate effects in $a_1\beta_2$ (444 %), $a_2\beta_3$ (415 %) and $a_5\beta_3$ (351 %) binary receptors and weak potentiation in γ_2 containing receptors. CBD showed strong and highly variable potentiation in $\alpha_4\beta_3\delta$ injected cells. In $\alpha_6\beta_3\delta$ and $\alpha_1\beta_3\delta$ the potentiation was 339% and 333% respectively. In γ_2 containing receptors, CBD displayed weak positive modulatory effects. Following observations were made, interpreting the screening data shown in **Table 3**.

1. THC coapplied with GABA enhanced currents in $\alpha_x\beta_3$ containing binary receptors compared to $\alpha_x\beta_3\gamma_2$ containing receptors at GABA EC3-5.
2. Incorporated β_1 subunits decreased the Cl currents compared to a GABA control.
3. CBD coapplied with GABA enhanced currents in delta containing receptors stronger compared to binary and γ_2 containing receptors.
4. CBD and THC effects discriminate in $\alpha_4\beta_3\delta$ and $a_2\beta_3$ receptors.
5. Potentiation varied with incorporated α (1,2,3,5) subunit.

Accordingly, more specific experiments were designed to obtain more mechanistic insights. A comparison of β_1 an β_3 should confirm the β isoform dependent effects of the compounds. In addition, the substance effects were tested at high concentrations (10µM) coapplied across an entire GABA DR curve to clarify the modulatory effects in dependence of GABA concentration. We investigated THC actions in various receptors.

Overview of THC Effects

This plot gives an overview of the effects of 10µM THC on GABA EC_{50} and maximum currents of different receptor subtypes. First a GABA dose response curve was obtained. Afterwards, the same GABA concentrations, were coapplied with 10 µM of THC.

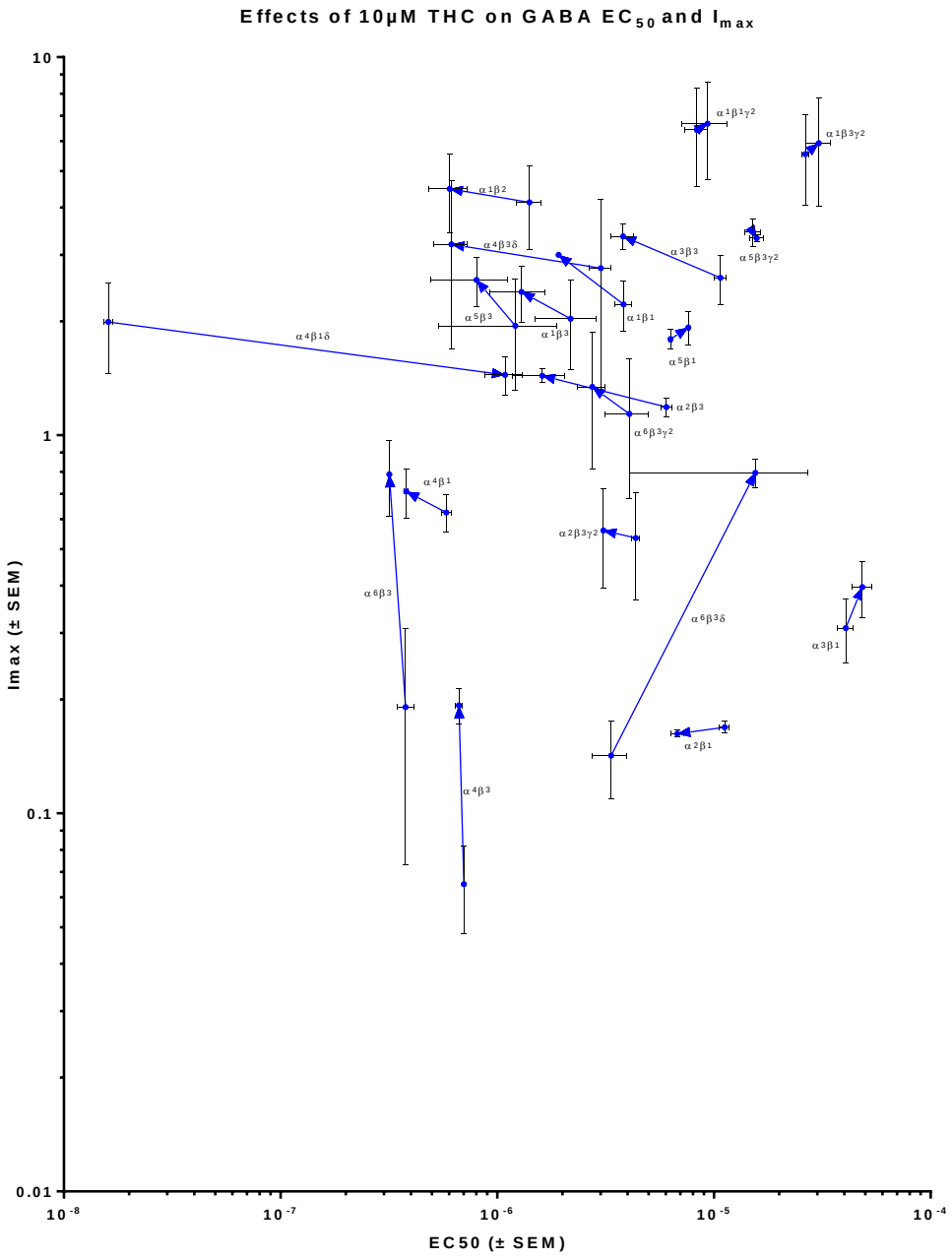

Figure 11: This plot shows the effects of 10µM THC coapplied on EC_{50} of GABA and maximum GABA currents of individual receptor subtypes.

	GABA EC50 [µM]	*CI95% [µM]*	*GABA EC50 [µM] + THC 10µM*	*CI95% [µM]*
$\alpha_1\beta_1$	*3,9*	*3,27 - 4,58*	*3,0*	*1,18 - 7,78*
$\alpha_1\beta_1\gamma_2$	*8,4*	*6,64 - 10,5*	*9,0*	*5,87 - 13,7*
$\alpha_1\beta_2$	*1,4*	*0,98 - 1,85*	*0,5*	*0,34 - 0,82*
$\alpha_1\beta_3$	*1,9*	*1,28 - 2,86*	*1,2*	*0,47 - 3,31*
$\alpha_1\beta_3\gamma_2$	*21,3*	*18,67 - 24,4*	*30,4*	*26 - 35,5*
$\alpha_2\beta_1$	*11,0*	*7,72 - 15,8*	*6,7*	*5,33 - 8,51*
$\alpha_2\beta_3$	*6,0*	*4,87 - 7,43*	*1,4*	*0,67 - 2,88*
$\alpha_2\beta_3\gamma_2$	*4,3*	*3,31 - 5,72*	*3,1*	*2,42 - 3,96*
$\alpha_3\beta_1$	*39,9*	*32,39 - 49,1*	*47,6*	*36,14 - 62,8*
$\alpha_3\beta_3$	*10,6*	*9,44 - 11,9*	*3,8*	*2,89 - 4,92*
$\alpha_4\beta_1$	*0,6*	*0,38 - 0,88*	*0,4*	*0,27 - 0,51*
$\alpha_4\beta_1\delta$	*0,02*	*0,01 - 0,02*	*1,2*	*0,20 - 6,66*
$\alpha_4\beta_3\delta$	*3,0*	*2,42 - 3,72*	*0,4*	*0,19 - 0,99*
$\alpha_5\beta_1$	*6,3*	*4,94 - 7,97*	*7,6*	*6,21 - 9,31*
$\alpha_5\beta_3$	*1,0*	*0,30 - 3,28*	*0,8*	*0,27 - 2,56*
$\alpha_5\beta_3\gamma_2$	*14,9*	*13,4 - 16,7*	*15,6*	*13,32 - 18,3*
$\alpha_6\beta_3\gamma_2$	*3,6*	*2,44 - 5,42*	*2,6*	*1,631 - 3,99*
$\alpha_6\beta_3\delta$	*3,2*	*1,91 - 5,23*		

Table 4: EC_{50}. Table of EC_{50} values of all GABA dose response curves with and without compounds, including the confidence interval.

β selectivity of THC

Indeed, the screening results indicate possible difference in effects through β isoform dependency of THC. Based on these findings, additional experiments with $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ were performed to test beta selectivity. All β isoforms were co-expressed with α_1 respectively. Effects were dependent on the β isoform. In summary, the β_1 containing receptors shows minimal or negative modulatory effects at low $EC_{3\text{-}5}$ and weak positive modulatory effects at higher GABA concentrations, shown in **Figure 12A**. The GABA dose response curve of the β_2 containing subtype coapplied with 10µM THC was shifted to the left compared to GABA alone, which indicates a positive modulatory effect shown in **Figure 12B**.

In β_3 containing receptors 10µM THC enhanced GABA currents across the entire DR curve, shown in **Figure 12C**.

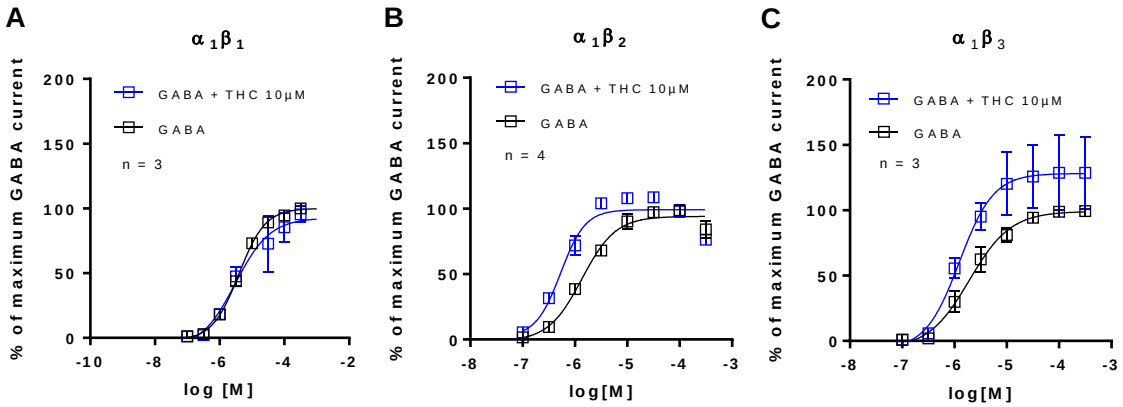

Figure 12: Impact of β-subunit on THC effect. ***[A]*** *10µM THC coapplied with different GABA concentrations on $\alpha_1\beta_1$ compared to GABA alone, normalized to the maximum GABA response.* ***[B]*** *10µM THC coapplied with different GABA concentrations on $\alpha_1\beta_2$ compared to GABA alone, normalized to the maximum GABA response.* ***[C]*** *10µM THC coapplied with different GABA concentrations on $\alpha_1\beta_3$ compared to GABA alone, normalized to the maximum GABA response.*

The modulatory effect of THC in β_3 containing binary receptors is α subunit dependent.

To test the influence of different α subunits, all α subunits were expressed together with the β_3 subunit respectively. THC modulation was affected by the type of the incorporated α subunit in β_3 containing binary receptors, shown in **Figure 13**. In all tested combinations, the maximum GABA response was increased, more so in $\alpha_4\beta_3$ and $\alpha_6\beta_3$ receptors, shown in **Figure 13D and 13E**. $\alpha_4\beta_3$ and $\alpha_6\beta_3$ receptors produce currents below 0.3µA when stimulated with high concentrations of GABA (1mM), data is not shown. This could be an explanation for the strong potentiation of currents by the substance at low and high GABA concentrations. However, the enhancement of maximum GABA currents was observed in all β_3 containing binary receptors, shown in **Figure 13A-E**. Additionally, moderate left shifts were displayed in $\alpha_2\beta_3$ and $\alpha_3\beta_3$ shown in **Figure 13B and 13C**

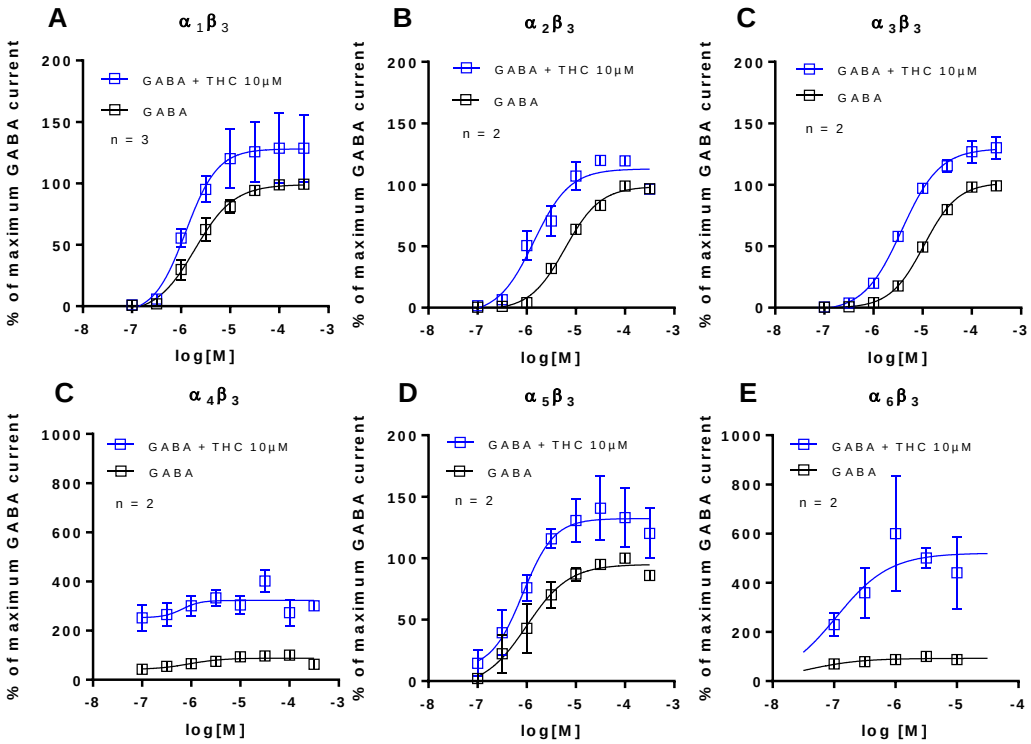

Figure 13: Influence of type of α subunit in THC effects. ***[A]*** *Actions of 10µM THC (EC50 1.24µM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC50 1.9µM) in $\alpha_1\beta_3$.* ***[B]*** *10µM THC (EC50 1.39µM) compared to GABA (EC50 6.0µM) in $\alpha_2\beta_3$.* ***[C]*** *10µM THC (EC50 3.7µM) compared to GABA (EC50 10.6µM) in $\alpha_3\beta_3$.* ***[D]*** *10µM THC (maximum current potentiation ~300%) compared to GABA maximum current in $\alpha_4\beta_3$.* ***[E]*** *10µM THC (EC50 0.82µM) compared to GABA (EC50 0.99µM) in $\alpha_5\beta_3$.* ***[F]*** *10µM THC (maximum current potentiation ~500%) compared to GABA maximum current in $\alpha_6\beta_3$*

The incorporation of the γ_2 subunit into β_3 containing receptors diminished the effects of 10µM THC, shown in **Figure 14A-D**. A weak positive modulatory effect was observed in $\alpha_6\beta_3\gamma_2$. This is in line with the strong modulation in the $\alpha_6\beta_3$ binary receptor subtype. The effects were not completely reversed by the incorporation of the γ_2 subunit, shown in **Figure 14D and 16B**.

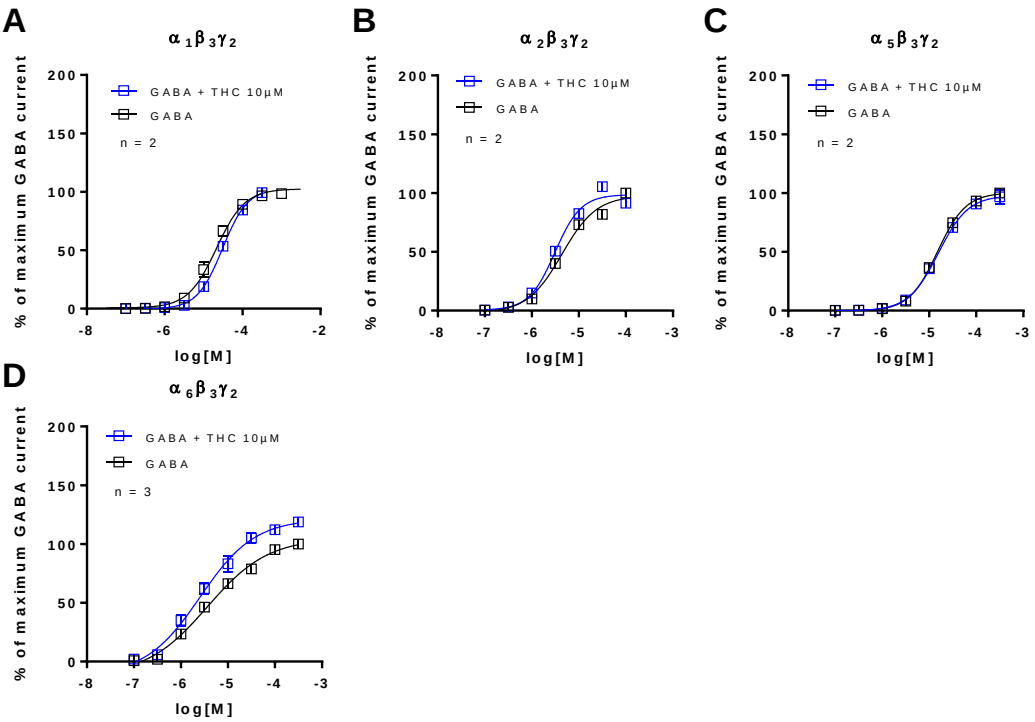

Figure 14: THC $\alpha_x\beta_3\gamma_2$. ***[A]*** *Effects of 10μM THC (EC50 30.36μM) on GABA evoked chloride currents when coapplied with GABA at different GABA concentrations compared to GABA alone (EC 50 21.28μM) in $\alpha_1\beta_3\gamma_2$.* ***[B]*** *10μM THC (EC50 3.0μM) compared to GABA (EC50 4.3μM) in $\alpha_2\beta_3\gamma_2$.* ***[C]*** *10μM THC (EC50 15.6μM) compared to GABA (EC50 14.9μM) in $\alpha_5\beta_3\gamma_2$.* ***[D]*** *Actions of 10μM THC (EC 50 2.5μM) coapplied with four different GABA concentrations compared to a GABA control (EC 50 3.638μM) in $\alpha_6\beta_3\gamma_2$ receptors.*

Finally, testing the hypothesis that γ_2 subunit exerts a diminishing effect in β_1 containing receptors, $\alpha_1\beta_1$ was compared to $\alpha_1\beta_1\gamma_2$. The weak negative modulatory effect of THC on $\alpha_1\beta_1$ was completely eliminated in $\alpha_1\beta_1\gamma_2$, shown in **Figure 15**.

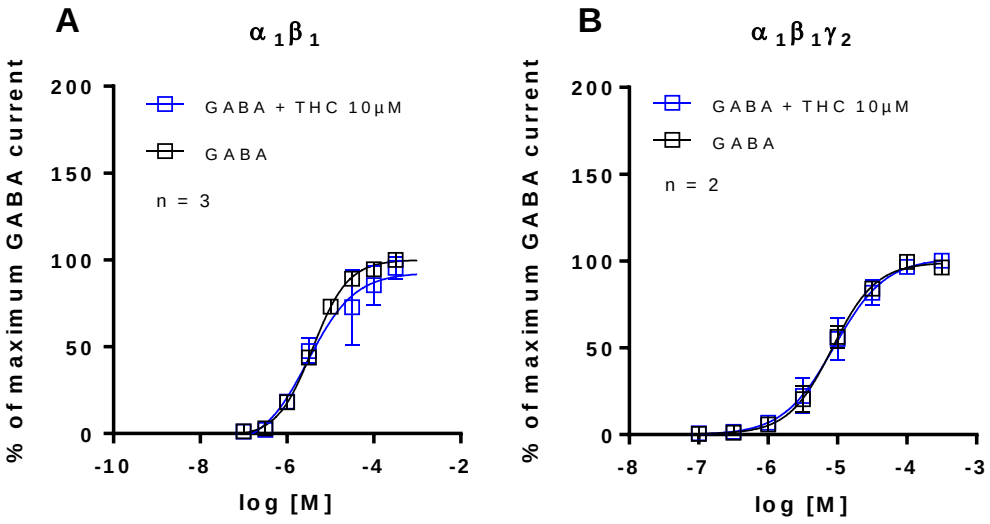

Figure 15: THC effects in $\alpha_1\beta_1$ vs. $\alpha_1\beta_1\gamma_2$. ***[A]*** *Actions of 10µM THC (EC50 3.0µM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC50 3.8µM) in $\alpha_1\beta_1$.* ***[B]*** *Actions of 10µM THC (EC50 8.9µM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC50 8.3µM) in $\alpha_1\beta_1\gamma_2$.*

Enhancement of GABA currents in α_6 containing receptors

When coapplied with THC, currents in α_6, containing receptors were strongly enhanced. In binary α_6 containing receptors, 1µM THC indicated saturation and displayed enhancements of 500% of maximum GABA currents, shown in **Figure 16C**. Additional experiments in $\alpha_6\beta_3\delta$ 10µM THC coapplied, potentiated currents at low GABA concentrations stronger compared to 10µM CBD, shown in **Figure 16A**. Modulation at high GABA concentrations is greater than 300% for THC. Measurements of compound dose dependent effects of the substance at low GABA concentrations were not possible due to low GABA currents. Nevertheless, the data suggest a higher potency of THC compared to CBD but needs to be shown in further experiments. Moreover, 10µM THC displayed weak positive modulatory effects in $\alpha_6\beta_3\gamma_2$ injected receptors (probably due to an $\alpha_6\beta_3$ pool of receptors), shown in **Figure 16B and 14D**.

A representative trace of CBD effects in $\alpha_6\beta_3\delta$ receptors was shown in **Figure 16D.** To investigate the effects of CBD on δ containing GABAA receptors, 10µM CBD was coapplied to different GABA concentrations and compared to the

effects of GABA alone. CBD convincingly potentiates GABA induced chloride currents in $\alpha_6\beta_3\delta$ receptors at low GABA concentrations. In addition, maximum GABA response was potentiated more than 3x in $\alpha_6\beta_3\delta$, shown in **Figure 16A and D**.

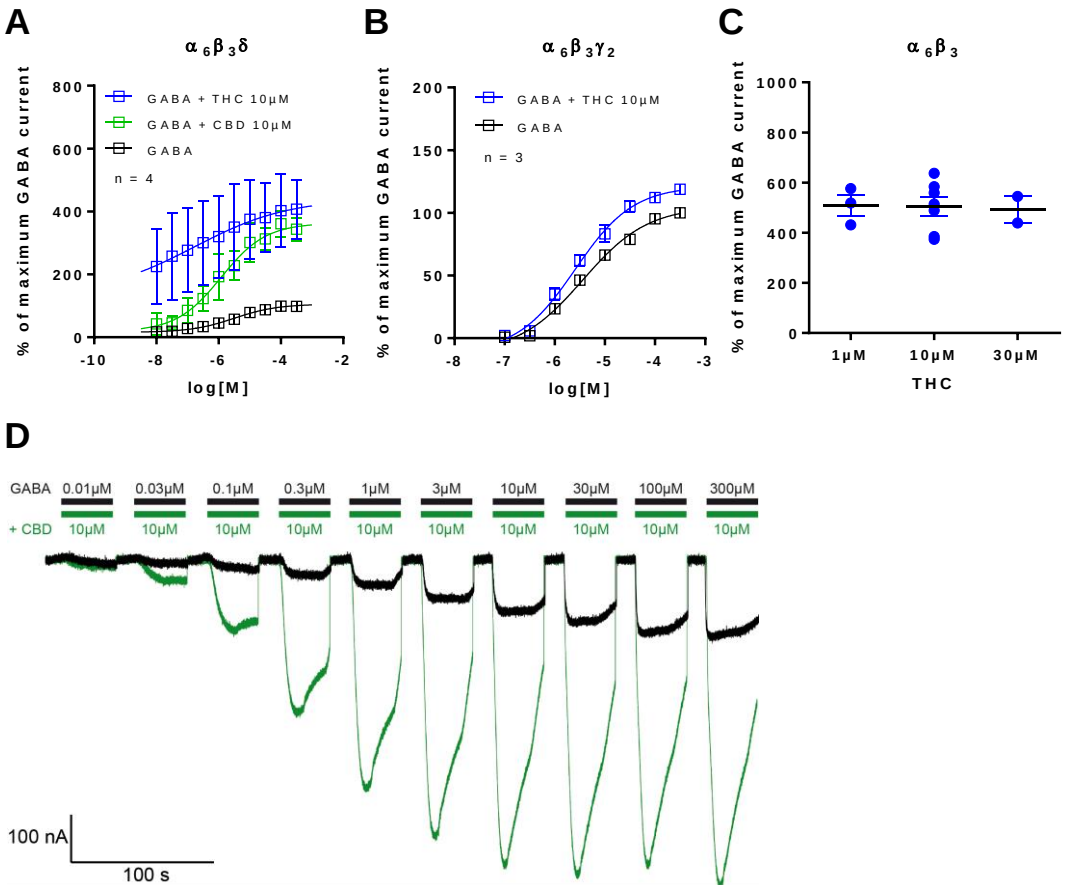

Figure 16: THC in a6 containing receptors. ***[A]*** *A comparison of 10µM THC (EC50 0.16µM) and 10µM CBD (EC50 1.2µM) actions coapplied with different GABA concentrations normalized to a GABA control (EC50 3.16µM) in $\alpha_6\beta_3\delta$.* ***[B]*** *Actions of 10µM THC (EC 50 2.5µM) coapplied with different GABA concentrations compared to a GABA control (EC 50 3.638µM) in $\alpha_6\beta_3\gamma_2$ receptors.* ***[C]*** *Effects of 1, 10 and 30µM concentrations of THC coapplied with GABA normalized to the maximum GABA current in $\alpha_6\beta_3$ receptors.* ***[D]*** *A representative trace of CBD 10µM coapplied with different GABA concentrations on $\alpha_6\beta_3\delta$ receptors is shown in green. GABA concentrations without compound are shown in black.*

Possible binding site of CBD and THC

The data suggest a different structure of the binding site in α_4 and α_6 compared to α_1 α_2 α_3 and α_5 $GABA_AR$ subunits. An alignment of GlyR-α_3 and several $GABA_A$

receptor sequences shed light on several specific amino-acids inside highly conserved regions, identified as 2-AG binding site on the β_2 subunit of $GABA_A$ receptors. A docking approach of CBD and THC for the homologous site in α_6 yields promising results. Indeed, a pose were CBD and THC are closely overlapping has been identified. In that position, CBD is capable forming a hydrogen bond to SER287, similar, to what is proposed by Xiong et al. in GlyR-α_3. THC and CBD can form a second hydrogen bond to a leucin backbone on the opposite side of the binding site, shown in **Figure 17**. This LEU316 is conserved in α_4 and α_6 but different in all other α subunits (Puthenkalam, Hieckel et al. 2016).

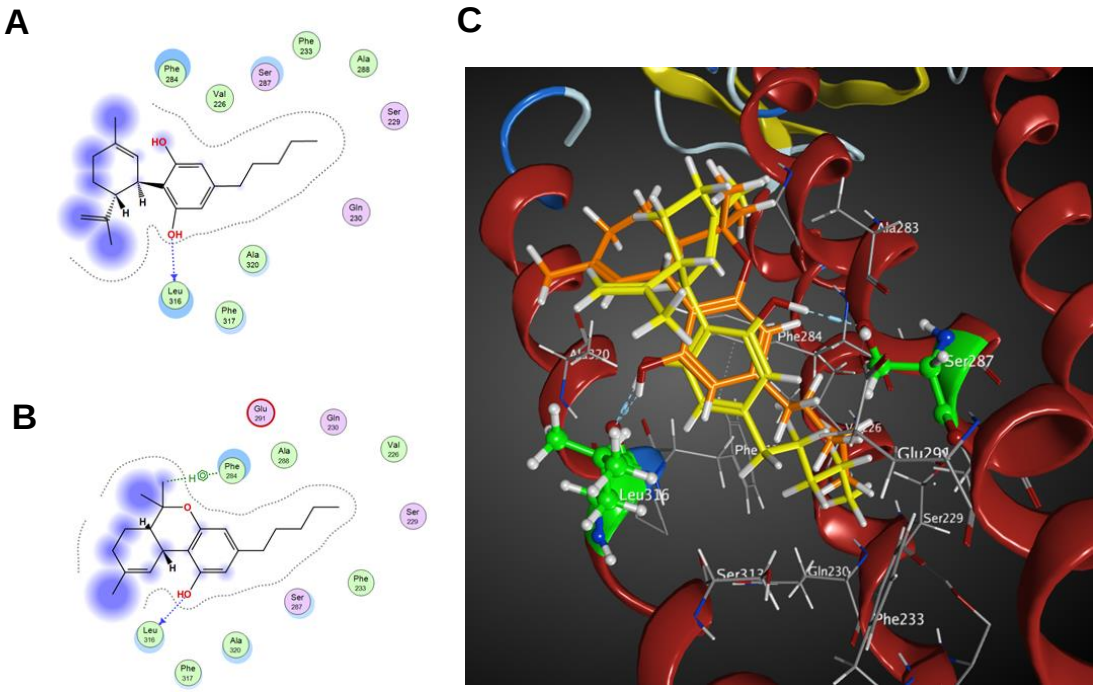

Figure 17: Docking of CBD and THC into an a6 subunit structural model. ***[A]*** *Surrounding of CBD in a possible binding pose. CBD formed hydrogen bond to LEU316.* ***[B]*** *Surrounding of THC in another possible binding pose. THC formed hydrogen bond to LEU316 and PHE284.* ***[C]*** *Docking of CBD and THC into the proposed 2-AG binding site in the TMD of a* α_6 $GABA_A$ *receptor subunit homology model. CBD formed hydrogen bonds with LEU316 and SER287, THC with LEU316.*

However, this binding hypothesis is based on literature findings, and in silico analysis. Of course, further investigation by site directed mutagenesis is necessary to clarify the findings. A docking into a α_4 subunit could contribute to further understanding.

Actions of THC and CBD on α4 containing receptors.

Identifying the actions of THC on α_4 containing receptors, $\alpha_4\beta_1$ was compared to $\alpha_4\beta_3$, $\alpha_4\beta_1\delta$ and $\alpha_4\beta_3\delta$. In contrast to findings in $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ binary receptors (shown in Appendix), $\alpha_4\beta_1$ shows small negative modulatory effects only at low GABA concentrations but positive modulation in the presence of higher GABA concentrations, shown in **Figure 18A**. Interestingly, incorporation of δ subunits, induced a strong negative modulatory effect in the presence of both, low and high GABA concentrations, displayed in **Figure 18B**. In line with positive modulatory effects of THC in presence of β_3 subunits, increased currents were observed in $\alpha_4\beta_3$ binary receptors in the presence of all GABA concentrations, shown in **Figure 18C**. An additional δ subunit, decreased the modulation compared to the binary receptors and is distinct to the observed negative modulation found in $\alpha_4\beta_1\delta$, shown in **Figure 18D**. 10μM CBD coapplied with GABA also displayed negative modulatory effects in $\alpha_4\beta_1\delta$ receptors, shown in **Figure 18E.** 10μM CBD coapplied with GABA displayed strong positive modulatory effects in $\alpha_4\beta_3\delta$ receptors, shown in **Figure 18F.**

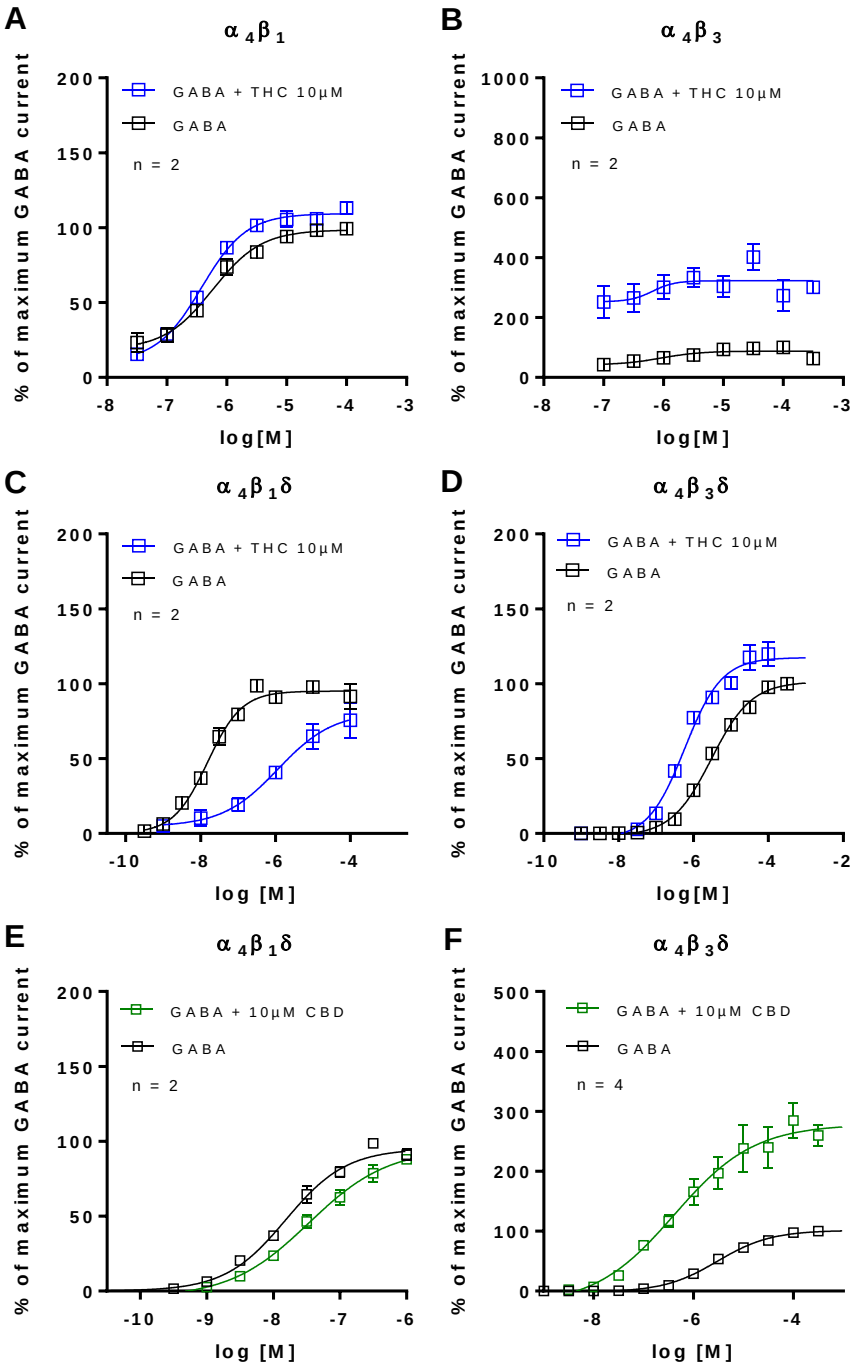

Figure 18: THC effects in α_4 containing receptors. **[A]** Actions of 10μM THC (EC_{50} 0.37μM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC_{50} 0.58μM) in $\alpha_4\beta_1$. **[B]** Actions of 10μM THC (EC_{50} 1.1μM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC_{50} 0.015μM) in $\alpha_4\beta_1\delta$. **[C]** Actions of 10μM THC (maximum current potentiation ~300%) coapplied with different GABA concentrations compared to GABA concentrations as control in $\alpha_4\beta_3$. **[D]** Actions of 10μM THC (EC_{50} 0.58μM) coapplied with different GABA concentrations compared to GABA concentrations as control (EC_{50} 3μM) in $\alpha_4\beta_3\delta$. ***[E]*** CBD 10μM was coapplied at different concentrations of GABA

(EC50 0.408µM) and compared to GABA alone (EC_{50} 3.0µM) on $\alpha_4\beta_3\delta$ receptors. The potentiation of efficacy was more than 200% of maximum GABA current and quantified in detail in Figure 24. **[F]** CBD 10µM was coapplied at different concentrations of GABA (EC50 0.034µM) and compared to GABA alone (EC50 0.015µM) on α4β1δ receptors. The efficacy was decreased, but was not quantified in detail.

Comparison of CBD with THC in $\alpha_4\beta_3\delta$ receptors

Effects of CBD and THC in $\alpha_4\beta_3\delta$ receptors were compared in **Figure 19A**. Actions of CBD in $\alpha_4\beta_3\delta$ receptors were found to be significantly stronger at EC_{3-5} and EC_{90-100} compared to the effects of THC, displayed in **Figure 19B**. Furthermore, the dose dependent effects of CBD and THC at low EC_{3-5} were tested in $\alpha_4\beta_3\delta$ receptors, which concludes a higher potency of CBD, shown in **Figure 19C**. Dose dependent effects of THC in $\alpha_4\beta_3\delta$ receptors were shown in **Figure 19D**. THC didn't show saturation at 30µM and the EC_{50} was not estimated.

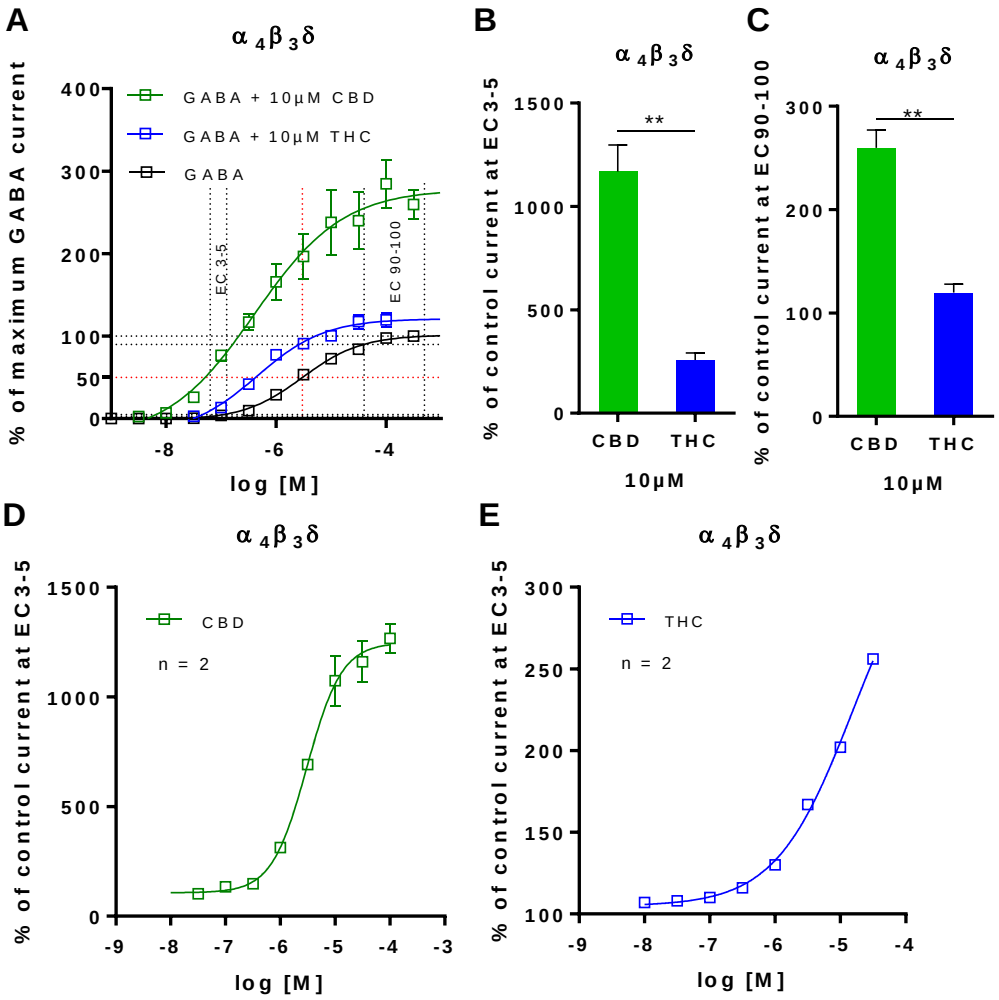

Figure 19: Comparison of THC and CBD in $\alpha_4\beta_3\delta$ receptors. ***[A]*** *Comparison of 10 µM CBD (EC50 0.408µM) and 10 µM THC (EC50 0.583µM) coapplied with GABA in $\alpha_4\beta_3\delta$ receptors, respectively (n=4).* ***[B]*** *Comparison of 10 µM CBD (1170 ± 127.2 n=12) and 10 µM THC (257.0 ± 33.86 n=3) coapplied with GABA at EC3-5 in $\alpha_4\beta_3\delta$ receptors, respectively. Quantification results in significant differences between CBD and THC (p=0.0040, CI95% 346.6 to 1479).* ***[C]*** *Comparison of 10 µM CBD (259.7 ± 17.42 n=4) and 10 µM THC (119.8 ± 8.153 n=2) coapplied with GABA at EC90-100 in $\alpha_4\beta_3\delta$ receptors. Quantification results in significant differences between CBD and THC (p=0.0063, CI95% 66.02 to 213.7).* ***[D]*** *Potentiation of GABA evoked chloride currents when different concentrations of CBD were coapplied with GABA at low EC3-5 (EC50 2.25µM to 4.10µM CI95%, n=2).* ***[E]*** *Potentiation of GABA evoked chloride currents when different concentrations of THC were coapplied with GABA at low EC3-5 (n=1).*

δ containing receptors in HEK293 cells

Human $\alpha_1\beta_3\delta$ receptors were expressed in HEK293 cells. CBD was pre-applied for 15s and then coapplied with 1mM GABA. Voltage clamp recordings displayed that CBD potentiated the maximum GABA current similar as LAU177. 3µM CBD potentiated GABA currents coapplied with 1µM GABA. CBD potentiated maximum GABA currents of $\alpha_1\beta_3\delta$ receptors in *X. oocytes (data not shown)*. Results were not quantified and not compared to oocyte data.

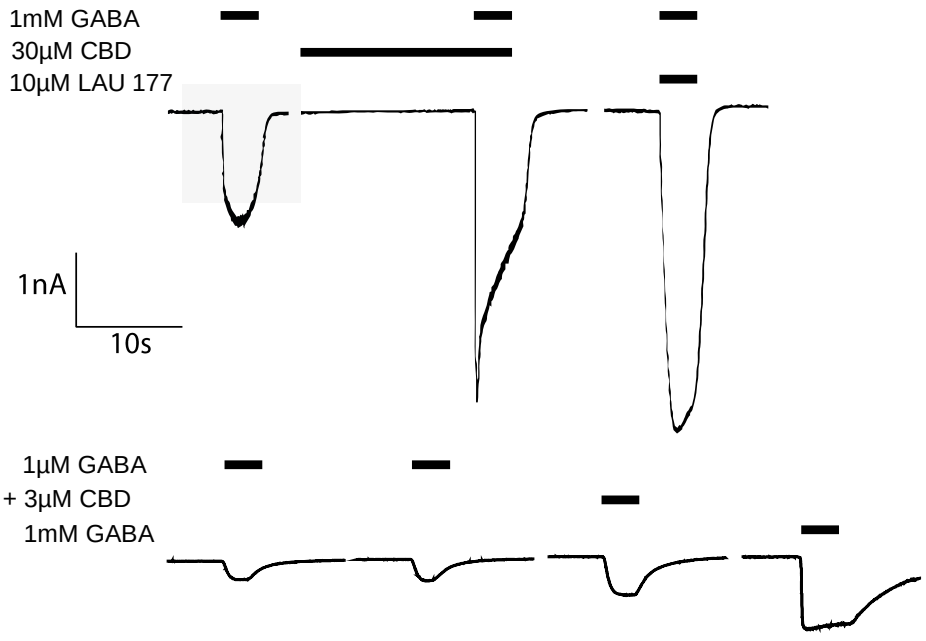

Figure 20: CBD in HEK cells expressing $\alpha_1\beta_3\delta$. Representative traces of human $\alpha_1\beta_3\delta$ maximum GABA currents modulated by 30µM CBD and 10µM LAU177 (top) in HEK293 cells. Representative traces of human $\alpha_1\beta_3\delta$ GABA currents (1µM GABA) potentiated by 3µM CBD (bottom) in HEK293 cells.

Effects of different phytocannabinoids, on the $\alpha_1\beta_3\gamma_2$ rat $GABA_A$ receptor, a postulated major receptor isoform.

We investigated the different effects of the phytocannabinoids THC, CBD, CBG and THCV on a frequently studied $GABA_A$ receptor subtype $\alpha_1\beta_3\gamma_2$. While 10 µM THC (GABA + substance EC_{50}: 30.36µM) and 10µM CBD (GABA + substance EC_{50}: 15.76µM) coapplied with GABA, seemingly display no or weak potentiation, 10 µM CBG (GABA + substance EC_{50}: 12.49µM) coapplied with GABA exhibits a left shift of the EC_{50} concentration referred to GABA (GABA EC_{50}: 21.28µM) alone. Moreover, THCV (GABA + substance EC_{50}: 14.64µM) displayed a left shift by 10µM substance of the curve but dose response curves (**Figure 22**) conclude a weak potency. Saturation was not reached by 30µM THCV at $EC_{3\text{-}5}$.

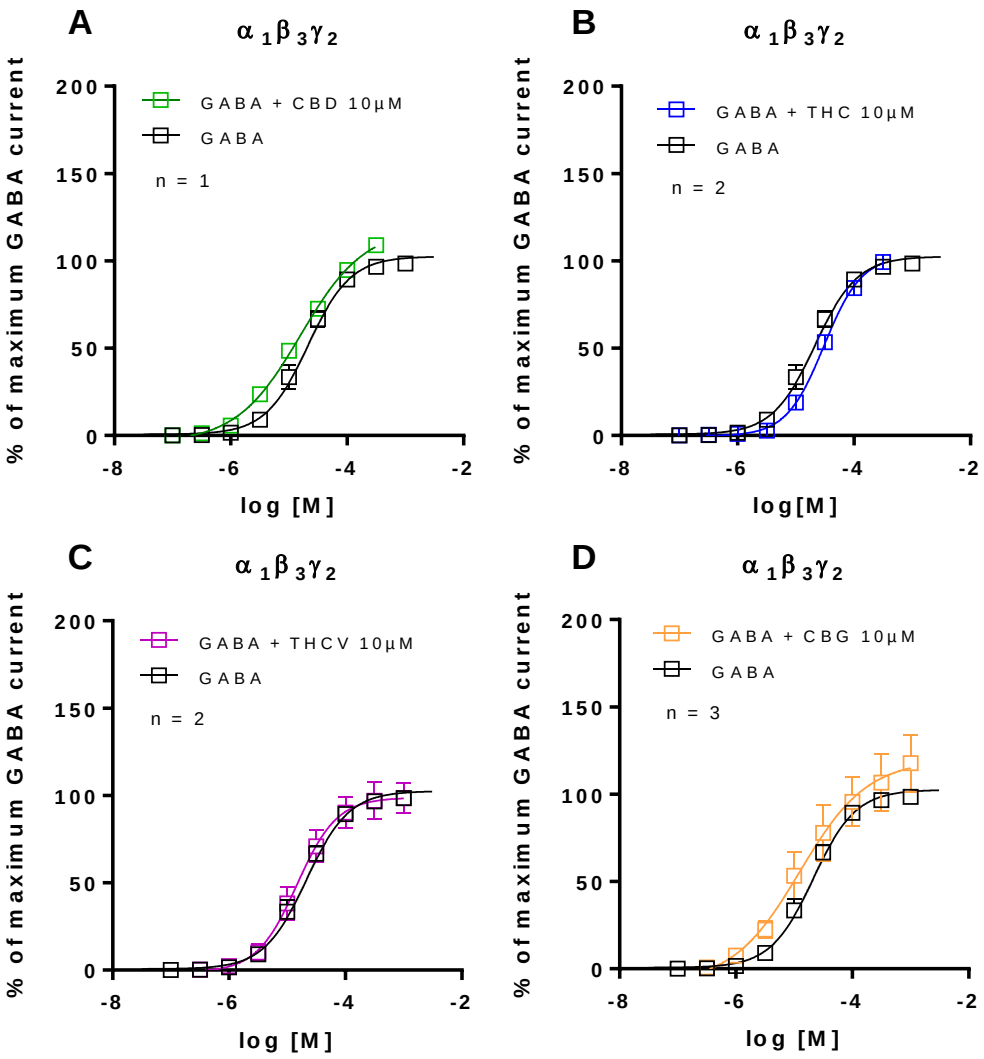

Figure 21: Phytocannabinoids in $\alpha_1\beta_3\gamma_2$ receptors. ***[A]*** *Effects of 10µM CBD (GABA + substance EC 50 15.76µM) on GABA evoked chloride currents when coapplied with GABA at different GABA concentrations compared to GABA alone (GABA EC 50 21.28µM).* ***[B]*** *Effects of 10µM THC (GABA + substance EC50 30.36µM) on GABA evoked chloride currents when coapplied with GABA at different GABA concentrations compared to GABA alone (GABA EC 50 21.28µM).* ***[C]*** *Effects of 10µM THCV (GABA + substance EC50 14.64µM) on GABA evoked chloride currents when coapplied with GABA at different GABA concentrations compared to GABA alone (GABA EC 50 21.28µM).* ***[D]*** *Effects of 10µM CBG (GABA + substance EC50 12.48µM) on GABA evoked chloride currents when coapplied with GABA at different GABA concentrations. compared to GABA alone (GABA EC 50 21.28µM)*

Furthermore, the phytocannabinoids CBD, THC, CBG and THCV have been tested at low EC_{3-5}. CBG showed the highest enhancement of GABA evoked chloride currents. As Sigel, E., R. Baur, et al. 2011 demonstrated, THC has weak modulatory properties in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Sigel, Baur et al. 2011). We find the same for $\alpha_1\beta_3\gamma_2$. In consideration to our results we conclude weak modulatory effects, but those are limited to presence of low GABA concentrations.

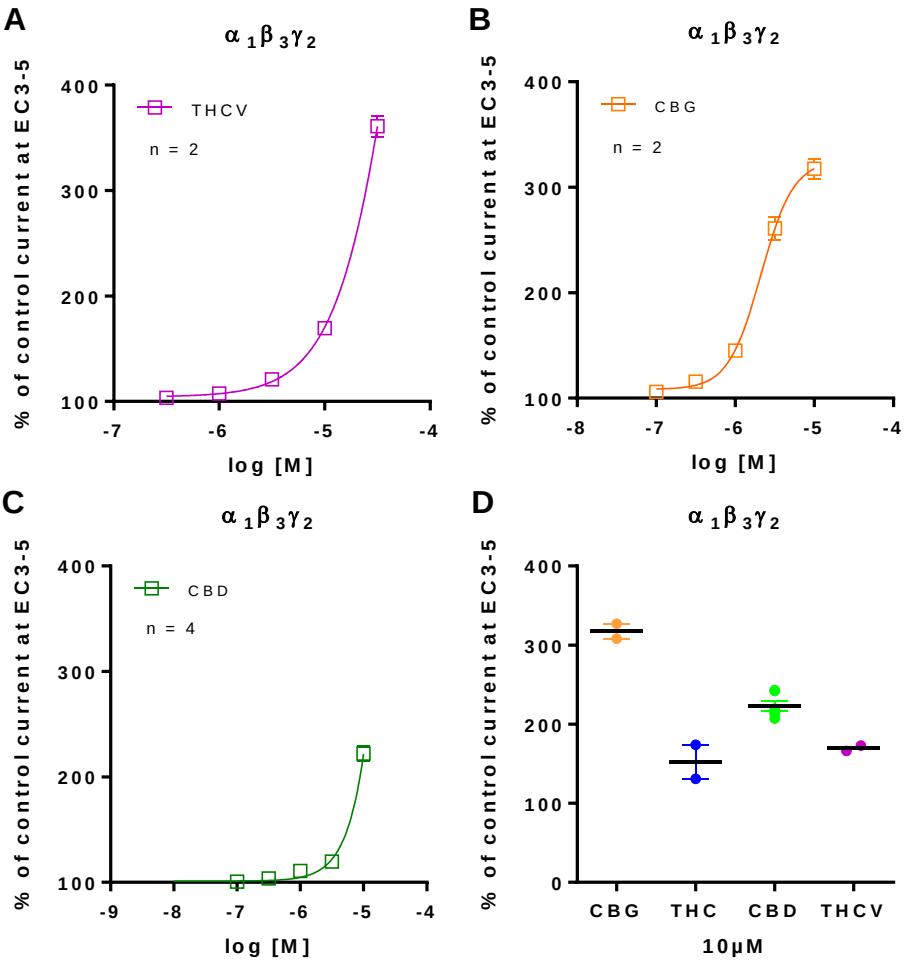

Figure 22: Dose dependent effects of phytocannabinoids in $\alpha_1\beta_3\gamma_2$ receptors. ***[A]*** *Dose dependent effects of THCV on GABA evoked chloride currents when coapplied with GABA at EC_{3-5}.* ***[B]*** *Dose dependent effects of CBG on GABA evoked chloride currents when coapplied with GABA at EC_{3-5}.* ***[C]*** *Dose dependent effects of CBD on GABA evoked chloride currents when coapplied with GABA at EC_{3-5}.* ***[D]*** *Comparison of 10µM compound effects of CBG, THC, CBD and THCV coapplied with GABA at EC_{3-5}.*

CBG displayed positive modulatory action in $\alpha_6\beta_2\gamma_2$ and $\alpha_6\beta_3\delta$ receptors coapplied with different GABA concentrations, shown in **Figure 24A and 24D**. Interestingly, rebound effects were noticed suggesting an influence of CBG in receptor kinetics, displayed in **Figure 24B**.

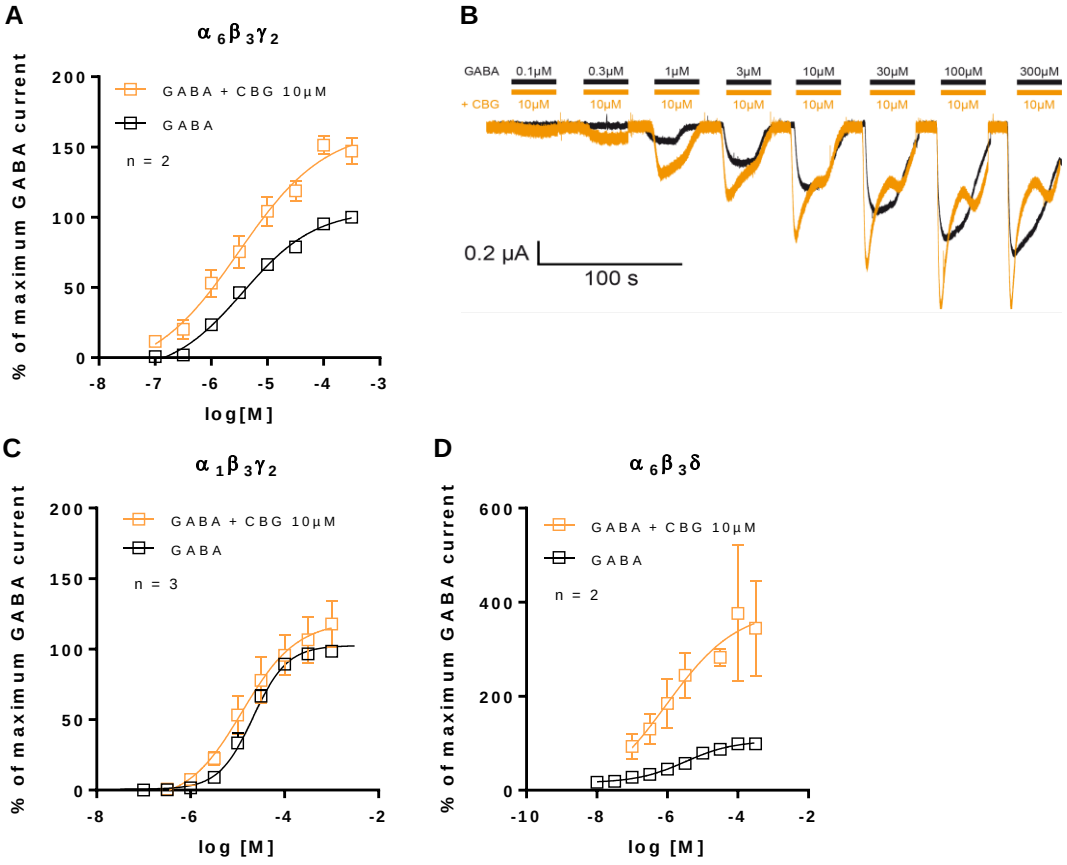

Figure 24: Effects of CBG. ***[A]*** *CBG 10µM was coapplied to different concentrations of GABA (EC50 3.084µM) and compared to GABA alone (EC50 3.638µM) on $\alpha_6\beta_3\gamma_2$ receptors. The potentiation of efficacy was around 150% of maximum GABA current but not further quantified in detail.* ***[B]*** *A representative trace of CBG 10µM coapplied with different GABA concentrations on $\alpha_6\beta_3\gamma_2$ receptors, are shown in orange. GABA currents without compound are shown in black.* ***[C]*** *CBG 10µM was coapplied at different concentrations of GABA (GABA + substance EC50 12.48µM) and compared to GABA alone (GABA EC50 21.28µM) on $\alpha_1\beta_3\gamma_2$ receptors. The potentiation was more than 200% of maximum GABA current.* ***[D]*** *CBG 10µM was coapplied at different concentrations of GABA and compared to GABA alone on $\alpha_6\beta_3\delta$ receptors. The efficacy was increased, but not further quantified in detail.*

	GABA EC50 [µM]	*CI95% [µM]*	*GABA EC50 [µM] + CBD 10µM*	*CI95% [µM]*	*GABA EC50 [µM] + CBG 10µM*	*CI95% [µM]*	*GABA EC50 [µM] + THCV 10µM*	*CI95% [µM]*
$\alpha_1\beta_1$	*3,9*	*3,27 - 4,58*						
$\alpha_1\beta_1\gamma_2$	*8,4*	*6,64 - 10,5*						
$\alpha_1\beta_2$	*1,4*	*0,98 - 1,85*						
$\alpha_1\beta_3$	*1,9*	*1,28 - 2,86*						
$\alpha_1\beta_3\gamma_2$	*21,3*	*18,67 - 24,4*	*15,76*	*11,06 - 22,5*	*12,49*	*3,83 - 40,79*	*14,6*	9,28 - 23,1
$\alpha_2\beta_1$	*11,0*	*7,72 - 15,8*						
$\alpha_2\beta_3$	*6,0*	*4,87 - 7,43*						
$\alpha_2\beta_3\gamma_2$	*4,3*	*3,31 - 5,72*						
$\alpha_3\beta_1$	*39,9*	*32,39 - 49,1*						
$\alpha_3\beta_3$	*10,6*	*9,44 - 11,9*						
$\alpha_4\beta_1$	*0,6*	*0,38 - 0,88*						
$\alpha_4\beta_1\delta$	*0,02*	*0,01 - 0,02*	*0,034*	*0,022 - 0,054*				
$\alpha_4\beta_3\delta$	*3,0*	*2,42 - 3,72*	*0,41*	*0,10 - 1,69*				
$\alpha_5\beta_1$	*6,3*	*4,94 - 7,97*						
$\alpha_5\beta_3$	*1,0*	*0,30 - 3,28*						
$\alpha_5\beta_3\gamma_2$	*14,9*	*13,4 - 16,7*						
$\alpha_6\beta_3\gamma_2$	*3,6*	*2,44 - 5,42*			*3,084*	*0,97 - 9,77*		
$\alpha_6\beta_3\delta$	*3,2*	*1,91 - 5,23*	*1,194*	*0,16 - 8,65*				

Table 5: Table of EC_{50} values of all GABA dose response curves with and without different phytocannabinoids, including the confidence interval.

Terpenes in extracts of cannabis sativa on $GABA_A$ receptors

Pilot experiments with whole plant extracts from *Cannabis sativa*, kindly provided by the company “cannhelp”, displayed different responses compared to different extract fractions containing mainly cannabinoids (CBD, THC, and low amounts of other cannabinoids). Redoil, a fraction containing ~90% CBD, ~10% THC and traces of other compounds was compared to pure CBD and a crude cannabis extract (~25% CBD). While CBD and Redoil displayed dose dependent negative modulatory effects, the crude extract showed positive modulatory action in $\alpha_1\beta_1$ receptors, shown in **Figure 25**.

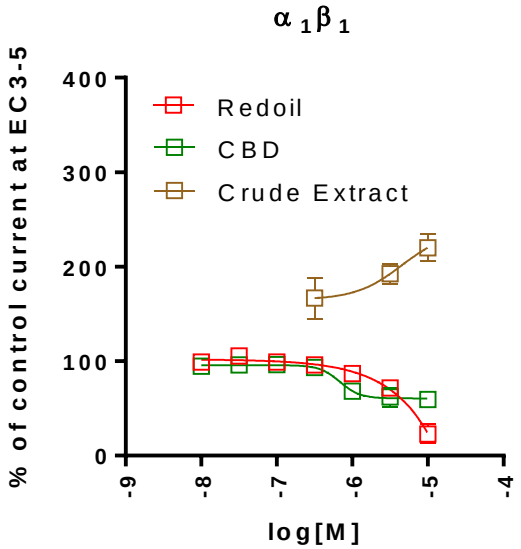

Figure 25: Extract fractions on $GABA_A$ receptors. Effects of different concentrations of fractions of cannabis extract coapplied with GABA on $\alpha_1\beta_1$ receptor at $EC_{3\text{-}5}$. The log[M] of the x-axis reflects the concentration of CBD in the fractions.

Terpenoids	PMID	Sample 1:	Sample 1:
Myrcene	16569037	1,47%	5,50%
Limonene	25532295	0,60%	1,36%
Linalool	12083865	N.D.	N.D.
trans-Ocimene	-	0,31%	0,17%
beta-Pinene	-	1,05%	1,02%
alpha-Pinene	21475467	2,90%	2,62%
beta-Caryophyllene	-	42,92%	19,02%
delta-3-Carene	-	0,06%	0,10%
trans-gamma-Bisabolene	-	N.A.	N.A.
trans-alpha-Farnesene	-	N.A.	N.A.
beta-Fenchol	-	N.A.	N.A.
beta-Phellandrene	-	N.A.	N.A.
alpha-Humulene	7649852	12,74%	0,76%
Guajol	-	0,09%	N.D.
alpha-Guaiene	-	N.A.	N.A.
alpha-Eudesmol	-	N.A.	N.A.
Terpinolene	-	0,05%	0,64%
alpha-Selinene	11824542	N.A.	N.A.
alpha-Terpineol	18593637	N.D.	0,23%
Fenchone	-	N.A.	N.A.
Camphene	-	0,06%	0,04%
cis-Sabinene hydrate	11308346	N.A.	N.A.
cis-Ocimene	-	0,08%	N.D.
beta-Eudesmol	-	N.A.	N.A.
beta-Selinene	11824542	N.A.	N.A.
alpha-trans-Bergamotene	-	N.A.	N.A.
gamma-Eudesmol	-	N.A.	N.A.
Borneol	15763546	N.A.	N.A.
cis-beta-Farnesene	-	N.A.	N.A.
gamma-Curcumene	-	N.A.	N.A.
cis-gamma-Bisabolene	-	N.A.	N.A.
alpha-Thujene	24486357	N.A.	N.A.
epi-alpha-Bisabolol	-	0,14%	N.D.
Ipsdienol	-	N.A.	N.A.
alpha-Ylangene	-	N.A.	N.A.
beta-Elemene	-	N.A.	N.A.
alpha-cis-Bergamotene	-	N.A.	N.A.
gamma-Muurolene	-	N.A.	N.A.
alpha-Cadinene	-	N.A.	N.A.
alpha-Longipinene	-	N.A.	N.A.
Caryophyllene oxide	-	3,24%	1,31%
gamma-Terpinene	-	0,08%	0,72%
Trans-nerolidol	-	0,19%	0,39%
p-Cymene	-	N.D.	0,20%
N.D. not dedectable			
N.A. not analysed			

Table 6: Terpenes of cannabis sativa. Table of cannabis derived terpenes and quantified amounts (percentages of total terpenes). PMID of publications indicate direct modulation of $GABA_A$ receptors.

Samples of distilled terpenes were analyzed by “fundacion canna” (a research facility of the company “canna”). A literature search in pubmed was performed and terpenes that directly act on $GABA_A$ receptors were marked with the PMID for further investigation, shown in **Table 6**. Seemingly crude hemp extract ingredients modulate the function of $GABA_A$ receptors. Terpenes were identified to have modulatory properties. Besides cannabinoids and terpenes there is the possibility of modulation by other compounds as flavonoids and fat derivates.

Discussion

α_6 containing receptors

Comprehensively studied, the α_6 subunit is primarily expressed in granule cells of the cerebellum, in the cochlear nucleus, in axons of the olfactory nerve, in the dorsal horn, in trigeminal neurons and hippocampal interneurons (Gutiérrez, Khan et al. 1996; Hayasaki, Sohma et al. 2006; Hortnagl, Tasan et al. 2013; Sieghart 2015; Tong, Peng et al. 2015; Yang, Xu et al. 2016). The α_6 subunit containing receptors were localized in both synaptic and extrasynaptic regions in cerebellar granule cells and contribute to both, phasic and tonic inhibitory responses in brain slices (Nusser, Sieghart et al. 1996; Santhakumar, Hanchar et al. 2006). Ectopic expression of α_6 subunits in hippocampal pyramidal neurons and SOM interneurons displayed increased extrasynaptic tonic inhibition. α_6 - seems to be contributing particularly to extrasynaptic $GABA_A$ receptors (Wisden, Cope et al. 2002) (Tong, Peng et al. 2015).

α_6 has been linked to several neuropsychiatric disorders, including depression, autism, motor tic disorders and neuropathic pain (more detailed below).

In post mortem lateral cerebellar tissue of patients with major depression, an upregulation of α_6 subunits protein but not mRNA was noticed (Fatemi, Folsom et al. 2013). Furthermore, in rat pups exposed to maternal separation, reduced expression of α_6 subunits in the hippocampal interneurons was observed, which was suggested to contribute to depressive behaviour such as anhedonia in this model (Yang, Xu et al. 2016).

In patients with autism, a downregulation of α_6 subunit protein and increase of α_6 subunit mRNA in the superior frontal cortex was observed (Fatemi, Reutiman et al. 2014).

In a motor tic disorder case study, a plant extract containing hispidulin, a weak PAM of $\alpha_6\beta_3\gamma_2$ receptors remitted symptoms. Furthermore, in gerbils, hispidulin has been show to cross the blood brain barrier and has anticonvulsant properties (Kavvadias, Sand et al. 2004). In mice, methamphetamine induced hyperlocomotion was alleviated by intracerebellar injections of hispidulin,

suggesting a role for α_6 subunit containing PAM in hyperdopaminergic disorders (Kavvadias, Sand et al. 2004; Liao, Lee et al. 2016). Moreover, reduced α_6 subunits expression in granule cells was observed in ataxic mouse models (Payne, Connelly et al. 2007).

The role of α_6 subunit containing receptors in neuropathic pain is not fully understood. α_6 subunit containing $GABA_A$ receptors were found to inhibit trigeminal ganglia nociceptive sensory afferents (Kramer and Bellinger 2013). Preliminary experiments with α_6 subunit selective drugs seemingly reduce neuropathic pain in animals (Dina Vasović 2017). Furthermore, a knockdown of α_6 subunit expression in trigeminal ganglia neurons enhanced the sensitivity to inflammatory nociception (Puri, Vinothini et al. 2012).The mechanism itself is unclear but could be based on reduced shunting inhibition and primary afferent depolarization (PAD) at central terminals of trigeminal neurons.

Neuropathic pain is a form of chronic pain and hard to alleviate by common drugs without severe side effects. For centuries, cannabis has been used for pain relief, but the molecular basis for the relieving of symptom is not yet explained in detail. In the last decades, more and more actions of the most abundant non-psychoactive and therapeutic used phytocannabinoid CBD had been concluded (Izzo, Borrelli et al. 2009; Ibeas Bih, Chen et al. 2015).

Especially the TRPV1 receptor and the GlyR have been reported as main targets in neuropathic pain models. Both targets were tested independently of each other but both seemingly alleviate neuropathic pain in a CB1 receptor independent manner in vivo. Moreover, the 5HT1A receptor has been implicated in the alleviation of chemotherapy induced neuropathic pain by cannabidiol (Chiara, Jounaidi et al. 2016). CBD reverses both mechanical and thermal hyperalgesia in different mice models of persistent, neuropathic and inflammatory pain (Costa, Trovato et al. 2007). Sativex (GW Pharmaceuticals), a phytocannabinoid based buccal spray approved for treatment of multiple sclerosis, has also been suggested to alleviate MS-induced neuropathic pain (Russo, Naro et al. 2016).

The link that $GABA_A$ receptors were modulated by CBD was not given yet. The findings reported here suggests to further study the effects of CBD in an α_6 knock

out mouse. At this point, not even the involvement of α_6 in neuropathic pain can be supported safely, but the modulatory effects of CBD on α_6 receptors provide a plausible mechanism, or at least can contribute to the effect.

Recombinant expression in oocytes revealed that α_6 containing binary and delta containing subtypes show low currents when stimulated with GABA. Nevertheless, measurements at different GABA concentrations displayed a sigmoid GABA dose response curve. Measurements at EC_{3-5} were hard to establish and often inconsistent. The currents are small and in some cases below the detection limit. Nevertheless, the results clearly show a strong positive modulatory effect of THC and of CBD. In contrast, the $\alpha_6\gamma_2$ containing receptors display higher currents and more stable GABA responses.

Future perspective

Due to the positive modulation of α6 containing receptors of CBD and THC (**Figure 16A**), experiments with those phytocannabinoids in models of pain, depression and motor tic disorders appear promising. Using different knockout mice for such models might help in clarify the contributions of different proposed targets. The effectiveness of CBD in epilepsy was shown in several studies. Epidiolex (GW Pharmaceuticals) successfully undergoes first phase 3 trials in USA for dravet syndrome. CBD was listed as pharmaceutical since October 2016 in Germany and can therefore be used in clinical trials of all kind.

Relevance of $\alpha_4\beta\delta$ receptors

In short, $\alpha_4\beta\delta$ containing receptors are found perisynaptically and show high GABA sensitivity. They are found in distinct regions listed below (Hortnagl, Tasan et al. 2013):

α4β3δ subunit co-localization in mouse brain

- dentate gyrus granule cells in hippocampus
- cerebral cortex
- Basal ganglia (N. accumbens, caudate putamen)
- Bulbus olfactorius

$\alpha_4\beta_2\delta$ subunit co-localization in mice brain

- as above and thalamus.

Pharmacologically, modulation and direct actions of endogenous and exogenous compounds were found. Histamine was identified to preferentially enhance α_4 subunit containing $GABA_A$ receptors (Bianchi, Clark et al. 2011). Furthermore, it was postulated that $\alpha_4\beta_3\delta$ $GABA_A$ receptors are major targets for general anesthetics (Chiara, Jounaidi et al. 2016). Moreover, endogenous neurosteroids and their synthetic analogues were identified as potent modulators for extrasynaptic $GABA_A$ receptors at low nanomolar concentrations. Furthermore, they can show agonistic properties at higher concentrations in the absence of GABA (Chisari, Eisenman et al. 2010).

Extrasynaptic $GABA_A$ receptors were found to mediate tonic inhibition in neuron. It was shown that neurosteroids enhance effects of endocannabinoid modulation in recombinant $GABA_A$ receptors (Sigel, Baur et al. 2011). Stress is a trigger for neurosteroid signaling and affects stress response of HPA (hypothalamic-pituitary-adrenocortical axis) in vivo, where $GABA_A$ receptors play a major role in regulation (Gunn, Cunningham et al. 2015). In fact, chronic stress impairs GABAergic control of the amygdala by suppression of tonic inhibition (Liu, Song et al. 2014).

Moreover, it has been implicated that fluctuation of neurosteroid levels are gender specific and vary during the female menstruation cycle (Smith, Shen et al. 2007). Expression of α_4 subunit was upregulated in female mice during progesterone withdrawal, a model of premenstrual anxiety in the amygdala but not in males. Further enhanced ASR (acoustic startle response) was evaluated in female mice (Gulinello, Orman et al. 2003). Premenstrual dysphoric disorder (PMDD) is a recently designated disorder (DSM-5) and is associated with the following symptoms: mood swings, tearfulness, sensitivity to rejection, marked depressed moods, anxiety and many more in women during their menstruation cycle. The anxiety-like symptoms are associated with diminished allopregnanolone levels in the amygdala, hippocampus and medial prefrontal cortex (Hantsoo and Epperson 2015). Progesterone withdrawal increases anxiety and seizure susceptibility via

declining levels of allopregnanolone and leads to upregulation of α_4 subunits in hippocampus. Furthermore, pubertal upregulation of $\alpha_4\beta\delta$ reduced seizure-like discharges in CA1 of hippocampus. This suggests anticonvulsant properties in adolescence (Yang, Shen et al. 2016).

Deletion of δ subunits in parvalbumin positive interneurons in the CA3 stratum pyramidale, which play a fundamental role in generation of γ oscillation leads to an increase in frequency. A reduction of oscillation frequency was achieved by the neurosteroid allopregnanolone but not by DS2 (Ferando and Mody 2015). Moreover, THDOC increases PKC mediated phosphorylation of $GABA_A$ receptor subunits and increased surface expression of $\alpha_4\beta$ containing extrasynaptic $GABA_A$ receptors (Modgil, Parakala et al. 2017). $\alpha_4\beta\delta$ $GABA_A$ receptor expression is increased by exposure to allopregnanolone in cultured hippocampal neurons. Surface expression was decreased by rottlerin (a protein kinase inhibitor) suggesting a role for PKC-d. In addition, flumazenil decreases $\alpha_4\beta\delta$ expression in HEK cells and in vivo (Kuver, Shen et al. 2012). Interestingly, cortical allopregnanolone levels were increased after acute administration of THC and cocaine but not by morphine (Grobin, VanDoren et al. 2005).

The effects of CBD on $\alpha_4\beta\delta$ $GABA_A$ receptors in oocytes suggest a possible physiological relevance by modulation of tonic inhibition in neurons. The hypothesis need to be tested in follow up experiments in brain slices and in behavior experiments. An α_4 knock out mouse could contribute to further investigations. Furthermore, a gender specificity is indicated according neurosteroid fluctuations.

The difference of THC actions in $\alpha_4\beta_1$ and $\alpha_4\beta_1\delta$ compared to $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$ (**Figure 18**) suggests a secondary effect dependent on the incorporation of δ subunits into the receptor or another allosteric binding site at an interface. The different GABA sensitivity of $\beta^+\delta^-$ GABA binding site and $\beta^+\alpha^-$ GABA binding site could contribute to the observed effects. Identification of binding sites of THC and CBD could contribute to the understanding of modulatory actions. Furthermore, the different Hill coefficients of the curves of $\alpha_4\beta_1$ and $\alpha_4\beta_1\delta$ indicate a possible loss or substitution of GABA binding sites. Interestingly, the current enhancement

of THC co-applied to GABA in $\alpha_4\beta_3$ receptors compared to GABA alone didn't change the Hill coefficient of the curve. This suggests a possible agonistic additive THC effect in this receptor subtype. Unfortunately, THC was not tested without GABA in this subtype neither in $\alpha_6\beta_3\delta$ to test this hypothesis.

β selectivity of THC

Distinct interfaces between α and β occur when β_1, β_2 or β_3 subunits are incorporated. In addition, a binding site could be placed on a subunit and not in the interface. As Sigel, Baur et al. 2011 indicated, endocannabinoids such as 2-AG selectively bind a pocket at β_2 TM domain. The results in **Figure 11** indicate a functional THC preference for β_2 and β_3 compared to β_1, whether the differences between β_2 and β_3 containing subtypes were only shown for α_1 containing receptors. Furthermore, compared to β_2 containing receptors, the β_3 containing subtype showed higher efficacy. If so in vivo, the β_3 containing subtypes in synaptic receptors could be a promising target for phytocannabinoids while the endocannabinoids are selective for β_2 containing subtypes. If peak concentrations in the millimolar range occur in synaptic transmission of GABA, THC could enhance the hyperpolarization or receptor desensitization. Other β_1 containing receptors were compared in **Figure 27** in the Appendix. Interestingly, in some receptors subtypes THC decreased the GABA elicited currents at low concentrations of GABA and enhanced them at higher concentrations.

Modulation of CBG

CBG is after CBD and THC, the most abundant phytocannabinoid and reaches concentrations up to 1 % in *Cannabis sativa*. Moreover, it is an ingredient of Sativex (GW Pharmaceuticals, GB) a plant derived medicine based on high phytocannabinoid (THC and CBD) content. Sativex is used to alleviate symptoms of spasticity and neuropathic pain in multiple sclerosis patients. Despite effects on different other receptors among humans the pharmacological profile is sparsely studied. The described effects on $GABA_A$ receptors in this thesis are new findings and could contribute to pharmacological effects of Sativex or other cannabis based medicine. Especially the great enhancement in $\alpha_6\beta\delta$ receptors

(**Figure 24**) could have an effect in the alleviation of neuropathic pain. In general, the effects of CBG were greater than all other tested cannabinoids. This suggest future experiments and further characterization of the effects of CBG on $GABA_A$ receptors.

$\alpha_1\beta_3\delta$ receptor combination in HEK293 and CHO cells

The primary goal of the experiments was a comparison of human and rat $\alpha_1\beta_3$ subtypes. It was not possible to achieve sigmoid GABA dose response curves with the used application method. Seemingly the gravity and the tubing lead to a mixture of GABA concentrations in the manifold. Only high concentrations of GABA (1mM) were used to compare substance effects. Additional results were produced, measuring the effects of CBD on human $\alpha_1\beta_3\delta$ receptor subtypes in HEK293 and CHO cells by whole cell recordings. Unfortunately, the results show broad distribution because of the application device. Time-dependent pre-application of CBD changed the response of GABA and receptor kinetics. Rapid desensitization but enhancement of maximum GABA current was observed after application of 30µM CBD for 10 and 20s. Different concentrations ratios of subtype DNA were used for transfection. Transfection time changed the response to GABA but was not methodological tested. Only a few traces were used in this thesis to show the effects of CBD which were not quantified in detail. Clearly, the setup and protocol would require detailed optimization.

Structural hypothesis

A docking into the novel CB1 receptor crystal structure displayed positions of THC. Interestingly, the hydrophobic tail of THC was found to be deeply inserted, between the two helices VI and VII (Hua, Vemuri et al. 2016). This is in line with our identified pose at the proposed THC binding site (**Figure 16**) on α_6 subunit, where the hydrophobic tail is deeply inserted as well.

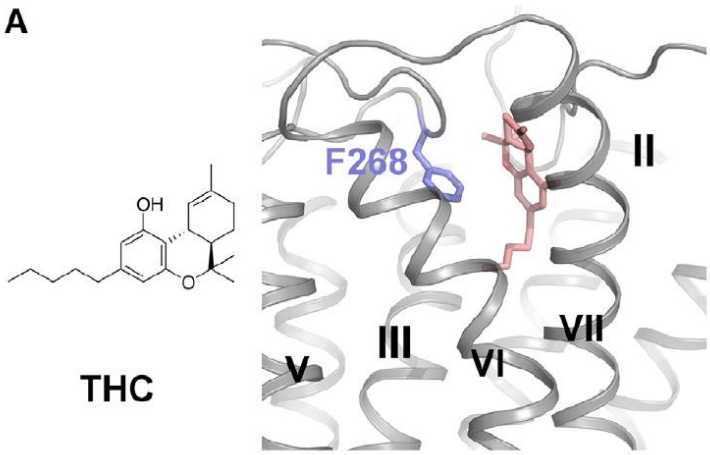

Figure 26: Docking of THC into the CB1 receptor. The docking pose suggesting that the hydrophobic tail of THC is deeply inserted into the receptor (Hua, Vemuri et al. 2016).

Conclusion

Phytocannabinoids such as THC and CBD directly act on recombinantly expressed $GABA_A$ receptors in *X. oocytes and* HEK293 cells. Effects of THC were higher in α_6 or α_4 containing perisynaptic receptors and binary receptors, in an additionally β subunit selective manner. CBD but not THC, is a potent allosteric modulator of $\alpha_4\beta_3\delta$ $GABA_A$ receptors. Furthermore, CBD shows PAM effects in delta containing receptors. Moreover, CBG affects GABA response in delta or γ_2 containing receptors. Measurements with oocytes can easily picture the properties of substances on receptors and their kinetics in a recombinant system. It's a straightforward method to characterize compounds on receptor subtypes. A general problem is the missing knowledge of subtype compositions. Further investigations in brain slices and animal models are necessary to verify the effects under physiological conditions. The use of CBD in future treatments for PMDD, chronic stress, epilepsy, chronic pain, drug withdrawal, anxiety and forms of depression should be studied in detail.

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List of Abbreviations

2-AG	2 arachidon glycerol
5HT1A	5-hydroxy tryptamine 1A receptor
AβPP	amyloid beta precursor protein
AEA	anandamide
ASM	aerial smooth muscle cells
ATP	adenosine triphosphate
BRET	bioluminescence resonance energy transfer
CaCl	calcium chloride
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBC	cannabichromene
CBD	cannabidiol
CBDA	cannabidiolic acid
CBG	cannabigerol
CBGA	cannabigerolic acid
CBNA	cannabinolic acid
CCK	cholecystokinin
CD	cluster of differentiation
CHO	chinese hamster ovary cells
CNS	central nervous system
CO2	carbon dioxide
COPD	chronic obstructive pulmonic disease
CsCl	cesium chloride
CsOH	cesium hydroxide
CYP	cytochrome
CYS	cysteine
D2	dopamine receptor 2

DC	dendritic cells
DHP	dihydropyran
DMEM	dulbeccos modified eagle media
DS2	delta selective compound 2
EAE	experimental autoimmune encephalomyelitis
EC	effective concentration
ECD	extracellular domain
EGTA	ethylenglycol-bis(aminoethylether)-N,N,N′,N′-tetraessigsäure
FAAH	fatty acid amide hydroxylase
FBS	fetal bovine serum
GABA	gamma aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
GlyR	glycine receptor
GPR	G-protein coupled receptor
GSH	glutathione
H	hydrogen
HCl	hydrochloric acid
HEK293	human embryonic kidney cells
IC	inhibitory concentration
ICD	intracellular domain
IPP	isopentenylpyrophosphat
KCl	potassium chloride
Ki	dissociation constant
LEU	leucine
LOX	lipoxygenase
MAGL	monoacyl glycerol lipase
Mg	magnesium
MgCl	magnesium chloride
n7-AChR	nicotinic 7 acetylcholin receptor

NaCl	sodium chloride
NAD	nicotine amid dinucleotide
NAM	negative allosteric modulator
NaOH	sodium hydroxide
NE	noladine ether
NE	norepinephrine
OCD	obsessive compulsive disorder
PAM	positive allosteric modulator
PHE	phenylalanine
PMID	pubmed ID
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
S	serine
SEM	standard error of mean
SER	serine
SOD	superoxide dismutase
STC	stanniocalcin
TEV	two electrode voltage clamp
THC	tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
THCV	tetrahydrocannabivarin
THDOC	tetrahydrodeoxycorticosterone
TM	transmembrane
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid 1
XR	xenopus ringer
Zn	zinc

Appendix

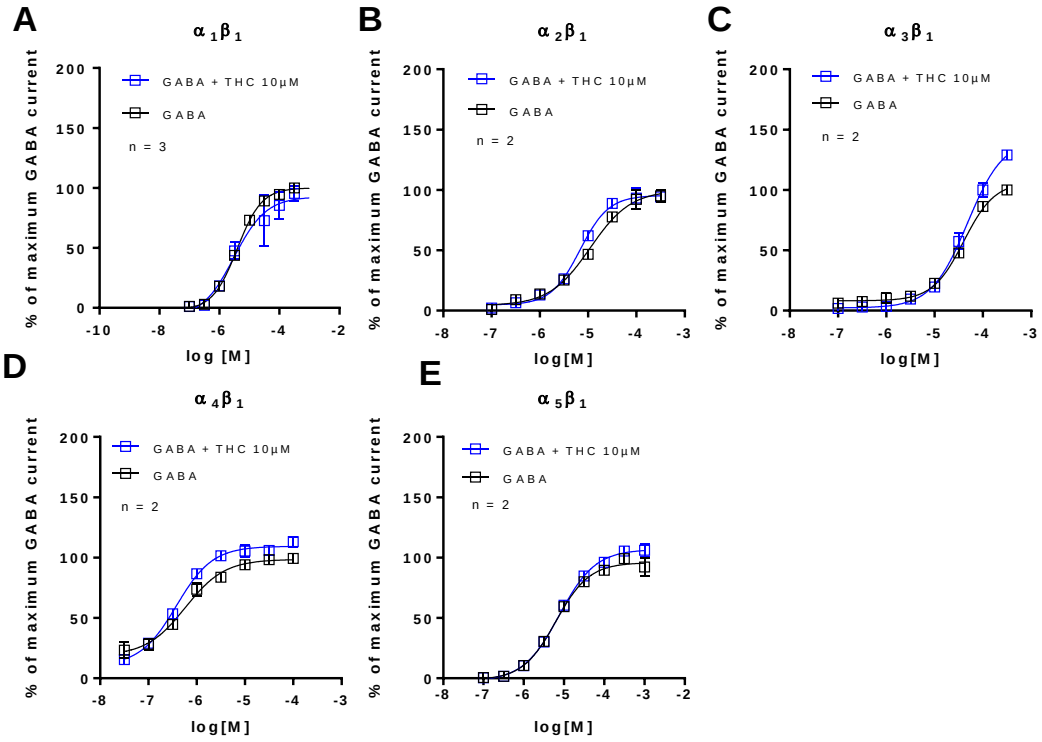

Figure 27: Comparison of β_1 containing subtype combinations.

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Abstract

Phytocannabinoids had been identified as promising cannabis derived pharmacologically active compounds. Their actions are not limited to molecular targets of the endocannabinoid system as CB1, CB2 and GPR55 receptors. Instead, TRP channels were found to be main targets of CBD, the non-psychoactive phytocannabinoid with an astonishing pharmacological profile. Increasing evidence of modulatory effects on Cys-loop receptors as GlyR, 5HT3A and n7-AChR had been shown. $GABA_A$ receptors, members of Cys-loop receptors display complex structural and pharmacological properties and are distributed in different compositions in many cell types but mainly in neurons. 19 different subunits form pentameric chloride ion channels and most of them were activated by GABA. Their main function is inhibition due to hyperpolarization by synaptic and extrasynaptic receptors. Many endogenous and exogenous compounds influence the function of $GABA_A$ receptors. We demonstrate direct positive allosteric modulation of CBD and THC in distinct subtypes of $GABA_A$ receptors. We identified a novel action of CBD and not THC as potent positive allosteric modulator (PAM) of $\alpha_4\beta_3\delta$ receptors. Moreover, delta containing receptors displayed strong response to phytocannabinoids compared to gamma containing receptors. Actions of CBD on delta containing receptors suggest pharmacological relevance, based on the enhancement of tonic inhibition in neurons.

Abstrakt

Phytocannabinoide, natürlich vorkommende pharmakologisch aktive Substanzen der Gattung Cannabis Sativa, werden vielversprechende medizinische Wirkungen nachgesagt. Deren Wirkung erfolgt jedoch nicht nur über Cannabinoid Rezeptoren (CB1, CB2 und GPR55) sondern auch über vielen anderen Rezeptoren, Enzymen und Transporter. Cannabidiol (CBD) ein schwach psychoaktiv wirkendes Phytocannabinoid wirkt unter anderem an Cys-Loop Rezeptoren wie dem GlyR, 5HT3A und dem n7-AChR. $GABA_A$ Rezeptoren, weitere Mitglieder der Cys-Loop Rezeptor Familie, sind die wichtigsten hemmenden Neurotransmitter Rezeptoren im Nervensystem. Sie formen Pentamere, die sich aus 19 unterschiedlichen Untereinheiten bilden können. Dementsprechend gibt es eine Vielzahl von $GABA_A$ Rezeptoren mit unterschiedlicher Struktur und Pharmakologie. Diese Arbeit untersucht die Wirkung von Cannabidiol (CBD) und Tetrahydrocannabinol (THC) an verschiedenen rekombinant exprimierten $GABA_A$ Rezeptoren in Xenopus Oocyten. Wir konnten direkte positive allosterische Modulation durch CBD und THC an verschieden $GABA_A$ Rezeptoren zeigen. Die größten Effekte wurden in Rezeptoren mit Delta Untereinheit gefunden. Eine potente positive allosterische Modulation von CBD aber nicht von THC konnte in $\alpha_4\beta_3\delta$ Rezeptoren gezeigt werden. Dieser Rezeptor ist physiologisch betrachtet als extrasynaptischer Rezeptor für die Entstehung von tonischen Strömen in Neuronen mitverantwortlich und daher an einer Vielzahl von Neuropsychiatrischen Erkrankungen beteiligt.