Modulation of inhibitory receptors by cannabidiol and cannabidivarin

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Thesis submitted for the degree of Doctor of Philosophy
I, Joana Assis Manuel certify that the research presented herein is my own and that any work conducted in collaboration is clearly indicated. I confirm that I have written all the text in this thesis and that I have indicated by way of references any ideas or findings derived from other sources.

Signature:                      Date:  07-03-2019
Acknowledgements

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Outside of work I am extremely thankful to Amodini Palipane for having turned my napkin vision for a figure into a reality and magically making an assortment of PowerPoint shapes resemble a microscope! Thank you Dr Ryan Walker-Gray for all of your love, near and far.
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Abstract

Type A γ-aminobutyric acid (GABA<sub>A</sub>) and glycine receptors are ligand-gated ion channels that mediate fast inhibitory neurotransmission in the nervous system. As such, they represent important targets in the management of epilepsy, a hyperexcitability disorder where pharmacological manipulation of inhibitory synaptic transmission can lead to viable therapeutic treatments. Research into new drugs for intractable epilepsy is ongoing, with cannabinoids emerging as a new class of anticonvulsants. Indeed, the non-psychotropic phytocannabinoids cannabidiol (CBD) and cannabidivarin (CBDV) have shown promising results in preclinical models of epilepsy. Moreover, clinical trials leading to the recent approval of a CBD-containing medicine by the Food and Drug Administration in the United States have further highlighted the therapeutic potential of CBD in paediatric epilepsy disorders characterized by drug-resistant seizures. However, the precise mechanisms by which these non-psychoactive phytocannabinoids exert their anticonvulsant effects are unknown though several plausible targets have previously been described.

A number of studies have shown a direct modulation of GABA<sub>A</sub> and glycine receptors by cannabinoids, though the action of CBD and CBDV on these receptors has not been systematically examined. Thus, the objective of this research was to assess the effect of CBD and CBDV on Cl<sup>-</sup> currents mediated by GABA or glycine (I<sub>GABA</sub> or I<sub>Gly</sub>). Whole-cell patch-clamp recordings from HEK293 cells transiently expressing different GABA<sub>A</sub> receptor subtypes, including the most common isoforms α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub>, revealed a transient potentiation of I<sub>GABA</sub> in the presence of CBD or CBDV. This effect was not dependent on the presence of the GABA<sub>A</sub> receptor γ<sub>2</sub> subunit. Moreover, site-directed mutagenesis indicated that I<sub>GABA</sub> potentiation by CBD and CBDV was independent of the benzodiazepine binding site. This was in line with data demonstrating an additive modulation of I<sub>GABA</sub> by phytocannabinoids applied in combination with the benzodiazepine diazepam. In addition, both CBD and CBDV potentiated δ-containing GABA<sub>A</sub> receptors, which are known to mediate tonic
inhibition. In contrast to the transient potentiation of $I_{\text{GABA}}$, the phytocannabinoids induced a progressive and sustained enhancement of $I_{\text{gly}}$ mediated by homomeric glycine receptors containing the $\alpha_1$, $\alpha_2$ or $\alpha_3$ subunits. Finally, the effect of CBD and CBDV on $I_{\text{GABA}}$ was examined in acute hippocampal slices from young adult mice. In contrast with the data obtained in HEK293 cells, superfusion of CBD had no effect on GABAergic currents elicited in CA1 pyramidal neurones, whilst CBDV had a slight inhibitory effect.

Overall, the findings presented in this thesis uncover a direct enhancement of the function of inhibitory receptors by CBD and CBDV, thus offering new opportunities for therapeutic intervention in the nervous system and shedding light on the mechanism of action of non-psychoactive phytocannabinoids.
Numerous reports have highlighted the medicinal properties of the cannabis plant, with some dating back several millennia (Russo 2005, Nagy et al. 2008). Yet contemporary society predominantly associates the use of cannabis with purely recreational applications. That said, a shift in attitudes is underway in a number of countries, both within the general public and within governments. This is clear from the recent approval of the first cannabis-derived medicine in the United States (US Food & Drug Administration 2018) to a change in regulations in the United Kingdom allowing specialist doctors to prescribe cannabis-based products for medicinal use (Home Office and The Rt Hon Sajid Javid MP 2018). This change in opinion follows a number of clinical trials for the use of cannabidiol in treatment-resistant epilepsy disorders. The therapeutic potential of this compound was evident as the number of seizures suffered by trial participants was reduced (Devinsky et al. 2017, Devinsky et al. 2018a, Thiele et al. 2018). It is estimated that 30% of epilepsy patients worldwide are unable to control chronic recurrent seizures with currently available antiepileptic drugs (Kwan and Brodie 2000). This makes the development of alternative treatments with improved efficacy and reduced side effects all the more pressing, and emphasises the significance of the recent cannabidiol clinical data. Cannabidiol is one of a number of molecules extracted from the cannabis plant, or phytocannabinoid, that lacks psychotropic activity (Mechoulam et al. 1970). In addition to cannabidiol, cannabidivarin has also shown important anticonvulsant properties in preclinical epilepsy models (Hill et al. 2012b). A number of targets for these phytocannabinoids have been identified and include T-type voltage-gated Ca\textsuperscript{2+} channels (Ross et al. 2008), G-protein coupled receptor GPR55 (Ryberg et al. 2007, Anavi-Goffer et al. 2012), and several transient receptor potential channels (De Petrocellis et al. 2011, Iannotti et al. 2014). Activity at these sites is likely to contribute to the mechanism of action of these compounds. Additionally, modulation of inhibitory receptors could underpin the anticonvulsant properties of CBD and CBDV. Both type A γ-aminobutyric acid (GABA\textsubscript{A}) and glycine receptors play a crucial role in maintaining the physiological net balance of excitation-inhibition.
Dampening hyperexcitability in the epileptic brain by augmenting inhibitory signalling is an established strategy for treating a number of epilepsy disorders (Porter et al. 2012). Moreover, a number of studies have highlighted the potentiating effect of CBD on both GABAergic and glycinergic signalling (Sigel et al. 2011, Xiong et al. 2012a, Bakas et al. 2017).

The study described herein further assessed the effect of CBD at the most widely expressed GABA\(_A\) receptors and at all homomeric glycine receptors. The effect of CBDV at these receptors was also examined for the first time. The data presented further our understanding of the mechanisms of action of these clinically relevant phytocannabinoids, helping to explain how they might bring about their anticonvulsant effects. Such mechanistic knowledge has a wide-ranging impact: it can better inform practice and policy on cannabis-derived medicinal products, it can support healthcare professionals prescribe suitable combination treatments, and it can contribute to future drug development efforts to design related products with improved efficacy.
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<tr>
<td>2-AG</td>
<td>2-arachidonoyl glycerol</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>AEA</td>
<td>Arachidonoyl ethanolamide, anandamide</td>
</tr>
<tr>
<td>AED</td>
<td>Anti-epileptic drugs</td>
</tr>
<tr>
<td>AJA</td>
<td>Ajulemic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BNF</td>
<td>British National Formulary</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>CBDV</td>
<td>Cannabidivarin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CLB</td>
<td>Clobazam</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DS</td>
<td>Dravet Syndrome</td>
</tr>
<tr>
<td>DS2</td>
<td>Delta selective compound 2</td>
</tr>
<tr>
<td>DSE</td>
<td>Depolarisation-induced suppression of excitation</td>
</tr>
<tr>
<td>DSI</td>
<td>Depolarisation-induced suppression of inhibition</td>
</tr>
<tr>
<td>DZP</td>
<td>Diazepam</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EC50</td>
<td>Half-maximal effective concentration</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABAA-R</td>
<td>γ-aminobutyric acid type A receptors</td>
</tr>
<tr>
<td>GABAB-R</td>
<td>γ-aminobutyric acid type B receptors</td>
</tr>
<tr>
<td>GlyR</td>
<td>Glycine receptor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HEK293 cells</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>$I_{\text{GABA}}$</td>
<td>GABA-mediated Cl- current</td>
</tr>
<tr>
<td>$I_{\text{Gly}}$</td>
<td>Glycine-mediated Cl- current</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>LB</td>
<td>Lennox L broth base</td>
</tr>
<tr>
<td>LGIC</td>
<td>Ligand-gated ion channel</td>
</tr>
<tr>
<td>LGS</td>
<td>Lennox Gastaut Syndrome</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA-Gly</td>
<td>N-arachidonoyl-glycine</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBPS</td>
<td>tert-butylibyclopshorphorothionate</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta 9$-tetrahycannabinol</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Introduction
1.1 Pharmacology of cannabinoids

Throughout history, both the recreational and medicinal benefits of the cannabis plant have attracted significant interest. Increasing number of studies on the endocannabinoid system and indeed on cannabinoid molecules – whether plant derived (phyto), endogenous or synthetic – have revealed this topic to be an exciting area of research with tremendous therapeutic promise. The phytocannabinoid cannabidiol (CBD) for instance exhibits a plethora of clinically relevant actions including anxiolytic, anticonvulsive, antiemetic and antipsychotic effects (Izzo et al. 2009). This compound is one of over 100 closely related cannabinoid molecules present in *cannabis sativa* and unlike the major constituent of cannabis, Δ⁹-tetrahydrocannabinol (THC), CBD lacks psychotropic activity making it essentially non-toxic (Mechoulam et al. 1970).

Endocannabinoids are long-chain fatty acid derivatives and as such, their mode of action was first thought to be non-specific, mediated by the disruption of cell lipid membranes. However, the characterisation of the first cannabinoid receptor, CB₁, in the 1980s (Devane et al. 1988) and molecular cloning shortly thereafter (Matsuda et al. 1990) identified a protein sequence with the features of a G-protein-coupled receptor (GPCR), indicating a distinct signal transduction mechanism was at play.

The discovery of the CB₁ GPCR prompted the realisation that endogenous cannabinoid ligands must exist and indeed, arachidonoyl ethanolamide (termed anandamide or AEA) was first isolated and its structure deduced by the Mechoulam group (Devane et al. 1992). Parallel to this, sequence homology studies identified a second receptor, CB₂, expressed in the periphery (Munro et al. 1993). This suggested the presence of a peripheral endogenous cannabinoid and rapidly, 2-arachidonoyl glycerol (2-AG) was isolated from intestinal tissue (Mechoulam et al. 1995), although it has since also been found in the central nervous system (CNS) (Stella et al. 1997). Together, the endocannabinoids, their receptors, and their biosynthetic and biodegrading enzymes make up the endocannabinoid system (ECS) which itself has been deemed important in the regulation of various physiological processes such as
learning and memory, mood, pain and inflammation, immune regulation, and energy metabolism (reviewed in Ligresti et al. 2016).

$\text{CB}_1$ and $\text{CB}_2$ receptors are differentially expressed. $\text{CB}_1$ is widely expressed presynaptically in the hippocampus as well as the neocortex, basal ganglia, cerebellum (Egertova and Elphick 2000) and testes (Gerard et al. 1991). $\text{CB}_1$ receptors expressed on cholecystokinin-positive GABAergic interneurones have been shown to modulate GABAergic neurotransmission; activation of these receptors results in reduced GABA$_A$ receptor-mediated inhibitory postsynaptic currents (Katona et al. 1999). On the other hand, $\text{CB}_2$ is primarily expressed in peripheral tissue such as the spleen and in immune cells (Munro et al. 1993, Klein et al. 1998) with evidence also supporting expression in the brain, albeit at much lower levels than $\text{CB}_1$ (Onaivi et al. 2006). A recent study reported that $\text{CB}_2$ receptors expressed in CA2 and CA3 hippocampal pyramidal neurones play a part in modulating cell-type specific excitability: the authors show that $\text{CB}_2$ receptor activation leads to a long-lasting hyperpolarisation of the neurone via a sodium-bicarbonate co-transporter mechanism (Stempel et al. 2016).

It is thought that endocannabinoids are not stored in cells but rather synthesised on demand. Studies have shown endocannabinoid biosynthesis to be calcium dependent, stimulated by an increased intracellular level of calcium during depolarisation of the postsynaptic neurone (Di Marzo et al. 1994, Stella et al. 1997). Following synthesis in the postsynaptic neurone, these endogenous cannabinoids can act in a retrograde fashion at presynaptic terminals and ultimately lead to inhibition of neurotransmitter release. This phenomenon, termed depolarisation-induced suppression of inhibition/excitation (DSI/DSE), rapidly modulates neurotransmitter release. Such an endocannabinoid function has been described both in the hippocampus (Ohno-Shosaku et al. 2001, Wilson and Nicoll 2001, Foldy et al. 2006) and the cerebellum where researchers found DSI/DSE to be due to an inhibition of presynaptic calcium influx (Kreitzer and Regehr 2001). This fits in well with studies demonstrating that activation of the $\text{CB}_1$ receptor in cultured hippocampal neurones inhibits N- and P/Q-type voltage-sensitive calcium channels (Twitchell et al. 1997). These calcium channels are responsible for coupling
depolarisation of the presynaptic terminal with neurotransmitter release via SNARE proteins. As such, inhibition of the calcium channels is likely to lead to reduced neurotransmitter release.

Thus, the ECS has garnered interest for its physiological function as a regulator of neuronal network excitability, and for the potential therapeutic target it represents in disorders where neuronal activity might be dysregulated. Similarly, increasing number of studies have highlighted the therapeutic properties of phytocannabinoid compounds, consigning them to a status beyond their recreational “drugs of abuse” classification and supporting the need for further controlled testing.

The first medicine derived from cannabis plant extracts was licensed in the United Kingdom in 2010. GW Pharmaceuticals developed Sativex®, a 1:1 mixture of THC:CBD, to treat spasticity associated with multiple sclerosis (GW Pharmaceuticals 2010). Decades prior, the synthetic cannabinoid Nabilone was licensed for use in treatment resistant nausea and vomiting caused by chemotherapy (Medicines & Healthcare products Regulatory Agency 2017). This compound, first developed by Eli Lilly, has a structure similar to THC. However, clinical application of cannabinoids and in particular THC has been largely constrained by known psychoactive effects, varied patient responsiveness and limited mechanistic understanding of the mode of action, leading to country-specific legal restrictions. The investigation of alternative cannabinoids lacking psychotropic activity resulted in GW Pharmaceuticals developing Epidiolex® and GWP42006 for use in various cases of paediatric epilepsy (GW Pharmaceuticals 2013). The active ingredient in Epidiolex® is CBD whereas the primary phytocannabinoid in GWP42006 is cannabidivarin (CBDV). Indeed, chronic administration of high-THC containing drugs to children with seizure disorders is not recommended because of the susceptibility of the developing brain to the psychoactive effects of THC (Rubino and Parolaro 2008, Meier et al. 2012, Camchong et al. 2017).

making them an attractive treatment for drug-resistant epilepsy disorders. More recently, GW Pharmaceuticals completed Epidiolex phase III clinical trials in patients with Dravet Syndrome (DS) or Lennox-Gastaut Syndrome (LGS), with positive clinical outcomes (Devinsky et al. 2017, 2018a, Thiele et al. 2018). This led to a recent Food and Drug Administration (FDA) approval in the United States, making Epidiolex the first FDA-approved drug containing a purified marijuana-derived substance. This medication is also the first FDA-approved for the treatment of DS (US Food & Drug Administration 2018).

The following section summarises key points regarding this neurological disorder and epilepsy in general, and the use of CBD and CBDV as a therapeutic treatment. This provides context to the purpose of my research which aims to elucidate the mechanisms of action of these phytocannabinoids and understand how they might have anti-seizure properties.

1.2 Non-psychoactive phytocannabinoids exhibit anti-epileptic properties

It is estimated that 68 million people worldwide live with epilepsy (Ngugi et al. 2010), making it one of the most common neurological disorders globally. Affected individuals suffer from seizures and tend to have a poorer quality of life and comorbidities such as learning difficulties or psychiatric disorders that include anxiety and depression (Tellez-Zenteno et al. 2007). Epilepsy is characterised by hyperexcitability in certain regions of the brain. This abnormal neuronal activity can result in different types of seizure, manifested as transient jerking, stiffening or loss of awareness for instance. Anti-epileptic drugs (AEDs) are usually aimed at managing and controlling such seizures. However, more than 30% of people diagnosed with epilepsy do not respond to available treatments (Kwan and Brodie 2000), representing a large unmet therapeutic need. Individuals affected with DS or LGS fall within this category. In the majority of cases, the aetiology of DS is de novo loss of function mutations in the voltage-gated sodium channel Na\textsubscript{v}1.1 (Marini et al. 2011). Reduced sodium currents in GABAergic interneurones signify a decrease in inhibitory
neurone excitability and consequently an increase in circuit excitability that leads to seizures (Cheah et al. 2012). The pathogenesis of LGS is more elusive, with approximately 75% of cases thought to arise from cerebral malformations or injuries, and the remaining having no apparent cause (Camfield 2011). Both of these rare epilepsy syndromes are childhood onset, often leading to cognitive impairment, and are characterised by multiple types of seizure that are refractory to AEDs. A new class of compounds, in the form of non-psychoactive cannabinoids, would thus provide an additional tool with which to manage pharmacoresistant seizures. Indeed, following the promising GW Pharmaceutical clinical trials, such compounds represent an exciting novel treatment option.

1.2.1 CBD: a promising novel epilepsy treatment

CBD was one of the first phytocannabinoids to be isolated from the cannabis plant (Adams et al. 1940) and to have its structure determined (Mechoulam and Shvo 1963). It was also shown to be devoid of psychotomimetic effects (Mechoulam et al. 1970, Hollister 1973, Perez-Reyes et al. 1973, Cunha et al. 1980). That said, a large number of reports quickly went on to highlight the neuroactive effects of this cannabinoid that include anticonvulsive actions. Studies in the early 1970s demonstrated a decreased susceptibility of CBD-treated rat hippocampus to seizure discharges caused by afferent stimulation (Izquierdo et al. 1973), a protective anticonvulsant effect of the cannabinoid in a maximal electroshock seizure mouse model (Karler et al. 1973), as well as a protective effect of CBD against pentylenetetrazol-induced convulsions in mice (Carlini et al. 1973). The authors of the latter study, encouraged by their findings and a preliminary clinical study highlighting a lack of hallucinogenic and toxic effects of CBD in humans, concluded that “a trial of CBD [is] worthwhile for the treatment of epilepsy” (Carlini et al. 1973). However, it took several decades, numerous investigations in a variety of preclinical animal models of seizure (reviewed in Rosenberg et al. 2017), as well as anecdotal reports and clinical case studies (Cunha et al. 1980) for the first large randomised, double blind, placebo-controlled clinical trials of CBD for the treatment of drug-resistant seizures to be undertaken. In DS patients, CBD treatment (two weeks of dose escalation and 12 weeks of dose maintenance at 20 mg/kg/day) was
found to reduce convulsive seizure frequency by 39% overall, a significantly better outcome than placebo treated patients. Specifically, 43% of the CBD-treated group experienced a 50% reduction in convulsive seizure frequency (Devinsky et al. 2017). Two phase III clinical trials for CBD use in LGS were also recently completed. In the first, CBD treatment (two weeks of dose escalation and 12 weeks of dose maintenance at 20 mg/kg/day) resulted in a 44% reduction in the frequency of drop seizures – a significant decrease compared to placebo treated patients. Similarly to the Dravet study, a 50% or more reduction in drop seizure frequency was observed in 44% of CBD-treated LGS patients (Thiele et al. 2018). In the second trial, patients treated with a higher (20 mg/kg/day) or lower (10 mg/kg/day) dose of CBD experienced a 42% or 37% reduction in drop seizure frequency, respectively. Again, a 50% or more reduction in drop seizure frequency was observed in 39% of CBD-treated LGS patients (Devinsky et al. 2018a). A dose-ranging safety trial in children with DS reported a peak CBD plasma concentration of 380 ng/ml at the end of a three-week treatment period with 20 mg/kg/day CBD oral solution, roughly translating into 1.2 µM (Devinsky et al. 2018b). Personal communication from GW Pharmaceuticals reported a steady state CBD plasma concentration of 330 ng/ml in trial patients on a 20 mg/kg/day regimen, which is approximately equivalent to 1 µM. Though both the DS and LGS clinical trial studies reported mild to moderate CBD-induced adverse events such as vomiting, diarrhoea, somnolence and decreased appetite in at least 10% of treated patients, the clinically meaningful reduction in seizure frequency observed in each clinical trial points towards CBD being an attractive adjunct therapy for these treatment-resistant patient populations. Moreover, a study found that CBD does not produce any signals of abuse liability, meaning the potential for addiction is minimal (Babalonis et al. 2017).

The clinical trials detailed above showed CBD is effective at preventing different types of seizures, whether convulsive, drop or other types reported in the studies, suggesting that this cannabinoid has a broad spectrum effect on seizure reduction. However, the mechanism and molecular targets through which CBD exerts these therapeutic effects are currently unclear.
Unlike THC, CBD does not have appreciable physiological affinity for the cannabinoid receptors CB₁ and CB₂ as revealed from radioligand binding studies (Showalter et al. 1996, Thomas et al. 1998, Jones et al. 2010). In line with this finding, the protective anticonvulsant activity of CBD in the maximal electroshock mouse model was not shown to be inhibited by a CB₁ receptor antagonist, suggesting CBD action is not mediated by this receptor (Wallace et al. 2001). Another study also proposed a CB₁ receptor-independent activity of CBD (Jones et al. 2010). It is possible that this cannabinoid potentiates the effect of AEDs, such as those taken by the participants of the clinical trials detailed above. Indeed, CBD has been shown to enhance anticonvulsant activity of phenytoin in rats (Consroe and Wolkin 1977) and increase plasma concentration of the active metabolite of clobazam in patients treated for refractory epilepsy (Geffrey et al. 2015). However, numerous in vitro and animal studies have observed anti-seizure properties of CBD without the presence of other AEDs, demonstrating that CBD alone is sufficient to exert a therapeutic action and suggesting that this phytocannabinoid has specific physiological targets.

CBD has been shown to act at a variety of sites that include ion channels, receptors, transporters and enzymes (reviewed in Ibeas Bih et al. 2015) and regulation of intracellular Ca²⁺ may contribute to the anticonvulsant mechanism of action of CBD. For instance, CBD was shown to inhibit T-type voltage-gated Ca²⁺ channels known to have a role in modulation of neuronal excitability (Ross et al. 2008). Another study found that CBD provides a bidirectional regulation of Ca²⁺ homeostasis in hippocampal cultures, and this effect was mediated by the mitochondrial Na⁺/Ca²⁺-exchanger: under normal physiological conditions CBD caused a subtle rise in cytosolic Ca²⁺, whilst in highly excitable neurones (in the presence of high extracellular K⁺), CBD reduced cytosolic Ca²⁺ levels (Ryan et al. 2009). Yet another study found that CBD inhibits the GPCR GPR55 (Ryberg et al. 2007). GPR55 mobilises presynaptic intracellular Ca²⁺ stores in the hippocampus, contributing to excitatory neurotransmitter release. CBD inhibition of the receptor would essentially restrict excitatory output from pyramidal cells (Sylantyev et al. 2013). Indeed, a recent study in a mouse model of DS demonstrated that the effects of CBD, notably, a reduction in action potentials generated in dentate granule cells during injection of a subthreshold stimulating current, could be mimicked by a GPR55 antagonist (Kaplan...
et al. 2017). Several transient receptor potential (TRP) channels have also been shown to be activated, and desensitised, by CBD (De Petrocellis et al. 2011, Iannotti et al. 2014). These ion channels are non-selectively permeable to cations and cannabinoid-induced desensitisation could lead to reduced neuronal activity. CBD was reported to act as an agonist at serotonin (5-hydroxytryptamine, 5-HT) GPCRs (Russo et al. 2005). However, although these receptors may play a role in the pathophysiology of epilepsy, a study found that the anticonvulsant effect of CBD is not mediated by activation of 5-HT₁A or 5-HT₂A receptors (Pelz et al. 2017). Activity at voltage-gated sodium channels has been described, with CBD inhibiting resurgent currents generated by Naᵥ1.6 channels expressed in HEK293 cells and reducing peak resurgent currents in striatal neurones (Patel et al. 2016). Additional clinically relevant molecular targets of CBD are inhibitory ligand-gated ion channels that include the glycine and γ-aminobutyric acid type A receptors (GlyRs and GABAₐRs). These are the focus of my research and a more detailed review is presented in sections 1.3-1.6.

1.2.2 The phytocannabinoid CBDV also exhibits anticonvulsant effects

Although not as extensively studied as THC or CBD, CBDV is slowly gaining traction as a clinically relevant non-psychoactive cannabinoid. It was isolated in 1969 as a CBD analogue (Vollner et al. 1969); these two compounds vary only in the length of their aliphatic side chain (Figure 1). Little has been reported about the pharmacological properties of CBDV, though it was shown to suppress epileptiform activity in vitro and act as an anticonvulsant in different animal seizure models (Hill et al. 2012a, 2013). This study also highlighted a lack of CBDV effect on rat motor coordination, suggesting the anticonvulsant properties of the cannabinoid are not due to suppressed motor function. Moreover, this means CBDV does not cause any adverse motor side effects. In addition to decreasing seizure severity in a rat pentylenetetrazol-induced seizure model, CBDV treatment also counteracted changes in epilepsy-related gene expression occurring in this model (Amada et al. 2013).
These promising animal studies warranted clinical trials for CBDV use in patients with inadequately controlled focal seizures. However, preliminary results from a phase II placebo-controlled study revealed a 40% reduction of focal seizures in both CBDV and placebo-treated patients (GW Pharmaceuticals 2018b). This placebo response was greater than expected and the company will be investigating possible explanations and will continue to explore potential uses of CBDV in epilepsy disorders.

**Figure 1. Chemical structure of the phytocannabinoids CBD and CBDV. (A) CBD and (B) CBDV are two of over 100 closely related cannabinoid molecules present in Cannabis sativa and are not known to have psychomimetic effects. They have very closely related structures which only differ in the length of the alkyl group, indicated by the arrows.**

Much like CBD, the mechanism of action underlying the anti-seizure properties of CBDV is yet to be determined. CBDV was found to have limited affinity for the CB₁ receptor and is thus likely to act via a CB₁ receptor-independent mechanism (Hill et al. 2013). Though the cannabinoid has been reported to act indirectly via CB₂ (Scutt and Williamson 2007), and has been shown to have a low affinity for this receptor (Rosenthaler et al. 2014), this has not been validated in the context of anticonvulsant activity. Similarly to CBD, CBDV has been shown to stimulate and desensitise TRP channels, thereby modulating neuronal hyperexcitability in vitro (De Petrocellis et al. 2011, Iannotti et al. 2014) and potentially contributing to anticonvulsant actions. Other CBDV targets also common to CBD have been reported including GPR55 (Anavi-Goffer et al. 2012). Most recently, clinical improvement of a patient with epilepsy using self-medicated cannabis was suggested to be due to CBDV acting via a GABAergic mechanism (Morano et al. 2016).
GABA<sub>A</sub> and GlyRs are major inhibitory receptors found in the brain, brainstem and spinal cord. They are thus crucial players in the balanced interplay of excitation and inhibition that is required for the normal function of the CNS. This makes them an important target in the management of hyperexcitability disorders such as epilepsy. Currently, the British National Formulary (BNF) lists 26 AEDs for the treatment of various epilepsy seizure symptoms (BNF accessed through www.medicinescomplete.com). Although not all have well-defined mechanisms of action, up to eleven are thought to affect GABAergic signalling in some form. This is the case for the benzodiazepines clobazam, clonazepam and diazepam or the barbiturate phenobarbital that have a direct action on GABA<sub>A</sub>Rs (Porter et al. 2012). On the other hand, the GABA reuptake inhibitor tiagabine and the GABA transaminase inhibitor vigabatrin act to increase levels of GABA in the brain (Angehagen et al. 2003). Amongst the multiple pharmacological actions of valproate, topiramate and gabapentin, an increase in GABA levels might contribute to their anti-seizure effects (Godin et al. 1969, Kuzniecky et al. 2002). Thus, modulation of inhibitory GABAergic transmission is already a tried and tested strategy in the treatment of various epilepsy disorders. Recent studies have found cannabinoids to be active at inhibitory receptors (Sigel et al. 2011, Xiong et al. 2012a, Golovko et al. 2015, Bakas et al. 2017), discussed in the following sections 1.4 and 1.6. This includes CBD. As such, modulation of GABA<sub>A</sub> and GlyRs by CBD and its structural analogue CBDV might underlie the mechanisms of action of these compounds. However, precisely which subtypes respond to CBD or CBDV is as yet unclear.

1.3 GABA<sub>A</sub> receptors

GABA is the most widely distributed inhibitory neurotransmitter in the CNS with as many as 20% of synapses in the brain relying on this amino acid (Sieghart and Ernst 2005). GABA binds and activates GABA receptors, of which two distinct types exist: type A and type B. Though both receptor types ultimately lead to inhibition of neuronal activity in mature neurones, the former are ligand-gated ion channels (LGICs) underlying fast synaptic conductances, whereas the latter are metabotropic
G-protein coupled receptors operating over a slower timescale (Gassmann and Bettler 2012).

GABA_A Rs belong to the nicotinic acetylcholine receptor family of LGICs, also known as the Cys-loop superfamily – so called for the disulphide bond present between two cysteine residues in the extracellular domain of all members. Other members include the nicotinic acetylcholine (nAChR), glycine and serotonin type 3 (5-HT_3) receptors. Structural features of the LGICs, including the GABA_A R s, were originally deduced from electron microscopy analysis of the electric ray nAChR (Miyazawa et al. 2003) and the crystal structure of the invertebrate ACh-binding protein (Brejc et al. 2001). Later, high resolution crystal structures of the extracellular domain of the α_1 nAChR subunit (Dellisanti et al. 2007) and of the bacterial homologues GLIC and ELIC (Hilf and Dutzler 2008, Bocquet et al. 2009) provided more complete molecular models. In recent years, the crystal structure of the first GABA_A R was determined, that of the human β_3 GABA_A R homopentamer (Miller and Aricescu 2014), though such a homopentamer has yet to be identified in nature. Structural determination of heteropentameric receptors by crystallography is difficult due to the stringent reliance of crystal formation on the ordered, homogenous arrangement of molecules. Moreover, structural examination of the most common α subunits has not been possible as these do not form functional homopentameric assemblies. To circumvent this, two recent x-ray crystallography studies made use of chimeric receptors, a GLIC-GABA_A R α_1 and a β_3-α_1 chimera, to investigate the structural arrangement of the α subunit transmembrane domains (TMD) known to contain numerous modulator binding sites (Laverty et al. 2017, Miller et al. 2017). The use of antigen-binding fragments, however, enabled Zhu et al. (2018) to solve the first high resolution cryo-electron microscopy structure of the tri-heteropentameric α_3β_2Y_2 GABA_A R, the predominant isoform found at synapses. This study further clarified the amino acids important for binding of the agonist, GABA, at the β/α interface of the extracellular domain of the receptor as well as the benzodiazepine binding site at the α/γ interface. In parallel, the cryo-electron microscopy structure of the tri-heteropentameric α_1β_1Y_2 GABA_A R was elucidated (Phulera et al. 2018). The authors of this study suggested that a particular glycosylation site on the α subunit is a source of steric hindrance that can shed light on ion channel assembly. However,
both of these cryo-electron microscopy studies used a receptor embedded in a
detergent micelle in order to solve receptor structures (Phulera et al. 2018, Zhu et
al. 2018). This has the caveat that the TMD structure may represent a non-native
conformation, which in itself may affect the extracellular portion of the receptor. On
the other hand, the latest GABA\textsubscript{A}R structure studies published used lipid-filled
nanodiscs that allowed receptors to remain within a lipid bilayer (Laverty et al. 2019,
Masiulis et al. 2019). In this way, Masiulis et al. (2019) were able to establish a cryo-
electron microscopy structure of the full-length human $\alpha_1\beta_3\gamma_2$ GABA\textsubscript{A}R bound to
GABA, the channel-blocker picrotoxin, the competitive antagonist bicuculline as well
as a structure of the receptor bound to benzodiazepines. Similarly, Laverty et al.
(2019) resolved a cryo-electron microscopy structure of the same GABA\textsubscript{A}R in a
desensitised conformation. Overall, these studies have provided an important
blueprint for the future pharmacological targeting of GABA\textsubscript{A}Rs and a fuller
understanding of the structure of Cys-loop receptors in general.

Members of the Cys-loop superfamily all have a common pentameric structure with
the five subunits arranged symmetrically so as to form a central ion-selective pore.
Each subunit is folded into four TMD, TM1-TM4, such that TM2 lines the pore wall. A
large N-terminal extracellular domain (ECD) contains the agonist binding sites and a
large intracellular domain available for phosphorylation or intracellular protein
interactions exists between TM3 and TM4 (Lynch 2004). Structure-function studies
also recently described a desensitisation gate in GABA\textsubscript{A} and GlyRs (Gielen et al.
2015), further supported by a chimeric GABA\textsubscript{A}R crystal structure (Laverty et al.
2017), whereby structural rearrangements at the cytoplasmic end of the TM2/TM3
interface result in constriction at the base of the ion channel.
Figure 2. Pentameric structure of the Cys-loop superfamily of ligand-gated ion channels. (A) Five subunits come together to form a functional receptor with an ion-selective pore that spans the lipid bilayer. (B) Each subunit or polypeptide is folded into four transmembrane domains, a large intracellular domain between TM3 and TM4, and a large N-terminal extracellular domain that contains the orthosteric agonist binding site. One subunit has been removed for clarity. (C) The four transmembrane domains of the five subunits are depicted. These are arranged symmetrically with the TM2 of each subunit lining the channel pore (lighter colour). The N-terminal domain of each polypeptide has been removed for clarity.

GABA_ARs are selective for Cl⁻ ions, with some permeability for bicarbonate anions. GABA binding to the N-terminal extracellular domain results in pore opening and increased Cl⁻ conductance. In mature neurones, after the first postnatal week in rats, this leads to neurone hyperpolarisation as the membrane potential shifts towards the more negative Cl⁻ equilibrium potential, thereby shunting excitatory currents. The opposite occurs in immature (P0-P8) neurones where higher intracellular Cl⁻ concentrations engender an outward flow of Cl⁻ ions upon GABA_AR opening, resulting in neuronal depolarisation (Ben-Ari et al. 1989, Cherubini et al. 1990). The same is true for GlyR-mediated Cl⁻ currents (Ehrlich et al. 1999). The different Cl⁻ equilibrium potentials in the embryonic versus mature neurones (and thus the different GABA_AR and GlyR-mediated excitatory versus inhibitory action) comes down to the expression of a K⁺-Cl⁻ cotransporter, KCC2, responsible for lowering the internal Cl⁻ concentrations (Rivera et al. 1999).
GABA activation of the GABA$_A$R and subsequent Cl$^-$ channel opening allows synaptic GABA$_A$Rs to mediate fast (phasic) inhibitory neurotransmission in neurones. On the other hand, extrasynaptic receptors exposed to low or ‘spill-over’ levels of GABA mediate tonic inhibition, a persistent basal inhibitory conductance (Farrant and Nusser 2005). The widespread distribution of GABA$_A$Rs in the brain and their central role in limiting neuronal excitability means excessive GABAergic signalling can result in sedation and ataxia whereas too little GABAergic signalling can lead to anxiety and insomnia. The distribution, identity, and sub-cellular localisation of GABA$_A$Rs is thus paramount to a healthy excitatory-inhibitory balance within the CNS.

There is a large heterogeneity of GABA$_A$R subunit isoforms and unsurprisingly, both subunit composition and arrangement within the pentamer define the functional properties of the receptor (Minier and Sigel 2004). The five subunits of the channel can be assembled from any combination of the 19 subunits (and their splice variants) thus far identified: six α, three β, three γ, three ρ, and one each of ε, δ, θ, and π (Olsen and Sieghart 2008). That said, subunits do form preferred assemblies. A review by Olsen and Sieghart (2008) of recombinant receptor expression studies, pharmacological and electrophysiological studies, and co-localisation and co-immunoprecipitation studies of GABA$_A$R subunits has led to a list of receptor subtypes that are highly likely to be native to neurones. The report suggests only 11 subtypes have been conclusively identified with strong evidence for just another six subtypes, listed in Table 1.

The γ$_2$ subunit seems to play a central role in the clustering of GABA$_A$Rs at the synapse (Schweizer et al. 2003) and is present in the vast majority of receptor subtypes (Olsen and Sieghart 2008). Of the α subunits, α$_1$ is the most widely expressed (Fritschy and Mohler 1995) with a study showing that knockout of this subunit causes a 50% decrease in the total GABA$_A$R content in mouse brain (Sur et al. 2001). The same can be said about β$_2$ out of the β subunits (Sur et al. 2001) although it is difficult to determine the identity of the β component in a particular receptor subtype as often all three β subunits are co-precipitated with the other portions of the receptor (Olsen and Sieghart 2008). Thus, evidence points towards the major adult isoform as being composed of α$_1$, β$_2$, and γ$_2$ subunits in a 2:2:1
stoichiometry (Sieghart and Sperk 2002, Farrant and Nusser 2005, Olsen and Sieghart 2008). This receptor subtype mediates fast synaptic inhibition whereas receptors containing the δ subunit in association with the α₄ or α₆ and a β subunit seem to predominantly mediate extrasynaptic tonic inhibition (Farrant and Nusser 2005, Olsen and Sieghart 2008).

Although more receptor subtypes than the 17 listed in Table 1 have been investigated, these have been omitted according to the criteria used by Olsen and Sieghart (2008). Thus, a different set of criteria might result in certain receptor subtypes being listed as more prominent than others. Indeed, in a report by Whiting (2003), the α₂β₃γ₂ and α₃β₃γ₂ receptor subtypes are depicted as making up more than 25% of the GABAₐR population. In contrast, Olsen and Sieghart (2008) point out that not enough evidence has yet come to light over the preferred β subunit associated with α₂γ₂ and α₃γ₂.

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Table 1. List of naturally occurring GABAₐR subtypes in the central nervous system. This list is according to the Olsen and Sieghart review of human, mouse and rat data (Olsen and Sieghart 2008).

Naturally, such receptor subunit diversity gives the GABAₐRs a rich pharmacology. Different α subunits, for instance, exhibit varying affinities for the agonist GABA; GABAₐRs containing the α₃ subunit are reported to have the lowest sensitivity, followed by α₅, α₄, α₂, α₁, and α₆ in order of increasing sensitivity. This difference was attributed to a specific four amino acid motif present near the agonist binding pocket in the extracellular N-terminal domain (Bohme et al. 2004).

In addition to two orthosteric agonist binding sites, located at the extracellular interface of the α and β subunits, alcohol and a number of clinically important drugs
are able to bind and modulate GABAₐR function (Figure 3). The benzodiazepines, for instance, are widely used for anxiety, sleep, muscle relaxation and some forms of epilepsy disorders, and though chronic use of these compounds can lead to tolerance and dependence, they remain one of the most important class of drugs targeting the GABAₐR. Classical benzodiazepines act as positive allosteric modulators and they have been shown to bind a site located in the ECD, at the α and γ interface (Sigel 2002, Masiulis et al. 2019). In addition to the traditional benzodiazepine binding site, numerous other low-affinity binding sites have been identified (Sigel and Ernst 2018). For instance, a site exists at the α/β interface in the ECD and is thus present in GABAₐR subtypes with or without a γ subunit. Binding at this site is thought to interfere with the action of positive allosteric modulators at the classical site (Baur et al. 2008, Maldifassi et al. 2016). Yet another site appears to be located in the TMD and is also independent of the γ subunit (Walters et al. 2000, Middendorp et al. 2015, Masiulis et al. 2019). Numerous studies have attempted to separate the divergent actions of the benzodiazepines, whether anxiolytic, hypnotic, sedative or anticonvulsant. Research has identified the α₁ subunit as responsible for mediating the sedative effects and partly the anticonvulsant action (Rudolph et al. 1999, McKernan et al. 2000), whilst the α₂ subunit likely mediates anxiolytic activity (Low et al. 2000) with further studies showing a possible involvement in mediating anticonvulsant effects (Fradley et al. 2007).

The TMD of the GABAₐR harbors binding pockets for a range of structurally diverse compounds in addition to the non-classical benzodiazepine binding site. Such is the case for the intravenous anaesthetics, with etomidate predicted to bind at the β/α interfaces in the TMD (Li et al. 2006) and the barbiturates shown to interact with distinct homologous TMD pockets at the α/β and γ/β interfaces (Chiara et al. 2013). The recent structure elucidation of the GABAₐR β₃-α₁ chimera and the GLIC-GABAₐR α₁ chimera, both bound to a potentiating neurosteroid, identified neurosteroid binding pockets at β/α and α/α inter-subunit TMD sites (Laverty et al. 2017, Miller et al. 2017). Moreover, a molecular modelling and docking study based on these structures predicted five neurosteroid cavities are likely to exist in the α₃β₂γ₂ receptor, located at each subunit interface (Alvarez and Pecci 2018). On the other hand, inhibitory neurosteroids were proposed to bind a discrete intra-subunit site
within the TMD (Laverty et al. 2017). Anaesthetics and potentiating neurosteroids enhance GABA-evoked currents ($I_{\text{GABA}}$) and at high doses, both of these classes of compounds can cause the opening of the GABA$_A$R channel regardless of GABA binding (Belelli and Lambert 2005, Hosie et al. 2007, Garcia et al. 2010). Thus, barbiturates once commonly used as AEDs, were largely replaced in favour of the safer benzodiazepine-based anticonvulsants, which do not open the receptor channel directly but allosterically potentiate $I_{\text{GABA}}$.

Figure 3. Illustration of the orthosteric and major allosteric binding sites of an $\alpha\beta\gamma$ GABA$_A$R. The GABA$_A$R with a canonical 2$\alpha$:2$\beta$:1$\gamma$ stoichiometry contains two orthosteric agonist binding sites, located at the $\beta/\alpha$ interface in the extracellular domain. The classical benzodiazepine binding site is found in a homologous position, at the $\alpha/\gamma$ interface. A number of potentiating neurosteroid binding sites exist, located at several subunit interfaces within the transmembrane domains. In addition, inhibitory neurosteroids were proposed to bind a discrete intra-subunit site also in the transmembrane domain (not indicated on the diagram). Intravenous anaesthetics such as etomidate bind at the two $\beta/\alpha$ interfaces in the transmembrane domain whilst barbiturates bind at $\gamma/\beta$ and $\alpha/\beta$ inter-subunit sites. Picrotoxin binds within the channel pore, inducing and maintaining a closed pore conformation. Positive and negative allosteric modulators are emboldened and underlined, respectively.
The numerous binding sites present on GABA<sub>A</sub>Rs result in a complex and extensive pharmacology, and also render these receptors susceptible to modulation by natural products from plants and mushroom, such as the pore-blocking inhibitor picrotoxin, the competitive antagonist bicuculline, and the agonist muscimol. A recent cryo-electron microscopy study suggested picrotoxin inhibition is the result of binding TM2 residues lining the channel wall of GABA<sub>A</sub>Rs and inducing a closed pore conformation (Masiulis et al. 2019). In the same study, bicuculline was shown to bind the orthosteric sites, ultimately stabilising the TMDs in the closed state (Masiulis et al. 2019). Aside from these structurally established binding sites, a modelling study identified numerous solvent accessible cavities that conceivably constitute binding pockets for GABA<sub>A</sub>R modulators (Ernst et al. 2005), and as highlighted in a recent review, GABA<sub>A</sub>R structure models point towards the existence of a higher number of drug-binding sites than previously expected (Sieghart 2015). This is certainly a sensible inference to make from the numerous high- and low-affinity binding sites already detailed in the literature for the different classes of compounds acting at GABA<sub>A</sub>Rs. Hence, it is foreseeable for plant-derived cannabinoids to occupy one of the many binding pockets on the receptor.

1.4 Cannabinoid modulation of GABA<sub>A</sub> receptors

Endogenously released cannabinoids at postsynaptic neurones are known to modulate GABAergic neurotransmission via retrograde signalling that is dependent on the CB<sub>1</sub> receptor and that results in a decreased presynaptic release of GABA (Hajos et al. 2000, Ohno-Shosaku et al. 2001, Wilson and Nicoll 2001, Foldy et al. 2006). Surprisingly however, fewer studies have looked into the CB<sub>1</sub> receptor-independent direct effect of cannabinoids on GABA<sub>A</sub>Rs.

Sigel et al. (2011) were first to describe a direct molecular interaction between a cannabinoid and GABA<sub>A</sub>Rs, showing a potentiation of I<sub>GABA</sub> by 2-AG in Xenopus oocytes made to express recombinant GABA<sub>A</sub>Rs containing α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>. This potentiation was both 2-AG and GABA concentration-dependent, with higher concentrations of agonist resulting in lower levels of cannabinoid potentiation – a finding also
observed with the GlyRs, discussed later. Other cannabinoids tested in the same study, including THC and AEA, only weakly potentiated the response to GABA. Further analysis of which subunit mediates 2-AG potentiation of $I_{GABA}$ revealed this effect was not $\alpha$ subunit selective; receptors containing either $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_5$, or $\alpha_6$ responded similarly to 2-AG. In contrast, omission of the $\gamma$ subunit resulted in a reduction of current potentiation by 2-AG. This effect was also highly selective for $\beta_2$ containing receptors; substitution of $\beta_2$ by $\beta_1$ eliminated $I_{GABA}$ potentiation whereas $\beta_3$ resulted in a reduction of the $I_{GABA}$ potentiation by 2-AG. Interestingly, Hejazi et al. (2006) had already described a lack of $I_{GABA}$ potentiation by THC or AEA in oocytes expressing $\alpha_2\beta_3\gamma_2$. A 50% reduction was observed in receptors containing one $\beta_1$ and one $\beta_2$ leading the authors to suggest that two 2-AG sites may be located on the $\alpha_1\beta_2\gamma_2$ receptor. Treatment of $\alpha_1\beta_2\gamma_2$ expressing oocytes with a combination of neurosteroid and 2-AG or benzodiazepine and 2-AG resulted in an additive potentiation of $I_{GABA}$. Thus, the cannabinoid site of action is likely to be distinct from the neurosteroid and benzodiazepine binding site (Sigel et al. 2011).

A protein sequence alignment of the three $\beta$ subunits highlights four amino acids within the TM domains that are identical in $\beta_1$ and $\beta_3$ but different in $\beta_2$. Site-directed mutagenesis studies identified amino acid residues V436 and F439 in the inner membrane part of the $\beta_2$ TM4 domain as central for mediating the functional effect of 2-AG (Sigel et al. 2011). A follow on study looking to further characterise the 2-AG binding site identified five additional residues affecting $I_{GABA}$ potentiation by 2-AG, all located in the same region (Baur et al. 2013a). Though the authors pointed out that the amino acid mutations in the study can either interfere with the 2-AG binding site or affect subsequent conformational changes of the receptor, they rule out the latter option as neurosteroid modulation of mutant receptors was unaffected. Thus, they proposed a model whereby 2-AG docks at the cytosol interface of TM3 and TM4 of the $\beta_2$ subunit, slightly immersed in the membrane with the fatty acid moiety forming hydrophobic contacts with the identified hydrophobic amino acid residues. This model agrees with the finding that onset of 2-AG modulation is slow. Such a delay could be explained by the cannabinoid having to diffuse through the membrane before interacting with the receptor (Baur et al. 2013a).
Additional work from the same group demonstrated that N-arachidonoyl-glycine (NA-Gly) potentiated $I_{\text{GABA}}$ measured from oocytes expressing $\alpha_3\beta_2\gamma_2$. This compound is an endogenous lipid molecule that is structurally similar to the endocannabinoids, but does not have functional affinity for the cannabinoid receptors. Compared with 2-AG, NA-Gly was a stronger potentiator of $I_{\text{GABA}}$ mediated by $\alpha_1\beta_2\gamma_2$ and this effect was also dependent on the presence of the $\beta_2$ subunit (Baur et al. 2013a, 2013b). Site-directed mutagenesis work led the authors to suggest overlapping binding sites for NA-Gly and 2-AG, located at the cytosolic end of the TM4 (Baur et al. 2013b).

The selective CB$_1$ receptor inverse agonists SR141716 and AM251 were also found to act directly at GABA$_A$Rs and strongly potentiate $I_{\text{GABA}}$ mediated by $\alpha_1\beta_2\gamma_2$ (Baur et al. 2012). AM251 similarly potentiated currents from $\alpha_2\beta_2\gamma_2$ receptors, though this effect was slightly reduced in GABA$_A$Rs containing $\alpha_3$, $\alpha_5$, or $\alpha_6$, in binary $\alpha_1\beta_2$ receptors, and in extrasynaptic $\alpha_4\beta_2\delta$ receptors. On the other hand, the presence of a $\beta_3$ subunit enhanced $I_{\text{GABA}}$ potentiation, whilst incorporation of a $\beta_1$ subunit abolished this potentiation (Baur et al. 2012). The latter finding is similar to that previously observed with 2-AG (Sigel et al. 2011). Baur et al. (2012) also found that a benzodiazepine antagonist did not counteract $I_{\text{GABA}}$ potentiation by AM251, indicating different binding sites. Moreover, co-application of pentobarbital or a neurosteroid with AM251 resulted in an additive effect, also highlighting different binding sites for these compounds (Baur et al. 2012).

More recently, an opposite effect was reported for cannabinoid modulation of GABA$_A$Rs expressed in HEK293 cells. Golovko et al. (2015) described an inhibition of $I_{\text{GABA}}$ by 2-AG and AEA in cells expressing GABA$_A$Rs $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$. This inhibition was observed with high concentrations (1 mM) of GABA, mimicking the concentration found at synapses (Golovko et al. 2015). The cannabinoids also increased desensitisation of the GABA$_A$Rs with the authors reporting a 50% reduction in charge transfer in the presence of 2-AG.

Data from brain slices, whereby inhibitory postsynaptic currents (IPSCs) were recorded from pyramidal neurones upon stimulation of synaptically-connected
interneurones, showed a reduced IPSC amplitude following application of the synthetic cannabinoid WIN55,212-2. This suppression was independent of the CB₁ receptor and shown to be mediated postsynaptically. Similar results were obtained when endocannabinoid signalling was stimulated in the recorded cells. Thus, endogenously released and exogenously applied cannabinoids directly suppress GABAergic IPSCs at fast spiking interneurone-pyramidal connections in a CB₁ receptor-independent manner (Golovko et al. 2015). However, the authors point out that this direct modulation of postsynaptic GABAₐRs by the endocannabinoids is synapse specific, and does not occur at cholecystokinin (CCK)-positive interneurone synapses, where CB₁ receptor expression level is high.

The study also investigated 2-AG modulation of extrasynaptic GABAₐRs and demonstrated that endogenously released 2-AG increases a tonic GABAₐR-mediated conductance. This is in contrast to the effect observed at synaptic GABAₐRs. Moreover, ambient levels of endocannabinoid were shown to enhance pyramidal neurone extrasynaptic GABAₐR activity in a CB₁ receptor-independent manner. Such a finding is in agreement with Sigel et al. (2011) who demonstrated that oocytes expressing GABAₐRs containing the δ subunit – known to be present in extrasynaptic receptors in nature – respond to 2-AG potentiation of I_GABA. Thus, Golovko et al. (2015) suggest that cannabinoids exert a bidirectional modulation of GABAₐRs, inhibiting the receptors in the presence of high synaptic GABA concentrations and potentiating in conditions of low ambient GABA. The mechanism for such a dual effect of cannabinoids on neuronal GABAₐRs remains unknown.
<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>GABA&lt;sub&gt;A&lt;/sub&gt;R subtype</th>
<th>Preparation</th>
<th>Effect</th>
<th>EC/IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Reference</th>
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<td><strong>Endogenous</strong></td>
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<td>2-AG</td>
<td>α&lt;sub&gt;1,3/5-6&lt;/sub&gt;β&lt;sub&gt;2γ2&lt;/sub&gt; α&lt;sub&gt;1βγ2, α1β2&lt;/sub&gt; α&lt;sub&gt;1γδ&lt;/sub&gt;</td>
<td>Human GABA&lt;sub&gt;A&lt;/sub&gt;R expressed in <em>Xenopus</em> oocytes</td>
<td>Potentiation (Weak) potentiation No effect Potentiation</td>
<td>2.1 ± 0.5</td>
<td>3&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td>2.9 ± 1.8</td>
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<td></td>
<td>α&lt;sub&gt;1β2γ2, α1β2γ2&lt;/sub&gt; α&lt;sub&gt;1β2γ2, α4β2γ2&lt;/sub&gt; α&lt;sub&gt;1β2γ2, α4β2γ2&lt;/sub&gt; α&lt;sub&gt;1β2γ2, α2β2γ2&lt;/sub&gt; α&lt;sub&gt;1β2γ2, α1β2γ2&lt;/sub&gt;</td>
<td>Human GABA&lt;sub&gt;A&lt;/sub&gt;R expressed in <em>Xenopus</em> oocytes</td>
<td>Potentiation Potentiation Potentiation Potentiation Potentiation</td>
<td>2.4, 15.7</td>
<td>14.7, 3.9</td>
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<td>1.5, 6.4</td>
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<td></td>
<td>α&lt;sub&gt;1/2β2γ2&lt;/sub&gt; Native</td>
<td>GABAR&lt;sub&gt;A&lt;/sub&gt;R expressed in HEK293 cells Nucleated patch from mice pyramidal neurones</td>
<td>Inhibition (in presence of high [GABA]) Potentiation (in presence of low [GABA])</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>AEA</td>
<td>α&lt;sub&gt;1β2γ2&lt;/sub&gt;</td>
<td>Human GABA&lt;sub&gt;A&lt;/sub&gt;R expressed in <em>Xenopus</em> oocytes</td>
<td>(Weak) potentiation</td>
<td>3&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td>α&lt;sub&gt;1/2β2γ2&lt;/sub&gt; Native</td>
<td>GABAR&lt;sub&gt;A&lt;/sub&gt;R expressed in HEK293 cells Isolated rat hippocampal pyramidal neurones</td>
<td>Inhibition (in presence of high [GABA]) Inhibition (in presence of high [GABA])</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.5</td>
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<td>NA-Gly</td>
<td>α&lt;sub&gt;1β2γ2&lt;/sub&gt;</td>
<td>Human GABA&lt;sub&gt;A&lt;/sub&gt;R expressed in <em>Xenopus</em> oocytes</td>
<td>Potentiation</td>
<td>3&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td>α&lt;sub&gt;1β1γ2&lt;/sub&gt;</td>
<td>Human GABA&lt;sub&gt;A&lt;/sub&gt;R expressed in <em>Xenopus</em> oocytes</td>
<td>(Weak) potentiation</td>
<td>3&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Plant</td>
<td>Compounds</td>
<td>Expression</td>
<td>Potentiation</td>
<td>Values</td>
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<td>CBD</td>
<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td>Human GABA$_A$R expressed in <em>Xenopus</em> oocytes</td>
<td>Potentiation</td>
<td>6.5, 16.1</td>
<td>Bakas et al. (2017)</td>
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<tr>
<td></td>
<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td></td>
<td>Potentiation</td>
<td>10, 0.9</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td></td>
<td>Potentiation</td>
<td>14.8, 8.2</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td></td>
<td>Potentiation</td>
<td>3.7, 2</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
<td>17.4, 4.4</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
<td>23.1</td>
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<tr>
<td>CBDV</td>
<td>Native</td>
<td>Epileptic hippocampal tissue transplanted in <em>Xenopus</em> oocytes</td>
<td>Partially rescued $I_{GABA}$ run-down</td>
<td>0.05†</td>
<td>Morano et al. (2016)</td>
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<td>THC</td>
<td>$\alpha_1\beta_2\gamma_2$</td>
<td>Human GABA$_A$R expressed in <em>Xenopus</em> oocytes</td>
<td>(Weak) potentiation</td>
<td>3†</td>
<td>Sigel et al. (2011)</td>
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<tr>
<td></td>
<td>$\alpha_1\beta_3\gamma_2$</td>
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<td>$\alpha_1\beta_2\beta_3\gamma_2$</td>
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<tr>
<td>Synthesis</td>
<td>AM251</td>
<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td>(Strong) Potentiation</td>
<td>0.4 ± 0.13, 3†</td>
<td>Baur et al. (2012)</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
<td>Rat GABA$_A$R expressed in <em>Xenopus</em> oocytes</td>
<td>(Strong) Potentiation</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
<td>3†</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
<td>3†</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
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<td>$\alpha_1\beta_2\gamma_2, \alpha_2\beta_2\gamma_2$</td>
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<td>Potentiation</td>
<td>3†</td>
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<td>SR141716</td>
<td>$\alpha_1\beta_2\gamma_2$</td>
<td>Rat GABA$_A$R expressed in <em>Xenopus</em> oocytes</td>
<td>(Strong) Potentiation</td>
<td>7.3 ± 0.5</td>
<td>Baur et al. (2012)</td>
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</table>

Table 2. Cannabinoid effects on native and recombinant GABA$_A$Rs. 2-AG, 2-arachidonoyl glycerol; AEA, Arachidonoyl ethanolamide; NA-Gly, N-arachidonoyl-glycine; CBD, cannabidiol; CBDV, cannabidivarin; THC, Δ$^9$-tetrahydrocannabinol. † For studies where the EC/IC$_{50}$ value was not reported, the concentration of compounds tested is indicated.
The most recent study to look at the direct effect of cannabinoids on GABA\textsubscript{A}Rs expressed in *Xenopus* oocytes systematically assessed the modulation of synaptic \(\alpha_1\delta\beta_2\gamma_2\) and extrasynaptic \(\alpha_4\beta\delta\) GABA\textsubscript{A}Rs by CBD and 2-AG (Bakas et al. 2017). The authors found that both compounds acted as positive allosteric modulators at all synaptic receptors tested, with low micromolar potencies (half-maximal effective concentration, EC\textsubscript{50}, ranging from 0.9 to 23 \(\mu\)M). Currents mediated by \(\alpha_2\)-containing receptor subtypes were most potentiated by the cannabinoids, which is in contrast to the Sigel et al. (2011) study that did not find a 2-AG selectivity for the \(\alpha\) subunit. In the Bakas et al. (2017) study both CBD and 2-AG increased the potency of GABA at \(\alpha_2\beta_2\gamma_2\) receptors; a leftward shift of the GABA concentration response curve was observed in the presence of the cannabinoids, without affecting maximal I\textsubscript{GABA}. Moreover, potentiation of I\textsubscript{GABA} was both cannabinoid and GABA concentration-dependent, as previously reported by Sigel et al. (2011). Thus, currents evoked by lower concentrations of agonist (< GABA EC\textsubscript{50}) were most enhanced by CBD and 2-AG (Bakas et al. 2017). Currents mediated by the binary receptors \(\alpha_1\beta_2\) were also found to be potentiated by CBD, suggesting that the \(\alpha/\gamma\) subunit interface (and thus the classical benzodiazepine binding site) is not required for CBD action on the GABA\textsubscript{A}R (Bakas et al. 2017). Sigel et al. (2011) reported similar findings with 2-AG and \(\alpha_1\beta_2\).

Bakas et al. (2017) observed a \(\beta\) subunit selectivity in that CBD and 2-AG were weaker potentiators of GABAergic currents mediated by \(\beta_1\)-containing receptors. CBD modulated \(\beta_2\) and \(\beta_3\)-containing receptors to a similar extent, though this cannabinoid was seen to be more potent (exhibiting a lower EC\textsubscript{50}) at \(\alpha_2\beta_3\gamma_2\), leading the authors to conclude that CBD is selective for \(\beta_3\) over the other \(\beta\) subunits. The study also uncovered a similar EC\textsubscript{50} value for 2-AG at \(\beta_1\), \(\beta_2\), and \(\beta_3\)-containing receptors. Indeed, Bakas et al. (2017) reported a cannabinoid-induced I\textsubscript{GABA} potentiation at \(\beta_1\)-containing receptors, in contrast to what was previously described in the Sigel et al. (2011) study. The latter used the \(\alpha_1\) subunit in their experiments whilst the former used \(\alpha_2\); this could be a contributing factor to the differences observed. Such variance only adds to the already complex picture of cannabinoid modulation of GABA\textsubscript{A}Rs, and suggests for instance that the 2-AG binding site is different at \(\alpha_1\) and \(\alpha_2\)-containing receptors. Finally, Bakas et al. (2017)
also looked at the effect of CBD and 2-AG on extrasynaptic δ-containing receptors and found both cannabinoids enhanced GABA-evoked currents from α₄β₂δ to a greater magnitude than any other receptor subtype tested.

To date, no studies have investigated the direct effect of CBDV on specific GABA_A Rs subtypes. However, GABA_A Rs have already been described as a possible target for CBDV. Morano et al. (2016) used a *Xenopus* oocyte microtransplantation technique to study the effect of this cannabinoid on native GABA_A Rs. Membrane vesicles prepared from hippocampal tissue resected from drug-resistant temporal lobe epilepsy patients were injected into *Xenopus* oocytes. With this approach, the human membrane vesicles undergo fusion with the oocyte plasma membrane. In this way, the authors were able to characterise a run-down of GABA current, usually observed in epileptic tissue, that was partially rescued by prolonged exposure to CBDV (Morano et al. 2016). However, it should be noted that the transplanted native tissue is composed of different cell types, such as neurones and glia, and contains a variety of receptors and transporters. Thus, the effect described in this study could be due to a combination of CBDV targets and not necessarily caused by a direct action on GABA_A Rs.

In summary, a handful of studies have been published investigating the direct effects of a number of cannabinoids on GABA_A Rs (Table 2), and a complex landscape has emerged whereby agonist concentrations influence the resulting cannabinoid modulation of the receptor and receptor subunit composition also dictates the extent of I_GABA potentiation. A larger body of research has focused on the cannabinoid-mediated modulation of GlyRs, uncovering a similar level of complexity.

**1.5 Glycine receptors**

Like the GABA_A Rs, GlyRs mediate rapid synaptic inhibitory neurotransmission in the nervous system. Glycine binding to the ECD of the receptor causes opening of the Cl⁻ ion selective pore and subsequent hyperpolarisation of mature neurones (Lynch 2004). Traditionally, GlyRs were associated with the mammalian spinal cord and
brain stem, though it is now well established glycinergic inhibition is not confined to these areas (Legendre 2001).

GlyRs belong to the Cys-loop LGIC superfamily and share the same structural features as other members (Lynch 2004), notably, five subunits arranged around a central ion pore (Figure 2). In recent years, a number of GlyR structures have been reported. The first X-ray structure of a human GlyR $\alpha_3$ homomer in complex with strychnine (Huang et al. 2015), as well as an electron cryo-microscopy structure of the zebrafish GlyR $\alpha_1$ homomer bound to glycine (Du et al. 2015), shed light on the binding sites of agonist and antagonists and on the conformational changes that they induce in the receptor. Most recently, crystal structures of human GlyR $\alpha_3$ bound to analgesic potentiators and ivermectin were published, describing a novel allosteric site and further elucidating the binding mode and mechanism of activation of GlyRs (Huang et al. 2017b, 2017c).

Immunolabeling of this inhibitory receptor has shown wide expression in the brainstem and throughout the gray matter of the spinal cord, often co-localised with the anchoring protein gephyrin (Baer et al. 2003, 2009). GlyRs have also been observed in discrete locations within the basal ganglia, being most abundant in the substantia nigra (Waldvogel et al. 2007). Within such regions the receptors are usually present on the plasma membranes of neuronal somata and dendrites. These immunolabeling studies carried out on human tissue are in agreement with rodent findings, although additional studies identified GlyRs in further brain regions in the rat, such as the olfactory bulb, cerebellar cortex, thalamus and hypothalamus (Malosio et al. 1991). Distribution of the different GlyR subtypes in the rodent retina has also been mapped (Wassle et al. 2009) and these receptors have also been identified in the hippocampus, albeit at lower concentrations than GABA$_A$Rs, where they might form mixed GABA/glycine synapses and contribute to the modulation of hippocampal excitability (Chattipakorn and McMahon 2002, Danglot et al. 2004, Keck and White 2009). Indeed, inhibitory synapses able to co-release GABA and glycine are known to exist in the spinal cord and brainstem (Jonas et al. 1998, O’Brien and Berger 1999) and immunolabeling studies have shown co-localisation of
GlyRs with specific GABA<sub>A</sub>R subunits in the mouse hypoglossal nucleus, from before birth and up to adulthood (Muller et al. 2004).

In humans, four functional GlyR subunits are expressed: α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub> and β. A pseudogene for α<sub>4</sub> has also been identified (Simon et al. 2004) which was shown to form functional receptors in the mouse, chick, zebrafish, gorilla and chimpanzee (Harvey et al. 2000, Imboden et al. 2001a, 2001b, Leacock et al. 2018). Functional receptors can be homomeric, composed of just one type of α subunit, or heteromeric, in which case they are formed from a combination of an α subunit (α<sub>1</sub>, α<sub>2</sub> or α<sub>3</sub>) with the β subunit. The GlyR β subunit alone cannot form functional receptors but when assembled with the α subunits it alters the Cl<sup>-</sup> conductance of the resulting heteromeric receptors (Bormann et al. 1993), and confers picrotoxinin resistance (Pribilla et al. 1992). This provides a pharmacological means of distinguishing heteromeric from homomeric receptors. The β subunit was originally thought to only have a structural role, with the cytoplasmic loop between TM3 and TM4 providing a gephyrin binding site (Meyer et al. 1995) – gephyrin plays an important role in GlyR anchoring to the cytoskeleton and clustering at synapses (Kirsch and Betz 1995, Feng et al. 1998). However, in heteromeric αβ glycine receptors, the β subunit was shown to exist in a 2α:3β stoichiometry and also to contribute to agonist binding (Grudzinska et al. 2005). This diverges from the previously accepted 3α:2β stoichiometry (Langosch et al. 1988).

The various GlyR subunits show different developmental expression profiles with α<sub>2</sub> expression starting prenatally and decreasing after birth (Malosio et al. 1991). This is consistent with the role of α<sub>2</sub> subunit-containing receptors in regulating the formation of cortical circuits (Avila et al. 2013, Morelli et al. 2017). The diminishing α<sub>2</sub> expression coincides with an increasing postnatal expression of α<sub>1</sub> and α<sub>3</sub> subunits (Malosio et al. 1991). These subunits also show site-specific differential expression patterns. Through mRNA hybridisation studies, α<sub>3</sub> was detected in the olfactory bulb (Malosio et al. 1991). Further studies described the expression of this subunit in the brainstem as being important in the control of breathing in mice (Manzke et al. 2010). In addition, α<sub>3</sub> present in the dorsal horn has been shown to mediate inflammatory pain sensitisation (Harvey et al. 2004). The α<sub>2</sub> subunit is
present in the cerebral cortex and hippocampus and the $\alpha_1$ dominates the brainstem nuclei and spinal cord. The $\beta$ subunit on the other hand seems to have a more widespread distribution, even extending to areas devoid of $\alpha$ subunits (Malosio et al. 1991).

The relatively widespread distribution of the GlyRs in the CNS certainly highlights an important physiological role. Indeed, correct functioning of these receptors and their resulting inhibitory action is crucial for countering increases in excitability and thus maintaining balanced neuronal networks. Failing this, defects in glycineric neurotransmission in reflex circuits of the spinal cord and brainstem can lead to motor disorders characterised by hyperexcitability of motor neurones and muscle hypertonia (Lynch 2004). An example is hyperekplexia, also known as startle disease due to an excessive startle reaction to unexpected sensory stimuli. A role for these receptors in pain pathways has also been described. Prostaglandin E2-mediated inhibition of GlyRs located in the superficial layers of the spinal cord dorsal horn can lead to inflammatory pain sensitisation as transmission of nociceptive stimuli to the brain is effectively increased (Ahmadi et al. 2002, Harvey et al. 2004). Thus, allosteric potentiators targeting GlyRs could have important therapeutic value.
Figure 4. Illustration of the orthosteric and major allosteric bindings sites on homomeric GlyRs. Five orthosteric sites located at each subunit interface in the extracellular domain bind glycine as well as the competitive antagonist, strychnine. A recent structural study identified a novel cleft adjacent to this orthosteric site that binds a class of analgesic compounds. The antiparasitic agent ivermectin also binds at inter-subunit sites located within the transmembrane domain of the receptor and can directly activate GlyRs. Low concentrations of Zn$^{2+}$ can potentiate GlyR-mediated currents by binding at an extracellular site. On the other hand, high concentrations of Zn$^{2+}$ inhibit GlyRs by binding at a distinct location also in the extracellular domain. Volatile anaesthetics and ethanol are thought to share an intra-subunit binding site in the transmembrane domain. Homomeric GlyRs are sensitive to picrotoxin channel block, unlike heteromeric αβ receptors. Positive and negative allosteric modulators are emboldened and underlined, respectively.

Though GlyRs do not exhibit the same subtype heterogeneity as GABA$_A$Rs, they still possess a number of modulator binding sites that endows them with a varied pharmacological profile (Figure 4). The natural plant product strychnine is a competitive antagonist specific to GlyRs, binding the orthosteric site located at the interface of adjacent subunits in the ECD (Huang et al. 2015). On the other hand, ivermectin can directly activate GlyRs by binding to a site other than the orthosteric site, in a cleft within the TMD at the interface of adjacent subunits (Huang et al. 2017c). Volatile anaesthetics potentiate homomeric GlyRs α$_3$ and α$_2$ and have been suggested to bind an intra-subunit site shared with ethanol in the TMD (Mihic et al. 1997). The metal ion Zn$^{2+}$ modulates the activity of GlyRs in a biphasic
concentration-dependent manner, with low (<1 μM) and high (>10 μM) concentrations leading to potentiation and inhibition of GlyR-mediated currents, respectively (Bloomenthal et al. 1994). This biphasic modulation has been linked to different Zn$^{2+}$ binding sites on the GlyR, with the potentiation site located on the outer face of the ECD (Miller et al. 2005), and the inhibition site located on the inner side of the ECD, at subunit interfaces within the vestibule lumen (Nevin et al. 2003). Most recently, a binding site for a novel class of analgesic potentiators was identified by way of X-ray crystallography; the analgesic molecule bound a pocket near the top of each α subunit interface in the ECD, in close proximity to the orthosteric agonist binding site (Huang et al. 2017b). Thus, a number of GlyR modulators have already been described, binding several different cavities on the receptor. Also shown to potentiate these inhibitory receptors, and likely binding a distinct site, are the cannabinoids, discussed in the following section.

### 1.6 Cannabinoid modulation of glycine receptors

In the last decade, a number of studies have uncovered the direct action of cannabinoids on GlyRs. Lozovaya et al. (2005) first reported an inhibition of glycine-activated currents ($I_{\text{gly}}$) in isolated hippocampal and cerebellar neurones by the endogenous cannabinoids AEA and 2-AG, further showing this to be a CB$_1$ receptor-independent effect. In another study performed on Chinese hamster ovary (CHO) cells heterologously expressing GlyRs $\alpha_1$, the authors showed a 2-AG-mediated reduction in peak amplitude and rise time of $I_{\text{gly}}$, as well as an enhanced rate of desensitisation in outside-out recordings (Lozovaya et al. 2011). Though this overall inhibitory effect of cannabinoids on GlyRs is in disagreement with subsequent studies on the topic, Lozovaya et al. (2005) were first to identify cannabinoids as GlyR modulators.

Shortly after, Hejazi et al. (2006) tested the effect of AEA and THC on different GlyR subtypes. When co-applied with glycine, both cannabinoids increased $I_{\text{gly}}$ in *Xenopus laevis* oocytes heterologously expressing human homomeric $\alpha_1$ and heteromeric $\alpha_1\beta$ GlyRs. This potentiation was reversible, concentration-dependent, and not mediated
by the CB$_3$ receptor. The same effect was observed for native GlyRs in acutely
dissociated rat ventral tegmental area neurones. The authors further described the
THC- and AEA-induced potentiation of $I_{gly}$ to be glycine concentration-dependent;
higher concentrations of co-applied agonist resulted in lower magnitudes of
cannabinoid potentiation, an effect also observed with GABA$_A$Rs. This is consistent
with the idea that a potentiating compound cannot increase current amplitudes
already at maximal levels from saturating agonist concentrations. Hejazi et al. (2006)
further tested the hypothesis that THC and AEA act at a GlyR site in the $\alpha$ subunit
TM2 domain previously identified as critical for allosteric modulation by volatile
anaesthetics and alcohols. However, site-directed mutagenesis of this site had no
significant effect on the resulting cannabinoid-induced $I_{gly}$ potentiation. Thus,
cannabinoids are likely to act on GlyRs at a position distinct from alcohols and
anaesthetics.

Differences in the Lozovaya et al. (2005) and Hejazi et al. (2006) studies – the former
observed an inhibitory and the latter a potentiating effect of cannabinoids on GlyRs
– might be due to different GlyR subunit composition and different glycine
concentrations used. Research from a third group set out to resolve such
discrepancies by examining the effect of several cannabinoid agonists on different
GlyR isoforms heterologously expressed in HEK293 cells (Yang et al. 2008). In this
report, AEA was found to potentiate $I_{gly}$ in cells expressing homomeric $\alpha_1$
and heteromeric $\alpha_1$/$\beta$ GlyRs. This cannabinoid potentiation of $I_{gly}$ was further found to be
glycine concentration-dependent, in agreement with the Hejazi et al. (2006) study.
In contrast, AEA produced no significant effect on current amplitudes measured
from cells expressing $\alpha_2$ or $\alpha_3$ subunits (Yang et al. 2008).

Additional studies have found other cannabinoids to have a positive allosteric effect
on $\alpha_1$ and $\alpha_1$/$\beta$ GlyRs heterologously expressed in HEK293 cells, including CBD
(Ahrens et al. 2009a) and the synthetic compounds ajulemic acid (AJA) and HU-210
(Yang et al. 2008, Ahrens et al. 2009b, Demir et al. 2009). Moreover, it was reported
that high concentrations of AJA, HU-210 or CBD can directly activate GlyRs in the
absence of glycine (Ahrens et al. 2009a, 2009b, Demir et al. 2009). Such an effect
had not previously been described for the cannabinoids, possibly due to issues with
compound solubility at high concentrations. On the other hand, HU-308 inhibited α₁ and α₁β, whereas WIN55,212-2 had no significant effect on either receptor subtype (Yang et al. 2008). The overall similar pharmacological profiles of the GlyRs α₁ and α₁β described in several studies suggests the β subunit plays no part in GlyR sensitivity to cannabinoids. Homomeric GlyRs α₂ and α₃ also display comparable responses to the synthetic cannabinoids, with HU-210, HU-308, and WIN55,212-2 inhibiting I_{gly}. Interestingly, higher concentrations of applied agonist surmounted this inhibition, suggesting a competitive mode of action. However, such a highly conserved glycine binding site could not account for the differential cannabinoid effects observed amongst the different subunits (Yang et al. 2008).
<table>
<thead>
<tr>
<th>Cannabinoid</th>
<th>GlyR subtype</th>
<th>Preparation</th>
<th>Effect</th>
<th>EC/IC_{50} (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2-AG</td>
<td>Native</td>
<td>Isolated rat hippocampal and cerebellar neurones</td>
<td>Inhibition</td>
<td>1′</td>
<td>Lozovaya et al. (2005)</td>
</tr>
<tr>
<td>α₁</td>
<td>Human GlyR expressed in CHO cells</td>
<td>Inhibition</td>
<td>1′</td>
<td></td>
<td>Lozovaya et al. (2011)</td>
</tr>
<tr>
<td>AEA</td>
<td>Native</td>
<td>Isolated rat hippocampal and cerebellar neurones</td>
<td>Inhibition</td>
<td>0.3</td>
<td>Lozovaya et al. (2005)</td>
</tr>
<tr>
<td>Native</td>
<td>Isolated rat ventral tegmental area neurones</td>
<td>Potentiation</td>
<td>0.2</td>
<td></td>
<td>Hejazì et al. (2006)</td>
</tr>
<tr>
<td>α₁</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>0.038 ± 0.011</td>
<td></td>
<td>Yang et al. (2008)</td>
</tr>
<tr>
<td>α₁β</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>0.075 ± 0.020</td>
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<td></td>
</tr>
<tr>
<td>α₂, α₃</td>
<td>Human and rat GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>30'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>Cultured rat spinal neurones</td>
<td>Potentiation</td>
<td>5.5 ± 2</td>
<td></td>
<td>Xiong et al. (2012a)</td>
</tr>
<tr>
<td>α₁, α₁β</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Complex; potentiation and inhibition</td>
<td>10² and 30'</td>
<td></td>
<td>Yang et al. (2008)</td>
</tr>
<tr>
<td>α₃</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Inhibition</td>
<td>3.03 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁</td>
<td>HEK293 cells</td>
<td>Potentiation</td>
<td>10'</td>
<td></td>
<td>Yevenes and Zeilhofer (2011)</td>
</tr>
<tr>
<td>α₂, α₃</td>
<td>HEK293 cells</td>
<td>Inhibition</td>
<td>10'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plant</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CBD</td>
<td>α₁</td>
<td>HEK293 cells</td>
<td>Potentiation</td>
<td>12.3 ± 3.8</td>
<td>Ahrens et al. (2009a)</td>
</tr>
<tr>
<td>α₁β</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>18.1 ± 6.2</td>
<td></td>
<td>Xiong et al. (2012b)</td>
</tr>
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<td>Chemical</td>
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<td>Isolated rat ventral tegmental area neurones</td>
<td>Human GlyR expressed in <em>Xenopus</em> oocytes</td>
<td>Potentiation</td>
<td>Potentiation</td>
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</tr>
<tr>
<td>THC α₁, α₁β</td>
<td>Native</td>
<td>Cultured rat spinal neurones</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>Potentiation</td>
</tr>
<tr>
<td>THC α₁, α₃</td>
<td>Cultured rat spinal neurones</td>
<td>Human and rat GlyR expressed in HEK293 cells</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>(Strong) potentiation</td>
</tr>
<tr>
<td>THC α₂</td>
<td>Cultured rat spinal neurones</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>THC α₁</td>
<td>HEK293 cells</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Potentiation</td>
<td>Potentiation</td>
</tr>
<tr>
<td>THC α₁</td>
<td>HEK293 cells</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>THC α₁</td>
<td>HEK293 cells</td>
<td>Human GlyR expressed in HEK293 cells</td>
<td>Rat GlyR expressed in HEK293 cells</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>THC α₁</td>
<td>Native</td>
<td>Isolated rat hippocampal and cerebellar neurones</td>
<td>No significant effect</td>
<td>1'</td>
<td>Lozovaya et al. (2005)</td>
</tr>
<tr>
<td>THC α₁</td>
<td>Native</td>
<td>Isolated rat hippocampal and cerebellar neurones</td>
<td>No significant effect</td>
<td>30'</td>
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<tr>
<td>THC α₂</td>
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<tr>
<td>THC α₃</td>
<td>Native</td>
<td>Isolated rat hippocampal and cerebellar neurones</td>
<td>No significant effect</td>
<td>30'</td>
<td>Yang et al. (2008)</td>
</tr>
</tbody>
</table>

Table 3. Cannabinoid effects on native and recombinant GlyRs. 2-AG, 2-arachidonoyl glycerol; AEA, Arachidonoyl ethanolamide; NA-Gly, N-arachidonoyl-glycine; CBD, cannabidiol; THC, Δ⁹-tetrahydrocannabinol; AJA, ajulemic acid. † In studies where the EC/IC₅₀ value was not reported, the concentration of compounds tested is indicated.
Hejazi et al. (2006) had already described a potentiation of \( I_{\text{gly}} \) by AEA and THC in oocytes expressing GlyRs \( \alpha_3 \) or \( \alpha_3\beta \). Further studies by Xiong et al. (2011, 2012a) reported that the different GlyR subunits are differentially sensitive to these cannabinoids; \( \alpha_3 \) and \( \alpha_3 \) exhibit a high sensitivity that translates into an elevated \( I_{\text{gly}} \) potentiation whilst \( \alpha_2 \) shows only modest positive modulation. Intriguingly, potentiation levels observed in the Xiong et al. studies (2011, 2012a) were considerably larger than that reported in the Hejazi et al. paper (2006).

The endogenous compound NA-Gly presents yet another modulatory profile. It was shown to elicit complex effects, transiently potentiating before inhibiting \( I_{\text{gly}} \) in HEK293 cells expressing the \( \alpha_1 \) subunit. NA-Gly negatively modulated glycine-mediated currents in cells expressing the \( \alpha_2 \) or \( \alpha_3 \) subunit (Yang et al. 2008). Such complex effects were not described in another study though overall, a similar modulation was reported; NA-Gly potentiated \( I_{\text{gly}} \) through GlyRs \( \alpha_1 \) but inhibited currents through GlyRs \( \alpha_2 \) and \( \alpha_3 \) (Yevenes and Zeilhofer 2011). By testing a series of compounds, the same authors suggest that hydroxyl groups on the cannabinoid molecules are necessary for the positive modulation of GlyRs whereas carboxyl groups might be required for mediating inhibition of the receptors (Yevenes and Zeilhofer 2011).

Studies using site-directed mutagenesis have attempted to pinpoint particular residues in the GlyRs necessary for mediating cannabinoid modulation. A serine residue (S267) in the GlyR \( \alpha_1 \) TM2 domain, previously identified as necessary for the interaction of alcohols and anaesthetics with the GlyR, was characterised as crucial for mediating the modulatory effects of AJA, CBD and HU-210 (Foadi et al. 2010). However, mutation of the same site had already been shown not to have an effect on THC and AEA potentiation of \( I_{\text{gly}} \) (Hejazi et al. 2006). It would seem another serine residue, S296, in TM3 is at play in THC and AEA-induced potentiation of GlyRs (Xiong et al. 2011, 2012a). In yet another study, several transmembrane, intracellular and extracellular loop residues were shown to be involved in the positive and negative modulation of the GlyRs by NA-Gly (Yevenes and Zeilhofer 2011). The identification of such residues provides additional insight into GlyR modulation by cannabinoids.
However, it remains unclear whether these residues are responsible for cannabinoid binding or are involved in ion channel gating mechanisms.

Thus far, several reports have shown that cannabinoids modulate, either by potentiating or inhibiting, GlyR activity in a CB receptor-independent manner. Results from such studies point to the different GlyR subunits having distinct pharmacological profiles for the cannabinoids, emphasising the idea that these molecules interact with the GlyRs via specific binding interactions, likely found within weakly conserved regions. Deciphering the molecular sites and mechanisms that mediate GlyR modulation by the cannabinoids has thus been difficult.

1.7 Study Aims

Epilepsy is a complex spectrum of disorders that can be simplistically characterised as spells of hyperexcitability in critical neuronal circuits. This results in different types of seizures in affected individuals. Thus, the use of medicinal compounds that cause potentiation of inhibitory currents – which could dampen such pathological hyperexcitability – could form the basis of effective epilepsy treatments.

As detailed earlier, CBD and CBDV have shown promising effects in preclinical models of epilepsy and a favourable outcome, translated as a reduction in seizure frequency, was recently reported for CBD in clinical trials for different paediatric epilepsy syndromes (Devinsky et al. 2017, 2018a, Thiele et al. 2018). Despite the continuous examination of potential cannabinoid targets and the extensive interest in their medicinal use, the research community remains uncertain of how CBD and CBDV bring about their therapeutic action. One hypothesis is that they might be operating via inhibitory LGICs.

In this study, I aimed to look at the direct effect of CBD and CBDV on GABA_A and GlyRs to ascertain whether these ion channels are targets of the phytocannabinoids. This is an important initial step towards confirming or refuting the potential involvement of inhibitory receptors in the anticonvulsant action of CBD and CBDV.
and thus shedding light on the mechanism of action of these phytocannabinoids. This was investigated by carrying out electrophysiological recordings of \( I_{\text{GABA}} \) and \( I_{\text{gly}} \) from HEK293 cells expressing defined recombinant \( \text{GABA}_A \) and \( \text{GlyR} \) subtypes and subsequently measuring the current modulation by agonists with or without cannabinoids. Additional site-directed mutagenesis studies were used to help identify, or exclude, sites for cannabinoid binding on the receptors. Finally, the effect of CBD and CBDV on \( I_{\text{GABA}} \) was investigated in hippocampal slices, at CA1 pyramidal neurones stimulated by local application of GABA. Data gathered from this project will underpin efforts to develop and market future paediatric epilepsy treatments.
Materials and Methods
2.1 Molecular biology

All complementary deoxyribonucleic acid (cDNA) sequences were cloned into the pRK5 vector where their expression was under the control of the strong mammalian cytomegalovirus promoter – this ensured constitutive expression of the genes of interest once transfected into HEK293 cells. pRK5 constructs expressing GlyR subunits \( \alpha_1 \), \( \alpha_2 \), or \( \alpha_3 \) have previously been described (Chung et al. 2010) whilst GABA\(_A\)R subunit genes were cloned as detailed in the steps below. All centrifugation steps were carried out at 16,000 g in a bench-top centrifuge (Eppendorf), unless stated otherwise. Liquid and solid bacterial growth media were sterilised by autoclaving at 121°C for 15 min in a bench-top autoclave (Priorclave). All reagents were acquired from Sigma Aldrich except where noted.

2.1.1 First-strand cDNA synthesis

cDNA was generated from human cerebellum poly(A)+ RNA (Clontech) using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). The following components were combined in a 0.2 ml reaction tube: 1 \( \mu l \) RNA (1 mg.ml\(^{-1}\)), 1 \( \mu l \) oligo(dT)\(_{20}\) primer (50 \( \mu M \)), 1 \( \mu l \) annealing buffer, 5 \( \mu l \) RNase/DNase-free water. The reaction was incubated at 65°C in a SimpliAmp thermal cycler (Applied Biosystems) for 5 min and subsequently placed on ice where 10 \( \mu l \) first-strand reaction mix (2x) and 2 \( \mu l \) SuperScript® III/RNaseOUT™ enzyme mix were added. The reaction was then incubated in the thermal cycler at 50°C for 50 min and the reaction terminated by incubating at 85°C for 5 min before chilling on ice and storing at -20°C.

2.1.2 Polymerase chain reaction amplification

DNA sequences of the GABA\(_A\)R subunits were amplified from human cerebellum first-strand cDNA by polymerase chain reaction (PCR). A list of oligonucleotide primers (Eurofins Genomics) used to amplify the different receptor subunits can be found in Table 1 of the Appendix. Distilled water was added to each lyophilised primer to make 100 \( \mu M \) stock solutions and ten-fold diluted aliquots were
subsequently prepared. PCR samples containing 22 μl AccuPrime™ Pfx SuperMix (Invitrogen), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 0.5 μl water and 0.5 μl human cerebellum cDNA were placed in a thermal cycler. The following thermal cycling parameters were used to carry out the amplification reactions on a Veriti 96 well thermal cycler (Applied Biosystems): an initial 5 min denaturation step at 95°C followed by 40 cycles of a 15 s denaturation step at 95°C, a 30 s annealing step at 60°C and a 2.5 min extension step at 68°C, with a final 7 min elongation step at 68°C. PCR amplification products were stored at 4°C until further use.

2.1.3 Agarose gel electrophoresis

PCR amplification products were analysed on a 1 % (w/v) agarose gel prepared by microwaving UltraPure™ Agarose powder (Invitrogen) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA at pH 8) until fully dissolved and allowed to cool before addition of 1x SYBR® Safe DNA gel stain (Invitrogen). This was poured into a casting tray with an appropriate size comb and allowed to set before placing in an electrophoresis chamber and immersing in TAE buffer. 10x DNA loading dye (20 % (w/v) Ficoll®, 0.2% (w/v) bromophenol blue) was added to each sample and loaded onto the gel alongside a 1 kb molecular weight size marker (Thermo Scientific). Samples were electrophoresed at 90 V for one hour before visualisation of DNA bands in a blue light Transilluminator (Syngene).

2.1.4 DNA gel extraction

Using a Safe Imager blue light Transilluminator (Invitrogen), amplified DNA fragments of correct size were excised from the agarose gel with a scalpel to sterile Eppendorf tubes. Purification of fragments was carried out using the QIAquick Gel Extraction Kit (Qiagen) and carried out according to the supplier’s instruction manual.
2.1.5 Restriction digest

The pRK5 vector and purified PCR products were digested with the appropriate restriction endonucleases (see Appendix Table 1) so as to create compatible sticky ends. All enzymes and corresponding buffers were obtained from New England Biolabs. Double digests were performed with enzymes of compatible buffers; 20 μl reactions containing 15 μl purified PCR samples, 2 μl enzyme buffer (10x), 0.5 μl enzyme 1, 0.5 μl enzyme 2 and 2 μl water were incubated for one hour at the enzymes’ required working temperature, as indicated in the supplier’s notice. pRK5 digest reaction mix contained 1 μg vector DNA and the water volume was adjusted accordingly. Sequential digests were carried out for enzymes with incompatible buffers, in which case the reaction mix contained 15 μl purified PCR samples, 2 μl enzyme buffer (10x), 0.5 μl enzyme and 2.5 μl water. Following the incubation period, the digested DNA was purified by way of a phenol chloroform extraction (see below) and a digest with the second enzyme set up. Digested samples were then run on a 1 % (w/v) agarose gel and DNA fragments of interest were purified by way of gel extraction (see above).

2.1.6 Phenol chloroform extraction

Water to a final volume of 100 μl was added to the digested samples followed by 100 μl UltraPure™ Phenol:Chloroform:Isoamyl alcohol (Invitrogen). Samples were subsequently centrifuged for 10 min and the upper aqueous phase was transferred into a microcentrifuge tube containing 250 μl 96 % (v/v) ethanol (Fisher Scientific), 10 μl sodium acetate (3 M) and 1.5 μl mussel glycogen (20 μg.μl⁻¹). Samples were incubated on dry ice for 15-30 min before centrifugation, after which the supernatant was discarded and the pellet washed in 250 μl 80 % (v/v) ethanol. Samples were centrifuged for 1 min and the supernatant discarded before a final centrifugation. Lids of the microcentrifuge tubes were left open until evaporation of any residual ethanol. Pellets were subsequently resuspended in elution buffer (10 mM Tris-Cl, pH 8.5, Qiagen).
2.1.7 Ligation

Purified DNA inserts and plasmid vectors were ligated using T4 DNA ligase (Roche) in a reaction mix containing 7 μl digested DNA insert, 1 μl digested vector, 1 μl ligation buffer (66 mM Tris-HCl, 5 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, pH 7.5) and 1 μl T4 DNA ligase (1 U.μl⁻¹). Samples were incubated at 4°C overnight or 15 min at room temperature and subsequently transformed into competent *Escherichia coli* (*E. coli*) cells (see below).

2.1.8 *E. coli* transformation

This procedure was carried out next to a Bunsen burner flame so as to work in a sterile environment when handling bacterial cells and media. Competent DH5α *E. coli* cells (Invitrogen) were thawed on ice. 5 μl of ligation mixture was added to 100 μl of *E. coli* cells and incubated on ice for 30 min, followed by 45 s at 42°C and 2 min on ice. 250 μl Lennox L broth base (LB, Invitrogen) was added and the cells incubated at 37°C for one hour in a ThermoMixer® F1.5 (Eppendorf) set to 800 RPM. Subsequently, 100 μl of the transformation mix was plated onto Lennox L agar (Invitrogen) supplemented with ampicillin (100 μg.ml⁻¹, Merck Millipore) and incubated overnight at 37°C. The pRK5 vector codes for the ampicillin resistance gene which allows ampicillin plates to select for those cells expressing the plasmid. The following day, colonies were picked and used to inoculate 4 ml of fresh LB media supplemented with ampicillin (100 μg.ml⁻¹). These were incubated overnight at 37°C in an Excella E24 incubator shaker (New Brunswick Scientific) set to 250 RPM. This starter culture was either used for DNA extraction and purification (see below) or to inoculate 200 ml of fresh LB media supplemented with ampicillin. The latter was incubated in the shaker overnight at 37°C and used for large quantity DNA purification (see below).
2.1.9 Purification of plasmid DNA

Plasmid DNA was extracted and purified from *E. coli* cells grown overnight using a QIAprep* Spin Miniprep kit (Qiagen) and according to the supplier’s protocol. For extraction and purification of larger quantities of DNA, a HiSpeed* Plasmid Maxi kit (Qiagen) was used and the supplier’s protocol was followed.

2.1.10 Diagnostic digest

In order to confirm integration of the insert of interest into the pRK5 vector, diagnostic digests were carried out. Purified DNA was digested with enzymes known to result in specific DNA fragment sizes which were subsequently electrophoresed on a 1 % (w/v) agarose gel. Plasmids containing inserts with the correct size were externally sequenced by DNA Sequencing and Services (Medical Sciences Institute, University of Dundee, UK) for further confirmation.

2.1.11 Site-directed mutagenesis

The QuickChange Lightning Site-Directed Mutagenesis kit (Agilent) was used to introduce artificial mutations in various GlyR and GABA<sub>A</sub>R subunits previously cloned into the pRK5 vector. Oligonucleotide primer pairs containing the desired mutation were designed according to the Agilent online tool (www.agilent.com/genomics/qcpd) and acquired from Eurofins Genomics (see Table 2 of Appendix for specific mutagenesis primer sequences). The procedure, including the reaction mixture and PCR thermal cycling parameters, was carried out according to the supplier’s instruction manual. PCR products were digested with the restriction enzyme DpnI, provided in the kit, and subsequently transformed into competent *E. coli* and purified as previously described. External sequencing of the full coding region of the constructs confirmed the presence of the desired mutation.
2.2 Cell culture and acute brain slice preparation

2.2.1 HEK293 cell culture

HEK293 cells (ATCC® CRL-1573™) were cultured in T75 tissue culture flasks (Greiner Bio-One) in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10 % (v/v) foetal bovine serum (FBS, Gibco), 50 U.ml⁻¹ penicillin and 50 μg.ml⁻¹ streptomycin (Gibco) and maintained at 37°C in a humidified incubator under 5 % CO₂ / 95 % O₂. Cells were passaged every 48-72 hours: culture medium was removed and cells were washed with 10 ml Hank’s Balanced Salt Solution (HBSS, Gibco) before addition of 1 ml 0.25 % trypsin-EDTA 1x (Gibco) incubated for 1 min and resuspension of cells in 10 ml culture medium. Cell suspension was plated in a new T75 flask in a 1:10 ratio with fresh medium. For transfections, cells were plated in Ø 6 cm culture dishes (Thermo Scientific) with 5 ml fresh medium so as to be 40-50 % confluent on the day of transfection.

2.2.2 HEK293 cell transfection

Prior to transfection, culture medium on the HEK293 cells was removed and replaced with minimum essential medium (MEM, Gibco) supplemented with 10 % (v/v) FBS, 50 U.ml⁻¹ penicillin and 50 μg.ml⁻¹ streptomycin. The transfection mixture consisting of 2.86 μg total DNA, 5.71 μl FuGENE HD transfection reagent (Promega) and 100 μl Opti-MEM (Gibco) was incubated at room temperature for 15 min and subsequently added onto the cells which were placed in the 37°C incubator overnight. Total DNA was a combination of expression plasmid for a GlyR α subunit and pEGFP-C1 (Clontech) in a 3:1 ratio; GABAₐR α, β, γ subunits and pEGFP-C1 in a 1:1:1:1 ratio; GABAₐR α, β, δ and pEGFP-C1 in a 1:1:1:1 ratio; or GABAₐR α, β and pEGFP-C1 in a 1:1:1 ratio.
2.2.3 Mouse hippocampal slice preparation and maintenance

A sucrose-based dissection and maintenance solution was prepared according to the
recipe in Table 4 and placed at -80°C to partially freeze it. With the help of a blender,
this sucrose-based solution was crushed so as to create a slush. This sucrose slush
was oxygenated with 5 % CO₂ / 95 % O₂ gas mixture and was used to maintain the
brain tissue at 0°C during the dissection and slicing steps, which were carried out as
described in Bischofberger et al. (2006).

<table>
<thead>
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</tr>
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<td>MgCl₂</td>
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</tbody>
</table>

Table 4. Dissection and maintenance sucrose solution. This solution was continuously
gassed with 5 % CO₂ / 95 % O₂. The osmolarity and pH was adjusted to 320 mOsmol.L⁻¹ and
7.3, respectively.

Male C57BL/6 mice (Harlan Laboratories, UK) aged four to eight weeks were killed
by cervical dislocation and rapidly decapitated. The skull was exposed by a scalpel-
made skin incision and dissection scissors were used to cut the skull along the
midline, beginning at the foramen magnum. Additional medio-lateral cuts were
made at the level of the cerebellum and olfactory bulb to facilitate lateral removal of
the skull and exposure of the brain. A spatula was used to carefully dislodge the
brain and momentarily place it in oxygenated sucrose-based ice-cold solution. The
brain was transferred onto a Whatman filter paper (GE Healthcare) and the olfactory
lobes and cerebellum were removed by way of a clean coronal plane cut (Figure 5).
A mid-sagittal cut separated the brain hemispheres, which were subsequently
positioned with the temporal side facing upwards. A final cut was made along the
dorsal side of each hemisphere before transferring them, flattened dorsal side face down (ventral side up), onto a stage with a thin coating of superglue. A soft paint brush was used to gently hold and stick down each hemisphere before transferring the stage into the buffer tray of a vibratome (Leica VT 1200S, Leica Biosystems) filled with oxygenated sucrose-based solution. Transverse 300 μm thick slices were cut from both hippocampi in a lateral to medial direction and transferred to a maintenance chamber with the aid of a wide-lumen pipette. Slices were allowed to recover in oxygenated sucrose solution at 35°C for 30 min, after which the maintenance chamber was removed from the water bath and kept at room temperature (up to 6 hours) until slices were used for recordings.

**Figure 5. Schematic illustration of the brain cuts.** The brain is shown from a dorsal (top) angle and the dotted lines represent blade cuts. Following removal of the brain from the skull, two coronal plane cuts shown in image 1 removed the olfactory bulbs and cerebellum. A mid-sagittal cut shown in 2 separated the brain hemispheres. A section from the dorsal side of each hemisphere was removed, as shown in the top image of 3. To make this cut, each hemisphere was rolled onto its medial side, represented by the bottom image of 3. Both brain hemispheres were then glued onto the stage of the vibratome, dorsal side facing down. The procedure for removing the brain from the skull and the subsequent cuts were quickly carried out so as to minimise the time the brain was left un-oxygenated.
2.3 Electrophysiology

2.3.1 Recording equipment

HEK293 cells plated onto coverslips or individual hippocampal slices were placed in a recording glass chamber continuously perfused with the appropriate saline solution at a gravity-driven rate of 5 ml.min\(^{-1}\). A manifold tube system, connected to a computer controlled magnetic pinch-valve (Nanion), was used to exchange solutions containing different drugs (Figure 6).

**Figure 6. Diagram of experimental set up and signal flow.** The bottom illustration depicts a cell expressing GABA\(_A\)Rs in whole-cell patch-clamp with the recording electrode on the right and a GABA puff pipette on the left. Equipment details can be found in the text.
HEK293 cells or hippocampal neurones were visualised under an infra-red differential interference contrast microscope with a water-emersion high magnification (60x) objective (Olympus). The microscope was equipped with a UV lamp and a bandpass emission filter to allow for the identification of EGFP-expressing cells when required. Patch pipettes were pulled from borosilicate glass capillaries with filament (outer diameter 1.5 mm, inner diameter 0.86 mm, Warner Instruments) on a Flaming/Brown micropipette puller (Sutter Instrument). Local pressure application of agonist (1 mM GABA or glycine in perfusion solution, unless otherwise indicated) was delivered every 20 s, in pulses of 4-5 ms duration and 10-20 psi, via a patch pipette connected to a Picospritzer (Parker Hannifin). The pipette was positioned roughly 10-20 μm away from the recorded cell by means of a micromanipulator (Luigs & Neumann). Pressure application of compounds allows for rapid agonist delivery, minimising desensitisation of ligand-gated receptors. This method of application does not allow clamping of the agonist concentration meaning that the final concentration reaching the cell is unknown. Nevertheless, this delivery system does allow the recording of reproducible and rapid agonist-induced currents, as shown in Figure 7A. Moreover, the current amplitude is directly proportional to the puff duration and pressure (Figure 7).

**Figure 7.** Pressure application of GABA to HEK293 cells expressing GABA_A R α2β2γ2. (A) Exemplar current traces induced by 1 mM GABA (4 ms, shown as black dot) applied with increasing pressure (12-44 psi). Traces shown in red correspond to the red data points in the current-pressure graph. (B) Incrementing the pressure in the puff pipette proportionally increased peak current.
Currents were recorded with a Multiclamp 700-B amplifier (Molecular devices) and digitised using a National Instrument data acquisition board installed on a personal computer. Data were filtered at 2 kHz (internal 4-pole low-pass Bessel filter) and sampled at 10 kHz. Customised data acquisition and analysis was performed using virtual instruments written in LabVIEW™ 8 (National Instruments).

2.3.2 Drugs and chemicals

All drugs were dissolved in distilled water or dimethyl sulfoxide (DMSO) and dissolved in perfusion solution to achieve their final experimental concentration. Specifically, 100 mM stock solutions of CBD and CBDV were prepared in DMSO and diluted in perfusion solution such that the final DMSO concentration was less than 0.01 % (v/v).

<table>
<thead>
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<th>Reagent</th>
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<td>Sucrose</td>
<td>Sigma</td>
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</table>

Table 5. List of drugs and chemicals used for patch-clamp experiments.
2.3.3 Recordings from HEK293 cells

Borosilicate coverslips (Ø 13 mm, VWR International) were coated by immersion in a 100 µg.ml⁻¹ Poly-D-Lysine solution for one hour, after which they were rinsed three times with deionized water and air dried overnight at room temperature. 16-20 hours post transfection, HEK293 cells were split and plated at low density onto previously coated coverslips and incubated for three to four hours at 37°C to allow for cell recovery and attachment. Coverslips were subsequently placed in a recording glass chamber filled with perfusion solution (Table 6). Glass microelectrodes (2-4 MΩ) were filled with intracellular solution (Table 7) and whole-cell recordings were obtained at room temperature from isolated EGFP-positive HEK293 cells voltage-clamped at -80 mV. This combination of perfusion and intracellular solutions resulted in a Cl⁻ equilibrium potential around 0 mV. The access resistance, monitored throughout the recordings by a 5 mV voltage step, was < 20 MΩ. Drugs applied in these experiments, at the concentrations indicated in the text included, CBD, CBDV, DZP, CLB, AEA, 2-AG, and DS2.

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<td>HEPES</td>
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</table>

Table 6. HEK293 perfusion solution. The osmolarity was adjusted to 298 mOsmol.L⁻¹ with distilled water and the pH was adjusted to 7.4 with 1 M NaOH.
### Table 7. Intracellular solution for HEK293 whole-cell patch-clamp recordings

<table>
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</tr>
<tr>
<td>Na₂phosphocreatine</td>
<td>10</td>
</tr>
</tbody>
</table>

The osmolarity was adjusted to 290 mOsmol.L⁻¹ with distilled water and the pH was adjusted to 7.2 with 1 M KOH.

### 2.3.4 Recordings from mouse hippocampal slices

Individual hippocampal slices were transferred to the recording glass chamber which was constantly perfused with oxygenated physiological saline (Table 8) at room temperature. Slices were held in place with a platinum wire and nylon strings and individual CA1 pyramidal neurones were identified on the basis of location and appearance (Figure 8). Glass pipettes with a resistance of 4-6 MΩ were filled with a caesium-based intracellular solution (Table 9). Neurones were clamped at -60 mV and currents evoked from pressure application of GABA in proximity to the cell were recorded in the presence of 5 μM CGP 52432, a selective GABA<sub>B</sub>R antagonist. The access resistance was < 40 MΩ. Drugs applied in these experiments, at the concentrations indicated in the text, included CBD, CBDV, DZP and picrotoxin.
<table>
<thead>
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<tr>
<td>MgCl₂</td>
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</table>

Table 8. Physiological saline for slice recordings. This solution was gassed continuously with 5 % CO₂ / 95 % O₂. The osmolarity and pH were adjusted to 320 mOsmol.L⁻¹ and 7.3, respectively.

<table>
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<td>MgATP</td>
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</tr>
<tr>
<td>Na₃GTP</td>
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</table>

Table 9. Intracellular solution for whole-cell patch-clamp of CA1 pyramidal neurones. The osmolarity was adjusted to 310 mOsmol.L⁻¹ with distilled water and the pH was adjusted to 7.2 with CsOH.
Figure 8. Illustration of a transversal hippocampal slice. CA1 pyramidal cells were identified on the basis of their appearance and location in relation to the dentate gyrus (DG). GABA was applied by pressure ejection from a pipette placed in proximity to the cell.

2.4 Data analysis and statistics

DNA sequences were analysed using the software Sequencher 5.0 (Gene Codes Corporation) by alignment with reference sequences obtained from Ensembl (http://www.ensembl.org/) or National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

Statistical analysis and graph figures were produced with GraphPad Prism 7 (GraphPad Software Inc.). Current amplitude in the presence of drug was normalised to control values in perfusion solution alone as follows:

\[ I_{\text{GABA/glycine}} = \left( \frac{I_{\text{drug}}}{I_{\text{control}}} \right) \times 100 \]

where \( I_{\text{GABA/glycine}} \) is the normalised response and is calculated as a percentage, \( I_{\text{drug}} \) is the peak current amplitude (pA) in the presence of a given concentration of drug and \( I_{\text{control}} \) is the peak amplitude of control \( I_{\text{GABA}} \) or \( I_{\text{gly}} \) (pA) prior to drug application. Control current amplitude was designated 100% in all cases. Normalised responses
for individual cells were pooled (mean ± standard error of the mean, SEM) for each time point. With the exception of Figures 14A, 18A, 19A, 20A, 21A, 23A, 24A, and 32, each plotted time point is the average of three consecutive time points. For each experiment, traces shown as representative examples are the average of two to three individual responses from the same cell.

The decay rate of potentiated $I_{GABA}$ presented in Figures 16 and 33 was calculated as $I_{GABA} \div I_{\text{max.GABA}}$ for each individual cell where $I_{GABA}$ is the normalised response and $I_{\text{max.GABA}}$ is the maximal potentiated response. Cells were then pooled (mean ± SEM) and graphed over time, with each time point representing the average of three consecutive time points.

For each time course presented, the maximum value of current potentiation in the presence of phytocannabinoid was used for statistical comparison against control current amplitude. Where the current amplitude progressively increased over the duration of phytocannabinoid application, the current amplitude after ten minutes of exposure to the phytocannabinoids was used for statistical analysis. This ten-minute time point was arbitrarily chosen to represent the average current modulation induced by the phytocannabinoids. The Shapiro-Wilk normality test was used to assess whether data were normally distributed: data were accepted to follow a normal distribution when the $P$ value of the Shapiro-Wilk test was greater than 0.05. Unpaired or paired comparisons for normally distributed data were performed using a two-tailed Student’s $t$-test. One-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test was used to compare more than two samples. Data that were not normally distributed were submitted either to a Mann-Whitney test (independent samples) or Wilcoxon signed-rank test (dependent samples). Statistical significance was accepted where $P < 0.05$. 
Results
3.1 CBD and CBDV modulation of GABA<sub>A</sub> receptors

Various cannabinoids, whether endogenous, synthetic or plant-derived, have been shown to modulate inhibitory currents through a direct effect on inhibitory receptors. This has important therapeutic implications in hyperexcitability disorders. However, the effect of CBD and CBDV on these receptors has not yet been studied in depth. The research presented here aims to determine whether $I_{GABA}$ and $I_{gly}$ currents are modulated by these phytocannabinoids and to uncover the possible site of action of these compounds.

To assess the effect of CBD and CBDV on GABA<sub>A</sub>Rs, a molecular approach was taken whereby human GABA<sub>A</sub>R subunits were transiently co-transfected and expressed in HEK293 cells. These were subsequently patched in whole-cell voltage-clamp mode and the resulting $I_{GABA}$ modulation by the cannabinoids was examined. Several GABA<sub>A</sub>Rs subtypes were studied to assess possible subtype-selectivity of the compounds.

3.1.1 GABA<sub>A</sub> receptor cloning

Human GABA<sub>A</sub>R subunits $\alpha_{1-5}$, $\beta_{1-3}$, $\gamma_2$ and $\delta$ were cloned into the expression vector pRK5 which harbours a cytomegalovirus (CMV) promoter, thus ensuring constitutive expression once transfected into HEK293 cells. For several GABA<sub>A</sub>R subunits, alternative splicing of exons results in multiple isoforms of the same subunit. Thus, of the three $\alpha_2$ and $\alpha_4$ isoforms identified to date, the shorter $\alpha_2$ isoform a and the longer $\alpha_4$ isoform 1 were cloned. The shorter $\beta_2S$ subunit, differing from $\beta_2L$ by 38 amino acids, was also cloned. Of the four known isoforms, $\beta_3$ isoform 1 was cloned and is considered the canonical sequence. This isoform is of the same length as isoform 2, however, these subunit variants have different N-termini. Finally, of the three known $\gamma_2$ isoforms, isoform 1 was cloned, corresponding to $\gamma_2L$ in the literature (Whiting et al. 1990). Throughout this manuscript, $\gamma_2$ indicates the $\gamma_2L$ isoform.
<table>
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Table 10. Human GABA<sub>A</sub>R subunits cloned. The particular subunit isoforms cloned for this project are highlighted in grey, along with a list of other known isoforms. The isoform names were taken from the NCBI and may differ from other databases. Where two NCBI transcript references are given for the same isoform, the different transcripts differ in the 5’ UTR region. † Several transcript variants, not listed here, have been identified for the GABA<sub>A</sub>R α1 subunit that differ in the 5’ UTR but encode the same protein.

3.1.2 CBD and CBDV potentiate I<sub>GABA</sub> mediated by α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub> receptors

The GABA<sub>A</sub>R α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> subunit combination is thought to be the major receptor subtype mediating fast synaptic inhibition in the mature brain. I thus examined the effect of the cannabinoids on this receptor subtype. The α and β subunits are required for agonist binding and the resulting GABA-mediated current. Hence, cells that responded to GABA were considered to express both α and β subunits. The γ<sub>2</sub> subunit is necessary to form the classical benzodiazepine binding site located at the interface of the α and γ subunits (Pritchett et al. 1989, Sigel and Ernst 2018). As such, diazepam (DZP) was used to confirm the presence of this subunit in recorded
cells. DZP treatment resulted in a reversible potentiation of \( I_{\text{GABA}} \) in those cells expressing the \( \gamma_2 \) subunit.

A time course of CBD- and CBDV-mediated facilitation of \( I_{\text{GABA}} \) at different cannabinoid concentrations is shown in Figure 9. For clarity, DZP-induced potentiation of \( I_{\text{GABA}} \) is not shown on these graphs. At 10 \( \mu \text{M} \), CBD had a significant effect on \( I_{\text{GABA}} \), enhancing the current amplitude to \( 225 \pm 21\% \) of the control amplitude (paired \( t \)-test, \( P = 0.0002 \), \( n = 10 \), Figure 9A). However, this potentiation was transient, as current amplitude steadily decreased despite continuous superfusion of CBD. On the other hand, lower concentrations of CBD did not result in a significant effect. That said, prolonged exposure to these low cannabinoid concentrations appeared to induce a small and sustained \( I_{\text{GABA}} \) potentiation, best observed with 3 \( \mu \text{M} \) CBD. After 10 min of CBD (3 \( \mu \text{M} \)) exposure, \( I_{\text{GABA}} \) was potentiated to \( 140 \pm 12\% \) of the control amplitude (paired \( t \)-test, \( P = 0.0196 \), \( n = 6 \), Figure 9A). In contrast, 10 \( \mu \text{M} \) CBD at the same 10 min time point no longer had a potentiating effect on \( I_{\text{GABA}} \) (110 \( \pm \) 20\% of control amplitude, paired \( t \)-test, \( P = 0.498 \), \( n = 10 \)).

CBDV had a stronger potentiating effect on \( \alpha_1\beta_2\gamma_2 \)-mediated \( I_{\text{GABA}} \) compared with CBD (unpaired \( t \)-test, \( P = 0.011 \)) as 10 \( \mu \text{M} \) CBDV enhanced the current amplitude to \( 423 \pm 70\% \) of the control (paired \( t \)-test, \( P = 0.0017 \), \( n = 9 \), Figure 9B) – nearly double the effect of CBD. Again, this cannabinoid-mediated potentiation was transient, despite the continuous presence of CBDV. Unlike with CBD, however, \( I_{\text{GABA}} \) potentiation was also present at lower concentrations of CBDV and the effect of this cannabinoid was dose-dependent, with 3 \( \mu \text{M} \) CBDV potentiating \( I_{\text{GABA}} \) to \( 157 \pm 19\% \) of control amplitude (paired \( t \)-test, \( P = 0.021 \), \( n = 6 \), Figure 9B) and 5 \( \mu \text{M} \) enhancing currents to \( 238 \pm 43\% \) (paired \( t \)-test, \( P = 0.015 \), \( n = 7 \), Figure 9B). Moreover, both of these concentrations resulted in a sustained enhancement of \( I_{\text{GABA}} \), in contrast to the declining effect observed with 10 \( \mu \text{M} \) CBDV. After 10 min of continuous exposure to 3 and 5 \( \mu \text{M} \) CBDV, \( I_{\text{GABA}} \) was potentiated to \( 178 \pm 16\% \) (paired \( t \)-test, \( P = 0.005 \), \( n = 6 \)) and \( 189 \pm 31\% \) of the control amplitude (paired \( t \)-test, \( P = 0.024 \), \( n = 7 \)), respectively.
Higher concentrations of either CBD or CBDV could not be tested due to limited solubility of the compounds in the external bath solution. Moreover, these compounds are highly lipophilic which made them difficult to washout. Consequently, a full dose-response curve could not be generated. CBD clinical plasma exposures are likely to be in the range of 1 μM (estimated from data presented in GW Pharmaceuticals (2018a) and Devinsky et al. (2018b)). However, phytocannabinoid concentrations lower than 10 μM did not have an immediate and pronounced effect on $I_{\text{GABA}}$ (Figure 9). As such, any difference in effects between CBD and CBDV, or any variations across different $\text{GABA}_A\text{R}$ subtypes might not be detected. Moreover, Bakas et al. (2017) described CBD modulation of $I_{\text{GABA}}$ at 10 μM. Thus, a concentration of 10 μM CBD or CBDV was used in all subsequent experiments as this gave rise to a clear, strong and transient $I_{\text{GABA}}$ potentiation by both compounds (Figure 9).
Figure 9. Time course of CBD and CBDV-induced modulation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the GABA₆R α₁β₂γ₂ subtype. (A, B) CBD and CBDV were continuously bath applied at different concentrations. DZP potentiation preceding cannabinoid treatment is not shown. Data shown as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 (paired t-test). CBD: 1 µM (n = 7), 3 µM (n = 6), 5 µM (n = 7), 10 µM (n = 10); CBDV: 1 µM (n = 5), 3 µM (n = 6), 5 µM (n = 6), 10 µM (n = 9).

The full time course for the 10 µM CBD and CBDV responses, including the control DZP effect observed prior to cannabinoid treatment, is shown in Figure 10. The maximum potentiation of GABA-evoked currents was reached within 2-3 min following the start of cannabinoid application. Whereas CBD enhanced I_GABA to a similar level as DZP (225 ± 21% versus 193 ± 6%, paired t-test, P = 0.27, n = 10, Figure 10B), CBDV treatment resulted in a current with twice the amplitude of the DZP enhanced current (423 ± 70% versus 185 ± 15%, paired t-test, P = 0.0083, n = 9, Figure 10C). The transient cannabinoid-induced I_GABA potentiation previously highlighted contrasted with the sustained I_GABA enhancement by DZP (Figure 10A).
Figure 10. Time course of DZP, CBD and CBDV-induced potentiation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the GABA₉R α₁β₁γ₂ subtype. (A) Bath application of 1 μM DZP resulted in a sustained and reversible I₉GABA potentiation (n = 8). Representative current traces prior to and during DZP application are shown in the right panels. (B, C) DZP was applied for 3 min prior to cannabinoids in all experiments as a test for the expression of the γ₂ subunit. Bath application of 10 μM CBD (n = 10) or 10 μM CBDV (n = 9) produced a transient potentiation of I₉GABA despite continuous cannabinoid application. Representative current traces prior to and during cannabinoid application are shown in the right panels. For both sets of data, the x-axis was discontinued during the washout of DZP, as indicated. Data were re-normalised to control values preceding cannabinoid exposure and are shown as mean ± SEM; **P < 0.01, ***P < 0.001 (paired t-test).
3.1.3 CBD and CBDV potentiate $I_{\text{GABA}}$ mediated by $\alpha_2\beta_2\gamma_2$ GABA$_A$ receptors

Next, the time course of CBD and CBDV-mediated facilitation of $I_{\text{GABA}}$ was examined in HEK293 cells expressing the GABA$_A$R $\alpha_2\beta_2\gamma_2$ subtype. A dose-dependent effect was observed, with increasing concentrations of CBD and CBDV resulting in a greater potentiation of $I_{\text{GABA}}$ (Figure 11). DZP-induced potentiation of $I_{\text{GABA}}$, used as a control for the presence of the $\gamma_2$ subunit, is not shown in Figure 11. At 10 μM, CBD enhanced the $I_{\text{GABA}}$ amplitude to 190 ± 36% of the control amplitude (paired t-test, $P = 0.033$, $n = 9$, Figure 11A). This CBD-induced potentiation appeared to be more sustained than that observed in HEK293 cells expressing $\alpha_1$-containing GABA$_A$Rs, where $I_{\text{GABA}}$ returned to control levels 7 min after the start of CBD application (Figure 10B). Nonetheless, the potentiation of $I_{\text{GABA}}$ in HEK293 cells expressing $\alpha_2\beta_2\gamma_2$ steadily decreased back to control levels after 12 min of continued CBD application (Figure 11A). Lower CBD concentrations had no immediate significant effect on $I_{\text{GABA}}$. However, prolonged exposure to these low cannabinoid concentrations appeared to result in a slow and gradual potentiation of $I_{\text{GABA}}$, best observed with 3 μM CBD. After 10 min of exposure to 3 μM CBD, $I_{\text{GABA}}$ was potentiated to 125 ± 9% of the control amplitude (paired t-test, $P = 0.0235$, $n = 5$, Figure 11A).

As previously noted with $\alpha_1$-containing GABA$_A$Rs, CBDV had a stronger potentiating effect on $\alpha_2\beta_2\gamma_2$-mediated $I_{\text{GABA}}$ than CBD (unpaired t-test, $P = 0.023$), with 10 μM CBDV enhancing the current amplitude to 303 ± 26% of control (paired t-test, $P < 0.0001$, $n = 9$, Figure 11B). The maximum potentiation of GABA-evoked currents was reached within 2-3 min following the start of CBDV application. Again, this cannabinoid-mediated potentiation was transient despite the continuous presence of CBDV, with $I_{\text{GABA}}$ returning to control levels 10 min following CBDV application (125 ± 17%, paired t-test, $P = 0.15$, Figure 11B). This transient effect contrasted with the sustained $I_{\text{GABA}}$ potentiation observed with DZP (Figure 12A). Lower concentrations of 3 and 5 μM CBDV also enhanced $I_{\text{GABA}}$ to 143 ± 14% (paired t-test, $P = 0.0174$, $n = 6$) and 255 ± 28% of control (paired t-test, $P = 0.0017$, $n = 7$), respectively (Figure 11B). Currents remained potentiated 10 min after the start of 3 and 5 μM CBDV application at 157 ± 17% (paired t-test, $P = 0.014$) and 195 ± 14% of control amplitude (paired t-test, $P = 0.0004$), respectively.
CBD had a smaller potentiating effect on $I_{\text{GABA}}$ mediated by $\alpha_2$-containing $\text{GABA}_A$Rs when compared with DZP (190 ± 36% versus 376 ± 33%, paired t-test, $P < 0.0001$, $n = 9$, Figure 12B). However, CBDV potentiated $I_{\text{GABA}}$ to the same extent as DZP (303 ± 26% and 350 ± 29%, paired t-test, $P = 0.196$, $n = 9$, Figure 12C).
Figure 12. Time course of DZP, CBD and CBDV-induced potentiation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the GABA<sub>A</sub>R α₂β₂γ₂ subtype. (A) Bath application of 1 µM DZP resulted in a sustained and reversible I<sub>GABA</sub> potentiation (n = 9). Representative current traces prior to and during DZP application are shown in the right panels. (B, C) DZP was applied for 3 min prior to cannabinoids in all experiments so as to test for the expression of the γ₂ subunit. Bath application of 10 µM CBD (n = 9) or 10 µM CBDV (n = 9) produced a transient potentiation of I<sub>GABA</sub> despite continuous cannabinoid application. Representative current traces prior to and during cannabinoid application are shown in the right panels. For both sets of data, the x-axis was discontinued during the washout of DZP, as indicated. Data were re-normalised to control values preceding cannabinoid exposure and are shown as mean ± SEM; *P < 0.05, ***P < 0.001, ****P < 0.0001 (paired t-test).
3.1.4 $I_{GABA}$ potentiation by CBD and CBDV is independent of the benzodiazepine binding site

The different time course profiles observed for DZP and the phytocannabinoids CBD and CBDV, where the former showed a sustained DZP-induced $I_{GABA}$ potentiation and the latter a transient effect, suggest that the cannabinoid mechanism of action is distinct from that of benzodiazepines. Thus, in order to verify that the GABA$_{\alpha}$R benzodiazepine binding site is different to that mediating the action of CBD and CBDV, the cannabinoids were tested on a benzodiazepine-insensitive GABA$_{\alpha}$R. The classical benzodiazepine binding site is located at the interface of the $\alpha$ and $\gamma$ subunits (Sigel and Ernst 2018). GABA$_{\alpha}$Rs containing the $\alpha_4$ and $\alpha_6$ subunits show DZP insensitivity due to a specific arginine residue in the N-terminal region of the polypeptide (Wieland et al. 1992, Benson et al. 1998). Mutation of the homologous histidine residue in the $\alpha_2$, $\alpha_3$, $\alpha_5$ and $\alpha_5$ subunits to arginine renders the receptors insensitive to DZP (Wieland et al. 1992, Benson et al. 1998). Site-directed mutagenesis was used to generate a GABA$_{\alpha}$R $\alpha_1$ subunit with the H102R mutation (Figure 13) and the effect of CBD and CBDV on the mutant GABA$_{\alpha}$R $\alpha_1^{H102R}\beta_2\gamma_2$ was examined.

**Figure 13. Illustration of mutant GABA$_{\alpha}$R $\alpha_1^{H102R}\beta_2\gamma_2$.** The diagram illustrates the location of the amino acid residue critical for benzodiazepine binding on the $\alpha_1$ subunit. Site-directed mutagenesis was used to mutate this histidine residue into an arginine, forming a benzodiazepine-insensitive $\alpha$ subunit.
First, DZP was tested on HEK293 cells expressing GABA$_4$R $\alpha_2^{H102R}\beta_2\gamma_2$ to ensure the mutation successfully abolished benzodiazepine-mediated $I_{GABA}$ potentiation. Indeed, no increase in $I_{GABA}$ was observed following bath application of DZP. If anything, a slight decrease in $I_{GABA}$ was measured on DZP application which might be due to non-specific binding at non-classical benzodiazepine sites, though this was not significant (61 ± 12% of control amplitude, paired $t$-test, $P = 0.061$, $n = 4$, Figure 14A).

On the other hand, continuous application of either CBD or CBDV to HEK293 cells expressing $\alpha_1^{H102R}\beta_2\gamma_2$ resulted in a potentiation of $I_{GABA}$ that persisted for at least 12 min (Figure 14). CBD enhanced the current amplitude to 187 ± 13% of control levels (paired $t$-test, $P < 0.0001$, $n = 10$, Figure 14B) and CBDV enhanced $I_{GABA}$ to 314 ± 56% (paired $t$-test, $P = 0.0099$, $n = 7$, Figure 14C). This level of potentiation was similar to that observed with the wild-type $\alpha_1\beta_2\gamma_2$ receptor. This strongly suggests the GABA$_4$R benzodiazepine binding site does not mediate the action of CBD or CBDV. However, the prolonged cannabinoid-mediated $I_{GABA}$ potentiation seen with $\alpha_1^{H102R}\beta_2\gamma_2$ contrasted with previous data from wild-type $\alpha_1\beta_2\gamma_2$ receptors. In HEK293 cells expressing the latter receptors, CBD-induced $I_{GABA}$ potentiation declined to control levels following 7-8 min of cannabinoid application and CBDV-mediated $I_{GABA}$ potentiation decreased to 50% of peak potentiation following 12 min of cannabinoid exposure (Figure 10). The differential cannabinoid effects on GABA$_4$R $\alpha_1\beta_2\gamma_2$ compared to the $\alpha_1^{H102R}\beta_2\gamma_2$ mutant suggest that the benzodiazepine binding site is important in shaping the rate of decay of $I_{GABA}$ potentiation brought about by the cannabinoids.
Figure 14. Time course of CBD and CBDV-induced modulation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the mutant GABAₐR α₁⁺H102Rβ₂γ₂ subtype. (A) Bath application of 1 μM DZP did not lead to I₇GABA potentiation in HEK293 cells expressing the benzodiazepine-insensitive GABAₐR α₁⁺H102Rβ₂γ₂ subtype (n = 4). (B, C) Bath application of 10 μM CBD (n = 10) or 10 μM CBDV (n = 7) potentiated I₇GABA. Data presented as mean ± SEM; **P < 0.01, ****P < 0.0001 (paired t-test).
3.1.5 The γ₂ subunit is not necessary for CBD and CBDV potentiation of I_{GABA}

As indicated above, the benzodiazepine binding site, thought to be located at the interface of the α and γ subunits, is not required for cannabinoid-induced potentiation of I_{GABA}. This raises the question of whether the γ₂ subunit is necessary to mediate CBD and CBDV actions on GABAₐRs. The effect of these two compounds was thus tested on receptors composed only of the α and β subunit, α₂β₂.

![Figure 15](image-url)

**Figure 15.** Time course of CBD and CBDV-induced modulation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the GABAₐR α₁β₂ subtype. (A, B) Continuous bath application of 10 μM CBD (n = 8) or 10 μM CBDV (n = 9) resulted in a significant potentiation of I_{GABA} in HEK293 cells expressing the GABAₐR α₁β₂ subtype, which lacks the classical benzodiazepine binding site. Data presented as mean ± SEM; *P < 0.05, **P < 0.01 (paired t-test).
Both CBD and CBDV potentiated $I_{\text{GABA}}$ recorded from cells expressing $\alpha_1\beta_2$ GABA$_A$Rs. CBD potentiated the current amplitude to $188 \pm 27\%$ of the control amplitude (paired $t$-test, $P = 0.015$, $n = 8$, Figure 15A) and CBDV potentiated to $184 \pm 25\%$ of the control amplitude (paired $t$-test, $P = 0.0045$, $n = 9$, Figure 15B). This indicates that the $\gamma_2$ subunit is not required for cannabinoids to exert their effect on GABA$_A$Rs. Specifically, CBD had a similar action on benzodiazepine-insensitive receptors composed of $\alpha_1\beta_2$ or $\alpha_1^{H1028}\beta_2\gamma_2$, causing a more prolonged $I_{\text{GABA}}$ potentiation than that observed with the wild-type $\alpha_1\beta_2\gamma_2$ receptor (Figure 16A). This is highlighted in the plotted decay rates of the potentiated currents in Figure 16B. The benzodiazepine-insensitive $\alpha_1\beta_2$ or $\alpha_1^{H1028}\beta_2\gamma_2$ GABA$_A$Rs have a similar profile that differs from $\alpha_1\beta_2\gamma_2$. This suggests the benzodiazepine binding site is important for ending the potentiating effect of CBD on GABA$_A$Rs.

Although CBDV enhanced $I_{\text{GABA}}$ mediated by $\alpha_1\beta_2$ receptors, the maximal potentiation was significantly smaller than that observed for wild-type $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs ($184 \pm 25\%$ versus $423 \pm 70\%$, one-way ANOVA $F(2,22)=5.39$, $P = 0.012$, Bonferroni post hoc analysis, $P = 0.01$, Figure 16C). This result suggests the $\gamma_2$ subunit is important for the efficacy of CBDV. However, the profiles for the rate of decay of CBDV-induced potentiation of $I_{\text{GABA}}$ were similar for benzodiazepine-insensitive $\alpha_1\beta_2$ or $\alpha_1^{H1028}\beta_2\gamma_2$ and wild-type $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs (Figure 16D). This is unlike the CBD data and suggests the benzodiazepine binding site plays a different modulatory role, if any, on the CBDV response.
Figure 16. Time course of CBD and CBDV-induced modulation of GABA-mediated Cl− currents in HEK293 cells expressing wild-type or benzodiazepine-insensitive GABA_A Rs. (A) Continuous bath application of 10 μM CBD resulted in the potentiation of I_GABA in HEK293 cells expressing GABA_A R α1β2γ2 (n = 10), α1β2 (n = 8), and α1β2γ2 H102R (n = 10). As the time course shows, this effect is more sustained with the benzodiazepine-insensitive receptors. Note that all time course profiles included here were individually presented in previous figures. (B) Rate of decay of the effect of CBD on I_GABA measured in cells expressing different GABA_A Rs. (C) Continuous bath application of 10 μM CBDV resulted in the potentiation of I_GABA in HEK293 cells expressing GABA_A R α1β2γ2 (n = 9), α1β2 (n = 9) and α1β2γ2 H102R (n = 7). Note that all time course profiles included here were individually presented in previous figures. One-way ANOVA F(2,22)=5.39, P = 0.012, Bonferroni post hoc analysis α1β2γ2 versus α1β2, P = 0.01. (D) Decay rates of CBDV-induced I_GABA potentiation were similar across the benzodiazepine-insensitive and wild-type GABA_A Rs. Data presented as mean ± SEM.
3.1.6 Radioligand binding assays confirm that CBD and CBDV do not bind to the benzodiazepine binding site, but do displace picrotoxin site ligands

The data presented above indicate that the potentiating effects of CBD and CBDV on $I_{\text{GABA}}$ do not depend on the GABA$_{\alpha}$R benzodiazepine binding site. To substantiate this result, radioligand competition binding assays were undertaken by GW Pharmaceuticals. Increasing concentrations of both CBD and CBDV failed to displace radiolabelled $[^3]$H flunitrazepam from the benzodiazepine binding site on the $\alpha_3\beta_2\gamma_2$ receptor (Figure 17B). The compounds were also unable to displace radiolabelled $[^3]$H muscimol from the orthosteric GABA binding site, as well as radiolabelled $[^3]$H GABA bound to the GABA transporter (Figure 17A and 17D). On the other hand, both CBD and CBDV displaced radiolabelled $[^35]$S tert-butilbicyclophosphorothionate (TBPS), a high-affinity ligand for the picrotoxin site of the GABA$_{\alpha}$R (Figure 17C). Picrotoxin was previously thought to act by obstructing the ion channel pore, however, a recent cryo-electron microscopy study suggested that picrotoxin binding to the inner wall of the channel induces a closed pore conformation (Masiulis et al. 2019). Amino acids thought to participate in the binding of this noncompetitive antagonist are located at the cytoplasmic end of the channel wall in TM2 (Chen et al. 2006, Masiulis et al. 2019).
Figure 17. Radioligand competition binding assay. The data show specific binding of radioligand in the presence of increasing concentrations of either CBD or CBDV. (A) CBD and CBDV failed to displace \([^{3}H]\) muscimol bound to the orthosteric GABA binding site of GABA\(_{A}\)R \(\alpha_1\beta_2\gamma_2\) at concentrations up to 10 \(\mu M\); \(n = 2\). (B) CBD and CBDV failed to displace \([^{3}H]\) flunitrazepam bound to the benzodiazepine binding site of GABA\(_{A}\)R \(\alpha_1\beta_2\gamma_2\) at concentrations up to 10 \(\mu M\); \(n = 2\). (C) Both CBD and CBDV displaced \([^{35}S]\) TBPS bound to the GABA\(_{A}\)R \(\alpha_1\beta_2\gamma_2\) Cl\(^{-}\) channel with an \(IC_{50}\) of 2.57 \(\mu M\) and 2.47 \(\mu M\), respectively; \(n = 2\). (D) CBD and CBDV failed to displace \([^{3}H]\) GABA bound to the GABA transporter at concentrations up to 10 \(\mu M\); \(n = 2\). Data presented as mean ± SEM.
3.1.7 Additive modulation of $I_{GABA}$ by cannabinoids and diazepam

As established above, CBD and CBDV do not bind the benzodiazepine site. There is thus a potential for pharmacological interaction between the cannabinoids and benzodiazepines, whereby each class of compound binds distinct sites on the $\text{GABA}_A R$. Additive modulation, in particular, would be an interesting outcome in a clinical context where adjunct therapies could provide additional relief of symptoms. The effect of co-application of DZP and CBD or DZP and CBDV was thus examined on HEK293 cells expressing either the $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$ receptor subtype.

DZP was first applied in order to confirm cell surface expression of the $\gamma_2$ subunit, and record the resulting $I_{GABA}$ potentiation by benzodiazepine. Following the washout of DZP, CBD or CBDV was applied for 3 min followed by a mixture of cannabinoid and DZP. In HEK293 cells expressing $\alpha_1\beta_2\gamma_2$, CBD potentiated $I_{GABA}$ to a similar extent as DZP ($254 \pm 24\%$ versus $273 \pm 20\%$, $n = 12$, Figure 18). Co-application of CBD and DZP resulted in further enhancement of $I_{GABA}$, the magnitude of which was equivalent to the sum of the effects of DZP and CBD tested separately ($494 \pm 130\%$, repeated measures one-way ANOVA $F(1.07,11.74)=6.4$, $P = 0.0253$, $n = 12$). Thus, it appears both classes of drugs can simultaneously act on the receptor and have additive effects. However, the larger $I_{GABA}$ potentiation observed during co-application of CBD and DZP was transient, declining to basal levels within a few minutes of superfusing the mixture. Similar findings were observed in HEK293 cells expressing $\alpha_2\beta_2\gamma_2$, with the exception that CBD-induced potentiation of $I_{GABA}$ was lower than that observed with DZP alone ($157 \pm 23\%$ versus $354 \pm 50\%$, $n = 4$, Figure 19). A mixture of CBD and DZP potentiated $I_{GABA}$ to $489 \pm 98\%$ of control amplitude (repeated measures one-way ANOVA $F(1.13,3.39)=8.72$, $P = 0.0505$, $n = 4$).

It should be noted that previous experiments revealed the transient nature of the cannabinoid-mediated potentiation of $I_{GABA}$ in HEK293 cells expressing $\text{GABA}_A R$ subtypes $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$. Indeed, a current run-down was noticeable as early as 3 min after the start of CBD or CBDV application (Figure 10 and 12). In the additive experiments detailed above, a mixture of cannabinoid and DZP was applied following 3 min of cannabinoid application alone. The current run-down anticipated
to occur around this time point may have limited any additive effect caused by the mixture of drugs or even concealed any synergistic relation between DZP and CBD/CBDV. In theory, the order of DZP and cannabinoid application should be reversed such that CBD/CBDV is applied first and washed out before application of DZP. Application of the cannabinoid-DZP mixture would then follow. However, this approach was not taken due to the lipophilic nature of the cannabinoids and thus the unreliable wash out process. As a result, potentiation of $I_{GABA}$ by the mixture of cannabinoid and DZP may be an underestimate of the actual additive response.
Figure 18. Additive modulation of $I_{\text{GABA}}$ by CBD and DZP in HEK293 cells expressing the GABA$_{A}$R $\alpha_{1}\beta_{2}\gamma_{2}$ subtype. (A) Time course of $I_{\text{GABA}}$ potentiation induced by successive application of DZP, CBD and a mixture of DZP and CBD. DZP was applied for 3-4 min and led to an increase in $I_{\text{GABA}}$ in cells expressing $\alpha_{1}\beta_{2}\gamma_{2}$. Following the washout of DZP, CBD application produced a similar enhancing effect on $I_{\text{GABA}}$. CBD was applied alone for 3 min before perfusion of CBD and DZP together. This mixture further potentiated $I_{\text{GABA}}$. The red lines represent the average maximal $I_{\text{GABA}}$ potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell are shown in the top panels. (B) Bar graph summarising the level of $I_{\text{GABA}}$ potentiation obtained in the presence of DZP and CBD applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of DZP and CBD. Combined cannabinoid and DZP treatment resulted in further enhancement of $I_{\text{GABA}}$ when compared to either compound alone. Data were re-normalized to control values preceding CBD exposure and are presented as mean ± SEM, repeated measures one-way ANOVA $F(1.07,11.74)=6.4$, $P = 0.0253$, Bonferroni post hoc analysis *** $P < 0.001$, **** $P < 0.0001$ versus control, n = 12.
Figure 19. Additive modulation of $I_{\text{GABA}}$ by CBD and DZP in HEK293 cells expressing the GABA\(_A\)R \(\alpha_2\beta_2\gamma_2\) subtype. (A) Time course of $I_{\text{GABA}}$ potentiation induced by successive application of DZP, CBD and a mixture of DZP and CBD. DZP was applied for 3-4 min and led to an increase in $I_{\text{GABA}}$ in cells expressing \(\alpha_2\beta_2\gamma_2\). Following the washout of DZP, CBD application potentiated $I_{\text{GABA}}$ to a lesser extent than DZP. CBD was applied alone for 3 min before perfusion of CBD and DZP together. This mixture further potentiated $I_{\text{GABA}}$. The red lines represent the average maximal $I_{\text{GABA}}$ potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell are shown in the top panels. (B) Bar graph summarising the level of $I_{\text{GABA}}$ potentiation obtained in the presence of DZP and CBD applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of DZP and CBD. Combined cannabinoid and DZP treatment resulted in further enhancement of $I_{\text{GABA}}$ when compared to either compound alone. Data were re-normalized to control values preceding CBD exposure and are presented as mean ± SEM, repeated measures one-way ANOVA $F(1.13,3.39)=8.72$, $P = 0.0505$, $n = 4$. 
With regards to CBDV, no additive effect was observed upon co-application of the cannabinoid and DZP in cells expressing α₁β₂γ₂ (Figure 20). DZP potentiated I_GABA to 291 ± 43% of control amplitude and CBDV enhanced it to 486 ± 89%. A mixture of both compounds potentiated I_GABA to 608 ± 159% (repeated measures one-way ANOVA F(1,20,3.60)=9.53, P = 0.0407, n = 4). On the other hand, DZP and CBDV did have an additive effect on I_GABA, in cells expressing α₂β₂γ₂, that matched the sum of effects induced by each compound applied individually (Figure 21). DZP potentiated I_GABA to 411 ± 75% of control amplitude and CBDV potentiated currents to 359 ± 75%. A mixture of the two compounds enhanced I_GABA to 767 ± 102% (repeated measures one-way ANOVA F(1,63,6.499)=20.51, P = 0.0019, Bonferroni post hoc analysis, P = 0.016 versus control, n = 5, Figure 21). However, this potentiation was transient as I_GABA levels steadily decreased despite continuous application of both CBDV and DZP. It appeared that the presence of the cannabinoids, whether CBD or CBDV, curtailed the sustained I_GABA potentiation ordinarily observed with DZP.
Figure 20. Additive modulation of $I_{\text{GABA}}$ by CBDV and DZP in HEK293 cells expressing the GABA$_A$R $\alpha_1\beta_2\gamma_2$ subtype. (A) Time course of $I_{\text{GABA}}$ potentiation induced by successive application of DZP, CBDV and a mixture of DZP and CBDV. DZP was applied for 3-4 min and led to an increase in $I_{\text{GABA}}$ in cells expressing $\alpha_1\beta_2\gamma_2$. Following the washout of DZP, CBDV application potentiated $I_{\text{GABA}}$ to a similar extent. CBDV was applied alone for 3 min before perfusion of CBDV and DZP together. This mixture did not have a stronger effect on $I_{\text{GABA}}$. The red lines represent the average maximal $I_{\text{GABA}}$ potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell are shown in the top panels. (B) Bar graph summarising the level of $I_{\text{GABA}}$ potentiation obtained in the presence of DZP and CBDV applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of DZP and CBDV. Combined cannabinoid and DZP treatment did not enhance $I_{\text{GABA}}$ further when compared to either compound alone. Data were re-normalized to control values preceding CBDV exposure and are presented as mean ± SEM, repeated measures one-way ANOVA $F(1.20,3.60)=9.53$, $P=0.0407$, $n=4$. 
Figure 21. Additive modulation of $I_{\text{GABA}}$ by CBDV and DZP in HEK293 cells expressing the $\text{GABA}_{\alpha2\beta2\gamma2}$ subtype. (A) Time course of $I_{\text{GABA}}$ potentiation induced by successive application of DZP, CBDV and a mixture of DZP and CBDV. DZP was applied for 3-4 min and led to an increase in $I_{\text{GABA}}$ in cells expressing $\alpha2\beta2\gamma2$. Following the washout of DZP, CBDV application potentiated $I_{\text{GABA}}$ to a similar extent. CBDV was applied alone for 3 min before perfusion of CBDV and DZP together. This mixture further potentiated $I_{\text{GABA}}$. The red lines represent the average maximal $I_{\text{GABA}}$ potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell are shown in the top panels. (B) Bar graph summarising the level of $I_{\text{GABA}}$ potentiation obtained in the presence of DZP and CBDV applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of DZP and CBDV. Combined cannabinoid and DZP treatment had a stronger potentiating effect on $I_{\text{GABA}}$ when compared to either compound alone. Data were re-normalized to control values preceding CBDV exposure and are presented as mean ± SEM, repeated measures one-way ANOVA $F(1.63,6.499)=20.51$, $P = 0.0019$, Bonferroni post hoc analysis * $P < 0.05$, ** $P < 0.01$, n = 5.
3.1.8 Modulation of $I_{GABA}$ by CBD and clobazam

Clobazam (CLB) is a 1,5-benzodiazepine approved for the treatment of DS and LGS (Sankar 2012). Compared with the 1,4-benzodiazepine DZP, CLB appears to induce less severe side effects such as sedation and is thus favoured as an adjunct treatment for the long term management of paediatric epilepsy disorders (Sankar 2012). Indeed, up to 60% of patients in the recent CBD clinical trials for DS and LGS were on a CLB regimen (Devinsky et al. 2017, 2018a, Thiele et al. 2018). As such, CLB was tested on GABA_A Rs in combination with CBD so as to investigate any synergistic effects.

A prolonged application of 1 μM CLB resulted in a sustained $I_{GABA}$ potentiation in HEK293 cells expressing either $\alpha_1\beta_2\gamma_2$ (264 ± 47% of control amplitude, paired t-test, $P = 0.011$, n = 7, Figure 22A) or $\alpha_2\beta_2\gamma_2$ GABA_A Rs (340 ± 43%, paired t-test, $P = 0.0005$, n = 10, Figure 22B).

In subsequent experiments, CLB was first applied for 4 min and washed out. CBD was then applied alone for 4 min, before co-application of CBD with CLB. In HEK293 cells expressing $\alpha_1\beta_2\gamma_2$, superfusion of the two compounds potentiated $I_{GABA}$ to 250 ± 46% of control amplitude. This was similar to the potentiation induced by CLB alone (228 ± 39% of control amplitude). No additive effect was thus observed (repeated measures, one-way ANOVA $F(1.17,3.50)=4.62$, $P = 0.107$, n = 4, Figure 23). In cells expressing $\alpha_2\beta_2\gamma_2$, there was a trend for co-application of CLB and CBD to potentiate $I_{GABA}$ further than the potentiation observed with either compound applied alone (repeated measures, one-way ANOVA $F(1.399, 12.59)=19.97$, $P = 0.0003$, n = 10, Figure 24). In these cells, CLB potentiated currents to 291 ± 24% of control amplitude whilst CBD had a minimal effect (116 ± 11%). A mixture of CLB and CBD enhanced $I_{GABA}$ to 370 ± 57%, which was in the range of CLB-induced potentiation. Exposure to the cannabinoid, however, seemed to cause a steady decrease in $I_{GABA}$. 

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Figure 22. Time course of CLB-induced potentiation of GABA-mediated Cl currents in HEK293 cells expressing GABA_{\alpha}\beta_{\gamma} or \alpha_{2}\beta_{2}\gamma_{2} subtypes. (A, B) Continuous bath application of 1 \text{\mu M} CLB resulted in a sustained and reversible $I_{GABA}$ potentiation in HEK293 cells expressing $\alpha_{1}\beta_{2}\gamma_{2}$ ($n = 7$) or $\alpha_{2}\beta_{2}\gamma_{2}$ ($n = 10$) GABA_{\alpha}Rs. Data presented as mean ± SEM.

It should be noted that the effect of CBD in these experiments was lower compared to previous experiments where DZP, and not CLB, was first applied. This could be due to residual CLB altering the response of the receptor to CBD, for example, by causing receptor desensitisation.
Figure 23. Modulation of I_{GABA} by CBD and CLB in HEK293 cells expressing α1β2γ2 GABA_{A}Rs.

(A) Time course of I_{GABA} potentiation induced by successive application of CLB, CBD and a mixture of CLB and CBD. CLB was applied for 4 min before washout, indicated by the discontinued x-axis. Data were re-normalized to control values preceding CBD exposure. CBD was individually applied for 4 min before co-application with CLB. The red lines represent the average maximal I_{GABA} potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell, taken from each treatment phase indicated by the numbers 1-4, are shown in the top panels.

(B) Bar graph summarising the level of I_{GABA} potentiation obtained in the presence of CLB and CBD applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of CLB and CBD. Combined cannabinoid and CLB treatment did not enhance I_{GABA} to a greater level than CLB applied alone. Data presented as mean ± SEM, repeated measures one-way ANOVA F(1.17,3.50)=4.62, P = 0.107, n = 4.
Figure 24. Modulation of $I_{\text{GABA}}$ by CBD and CLB in HEK293 cells expressing $\alpha_2\beta_2\gamma_2$ GABA$_A$Rs.

(A) Time course of $I_{\text{GABA}}$ potentiation induced by successive application of CLB, CBD and a mixture of CLB and CBD. CLB was applied for 4 min before washout, indicated by the discontinued x-axis. Data were re-normalized to control values preceding CBD exposure. CBD was individually applied for 4 min before co-application with CLB. The red lines represent the average maximal $I_{\text{GABA}}$ potentiation for each treatment. These values were used to plot the bar graph. Representative current traces from one cell, taken from each treatment phase indicated by the numbers 1-4, are shown in the top panels. (B) Bar graph summarising the level of $I_{\text{GABA}}$ potentiation obtained in the presence of CLB and CBD applied separately or simultaneously. The superimposed bars represent the arithmetic sum of the effects of CLB and CBD. Data presented as mean ± SEM, repeated measures one-way ANOVA $F(1.399, 12.59)=19.97$, $P = 0.0003$, Bonferroni post hoc analysis ** $P < 0.01$, *** $P < 0.001$, $n = 10$. 
3.1.9 CBD and CBDV potentiate $I_{\text{GABA}}$ mediated by $\beta_1$- and $\beta_3$-containing GABA$_A$ receptors

The GABA$_A$R subtypes tested above all contained a $\beta_2$ subunit which is thought to be the most abundant and often associated with $\alpha_1$ and $\gamma_2$ subunits in vivo (Sur et al. 2001). A study reported that $I_{\text{GABA}}$ potentiation by the cannabinoid 2-AG is dependent on the $\beta$ subunit present. According to Sigel et al. (2011), 2-AG has little or no effect on GABA$_A$Rs containing the $\beta_1$ or $\beta_3$ subunit and is selective for $\beta_2$-containing receptors. Alignment of the three GABA$_A$R $\beta$ subunits, and scanning for differences between these, helped the authors pinpoint a particular residue in the $\beta_2$ subunit TM4, V436, as important for mediating the effect of 2-AG. Further, the authors found that the $\beta_2^{V436T}$ mutation rendered the receptor insensitive to 2-AG. This led me to interrogate whether CBD and CBDV-induced potentiation of $I_{\text{GABA}}$ is also dependent on the type of $\beta$ subunit present in the receptor. The effect of CBD on the mutant GABA$_A$R subtype $\alpha_1\beta_2^{V436T}\gamma_2$ was also examined.

In order to reproduce the results described in the Sigel et al. (2011) study, 2-AG was tested on HEK293 cells expressing either $\beta_1$- or $\beta_2$-containing GABA$_A$Rs. For these experiments, a GABA puff concentration of 10 $\mu$M was used instead of 1 mM so as to approximate the 1 $\mu$M GABA concentration used in Sigel et al. (2011). Initial testing of 10 $\mu$M 2-AG, bath applied to HEK293 cells expressing $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs, did not potentiate $I_{\text{GABA}}$ (95 ± 8% of control amplitude, paired $t$-test, $P = 0.77$, $n = 4$). The liquid form of 2-AG had greater solubility in aqueous conditions compared to CBD and CBDV, allowing it to be used at concentrations up to 100 $\mu$M. In HEK293 cells expressing $\alpha_1\beta_2\gamma_2$, bath application of 100 $\mu$M 2-AG potentiated $I_{\text{GABA}}$ to 372 ± 124% of the control amplitude (paired $t$-test, $P = 0.11$, $n = 4$, Figure 25). On the other hand, 100 $\mu$M 2-AG potentiated $I_{\text{GABA}}$ to 145 ± 8% of the control amplitude in cells expressing $\beta_1$-containing GABA$_A$Rs (paired $t$-test, $P = 0.032$, $n = 3$, Figure 25). This latter observation contrasts with Sigel et al. (2011) where $I_{\text{GABA}}$ potentiation by 2-AG was absent. Differences in the experimental procedures, notably the different concentrations of 2-AG used, might account for these opposite findings. The data presented in Figure 25 show a trend towards 2-AG being more efficacious at $\beta_2$-containing receptors, however, additional experiments would be required to
ascertain any 2-AG β subunit selectivity and confirm findings from the Sigel et al. (2011) study.

![Diagram](image_url)

Figure 25. Time course of 2-AG-induced modulation of GABA-mediated Cl− currents in HEK293 cells expressing GABAAR α₂β₁γ₂ or α₁β₂γ₂ subtypes. (A) 1 μM DZP was applied for 4 min before 2-AG to test for the expression of the γ₂ subunit. The x-axis was discontinued during the washout of DZP and data were re-normalized to control values preceding 2-AG exposure. Bath application of 100 μM 2-AG, applied for 6 min, resulted in a potentiation of I_{GABA} in cells expressing GABAAR α₁β₁γ₂ (n = 3) or α₁β₂γ₂ (n = 4). The arrow indicates maximal potentiation of I_{GABA} in the presence of 2-AG. (B) Maximal I_{GABA} potentiation induced by 2-AG in individual cells expressing either α₁β₁γ₂ or α₁β₂γ₂. Data presented as mean ± SEM; P = 0.18 (unpaired t-test).

Similarly to 2-AG, CBD had a potentiating effect on I_{GABA} mediated by α₁β₁γ₂, with 10 μM CBD enhancing the current to 170 ± 22% of control amplitude (paired t-test, P = 0.017, n = 7, Figure 26). The same experiment carried out in HEK293 cells expressing β₃-containing receptors resulted in an enhancement of I_{GABA} reaching 230 ± 34% of control (paired t-test, P = 0.0076, n = 8, Figure 26). There were no statistically
significant differences observed across the three β subunit group means as determined by one-way ANOVA (F(2,22)=1.41, P = 0.266), suggesting CBD is not selective for a particular β subunit.

Figure 26. Time course of CBD-induced modulation of GABA-mediated Cl⁻ currents in HEK293 cells expressing GABA<sub>A</sub>R α<sub>1</sub>β<sub>1</sub>γ<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> or α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> subtypes. (A) 1 μM DZP was applied for 3-4 min before CBD to test for the expression of the γ<sub>2</sub> subunit. The x-axis was discontinued during the washout of DZP and data were re-normalized to control values preceding CBD exposure. Continuous bath application of 10 μM CBD resulted in a transient potentiation of I<sub>GABA</sub> in cells expressing GABA<sub>A</sub>R α<sub>1</sub>β<sub>1</sub>γ<sub>2</sub> (n = 7), α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> (n = 10) and α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> (n = 8). The arrow indicates maximal potentiation of I<sub>GABA</sub> in the presence of CBD. (B) Maximal I<sub>GABA</sub> potentiation induced by CBD in individual cells expressing GABA<sub>A</sub>Rs containing either the β<sub>1</sub>, β<sub>2</sub>, or β<sub>3</sub> subunit. Data presented as mean ± SEM; one-way ANOVA F(2,22)=1.41, P = 0.266.

Similar findings were also observed for CBDV. Though the time course profiles presented in Figure 27A suggest CBDV is more efficacious at β<sub>2</sub>-containing receptors, the maximal potentiating effect of this cannabinoid was comparable across the
three receptor subtypes (one-way ANOVA $F(2,20)=2.66$, $P = 0.095$, Figure 27B). CBDV potentiated $I_{GABA}$ to 227 ± 30% of control amplitude in cells expressing $\alpha_1\beta_1\gamma_2$ (paired $t$-test, $P = 0.0083$, $n = 6$), 423 ± 70% in cells expressing $\alpha_1\beta_2\gamma_2$ (paired $t$-test, $P = 0.0017$, $n = 9$), and 314 ± 54% in cells expressing $\alpha_1\beta_3\gamma_2$ (paired $t$-test, $P = 0.0063$, $n = 8$). This enhancing effect was transient in all three cases.

![Figure 27](image)

**Figure 27.** Time course of CBDV-induced modulation of GABA-mediated $I_e$ currents in HEK293 cells expressing GABA$_A$R $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$ or $\alpha_1\beta_3\gamma_2$ subtypes. (A) 1 $\mu$M DZP was applied for 3-4 min before CBDV to test for the expression of the $\gamma_2$ subunit. The x-axis was discontinued during the washout of DZP and data were re-normalized to control values preceding CBDV exposure. Continuous bath application of 10 $\mu$M CBDV resulted in a transient potentiation of $I_{GABA}$ in cells expressing GABA$_A$R $\alpha_1\beta_1\gamma_2$ ($n = 6$), $\alpha_1\beta_2\gamma_2$ ($n = 9$) and $\alpha_1\beta_3\gamma_2$ ($n = 8$). The arrow indicates maximal potentiation of $I_{GABA}$ in the presence of CBDV. (B) Maximal $I_{GABA}$ potentiation induced by CBDV in individual cells expressing GABA$_A$Rs containing either the $\beta_1$, $\beta_2$, or $\beta_3$ subunit. Data presented as mean ± SEM; one-way ANOVA $F(2,20)=2.66$, $P = 0.095$. 

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Site-directed mutagenesis was used to introduce a valine to threonine point mutation in the β₂ subunit (Figure 28). The resulting mutant GABAₐR α₁β₂⁴₃₆₅γ₂ was shown to be insensitive to 2-AG by Sigel et al. (2011). However, CBD was still able to potentiate currents mediated by this mutant receptor (151 ± 20% of control amplitude, Wilcoxon test, \( P = 0.002 \), \( n = 10 \), Figure 29). This was to be expected considering CBD also had an effect on β₁-containing GABAₐRs. Nonetheless, the CBD effect observed with the mutant receptor was diminished compared to the wild-type receptor (151 ± 20% versus 225 ± 21%, Mann-Whitney, \( P = 0.029 \), Figure 29B).

Overall, these experiments highlight that CBD and CBDV are not selective for any of the β subunits. Both cannabinoids were able to potentiate \( I_{GABA} \) from GABAₐRs containing β₁, β₂, or β₃ in combination with α₁ and γ₂ subunits.
Figure 29. Time course of CBD-induced modulation of GABA-mediated Cl⁻ currents in HEK293 cells expressing wild-type α₁β₂γ₂ or mutant α₁β₂V436γ₂ GABA<sub>R</sub> subtypes. (A) 1 μM DZP was applied for 3-4 min before CBD to test for the expression of the γ₂ subunit. The x-axis was discontinued during the washout of DZP and data were re-normalized to control values preceding CBD exposure. Bath application of 10 μM CBD resulted in a transient potentiation of <i>I<sub>GABA</sub></i> in cells expressing GABA<sub>R</sub> α₁β₂γ₂ (n = 10) and a lower but more sustained enhancement of <i>I<sub>GABA</sub></i> in cells expressing the mutant α₁β₂V436γ₂ (n = 10). The arrows indicate maximal potentiation of <i>I<sub>GABA</sub></i> in the presence of CBD. (B) Maximal <i>I<sub>GABA</sub></i> potentiation induced by CBD in individual cells expressing either GABA<sub>R</sub> subtype α₁β₂V436γ₂ or α₁β₂γ₂. Individual data points are presented together with the median and interquartile range; * P = 0.029 (Mann-Whitney test).

3.1.10 CBD and CBDV transiently potentiate <i>I<sub>GABA</sub></i> mediated by δ-containing GABA<sub>A</sub> receptors

The GABA<sub>R</sub> subtypes expressed in this study thus far have contained either an α₁ or α₂ subunit. In vivo, these particular α subunits are most often located at the synapse and linked to phasic inhibition. On the other hand, GABA<sub>R</sub> subtypes containing α₄ or α₅ in
association with the δ subunit are known to mediate extrasynaptic tonic inhibition (Farrant and Nusser 2005). This type of GABAergic inhibition, activated by extracellular ambient low levels of GABA, mediates a steady-state dampening of neuronal excitability. Enhanced expression of the δ subunit, resulting in an increase in tonic inhibition, has been associated with a decrease in seizure susceptibility in rodents (Maguire et al. 2005). As the data in previous sections highlight, both CBD and CBDV are able to potentiate currents mediated by ‘synaptic’ GABA<sub>A</sub>Rs. Next, the effect of CBD and CBDV on the ‘extrasynaptic’ α<sub>4</sub>β<sub>2</sub>δ subtype was examined.

A study performed in Xenopus oocytes used the compound DS2 to test for cell-surface expression of the α<sub>4</sub>β<sub>2</sub>δ receptor subtype. This positive allosteric modulator of δ-containing GABA<sub>A</sub>Rs was applied at a concentration of 300 nM and resulted in an eightfold enhancement of EC<sub>50</sub> GABA-mediated currents (Bakas et al. 2017). The same concentration of DS2 was used in the experiments detailed here, where α<sub>4</sub>β<sub>2</sub>δ was expressed in HEK293 cells. However, a much smaller increase in I<sub>GABA</sub> amplitude was observed (123 ± 5% of control amplitude, paired t-test, P = 0.0041, n = 7, Figure 30B), likely due to the use of a higher concentration of GABA (1 mM). DS2 also prolonged the duration of I<sub>GABA</sub>, resulting in a significant increase in charge transfer compared to control currents (260 ± 28%, Bonferroni post hoc analysis, P = 0.0039, n = 7, Figure 30A). This was taken as a positive control for cells expressing δ-containing GABA<sub>A</sub>Rs at the plasma membrane. Subsequent application of 10 μM CBD potentiated I<sub>GABA</sub> to 370 ± 52% of control (paired t-test, P = 0.002, n = 7, Figure 30B). On average, this potentiation was larger than that observed with the αβγ subunit combinations, though it was a similarly transient effect. Within 6 min of CBD application, I<sub>GABA</sub> returned to control pre-CBD levels, followed by a complete rundown of the current within 10 min of cannabinoid exposure.
Figure 30. Time course of CBD-induced potentiation of GABA-mediated Cl⁻ currents in HEK293 cells expressing the GABA₅R α₄β₂δ subtype. (A) Bath application of 300 nM DS2, a positive allosteric modulator of δ-containing GABA₅Rs, did not result in a noticeable modulation of I_GABA amplitude. However, the decay rate of I_GABA was prolonged by DS2, visible in the representative current traces in the top right panel. The resulting increase in the area under the curve (AUC) was measured offline. This effect was used as a control for the presence of the δ subunit and only cells showing a DS2-induced increase in AUC were included in the analysis, repeated measures one-way ANOVA F(1.16,6.93)=13.17, P = 0.0075, Bonferroni post hoc analysis * P < 0.05, ** P < 0.01, n = 7. (B) DS2 was applied for 3 min before CBD and the x-axis was discontinued during washout. Bath application of 10 μM CBD resulted in I_GABA potentiation in cells expressing the GABA₅R α₄β₂δ subtype, followed by a run-down of the current below control levels. Data were re-normalised to control values preceding cannabinoid exposure and are shown as mean ± SEM, paired t-test, ** P < 0.01.

Comparable findings were observed with CBDV. Initial application of DS2 to HEK293 cells expressing the GABA₅R α₄β₂δ subtype prolonged the duration of I_GABA, translating into a significant increase in charge transfer compared to control (283 ± 44%, Bonferroni post hoc analysis, P = 0.01, n = 9, Figure 31A). The consecutive bath application of CBDV led to a very short-lived enhancement of I_GABA (822 ± 137%,
paired t-test, $P = 0.0008$, $n = 9$, Figure 31B). This potentiation was larger than the corresponding CBD-induced effect as well as the CBDV response recorded in cells expressing $\alpha\beta\gamma$ subunit combinations. Moreover, $I_{\text{GABA}}$ rapidly ran down within 7 min of CBDV application (Figure 31B), a considerably faster time course than previously observed with other GABA$_A$R subtypes.

**Figure 31. Time course of CBDV-induced potentiation of GABA-mediated Cl$^-$ currents in HEK293 cells expressing the GABA$_A$R $\alpha_4\beta_2\delta$ subtype. (A)** Bath application of 300 nM DS2, a positive allosteric modulator of $\delta$-containing GABA$_A$R$\delta$s, did not result in a significant modulation of $I_{\text{GABA}}$ amplitude. However, the decay rate of $I_{\text{GABA}}$ was prolonged by DS2, visible in the representative current traces in the top right panel. The resulting increase in the area under the curve (AUC) was measured offline. This effect was used as a control for the presence of the $\delta$ subunit and only cells showing a DS2-induced increase in AUC were included in the analysis, repeated measures one-way ANOVA $F(1.05,8.397)=29.35$, $P = 0.0005$, Bonferroni post hoc analysis ** $P < 0.01$, $n = 9$. **(B)** DS2 was applied for 3 min before CBDV and the x-axis was discontinued during washout. Bath application of 10 $\mu$M CBDV resulted in a short-lived $I_{\text{GABA}}$ potentiation in cells expressing GABA$_A$R $\alpha_4\beta_2\delta$, followed by a complete run-down of the current. Data were re-normalised to control values preceding cannabinoid exposure and are shown as mean ± SEM, paired t-test, *** $P < 0.001$. 

![AUC and Amplitude Graphs](image-url)
In order to assess whether this rapid CBDV-induced run-down of \( I_{\text{GABA}} \) is use dependent, puffs of GABA were paused upon CBDV application. The resulting modulation of \( I_{\text{GABA}} \) over time, shown in Figure 32, indicates that the effect of CBDV is not dependent on agonist-binding to the receptor. The time course depicted in red represents a cell expressing the \( \text{GABA}_A \) \( \alpha_4\beta_2\delta \) subtype where the GABA puffs were paused for 10 min following the start of CBDV bath application. Once restarted, no \( I_{\text{GABA}} \) could be recorded suggesting that over time CBDV inhibited the \( \alpha_4\beta_2\delta \) receptor.

![Figure 32. CBDV-induced potentiation of GABA-mediated Cl\(^-\) currents in HEK293 cells expressing \( \text{GABA}_A \) \( \alpha_4\beta_2\delta \) is not use dependent.](image)

The time course profiles of two individual cells expressing \( \alpha_4\beta_2\delta \) are shown. DS2 was applied for 3 min before CBDV and resulted in a prolonged duration of \( I_{\text{GABA}} \) in both cells, indicating expression of the \( \alpha_4\beta_2\delta \) receptor subtype at the cell surface. The x-axis was discontinued during washout of DS2. Upon the start of CBDV superfusion, pressure application of GABA was paused for 2 and 10 min in the cell depicted in blue and red, respectively. Data were re-normalised to control values preceding CBDV exposure.

3.1.11 CBDV transiently potentiates \( I_{\text{GABA}} \) mediated by the \( \text{GABA}_A \) receptor \( \alpha_4\beta_2 \) subtype

CBD and CBDV were also tested on \( \alpha_4\beta_2 \) receptors in order to shed light on the contribution of the \( \delta \) subunit to the rapid cannabinoid-induced run-down of \( I_{\text{GABA}} \) previously highlighted in Figure 30 and 31. Interestingly, CBD did not enhance \( I_{\text{GABA}} \) mediated by these receptors. Instead, the cannabinoid induced a steady run-down
of control $I_{\text{GABA}}$ (Figure 33A). The time course of this run-down was comparable to the potentiated $I_{\text{GABA}}$ decay observed with $\alpha_3\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$ (Figure 33B).

![Image of Figure 33A and B](image)

Figure 33. Time course of CBD and CBDV-induced potentiation of GABA-mediated Cl⁻ currents in HEK293 cells expressing $\text{GABA}_A\text{R}\alpha_4\beta_2$. (A) Continuous bath application of 10 μM CBD led to a steady decrease in $I_{\text{GABA}}$ in HEK293 cells expressing $\text{GABA}_A\text{R}\alpha_4\beta_2$ ($n = 6$). (B) The decay rate of CBD-induced $I_{\text{GABA}}$ potentiation for $\alpha_4\beta_2\delta$ was similar to that of the $\alpha_3\beta_2\gamma_2$ receptor subtype. (C) Bath application of 10 μM CBDV resulted in a transient potentiation of $I_{\text{GABA}}$ in cells expressing $\text{GABA}_A\text{R}\alpha_4\beta_2$ ($n = 3$). (D) The decay rate of CBDV-induced $I_{\text{GABA}}$ potentiation was faster for $\alpha_4$-containing $\text{GABA}_A\text{R}$s compared to $\alpha_1$-containing receptors. Data presented as mean ± SEM.

On the other hand, CBDV potentiated $I_{\text{GABA}}$ in HEK293 cells expressing $\alpha_4\beta_2$ to 231 ± 67% of control amplitude (paired $t$-test, $P = 0.19$, $n = 3$, Figure 33C). This was less than half of the enhancement observed with $\alpha_4\beta_2\delta$ (231 ± 67% versus 822 ± 137%, unpaired $t$-test, $P = 0.038$). Again, this potentiation was transient and $I_{\text{GABA}}$ was rapidly abolished within 6 min of CBDV application. The decay in $I_{\text{GABA}}$ potentiation was similar, and more rapid, for both $\alpha_4\beta_2$ and $\alpha_4\beta_2\delta$ compared with $\alpha_4\beta_2$ and $\alpha_3\beta_2\gamma_2$ receptor subtypes (Figure 33D). This suggests the $\delta$ subunit is not mediating the...
CBDV-induced run-down of $I_{\text{GABA}}$. Instead, it might be that the $\alpha_4$ subunit is precipitating this decline.

3.2 Effect of CBD and CBDV on $I_{\text{GABA}}$ in CA1 pyramidal neurones

The results detailed thus far, and obtained from HEK293 cells transiently expressing GABA$\alpha$Rs, indicate both CBD and CBDV are able to potentiate $I_{\text{GABA}}$ mediated by various GABA$\alpha$R subtypes, though this effect is generally short-lived. The use of HEK293 cells as a heterologous expression system provides a relatively blank canvas with which to study ion channel function and investigate direct modulatory effects of compounds on receptors of interest. However, results obtained from simple and isolated cells might not reflect the responses occurring in vivo, at systems level, where receptors are embedded within synapses and circuits. I thus examined the effects of CBD and CBDV on $I_{\text{GABA}}$ in CA1 pyramidal neurones using whole-cell recordings in ex vivo hippocampal slices from C57BL/6 mice. The CA1 network is a well studied area of the mammalian brain containing a high number of GABAergic synapses, making it a potential site of action for the cannabinoids. Moreover, for the purpose of this preliminary investigation, CA1 pyramidal neurones could easily be visualised and identified on the basis of location and appearance without the need for further immunohistochemical post hoc analysis and characterisation.

The same approach was taken to record $I_{\text{GABA}}$ in CA1 pyramidal neurones as with HEK293 cells, notably, the use of pressure ejection of GABA or ‘puff application’ in proximity to the voltage-clamped pyramidal neurone. This differs from the standard experimental design described in the literature which uses a stimulating electrode to measure a postsynaptic event. For the purpose of this study I was not concerned with examining how the cannabinoids might affect synaptic events, but rather how they may directly modulate GABA$\alpha$R activity in vivo. The puff application was thus employed. In addition, maintaining similar conditions to those used in the HEK293 experiments allowed for a more direct comparison.
3.2.1 CBD and CBDV do not potentiate $I_{GABA}$ in mouse CA1 pyramidal neurones

GABA can activate type A and type B GABA receptors, both present within the hippocampus. In order to specifically record GABA-evoked currents resulting from the activation of GABA$\alpha$Rs, a selective GABA$\alpha$R antagonist, CGP 52432, was present in the perfusion solution throughout the entire experiment. Figure 34A shows a trend for $I_{GABA}$ decrease in CA1 pyramidal neurones in the presence of 10 $\mu$M CBD, though this was not significant when values were analysed 10 min following the start of CBD application (82 ± 15% of control amplitude, paired t-test, $P = 0.22$, n = 6, Figure 34B). Nonetheless, this time course profile contrasts with the results obtained in HEK293 cells expressing $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, or $\alpha_4\beta_2\delta$ GABA$\alpha$Rs, where there was a clear initial $I_{GABA}$ potentiation induced by CBD. This transient increase in $I_{GABA}$ was not visible in CA1 pyramidal neurones. DZP was tested on four of these cells prior to CBD application and resulted in an enhancement of $I_{GABA}$ in each cell (163 ± 36% of control amplitude, paired t-test, $P = 0.21$, n = 4, Figure 34B). Although on average this was not significant, the trend for $I_{GABA}$ to increase in the presence of DZP indicated that GABA$\alpha$Rs typically associated with fast synaptic inhibition were present and being activated. However, puff application of GABA in slices is likely to be more susceptible to diffusional barriers as well as fast reuptake by GABA transporters, thereby reducing the GABA concentration experienced by hippocampal GABA$\alpha$Rs. It is thus possible that these low concentrations of GABA also activated extrasynaptic, diazepam-insensitive $\alpha_4$-containing receptors. The heterogeneity of GABA$\alpha$R subunits expressed in pyramidal neurones (Drexel et al. 2015) is likely to result in a mixture of responses and may underlie the weak diazepam-induced $I_{GABA}$ potentiation.
Figure 34. Time course of CBD modulation of GABA-mediated Cl⁻ currents in mouse CA1 pyramidal neurones. GABA was puff applied every 20 s in proximity to the voltage-clamped CA1 pyramidal neurone. The selective GABA<sub>B</sub>R antagonist, CGP 52432, was bath applied at a concentration of 5 µM all throughout the recording. (A) 500 nM DZP was first applied in four of six cells and washed out, as indicated by the discontinued x-axis. Data were re-normalised to control values preceding cannabinoid exposure. Continuous bath application of 10 µM CBD appeared to depress I<sub>GABA</sub> (n = 6). (B) Bar graph data were taken from 4 min after the start of DZP application (163 ± 36% of control amplitude, paired t-test, P = 0.21, n = 4) and 10 min after the start of CBD application (82 ± 15%, paired t-test, P = 0.22, n = 6). Representative traces taken from one cell are shown in the right panels. Data presented as mean ± SEM.

Similarly to CBD, application of 10 µM CBDV depressed I<sub>GABA</sub> in CA1 pyramidal neurones. Sustained perfusion of the cannabinoid resulted in a decrease in the amplitude of GABA-evoked currents which was 68 ± 13% of control amplitude, 10 min after the start of CBDV perfusion (one-way ANOVA F(2,24)=17.33, P < 0.0001, Bonferroni post hoc analysis, P = 0.021, n = 11, Figure 35B). Again, this was in sharp contrast to the effect observed in HEK293 cells expressing a homogeneous GABA<sub>B</sub>R
population, where CBDV treatment led to an initial transient potentiation of $I_{GABA}$. Such a brief enhancement may have been counteracted by the presence of a heterogeneous population of GABA$_A$Rs in slices. The phytocannabinoid-induced inhibition of $\alpha_4$-mediated currents, previously observed in HEK293 cells, might overshadow any transient $I_{GABA}$ potentiation mediated by other GABA$_A$R subunits and explain the modest decrease in $I_{GABA}$ seen in pyramidal neurone experiments. Additionally, the decrease in $I_{GABA}$ amplitude occurring during the prolonged exposure to CBD and CBDV could be due to receptor desensitisation. This gradual current decrease contrasted with the rapid inhibition of $I_{GABA}$ upon bath application of the GABA$_A$R channel blocker picrotoxin (14 ± 2% of control, Bonferroni post hoc analysis, $P < 0.0001$, $n = 5$, Figure 35B). Interestingly, picrotoxin did not fully abolish $I_{GABA}$. The residual Cl$^-$ current could be due to the presence of GABA$_A$Rs containing the $\rho_2$ subunit. The rodent $\rho_2$ subunit has been shown to exhibit picrotoxin resistance (Zhang et al. 1995) and has been found in the hippocampus (Didelon et al. 2002, Liu et al. 2004). This further highlights the heterogeneity of GABA$_A$Rs in vivo.
Figure 35. Time course of CBDV modulation of GABA-mediated Cl⁻ currents in mouse CA1 pyramidal neurones. GABA was puff applied every 20 s in proximity to the voltage-clamped CA1 pyramidal neurone. The selective GABAᵦR antagonist, CGP 52432, was bath applied at a concentration of 5 μM all throughout the recording. (A) Prolonged exposure to 10 μM CBDV resulted in a decrease in $I_{GABA}$. (B) Bar graph data were taken from 10 min after the start of CBDV application (68 ± 13% of control amplitude, one-way ANOVA F(2,24)=17.33, $P < 0.0001$, Bonferroni post hoc analysis, $P = 0.021$, n = 11). Following CBDV perfusion for a minimum of 20 min, 100 μM picrotoxin was bath applied in five of the cells. Average $I_{GABA}$ amplitude in the presence of picrotoxin was calculated from traces extracted 3 min following the start of picrotoxin application (14 ± 2%, Bonferroni post hoc analysis, $P < 0.0001$, n = 5). Representative traces taken from one cell are shown on the right. Data presented as mean ± SEM.
3.3 CBD and CBDV modulation of glycine receptors

GlyRs also mediate inhibitory neurotransmission and though present in lower numbers than the GABA<sub>A</sub>Rs, they have also been identified in the hippocampus. Moreover, a number of different cannabinoids have been shown to exert an effect on these receptors, including CBD. Thus, the same in vitro approach taken to study cannabinoid action on GABA<sub>A</sub>Rs was used for GlyRs: HEK293 cells transiently expressing homomeric human GlyRs were whole-cell voltage clamped and the resulting I<sub>gly</sub> modulation by the cannabinoids was examined.

3.3.1 Glycine receptor cloning

All GlyR subunits were cloned by previous members of the Harvey group. According to the NCBI database (https://www.ncbi.nlm.nih.gov), three human GlyR α<sub>1</sub> isoforms have been identified. Isoform 2, which differs from isoform 1 by 8 amino acid residues, was cloned and used in subsequent experiments. GlyR α<sub>2</sub> isoforms A and B differ by two amino acids in the N-terminal extracellular domain. The canonical isoform A was cloned along with both GlyR α<sub>3</sub> isoforms, denoted α<sub>3</sub>S and α<sub>3</sub>L in the literature. The latter differ by a stretch of 15 amino acids located in the TM3-TM4 cytoplasmic loop, which seems to affect the resulting desensitisation kinetics of the receptor (Nikolic et al. 1998). All cDNAs were cloned into the pRK5 vector whose expression is under the control of the strong CMV promoter.
### Table 11. Human GlyR subunits cloned

The particular subunit isoforms cloned by previous members of the Harvey group, and used in this project, are highlighted in grey, along with a list of known isoforms to date. The isoform names were taken from the NCBI and may differ from other databases. Where two NCBI transcript references are given for the same isoform, the different transcripts differ in the 5' UTR region.

<table>
<thead>
<tr>
<th>GlyR subunit</th>
<th>Isoform name</th>
<th>Protein size (amino acids)</th>
<th>NCBI transcript reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1)</td>
<td>Isoform 1</td>
<td>457</td>
<td>NP_001139512</td>
</tr>
<tr>
<td></td>
<td>Isoform 2</td>
<td>449</td>
<td>NP_000162</td>
</tr>
<tr>
<td></td>
<td>Isoform 3</td>
<td>366</td>
<td>NP_001278929</td>
</tr>
<tr>
<td>(\alpha_2)</td>
<td>Isoform A</td>
<td>452</td>
<td>NP_002054, NP_001112357</td>
</tr>
<tr>
<td></td>
<td>Isoform B</td>
<td>452</td>
<td>NP_001112358</td>
</tr>
<tr>
<td></td>
<td>Isoform C</td>
<td>363</td>
<td>NP_001165143</td>
</tr>
<tr>
<td>(\alpha_3)</td>
<td>Isoform A ((\alpha_3)L)</td>
<td>464</td>
<td>NP_006520</td>
</tr>
<tr>
<td></td>
<td>Isoform B ((\alpha_3)S)</td>
<td>449</td>
<td>NP_001036008</td>
</tr>
</tbody>
</table>

**3.3.2 CBD potentiates I\(_{\text{gly}}\) mediated by homomeric glycine receptors in a dose-dependent manner**

Puff application of 1 mM glycine on HEK293 cells expressing either homomeric \(\alpha_1\) or \(\alpha_2\) GlyRs evoked large inward currents (representative traces shown in Figure 36). CBD concentrations lower than 100 nM had little effect on these currents, visible from the time courses presented in Figure 36. There was a trend for 1 \(\mu\)M CBD to potentiate I\(_{\text{gly}}\) mediated by GlyR \(\alpha_1\) (167 \(\pm\) 54\% of control amplitude, paired t-test, \(P = 0.33, n = 3\), Figure 36A) and \(\alpha_2\) (132 \(\pm\) 14\%, paired t-test, \(P = 0.0496, n = 6\), Figure 36B). Perfusion of 10 \(\mu\)M CBD further enhanced the potentiation of I\(_{\text{gly}}\) mediated by \(\alpha_1\) (328 \(\pm\) 81\% of control amplitude, paired t-test, \(P = 0.0435, n = 5\)), and to a lesser extent \(\alpha_2\) (143 \(\pm\) 10\%, paired t-test, \(P = 0.008, n = 4\)), pointing to a differential action of CBD on distinct GlyR subunits. This is in agreement with previous studies showing a differential modulation of \(\alpha_1\) and \(\alpha_2\) by THC and AEA (Xiong et al. 2011, 2012a).

Higher concentrations of CBD could not be tested due to the insoluble nature of this compound in aqueous solution – a limiting factor also encountered in GlyR studies with other cannabinoids (Xiong et al. 2012a). As such, a dose-response could not be established. A concentration of 10 \(\mu\)M was chosen for further experimentation as it clearly revealed a selective effect of CBD on different GlyR subtypes. Furthermore,
this corresponds to the EC$_{50}$ value of 12.3 $\pm$ 3.8 $\mu$M calculated for CBD potentiation of GlyR $\alpha_1$-mediated currents (Ahrens et al. 2009a). Though it is now known, from completed clinical trials and dose-ranging safety studies, that the physiological plasma concentration of CBD is around 1 $\mu$M (Devinsky et al. 2018b, GW Pharmaceuticals 2018a), low CBD concentrations did not significantly modulate $I_{gly}$ in the present study. This made it difficult to detect differing CBD effects amongst different receptor subtypes and as such, low phytocannabinoid concentrations were not further explored. CBDV was also used at 10 $\mu$M in all subsequent experiments.
Figure 36. Time course of CBD-induced modulation of glycine-evoked Cl⁻ currents in HEK293 cells expressing α₁ or α₂ subunit GlyR. (A) Time course of I<sub>gly</sub> potentiation induced by 10 nM (n = 6), 100 nM (n = 7), 1 µM (n = 3) and 10 µM CBD (n = 5) in HEK293 cells expressing the GlyR α₁ subunit. Panel on the right shows representative current traces from a cell activated by 1 mM glycine, before (black), and following 10 min application of 10 µM CBD (grey). (B) Time course of I<sub>gly</sub> potentiation induced by 100 nM (n = 7), 1 µM (n = 6) and 10 µM CBD (n = 4) in HEK293 cells expressing the GlyR α₂ subunit. Panel on the right same as above. Data presented as mean ± SEM, paired t-test versus control current amplitude, * P < 0.05, ** P < 0.005.
3.3.3 CBD and CBDV differentially potentiate $I_{\text{gly}}$ mediated by homomeric glycine receptors

The time course of CBD and CBDV-mediated facilitation of $I_{\text{gly}}$ was examined for all homomeric GlyRs (Figure 37). The maximum potentiation of glycine-evoked currents was reached within 5-7 min following the start of cannabinoid application. Overall, CBD and CBDV had a positive modulatory effect on all GlyRs tested, though varying compound efficacies were observed amongst the different receptors, particularly with CBD. Continuous superfusion of 10 µM CBD for at least 10 min had a comparable effect on $\alpha_1$ and $\alpha_3$L GlyRs (Figure 37C), potentiating $I_{\text{gly}}$ to $328 \pm 81\%$ (paired $t$-test, $P = 0.0435$, n = 5) and $298 \pm 87\%$ of control amplitude (paired $t$-test, $P = 0.0599$, n = 6), respectively. The $\alpha_2$ GlyR was the least sensitive to CBD: $I_{\text{gly}}$ mediated by this receptor was potentiated to $143 \pm 10\%$ of the control amplitude (paired $t$-test, $P = 0.008$, n = 4). On the other hand, $\alpha_3$S was the most sensitive to the phytocannabinoid as $I_{\text{gly}}$ was enhanced to $678 \pm 97$ (paired $t$-test, $P = 0.0037$, n = 5). This effect was significantly larger than at $\alpha_3$L receptors (one-way ANOVA $F(3,16)=7.04$, $P = 0.0031$, Bonferroni post hoc analysis, $P = 0.022$).

The $I_{\text{gly}}$ potentiation observed with CBDV was more uniform across GlyRs (one-way ANOVA $F(3,30)=2.71$, $P = 0.063$, Figure 37D). Following 10 min superfusion of 10 µM CBDV, $I_{\text{gly}}$ mediated by $\alpha_1$, $\alpha_2$ and $\alpha_3$L was potentiated to $223 \pm 70\%$ (paired $t$-test, $P = 0.13$, n = 7), $195 \pm 27\%$ (paired $t$-test, $P = 0.013$, n = 9), and $329 \pm 67\%$ of the control amplitude (paired $t$-test, $P = 0.0054$, n = 12), respectively. CBDV appeared to have a stronger enhancing effect on $I_{\text{gly}}$ mediated by $\alpha_3$S receptors, however, this did not reach significance ($683 \pm 305\%$ of control amplitude, paired $t$-test, $P = 0.115$, n = 6).
Figure 37. Time course of CBD and CBDV potentiation of glycine-evoked Cl⁻ currents in HEK293 cells expressing different homomeric GlyRs. (A, B) CBD and CBDV were continuously bath applied at 10 µM. The enhancement of $I_{\text{gly}}$ reached a plateau within 5-7 min following the start of cannabinoid application. Cells expressing GlyR $\alpha_3S$ showed the greatest cannabinoid-induced $I_{\text{gly}}$ potentiation. The dotted box indicates 10 min after the start of cannabinoid perfusion and these time point values were used to plot the scatter plots. (C, D) $I_{\text{gly}}$ amplitude from individual cells after exposure to 10 µM CBD or CBDV for 10 min. Data presented as mean ± SEM. CBD: $\alpha_1$ (n = 5), $\alpha_2$ (n = 4), $\alpha_3S$ (n = 5), and $\alpha_3L$ (n = 6), one-way ANOVA $F(3,16)=7.04$, $P = 0.0031$. CBDV: $\alpha_1$ (n = 7), $\alpha_2$ (n = 9), $\alpha_3S$ (n = 6), and $\alpha_3L$ (n = 12), one-way ANOVA $F(3,30)=2.71$, $P = 0.063$.

3.3.4 CBD and CBDV likely act at a glycine receptor site distinct from AEA and THC

Previous investigations of the molecular basis of AEA and THC interactions with the GlyR have identified a particular amino acid in the TM3 of the receptor as critical in the cannabinoid modulation of $I_{\text{gly}}$ (Xiong et al. 2011, 2012a). The authors described a strong THC and AEA-induced potentiation of $I_{\text{gly}}$ mediated by GlyRs $\alpha_1$ and $\alpha_3$ (currents were enhanced to 700-1000% of the control currents) with $\alpha_2$ showing less...
sensitivity (\(I_{\text{gly}}\) was enhanced to around 200% of the control currents). A sequence alignment of the three subunits highlighted a serine at 296 and 307, present in GlyR \(\alpha_1\) and \(\alpha_3\) respectively, that is substituted to an alanine at 303 in GlyR \(\alpha_2\) (Figure 38). Site-directed mutagenesis rendered GlyR \(\alpha_1^{S296A}\) less sensitive to the cannabinoids whereas GlyR \(\alpha_2^{A303S}\) gained sensitivity (Xiong et al. 2011, 2012a).

In light of this finding, I generated the above GlyR mutants using site-directed mutagenesis. CBD and CBDV were tested on these mutants so as to determine the significance of S296 in mediating the effect of these two cannabinoids. AEA modulation of GlyR \(\alpha_1\), \(\alpha_2\), and their mutant counterparts was also assessed. These experiments aimed to replicate the results obtained by Xiong et al. (2012a) and thus served as a control.

10 \(\mu\text{M}\) AEA potentiated \(I_{\text{gly}}\) mediated by \(\alpha_1\) and \(\alpha_2\) GlyRs to levels that were not significantly different (169 ± 40%, \(n = 4\) versus 93 ± 2%, \(n = 3\), respectively, one-way ANOVA \(F(3,9)=4.09\), \(P = 0.044\), Bonferroni post hoc analysis, \(P = 0.70\)). The similar effect of AEA on these two subunits is unlike the observations by Xiong et al. (2012a). Specifically, Xiong et al. (2012a) reported a stronger potentiation of \(\alpha_1\)-mediated \(I_{\text{gly}}\) by 10 \(\mu\text{M}\) AEA (695 ± 76%), which was not observed in cells expressing
the α₂ subunit (127 ± 37%). In the present study, 10 µM AEA did not potentiate I_{gly} in cells expressing the mutant α₁^{S296A} GlyR (63 ± 21% of control amplitude, paired t-test, \( P = 0.22 \), \( n = 3 \)), and potentiation of α₂^{A303S} GlyR-mediated I_{gly} did not reach significance 10 min after the start of AEA application (204 ± 34% of control amplitude, paired t-test, \( P = 0.086 \), \( n = 3 \), Figure 39A and 39B).

Altered CBD-induced responses in the mutant receptors were not significant compared to wild-type GlyRs (Figure 39C and 39D). GlyR α₁ and α₁^{S296A} were similarly modulated by CBD (328 ± 81%, \( n = 5 \) versus 638 ± 118%, \( n = 8 \), respectively, one-way ANOVA F(3,18)=5.51, \( P = 0.0073 \), Bonferroni post hoc analysis, \( P = 0.169 \)). The same was true for the α₂ and α₂^{A303S} receptors (143 ± 10%, \( n = 4 \) versus 246 ± 38%, \( n = 5 \), respectively, Bonferroni post hoc analysis, \( P > 0.999 \)).

As previously detailed, and again highlighted in Figure 39E, CBDV did not differentially modulate α₁ and α₂ subunit GlyRs. This cannabinoid had a similar effect on I_{gly} mediated by either receptor (223 ± 70%, \( n = 7 \) and 195 ± 27%, \( n = 9 \), one-way ANOVA F(3,23)=0.27, \( P = 0.85 \)). As such, CBDV modulation of α₁^{S296A} and α₂^{A303S} -mediated I_{gly} (262 ± 108%, \( n = 5 \) and 255 ± 53%, \( n = 6 \), respectively) did not differ from the wild-type counterparts. Together, these results suggest that CBD and CBDV modulate GlyRs at a site distinct from that of THC and AEA. Indeed, the S296A mutation on the α₁ subunit had no significant effect on the behaviour of CBD and CBDV at the GlyR.
Figure 39. Effect of AEA, CBD, and CBDV on glycine-evoked Cl⁻ currents from wild-type and mutant GlyRs. (A) Time course of AEA-induced modulation of $I_{\text{gly}}$ in HEK293 cells expressing GlyRs $\alpha_1$, $\alpha_1^{S296A}$, $\alpha_2$ or $\alpha_2^{A303S}$. AEA was continuously bath applied at a concentration of 10 µM. The dotted box indicates 10 min after the start of cannabinoid perfusion and current amplitudes at this time point were used to plot the bar graph. (B) Summary bar graph highlights the modulation of each receptor by 10 AEA µM. (C, E) Same experiment as in (A) but with 10 µM CBD or CBDV. (D, F) Same as (B) but for CBD or CBDV. Data presented as mean ± SEM; n values are indicated within each bar.
Over the years, an increasing number of studies have reported on the potential medicinal benefits of phytocannabinoids, from antiemetic effects and pain attenuation in clinical oncology (Walsh et al. 2003), to spasticity relief in multiple sclerosis patients (Rice and Cameron 2018). Research into the therapeutic effects of phytocannabinoids in several disease areas such as cancer, irritable bowel syndrome, addiction and symptom amelioration in amyotrophic lateral sclerosis, dementia, Parkinson’s and Huntington’s disease has, as yet, provided insufficient evidence to support the use of cannabis-derived compounds in these diseases (National Academies of Sciences, Engineering, and Medicine 2017). Until recently, epilepsy had been categorised in this list. However, an anticonvulsive therapeutic action of CBD was confirmed in the first placebo-controlled clinical trials of a phytocannabinoid for DS and LGS (Devinsky et al. 2017, 2018a, Thiele et al. 2018), validating previous preclinical research. Such exciting results warrant further investigation into the mechanisms of action of CBD and its propyl analogue CBDV. Understanding how these compounds work and affect their specific endogenous targets would better inform patients and healthcare providers, help shape future practice and policy for the medical use of cannabinoids, and would enable the development of related molecules with improved efficacy.

Currently, commercially available AEDs in the United Kingdom include phenobarbital and various benzodiazepines, whose therapeutic actions are directly mediated by GABA$_A$Rs. Moreover, as detailed in sections 1.4 and 1.6, a number of studies have uncovered a direct action of cannabinoids on GABA$_A$ and GlyRs. Thus, phytocannabinoids CBD and CBDV may also be acting at these inhibitory receptors to bring about the anticonvulsive effects previously observed in preclinical models of epilepsy (Carlini et al. 1973, Karler et al. 1973, Consroe and Wolkin 1977, Consroe et al. 1982, Jones et al. 2010, 2012, Hill et al. 2012a, Kaplan et al. 2017).

The present study used patch-clamp recordings from HEK293 cells transiently expressing GABA$_A$ or GlyR subtypes to investigate any direct action of CBD and CBDV on these receptors. The phytocannabinoids had a transient potentiating effect on
l_{GABA} mediated by different GABA_{A}Rs and this effect was found to be independent of the benzodiazepine binding site. Both CBD and CBDV enhanced l_{gly} mediated by all homomeric GlyRs and this potentiation was sustained throughout the duration of phytocannabinoid application. The effect of both compounds was also examined on GABA_{A}R-mediated currents recorded in acute hippocampal slices. In stark contrast with the data obtained in HEK293 cells, superfusion of CBD had no effect on GABAergic currents elicited in CA1 pyramidal neurones, whilst CBDV had a slight inhibitory effect.

4.1 CBD and CBDV are positive allosteric modulators at all GABA_{A} receptor subtypes tested

4.1.1 α_{1} and α_{2}-containing GABA_{A} receptors are similarly modulated by CBD and CBDV

The data gathered from this project show that both cannabinoids acted as positive allosteric modulators of different GABA_{A}R subtypes in vitro. Indeed, a minimum effective concentration of 3 μM CBD or CBDV was able to potentiate l_{GABA} from both α_{1} and α_{2}-containing GABA_{A}Rs. This is in agreement with Bakas et al. (2017) who investigated the effects of a phyto- and endocannabinoid, CBD and 2-AG, on all six α subunits in combination with β_{2} and γ_{2}. They found that both compounds had a positive modulatory effect on these six GABA_{A}R subtypes. Similarly, Sigel et al. (2011) reported a current potentiation by 2-AG and this effect was comparable across GABA_{A}Rs containing different α subunits (α_{1,2,3,5,6}). Thus, these studies together with the findings presented here highlight a lack of cannabinoid selectivity for the different GABA_{A}R α subunits. That said, Bakas et al. (2017) described a higher efficacy of CBD and 2-AG at α_{2}-containing receptors, though this was only apparent at cannabinoid concentrations of 30 μM or higher. Indeed, 10 μM of the compounds had comparable effects on GABA_{A}Rs containing different α subunits. Similarly, I found no significant difference between α_{1}β_{2γ_{2}} and α_{2}β_{2γ_{2}} regarding the maximal level of current potentiation induced by 10 μM CBD or CBDV. Limited solubility of
the cannabinoids, together with the fact that higher concentrations are less likely to be relevant when translated into the clinic, meant that higher cannabinoid concentrations were not tested. To put things into context, a study on Huntington’s disease patients treated with an average daily oral dose of 700 mg CBD (10 mg.kg\(^{-1}\)) found that plasma CBD levels ranged from 5.9-11.2 ng.ml\(^{-1}\) over the six week administration period (Consroe et al. 1991). This roughly translates into 19-36 nM. Another study found plasma CBD levels in known cannabis smokers to range from 1.6 to 271.9 ng.ml\(^{-1}\) (mean of 77.9 ng.ml\(^{-1}\)) in the six hours following ingestion of an 800 mg CBD capsule (Haney et al. 2016), roughly translating into a maximal concentration of 0.9 \(\mu\)M. A pharmacokinetic study in healthy individuals reported peak CBD plasma concentrations of roughly 200 ng.ml\(^{-1}\) in the hours following a single dose of 1500 mg CBD oral solution, equalling 0.6 \(\mu\)M (GW Pharmaceuticals 2018a). Finally, a randomised, double-blind, placebo-controlled trial in children with DS reported a CBD plasma concentration of 380 ng.ml\(^{-1}\) at the end of a three-week treatment period with 20 mg.kg\(^{-1}\) CBD oral solution per day, roughly translating into 1.2 \(\mu\)M (Devinsky et al. 2018b). This together with a rat pharmacokinetic study that highlights bioavailability of CBD is lower in brain tissue compared to serum (Hlozek et al. 2017) suggests that concentrations of CBD acting on receptors in the brain of CBD-treated patients is likely to be in the very low micromolar range. The ability of Bakas et al. (2017) to test higher concentrations of cannabinoids without any solubility issues is likely due to the use of a higher DMSO concentration. Whereas the final experimental concentration of DMSO in my study was 0.01%, Bakas et al. (2017) reported a final 0.8% DMSO concentration. Nonetheless, both studies found 10 \(\mu\)M CBD can act at \(\alpha_1\) and \(\alpha_2\)-containing receptors. Research has shown that the \(\alpha_1\) subunit is responsible for mediating the sedative effects of benzodiazepines (Rudolph et al. 1999, McKernan et al. 2000) whilst the \(\alpha_2\) subunit is generally associated with the anxiolytic effects (Low et al. 2000). Both subunits have been linked to the anticonvulsant actions of the compounds (Rudolph et al. 1999, Fradley et al. 2007). Thus, the lack of CBD and CBDV differentiation between these \(\alpha\) subunits augments the risk of adverse events occurring in the treatment of epilepsy disorders. Indeed, GABA\(_a\)R-mediated side effects reported in the CBD clinical trials for DS and LGS included sedation, though this was likely due to the concomitant benzodiazepine medication of each participant (Devinsky et al. 2017, 2018a, Thiele
et al. 2018). The fact that the phytocannabinoids can exert an effect on multiple GABA$_A$R subtypes, as shown in this study and the Bakas et al. (2017) study, suggests their binding site is within a conserved location of the receptor such as the TMD. This renders the task of designing target-specific drugs more difficult, though perhaps in the context of treating complex epilepsy disorders, such target specificity may not be required.

4.1.2 CBD and CBDV do not exhibit β subunit selectivity

Several studies have shown that positive allosteric modulators of GABA$_A$Rs exhibit β subunit selectivity. For instance, the anticonvulsant compound loreclezole strongly potentiated currents from GABA$_A$R subtypes containing either a β$_2$ or β$_3$ subunit, causing a doubling of the response amplitude, but only weakly potentiated currents from β$_1$-containing receptors (Wafford et al. 1994). Varying levels of $I_{GABA}$ enhancement by the cannabinoid 2-AG have also been described, depending on which β subunit is present. Currents mediated by receptor subtypes containing a β$_2$ subunit showed the largest potentiation by 3 μM 2-AG (Sigel et al. 2011). This potentiation was reduced to one third in β$_3$-containing receptors whilst no effect of 2-AG was observed on GABA$_A$Rs incorporating a β$_1$ subunit. The study further pinpointed a valine residue at position 436 in the β$_2$ TM4 as necessary for mediating the positive modulatory action of 2-AG (Sigel et al. 2011). These experiments were carried out in Xenopus oocytes and I sought to replicate these findings in HEK293 cells. Thus, the action of 2-AG was initially examined on GABA$_A$Rs $\alpha_1\beta_1\gamma_2$ and $\alpha_1\beta_2\gamma_2$. I also investigated the β subunit selectivity of CBD and CBDV by testing the effect of these cannabinoids on $\alpha_1\beta_1\gamma_2$ and $\alpha_1\beta_3\gamma_2$, and further assessed the effect of CBD on the mutant $\alpha_3\beta_2^{V436T}\gamma_2$ receptor. Unlike data reported by Sigel et al. (2011), where 2-AG had no effect on β$_1$-containing receptors, I found that 100 μM 2-AG potentiated $I_{GABA}$ in HEK293 cells expressing $\alpha_1\beta_1\gamma_2$. It should be noted that the concentration of 2-AG used here was ten-fold higher than that used by Sigel et al. (2011). Nonetheless, Bakas et al. (2017) also described a positive modulatory action of 10 μM and 100 μM 2-AG on β$_1$-containing GABA$_A$Rs, albeit weaker than at receptors with a β$_2$ or β$_3$ subunit. Also in contrast to the Sigel et al. (2011) study, Bakas et al.
(2017) found that 2-AG had a similar effect on both $\beta_2$ and $\beta_3$-containing receptors. Though I did not find the action of 2-AG at $\alpha_i\beta_1\gamma_2$ receptors to be significantly different to that on $\alpha_1\beta_2\gamma_2$, the time course data show a trend towards 2-AG being a stronger positive modulator of the latter GABA$_A$R subtype (Figure 25). Indeed, a more prolonged application of 2-AG might highlight a $\beta$ subunit selectivity similar to that observed by Bakas et al. (2017).

Also in contrast to the differential effects observed by Sigel et al. (2011) with 2-AG, data from my study show that both CBD and CBDV can potentiate currents from GABA$_A$Rs containing any of the three $\beta$ subunits in combination with $\alpha_1\gamma_2$. This is in agreement with Bakas et al. (2017) who tested CBD on $\alpha_x\beta_1\gamma_2$, where $x$ was any of the $\beta$ subunits, and found that this phytocannabinoid can positively modulate all three receptor subtypes. Bakas et al. (2017) further described CBD to be less potent and efficacious at $\beta_1$-containing receptors. Moreover, although CBD showed a similar efficacy at $\alpha_2\beta_2\gamma_2$ and $\alpha_2\beta_3\gamma_2$, differences in the potency at these receptor subtypes led the authors to suggest CBD exhibits some degree of selectivity for $\beta_3$-containing receptors. It is interesting to note that Bakas et al. (2017) interchange the term ‘selectivity’ to mean highest potency or highest efficacy depending on which of these two definitions better showcase a degree of differentiation amongst the subunits. Indeed, the term selectivity denotes a preference of a compound for a particular target but it can often go undefined in studies. Thus, when analysing the preference of CBD for a particular $\alpha$ subunit, Bakas et al. (2017) selected $\alpha_2$ because of the higher efficacy of CBD at this subunit, despite CBD being the least potent on $\alpha_2$-containing GABA$_A$Rs. With CBD showing a similar efficacy at $\beta_2$ and $\beta_3$ subunits, Bakas et al. (2017) redefined selectivity to mean potency. I did not assess the potency of CBD and CBDV at GABA$_A$Rs as the highly lipophilic nature of these compounds made it difficult to washout and carry out dose-response experiments within a single cell recording. Thus, I found both phytocannabinoids potentiated $I_{\text{GABA}}$ mediated by $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_2\gamma_2$, and $\alpha_1\beta_3\gamma_2$ to a similar extent at the 10 $\mu$M concentration tested (Figure 26 and 27). This would suggest CBD and CBDV are not selective for any of the $\beta$ subunits. Differences in experimental procedures between studies might have contributed to the different results. Bakas et al. (2017) tested a
greater range of CBD concentrations (1 – 100 µM) to identify differences in phytocannabinoid potencies across GABA<sub>A</sub>Rs. The same study also used *Xenopus* oocytes as the expression system. The resulting higher levels of GABA<sub>A</sub>R expression might have acted to amplify slight differences amongst different receptor subtypes.

Finally, my data show that the V436T mutation on the β<sub>2</sub> subunit significantly reduced the effect of CBD at α<sub>1</sub>β<sub>2</sub><sup>V436T</sup>γ<sub>2</sub> receptors when compared to wild-type α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors, though it did not abolish cannabinoid-mediated current potentiation as previously described by Sigel et al. (2011). Bakas et al. (2017) also found that CBD and 2-AG were still able to potentiate currents mediated by α<sub>2</sub>β<sub>2</sub><sup>V436T</sup>γ<sub>2</sub>. They did so with a reduced efficacy but similar potency as compared to wild-type α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub>. My findings, together with those presented by Bakas et al. (2017), suggest that V436 is not involved in the binding of the cannabinoids 2-AG or CBD, and by extension CBDV. Instead, the reduced ability of these compounds to enhance I<sub>GABA</sub> at β<sub>2</sub><sup>V436T</sup>-containing receptors might be explained by altered conformational changes upon cannabinoid binding.

### 4.1.3 CBD and CBDV can potentiate currents mediated by binary GABA<sub>A</sub> receptors

The effect of CBD and CBDV on α<sub>1</sub>β<sub>2</sub> GABA<sub>A</sub>Rs was evaluated and both phytocannabinoids were found to potentiate currents mediated by this binary receptor. This is in agreement with Sigel et al. (2011) who reported a 2-AG-induced enhancement of I<sub>GABA</sub> at α<sub>1</sub>β<sub>2</sub>, and Bakas et al. (2017) who reported a CBD-induced enhancement of I<sub>GABA</sub> at α<sub>1</sub>β<sub>2</sub> and α<sub>2</sub>β<sub>2</sub> receptors. In all instances, CBD potentiated α<sub>1</sub>β<sub>2</sub>-mediated I<sub>GABA</sub> to a similar extent as α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>. This suggests the γ<sub>2</sub> subunit is dispensable for transducing the action of CBD. On the other hand, CBDV had a reduced effect at binary α<sub>1</sub>β<sub>2</sub> receptors compared with α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> indicating that the γ<sub>2</sub> subunit plays a role in mediating CBDV efficacy.
Binary αβ receptors were originally thought to be absent from neurones. However, functional data suggest that these receptors, with a high affinity for GABA, may account for up to 10% of extrasynaptic GABA_ARs in hippocampal pyramidal neurones (Mortensen and Smart 2006). Thus, cannabinoids acting at tonically-active GABA_ARs could result in an increase in background inhibitory tone which in turn could regulate excitatory networks and account for the reduction in seizure frequency in CBD-treated DS and LGS patients. However, as Mortensen and Smart (2006) point out, αβ receptors have a low conductance and short mean open times, only modestly contributing to tonic inhibition. As such, CBD and CBDV modulation of binary αβ GABA_ARs represents only a small facet of their multimodal action.

4.1.4 CBD and CBDV can potentiate currents mediated by δ-containing GABA_A receptors

In this study, both CBD and CBDV were found to potentiate I_GABA mediated by α_4β_2δ receptors. This current enhancement was larger than that detected at other GABA_A_R subtypes, an observation also made by Bakas et al. (2017). Both studies recorded a similar level of current potentiation by 10 µM CBD, with Bakas et al. (2017) further reporting a maximal eight-fold current enhancement by 100 µM CBD. It should be noted that the presence of a δ versus a γ subunit in itself alters intrinsic properties of the receptor, with the δ subunit imparting a better GABA gating efficiency on GABA_A_Rs (Ahring et al. 2016). This complicates the direct comparison of modulator strength at these different receptor subtypes. Nonetheless, CBD and CBDV both acted as positive allosteric modulators at α_4β_2δ receptors. GABA_A_R subtypes containing a δ subunit are thought to be one of the primary contributors to tonic inhibition in the CNS (Zheleznova et al. 2009) and as such play a critical role in regulating neuronal excitability. These receptors are predominantly located at extrasynaptic or perisynaptic sites (Nusser et al. 1998, Wei et al. 2003) where they can sense low ambient GABA concentrations. Altered extrasynaptic GABA_A_R expression has been implicated in epilepsy (Peng et al. 2004). Moreover, several AEDs currently available (for example, vigabatrin, tiagabine, and pregabalin) act to increase ambient GABA levels in the brain, leading to modulation of tonic inhibition.
Thus, by acting at $\delta$-containing $\text{GABA}_A$Rs, CBD and CBDV could also be upregulating tonic inhibition.

However, though these phytocannabinoids had the strongest effect at $\alpha_4\beta_2\delta$ $\text{GABA}_A$Rs out of all the subtypes tested in this study, they also induced a rapid desensitisation or inhibition of $\alpha_4\beta_2\delta$-mediated currents. Indeed, following a short-lived $I_{\text{GABA}}$ potentiation, continued superfusion of CBD or CBDV led to a swift decline of this enhanced current and within ten minutes of the start of phytocannabinoid application, $I_{\text{GABA}}$ was abolished (Figure 30 and 31). Current run-down did not appear to be use-dependent as temporary suspension of the agonist puff still resulted in CBDV negatively modulating the receptor (Figure 32). This rapid current decay is somewhat surprising considering that $\delta$-containing receptors display little desensitisation (Saxena and Macdonald 1994). The cannabinoids could either be driving the receptors into an inactive state or binding a secondary inhibitory site on $\alpha_4\beta_2\delta$ receptors. Essentially, such a mechanism would have a pro-epileptic effect by reducing GABA-mediated inhibitory tone in brain circuitry. That said, a study carried out in a pilocarpine model of epilepsy revealed that differential changes occur in the expression of the $\delta$ subunit (Peng et al. 2004). Whilst a decrease in the expression of this subunit was observed in principal cells of the dentate gyrus, an increase was identified in interneurones. Thus, by inhibiting persistently active $\delta$-containing receptors in interneurones, CBD and CBDV could enhance interneurone excitability, thereby increasing inhibitory drive to principal cells. In agreement with such a model is a study showing that CBD enhances the excitability of parvalbumin expressing interneurones that innervate the dendrites of CA1 pyramidal neurones (Khan et al. 2018). In this study, CBD reduced the threshold for evoking action potentials and increased neuronal input resistance. However, the $\delta$ subunit does not seem to participate in tonic inhibition at hippocampal interneurones based on the observation that zolpidem, a drug acting at $\gamma$-containing receptors, increases tonic currents (Semyanov et al. 2003). Additionally, immunohistochemical localisation of the $\alpha_4$-subunit showed no labelling in interneurones in the dentate gyrus in both control and pilocarpine-treated mice (Peng et al. 2004). Indeed, $\alpha_4\beta_2\delta$ $\text{GABA}_A$Rs are not considered to be associated with interneurones. Instead, they have been shown
to mediate tonic inhibition in glutamatergic neurones (Zheleznova et al. 2009). It is thus difficult to reconcile the observed cannabinoid-induced inhibition of $\alpha_4\beta_2\delta$ GABA$_A$Rs with the anticonvulsant properties of CBD and CBDV. It is possible that the expression of this receptor, as well as other GABA$_A$Rs, is altered in disorders such as DS and LGS and may in fact become expressed in a particular interneurone population as the conditions progress. Interestingly, various studies have shown that CBD and CBDV activate, and desensitise, receptors of the TRP family (Bisogno et al. 2001, De Petrocellis et al. 2008, De Petrocellis et al. 2011). However, it appears agonist-induced desensitisation of these receptors is a self-limiting mechanism dependent on Ca$^{2+}$ (Gordon-Shaag et al. 2008).

4.2 The effect of CBD and CBDV at GABA$_A$ receptors is not sustained

A positive modulatory effect of CBD and CBDV was observed at all GABA$_A$R subtypes tested in this study. However, the cannabinoid-induced enhancement of $I_{GABA}$ was not sustained in the continued presence of 10 $\mu$M of one or the other compound. Within ten minutes of the start of cannabinoid application, potentiated $I_{GABA}$ mediated by either $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$ returned to baseline current level. Such an observation has not previously been reported and indeed, no prolonged time course profile of the effect of cannabinoids on GABA$_A$Rs has been documented. Sigel et al. (2011) tested the effect of 2-AG on these receptors by pre-applying the cannabinoid for 30 seconds before co-applying with agonist. An unspecified washout period was incorporated between each subsequent drug and agonist application within the same experiment. Similarly, Bakas et al. (2017) pre-applied either CBD or 2-AG for 75-90 seconds before co-applying cannabinoid with GABA. A three to fifteen minute washout period was allowed between drug applications. Overall, these studies applied cannabinoids for no longer than two to three minutes. According to my time course data, this time span falls within peak cannabinoid action. Thus, the aforementioned studies would not have registered an $I_{GABA}$ decrease following maximal current potentiation, due to the relatively short cannabinoid application.
The study detailed here represents the only one thus far to have looked at the direct effect of a sustained cannabinoid application on GABA<sub>A</sub>Rs. This is particularly relevant considering that CBD and CBDV have been shown to have anti-epileptiform activity in vitro following a 30 minute cannabinoid application protocol (Jones et al. 2010, Hill et al. 2012a). Moreover, clinical outcomes from the trial for CBD treatment of DS and LGS are the result of a sustained level of the phytocannabinoid which was administered twice daily over a 14 week period (Devinsky et al. 2017, 2018a, Thiele et al. 2018). Thus, it would be important to design studies that investigate the direct effect of these compounds on particular receptors over a longer timescale. It is possible that the short-lived potentiation of I<sub>GABA</sub> induced by CBD and CBDV, described in this study, was due to receptor desensitisation. However, DZP enhanced I<sub>GABA</sub> and this effect was sustained for the duration of DZP application (at least ten minutes, Figure 10A and 12A). In addition, experiments with δ-containing receptors, where pressure application of agonist was temporarily suspended during CBDV application, still resulted in an inhibition of I<sub>GABA</sub>. This indicates that I<sub>GABA</sub> decay was due to cannabinoid-mediated alteration of the GABA<sub>A</sub>R rather than intrinsic receptor kinetics. Golovko et al. (2015) described a dual direct effect of 2-AG on neuronal GABA<sub>A</sub>Rs whereby this endocannabinoid inhibits I<sub>GABA</sub> in the presence of high GABA concentration (1 mM) and potentiates I<sub>GABA</sub> at low agonist concentration (1 µM). Thus, inhibition of GABA<sub>A</sub>Rs by a cannabinoid has previously been reported. However, in contrast to Golovko et al. (2015), I observed an initial cannabinoid-induced potentiation of I<sub>GABA</sub> with 1 mM GABA. Moreover, except for experiments using α<sub>4</sub>-containing receptors, CBD and CBDV did not inhibit I<sub>GABA</sub> to levels below control values. It should be noted that Golovko et al. (2015) investigated the effect of cannabinoids on native GABA<sub>A</sub>Rs, either in mouse brain slices or on nucleated patches pulled from neocortical pyramidal neurones. Thus, different populations of GABA<sub>A</sub>R subtypes are likely to be present and the dual action of the cannabinoids tested might not be contingent on the concentration of GABA but rather on the particular subunit composition of the receptors.

In the present study, a concentration of 10 µM CBD or CBDV was used in all experiments, which produced rapid and clear effects on I<sub>GABA</sub>, notably, a transient
potentiation. However, lower phytocannabinoid concentrations tested on cells expressing $\alpha_1\beta_2\gamma_2$ or $\alpha_2\beta_2\gamma_2$ receptors did not cause a surge in $I_{GABA}$. Instead, the time course profile of 3 $\mu$M CBD or CBDV at these receptors revealed a slight but gradual enhancement of the current. This $I_{GABA}$ potentiation could be described as sustained as it was still observed ten minutes after the start of phytocannabinoid application (Figure 9 and 11). It would thus be interesting to explore lower concentrations of the phytocannabinoids at other receptor subtypes, if these are more likely to induce longer lasting effects on GABA$_A$Rs. For the purpose of this study, the higher 10 $\mu$M concentration was favoured as this would best highlight, within a short amount of time, any differences in phytocannabinoid effects amongst various receptor subtypes. It was expected that any dissimilarities would provide insight into potential CBD or CBDV binding sites. Moreover, information regarding the clinical plasma exposures to CBD was not known at the start of this project. However, as previously discussed, therapeutic concentrations of CBD acting on receptors in the brain of clinical patients are expected to be in the low micromolar range. A mechanism could exist whereby low concentrations of CBD or CBDV induce a long term enhancement of $I_{GABA}$, but higher concentrations curtail this effect or even completely inhibit $I_{GABA}$, as was observed with $\alpha_4$-containing GABA$_A$Rs. Such a dual modulation by the phytocannabinoids would suggest more than one binding site on GABA$_A$Rs. Research shows the same compound can have opposing modulatory effects on particular GABA$_A$R subtypes. For instance, the non-nucleoside reverse transcriptase inhibitor efavirenz, an antiretroviral drug, was demonstrated to modestly potentiate $I_{GABA}$ at 10 $\mu$M in HEK293 cells transiently expressing $\alpha_6\beta_2\gamma_2$ receptors, but inhibited current amplitude at 30 $\mu$M. The authors further postulated that these opposing, concentration-dependent actions of efavirenz, also observed at other receptor subtypes, are mediated by separate binding sites (Huang et al. 2017a). In a separate study, a biphasic effect of midazolam on GABA$_A$Rs in acutely isolated sacral dorsal commissural nucleus neurones was described. Low concentrations (0.1 $\mu$M) of this benzodiazepine acted as an agonist via the benzodiazepine site, whereas higher concentrations (30-300 $\mu$M) resulted in an antagonistic action, likely mediated by a distinct binding site (Wang et al. 2003). The variable effects observed with the higher concentration of CBD and CBDV tested in the present study, specifically, $I_{GABA}$ potentiation followed by a current run-down,
could reflect receptor desensitisation induced by binding to a secondary site or even a progressive channel block. The latter would align with radioligand binding data that showed both CBD and CBDV can displace radiolabelled TBPS, known to bind the GABA$_A$R picrotoxin site. Indeed, site-directed mutagenesis data from Huang et al. (2017a) suggested that the antagonistic action of efavirenz may be mediated by known picrotoxin sites at the TM2. However, such a channel block would lead to CBD or CBDV fully inhibiting $I_{GABA}$, which did not occur in GABA$_A$Rs containing $\alpha_1$ or $\alpha_2$ subunits. Moreover, the phytocannabinoids displaced TBPS with an IC$_{50}$ in the low micromolar range, a concentration that caused a gradual increase in $I_{GABA}$ and not an inhibition. The TBPS displacement is thus more likely to be due to phytocannabinoid-induced conformational changes in the receptor, or ‘allosteric displacement’, rather than actual phytocannabinoid binding in the channel pore that would block ion flow. These conformational changes might also underlie the current run-down observed in the continued presence of CBD and CBDV.

Interestingly, research into cannabinoid modulation of GlyRs uncovered a complex effect of NA-Gly on $\alpha_1$ homomeric receptors (Yang et al. 2008). This endogenous non-cannabinoid compound was studied due to its structural similarity with AEA. The authors found that a lower concentration of NA-Gly (10 µM) potently potentiated $I_{gly}$ whereas a higher concentration (30 µM) induced a current potentiation whose magnitude progressively declined with successive NA-Gly applications. Thus, the initial $I_{gly}$ enhancement seen with 30 µM NA-Gly was abolished following multiple exposures of GlyRs to the compound (Yang et al. 2008). Though differences exist between this study and my own, notably, the different application protocols of the drugs, my data are reminiscent of the Yang et al. (2008) findings. Both studies showed a progressive inactivation of the current potentiating mechanisms in the continued presence of test compounds. More complex mechanisms of action may be at play with the phytocannabinoids and GABA$_A$Rs.
4.3 Inhibitory action of CBD and CBDV on GABA<sub>A</sub> receptors expressed in pyramidal neurones

CBD and CBDV modulation of GABAergic currents in pyramidal neurones of the mouse CA1 hippocampal region was also investigated, so as to examine the effect of these phytocannabinoids on native GABA<sub>A</sub>Rs. In stark contrast with HEK293 cells, the short-lived potentiation of I<sub>GABA</sub> induced by CBD and CBDV was not present in CA1 pyramidal neurones held in voltage-clamp. Overall, there was a trend for I<sub>GABA</sub> to decrease in the presence of these compounds (Figure 34 and 35). This would agree with observations from Golovko et al. (2015) who found that various cannabinoids inhibit I<sub>GABA</sub> from GABA<sub>A</sub>Rs expressed heterologously. Moreover, the study found that both the CB<sub>1</sub> receptor agonist CP 55,940 and endogenous 2-AG led to a reduction in evoked IPSCs, in a CB<sub>1</sub> receptor-independent manner. However, these findings were synapse specific, occurring at neocortical, fast spiking, parvalbumin-expressing interneurones innervating pyramidal neurones. The authors did not see a difference in IPSCs recorded from hippocampal cholecystokinin-positive interneurones in CA1 in the presence of CP 55,940 (Golovko et al. 2015). On the other hand, Khan et al. (2018) measured an increase in spontaneous inhibitory postsynaptic potentials (IPSPs) in pyramidal cells, elicited by both parvalbumin and cholecystokinin-positive interneurones following a challenge with CBD. This CBD-induced increase in spontaneous IPSP amplitude and frequency persisted in the presence of a CB<sub>1</sub>R antagonist (Khan et al. 2018). The modest I<sub>GABA</sub> inhibition induced by CBD and CBDV in CA1 pyramidal cells in the present study thus appears to be at odds with published findings. It is possible that the high artificial concentration of GABA used here, together with phytocannabinoid-mediated modulation of the receptor, engendered a GABA<sub>A</sub>R desensitisation or endocytosis that would not occur under normal physiological conditions. GABA<sub>A</sub>Rs are trafficked in and out of the plasma membrane, allowing for modulation of GABAergic neurotransmission (Luscher et al. 2011). DZP was recently shown to lead to long term down-regulation of surface GABA<sub>A</sub>Rs through a metabotropic signalling pathway. The authors suggested this dynamin-dependent receptor internalisation forms the basis of pharmacological tolerance to chronic benzodiazepine exposure.
(Nicholson et al. 2018). Thus, the phytocannabinoids could be activating a similar pathway, leading to a reduced number of receptors at the plasma membrane and a reduction in $I_{\text{GABA}}$. However, the DZP-induced endocytosis of GABA$_A$Rs described by Nicholson et al. (2018) followed a prolonged stimulation of the receptor with this benzodiazepine, with the first statistically significant reduction in cell-surface receptors visible following one hour incubation with DZP. Thus, a comparatively slow mechanism of receptor endocytosis is unlikely to underlie the present $I_{\text{GABA}}$ decay observed in experiments with HEK293 cells, and the modest current inhibition seen in pyramidal cells. Moreover, superfusion of picrotoxin following CBDV led to a strong inhibition of $I_{\text{GABA}}$, suggesting GABA$_A$Rs were present and active at the cell surface.

The phytocannabinoid-mediated increase in inhibitory neurotransmission that has been detailed in the literature (Kaplan et al. 2017, Khan et al. 2018), and the general anticonvulsant properties assigned to these compounds (Jones et al. 2010, 2012, Hill et al. 2012a), could be the result of activity at other molecular targets. A recent study suggested that the therapeutic effects of CBD could be mediated through antagonism of the lipid-activated GPCR GPR55 as all effects elicited by the phytocannabinoid were mimicked by a selective GPR55 antagonist (Kaplan et al. 2017). The authors found that the frequency of spontaneous, action-potential driven IPSCs in dentate granule cells of a DS mouse model was reduced compared to wild-type animals. Treatment with CBD increased the frequency of these spontaneous IPSCs, without affecting the amplitude. Blocking action potentials with tetrodotoxin abolished the effect of CBD. This led the authors to suggest that action potential generation in GABAergic interneurones is augmented in the presence of CBD, resulting in an increase in spontaneous IPSCs in granule cells (Kaplan et al. 2017). However, the authors do not raise the fact that if CBD action were to be postsynaptic, mediated by allosteric modulation of GABA$_A$Rs, the addition of tetrodotoxin would also eliminate the effect of CBD. Nevertheless, CBD treatment was found to increase the number of action potentials in fast spiking parvalbumin-positive interneurones upon depolarising current injection, indicating an increase in interneurone excitability. Similar experiments on dentate granule cells found that
CBD reduced the frequency of action potentials elicited by a wide range of depolarising current steps, which the authors interpreted as a reduction in intrinsic excitability. The GABA<sub>A</sub>R antagonist GABA<sub>Ant</sub> abolished these CBD effects, suggesting that an increase in GABAergic transmission was behind the reduced excitability of principal cells (Kaplan et al. 2017). This is in disagreement with the slight I<sub>GABA</sub> inhibition I observed in CA1 pyramidal cells upon phytocannabinoid application (Figure 34 and 35). However, the effect of these compounds at GPR55, and subsequent alteration of downstream signalling pathways, may prevail over the direct action of CBD and CBDV at GABA<sub>A</sub>Rs. GPR55 expression has been found in pyramidal cells and some interneurones of the rodent hippocampus (Hurst et al. 2017), making this GPCR a possible target of the phytocannabinoids. CBD has already been shown to have dual, opposing, effects on interneurone and principal cell excitability (enhancement versus reduction, respectively) (Kaplan et al. 2017, Khan et al. 2018). It is thus highly probable that CBD (and probably CBDV) act at several sites to produce such effects.

4.4 Cannabinoids and benzodiazepines

The different effects induced by the phytocannabinoids and DZP, notably, a transient versus a sustained potentiation of I<sub>GABA</sub>, pointed towards separate mechanisms of action likely underlined by distinct binding sites. Indeed, experiments showing that CBD and CBDV exerted an effect on benzodiazepine-insensitive GABA<sub>A</sub>Rs α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>1</sub>β<sub>2</sub> substantiated the idea of different binding sites. This was further confirmed by radioligand binding data indicating neither phytocannabinoid could displace flunitrazepam from the GABA<sub>A</sub>R benzodiazepine binding site. Similar conclusions were reached in previous studies showing 2-AG and CBD are able to positively modulate currents mediated by receptors lacking a γ subunit, and hence missing the classical benzodiazepine binding site (Sigel et al. 2011, Bakas et al. 2017).
The effect of CBD on $\alpha_1^{H102R}\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors was more prolonged than on $\alpha_1\beta_2\gamma_2$ (Figure 16A), consistent with the notion that the benzodiazepine binding site plays a part in modulating the action of this phytocannabinoid. If CBD were to interact with GABA$_A$Rs at multiple locations, binding at a site overlapping a benzodiazepine binding loop might be responsible for transducing negative allosteric changes to the channel pore. Multiple binding pockets have been identified on GABA$_A$Rs and indeed compounds have been shown to bind more than one location. This is true of the benzodiazepines which have been shown to interact with numerous cavities on GABA$_A$Rs (Sigel and Ernst 2018). These include the classical benzodiazepine binding site 1 located at the $\alpha/\gamma$ subunit interface, site 2 located at the $\alpha/\beta$ subunit interface in the ECD, and site 3 found in the TMD of GABA$_A$Rs. Binding of flurazepam at site 2 was shown to have a “null modulatory” effect; it prevented the potentiation of $I_{GABA}$ by benzodiazepines acting through the classical high affinity binding site 1 (Baur et al. 2008). On the other hand, compound SJM-3 was shown to be an antagonist of site 1, inhibiting DZP-induced potentiation of $I_{GABA}$ at this benzodiazepine binding pocket, but was seen to potentiate $I_{GABA}$ via binding at site 3 (Middendorp et al. 2015). It is thus conceivable that CBD occupy more than one binding site, and in so doing, mediate different modulatory actions on GABA$_A$Rs.

I found that CBDV had a reduced efficacy at $\alpha_1^{H102R}\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors compared to wild-type $\alpha_1\beta_2\gamma_2$; however, the current decay that followed maximal $I_{GABA}$ potentiation was similar in all three receptor subtypes (Figure 16C). This is in contrast to the CBD observations described above and would suggest that the classical benzodiazepine binding site plays a different role, if any, in mediating the CBDV effect. This also indicates that the two phytocannabinoids, despite their near identical structures, interact with the GABA$_A$Rs in a different manner. The significance of this is further discussed in section 4.6.

Having established that CBD and CBDV mediate their $I_{GABA}$ enhancing effect via a binding site other than the classical benzodiazepine site, I investigated the potential for an additive effect between the phytocannabinoids and DZP. Synergy between
these two classes of compounds could have substantial therapeutic benefits for disorders already treated with benzodiazepines, including DS and LGS. Sigel et al. (2011) previously demonstrated a supra-additive effect between 2-AG and DZP in *Xenopus* oocytes expressing α₁β₂γ₂ receptors. The current potentiation resulting from co-application of the compounds was larger than the sum of effects produced by each drug applied individually. The authors also found that the EC₅₀ for 2-AG was reduced in the presence of DZP, indicating an improved affinity of the receptor for this endocannabinoid (Sigel et al. 2011). Results from the study presented here show an overall trend for CBD and CBDV to act in concert with DZP and cause an additive potentiation of Iₜₜ in cells expressing either α₁β₂γ₂ or α₂β₂γ₂ receptors (Figure 18, 19, 20, and 21). The maximal current potentiation observed with a mixture of phytocannabinoid and DZP, however, did not surpass the effect of either compound applied alone, suggesting that there is no modulatory cooperativity. Indeed, previous *in vivo* work in a pilocarpine model of epilepsy found that CBDV administered in combination with phenobarbital, a barbiturate acting at GABAₐRs, had no additional benefits over phenobarbital treatment alone (Hill et al. 2012a). In the present study, a pronounced current decay was observed with the mixture of phytocannabinoid and DZP, similar to that previously seen in experiments with the phytocannabinoids alone. This is in contrast to the sustained potentiation of Iₜₜ that occurs in the presence of DZP. Similar to the mixture of CBD and DZP, a rundown of Iₜₜ was observed in experiments where CBD was co-applied with another benzodiazepine, CLB. This 1,5-benzodiazepine is favoured over DZP, a classical 1,4-benzodiazepine, as an adjunctive therapy for the treatment of epilepsy due to fewer and less severe adverse events (Sankar 2012). In the present study, a prolonged application of CLB on cells expressing either α₁β₂γ₂ or α₂β₂γ₂ receptors led to a sustained potentiation of Iₜₜ (Figure 22). Co-application of CBD did not induce a significantly larger current enhancement, though the presence of the phytocannabinoid did curtail the effect of CLB (Figure 23 and 24). Thus, in a situation where DZP or CLB is applied with either CBD or CBDV, the modulatory action of the phytocannabinoids prevails. This observation is consistent with the idea that these compounds bind at a secondary site on GABAₐRs that mediates negative allosteric modulation or accelerates desensitisation. However, the consequence of this would be limited benzodiazepine action at these receptors, which might be considered
counter beneficial for the treatment of epilepsy disorders. An early study investigating anticonvulsant properties of a combined CBD and AED treatment on experimentally induced seizures in rats found that CBD reduced the anticonvulsant potencies of the 1,4-benzodiazepines chlordiazepoxide and clonazepam (Consroe and Wolkin 1977). This observation is consistent with my finding that CBD and CBDV steadily reduced the benzodiazepine-induced potentiation of I\textsubscript{GABA}. No other preclinical studies have assessed the concurrent use of a phytocannabinoid (not acting via the CB\textsubscript{1} receptor) and benzodiazepine on epileptogenic activity. This would be particularly important considering that around 50% of participants in the recent CBD trials for DS and LGS remained on their regular CLB treatment (Devinsky et al. 2017, 2018a, Thiele et al. 2018). The anti-epileptic effects of CLB have been attributed to its active metabolite N-desmethyloclobazam (Kinoshita et al. 2007). Moreover, following long term dosing of CLB in humans, N-desmethyloclobazam is found in higher concentrations than CLB owing to its longer plasma half-life (Lundbeck LLC 2011). Clinical studies have revealed elevated levels of CLB and N-desmethyloclobazam in patients treated with their regular CLB regimen supplemented with CBD (Geffrey et al. 2015, Devinsky et al. 2018b). Thus, the reduction in seizure frequency observed in participants of the CBD trials for DS and LGS could be due to increased plasma concentrations of CLB and its active metabolite. In terms of the interaction of phytocannabinoids with benzodiazepines at the level of GABA\textsubscript{A}Rs, further investigations such as single channel studies would help elucidate how CBD and CBDV may be modulating the action of DZP and CLB.

4.5 CBD and CBDV act as positive allosteric modulators at glycine receptors

Research into the impact of CBD on GlyRs revealed that this phytocannabinoid can potentiate I\textsubscript{gly} mediated by all homomeric receptors, \(\alpha_1\), \(\alpha_2\), \(\alpha_3S\) and \(\alpha_3L\), with differing efficacies (Figure 37A). Currents from \(\alpha_1\) and \(\alpha_3L\) receptors were potentiated to similar levels. In contrast, \(\alpha_2\)-mediated I\textsubscript{gly} was the least affected by CBD. This is in line with previous studies by Xiong et al. (2011, 2012a) who
demonstrated that the GlyR subunits $\alpha_1$ and $\alpha_3$ are equally sensitive to THC and AEA, and that GlyR $\alpha_2$ subunits are significantly less sensitive. These particular observations led Xiong et al. (2011, 2012a) to investigate important amino acids in the GlyR structure that could underpin the contrasting cannabinoid sensitivities. Site-directed mutagenesis revealed that residue S296 in $\alpha_1$ TM3 is necessary for mediating THC and AEA effects whilst residue A303 in $\alpha_2$ is responsible for the low sensitivity of the receptor to the cannabinoids. However, in the present study, CBD action at mutant GlyRs $\alpha_1^{S296A}$ and $\alpha_2^{A303S}$ did not differ compared to wild-type receptors (Figure 39C). This suggests that CBD acts at a site distinct from THC and AEA.

The CBD-induced potentiation of $I_{gly}$ measured in this study developed gradually during continuous application of the phytocannabinoid. Again, this is in agreement with reports from Xiong et al. (2011, 2012a) which described THC and AEA modulation of glycine-gic currents. $I_{gly}$ potentiation by CBD was sustained throughout the 12 minute drug superfusion period, seemingly reaching a plateau within this time. This differed from the transient enhancement of GABA$_A$R-mediated currents. CBDV also induced a prolonged potentiation of $I_{gly}$ mediated by homomeric GlyRs $\alpha_1$, $\alpha_2$ and $\alpha_3L$. However, unlike CBD, THC and AEA, CBDV had a similar effect on these three receptors and did not display any subunit selectivity (Figure 37B). This indicates that CBDV does not act via the same site as THC and AEA. This was further confirmed by experiments with mutant GlyRs $\alpha_1^{S296A}$ and $\alpha_2^{A303S}$. These mutations had no effect on the overall $I_{gly}$ potentiation induced by CBDV (Figure 39E).

4.5.1 CBD and CBDV potentiate $I_{gly}$ in a sustained manner

The sustained action of both phytocannabinoids on GlyRs is likely to contribute to their anticonvulsant properties. Though these receptors are not as abundant in the CNS as GABA$_A$Rs, nor are they generally associated with epilepsy disorders, cases have been reported where patients with GlyR autoantibodies presented with
recurrent focal seizures (Wuerfel et al. 2014, Ude and Ambegaonkar 2016). This would suggest that in parallel to GABA_{A}Rs, GlyRs have an important role to play in maintaining neuronal excitability in check. Indeed, studies have highlighted the ability of GlyRs to depress hyperexcitability. Perfusion of hippocampal slices with high K\(^+\) solution or the GABA_{A}R antagonist bicuculline induces spontaneous discharges in the dentate gyrus resembling epileptic spikes. Chattipakorn and McMahon (2003) found that bath application of glycine in these models completely interrupted the bursting firing pattern recorded in granule cells, and this effect was abolished in the presence of the GlyR antagonist strychnine. This study further highlighted that GlyR-mediated inhibition is effective at depressing hyperexcitable circuits, regardless of the compromised GABAergic signalling. \(I_{gly}\) has also been recorded in pyramidal cells and interneurones of the hippocampus and these currents were shown to be mediated by both \(\alpha\) homomeric and \(\alpha\beta\) heteromeric GlyRs (Chattipakorn and McMahon 2002). Here, GlyR activation by exogenous glycine reduced action potentials generated by depolarising current steps and depressed synaptically-evoked excitatory postsynaptic potentials (EPSPs) in both CA1 pyramidal cells and interneurones (Song et al. 2006). In a separate study, extracellular glycine accumulation in hippocampal slices, brought about by inhibition of the glial glycine transporter GlyT1, resulted in a rightward shift of the EPSP-spike relation (Zhang et al. 2008). This indicated that, for a fixed EPSP, there was a reduced likelihood of action potential generation in CA1 neurones. Using a pentylenetetrazol-induced seizure model, the authors also found that the same GlyT1 inhibitor acted as an anticonvulsant, increasing the latency of seizure onset and decreasing seizure duration (Zhang et al. 2008). Altogether, these findings suggest that CBD and CBDV-induced potentiation of GlyR-mediated currents could underpin the anticonvulsant action of these phytocannabinoids.

Immunohistochemical studies have identified hippocampal interneurones immunopositive for both glycine and GABA, confirming an endogenous source of glycine within the hippocampus (Song et al. 2006, Muller et al. 2013). Moreover, GlyR immunoreactivity was observed at both interneurones and pyramidal neurones in rat primary hippocampal cultures and hippocampal slices from adult rats, where
these receptors dominantly localised at extrasynaptic sites, and partially at synaptic sites (Brackmann et al. 2004, Aroeira et al. 2011). Thus, the widespread presence of GlyRs in the hippocampus and the sustained CBD and CBDV-induced enhancement of $I_{\text{gly}}$ could compensate for the slight inhibitory effect of these compounds on $I_{\text{GABA}}$ previously detailed in the present study. Action of the phytocannabinoids at GlyRs could prevail over that at $GABA_A$Rs, leading to an overall increase in inhibitory signalling and dampened excitability of pyramidal cells. Such a net result would be in agreement with findings from Kaplan et al. (2017) and Khan et al. (2018) in their study of CBD.

Quantitative analysis of different GlyR subunit messenger ribonucleic acids (mRNA) in mature rat hippocampus revealed a higher expression of $\alpha_2$ and $\alpha_3$ compared with $\alpha_1$. Additional immunohistochemical studies found the low $\alpha_1$ expression to be synaptic, whilst $\alpha_2$ and $\alpha_3$ predominantly locate to extrasynaptic sites (Aroeira et al. 2011). The ability of CBD and CBDV to act at all homomeric GlyRs suggests they could play a part in both phasic and tonic glycineergic modulation, thereby ensuring the regulation of dysfunctional excitatory circuits in epilepsy. Furthermore, glycine has been shown to accumulate in hippocampal glutamatergic presynaptic terminals where it is stored in vesicles and released during synaptic activity (Muller et al. 2013). This together with the widespread distribution of GlyRs in the hippocampus, particularly at extrasynaptic sites of principal cells (Brackmann et al. 2004, Aroeira et al. 2011), suggests that phytocannabinoid action at glutamatergic synapses is primed to enhance tonic glycineergic inhibition, thereby reducing excessive excitability during epileptiform activity.

4.5.2 CBD differentially potentiates $I_{\text{gly}}$ mediated by $\alpha_3S$ and $\alpha_3L$

Alternative splicing results in two $\alpha_3$ subunit variants that only differ by 15 amino acids in the large cytoplasmic loop between TM3 and TM4. Functional differences between the two variants were only found in their desensitisation kinetics: incorporation of the 15 amino acid sequence in $\alpha_3L$ confers a slower desensitisation
on the receptor (Nikolic et al. 1998). Surprisingly, CBD had a pronounced differential effect on these two splice variants. The phytocannabinoid was significantly more efficacious at enhancing $I_{\text{gly}}$ mediated by $\alpha_3\text{S}$. The magnitude of potentiation at this receptor was twice that observed at $\alpha_3\text{L}$. Secondary structure analysis of the two $\alpha_3$ GlyR isoforms found that the 15 amino acid insert stabilises the fold of the TM3-TM4 cytoplasmic domain, with a resulting effect on channel gating (Breitinger et al. 2009). Thus, CBD binding to $\alpha_3\text{S}$ could be stabilising the receptor and facilitating channel gating. It would be interesting to examine the effect of CBD on the characteristically fast desensitisation profile of the receptor. In the hippocampus, mRNA expression of $\alpha_3\text{L}$ was found to outweigh that of $\alpha_3\text{S}$ (Eichler et al. 2009). The same study reported an up- and down-regulation of $\alpha_3\text{S}$ and $\alpha_3\text{L}$, respectively, in the resected hippocampus of patients with a severe course of intractable temporal lobe epilepsy. Considering the respective desensitisation kinetics of the splice variants, this altered expression might translate as a decrease in tonic glycinergic inhibition. However, the stronger CBD effect at $\alpha_3\text{S}$ GlyRs would be particularly favourable in such a situation. Indeed, if upregulation of $\alpha_3\text{S}$ expression on hippocampal principal cells is a general feature of DS or LGS, CBD action at these receptors would lead to an overall increase in glycinergic inhibition, contributing to the anticonvulsant properties of the phytocannabinoid.

4.6 Differences between CBD and CBDV

CBD and CBDV are structural homologues that only differ in the length of the alkyl group: CBD has a five carbon side chain whilst CBDV has a shorter three carbon side chain. However, this structural distinction is not trivial, as the two phytocannabinoids appear to have discrete actions and pharmacological profiles. This is most apparent with the effect of CBD and CBDV on fatty acid amide hydrolase (FAAH), the enzyme responsible for the degradation of the endocannabinoid AEA. CBD was shown to inhibit this amidase ($IC_{50}$ 15.2 μM) whilst CBDV had a minimal impact (De Petrocellis et al. 2011). On the other hand, the same study found CBD had no appreciable effect on the enzyme catalysing 2-AG biosynthesis, diacylglycerol
lipase, though CBDV acted to inhibit this particular enzyme (IC$_{50}$ 16.6 μM). It should be noted that inhibition of FAAH and diacylglycerol lipase by CBD and CBDV, respectively, occurred at micromolar concentrations not considered to be physiologically relevant. In addition, though neither phytocannabinoid is considered as having relevant physiological affinity for the cannabinoid receptors, the inhibition constant ($K_i$) of CBDV for the CB$_1$ receptor was reported to be ten-fold greater than CBD (Rosenthaler et al. 2014). Similarly, CBDV was also found to be a more potent inhibitor of lysophosphatidylinositol-activation of GPR55 downstream signalling (Anavi-Goffer et al. 2012). It should thus come as no surprise to find that CBD and CBDV exhibited different efficacies at GABA$_A$Rs. This was particularly visible at $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_4\beta_2\delta$ receptors where CBDV acted as a significantly better potentiator of $I_{GABA}$ than CBD. Similarly, assessment of the anticonvulsive effects of these compounds in an audiogenic seizure model revealed that CBDV is more potent at reducing clonic convulsions (Hill et al. 2013). I also found that the time course profiles of the two phytocannabinoids were different at benzodiazepine-insensitive GABA$_A$Rs (Figure 16). CBD had a more prolonged enhancing effect on $I_{GABA}$ mediated by $\alpha_1^{H102R}\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors, suggesting that the classical benzodiazepine binding site modulates the response of the receptor to CBD binding. Further, if CBD were to interact with GABA$_A$Rs at multiple sites, the altered profile of the benzodiazepine-insensitive receptors might indicate a secondary CBD binding site overlapping that of the benzodiazepines. On the other hand, the current run-down observed with prolonged application of CBDV was similar at $\alpha_1\beta_2\gamma_2$, $\alpha_1^{H102R}\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors. This finding would suggest that CBDV binding does not overlap the classical benzodiazepine binding site, nor is the latter site involved in modulating the long term effect of CBDV. The slight differences in the way the two phytocannabinoids interact with GABA$_A$Rs may have important implications for combination treatments. Indeed, if these compounds do not compete for the same binding sites, there is a potential for additive or synergistic effects upon co-application. In agreement with this idea are findings from an isobolographic study that indicated an additive anticonvulsant action of CBD and CBDV when co-administered in an audiogenic seizure mouse model (Hill et al. 2013). An isobolographic study uses individual dose-responses for agonist “A” and agonist “B” to determine a specified dose-effect value for each, for example, the dose that
results in 50% of the maximum effect, or EC\textsubscript{50}. The EC\textsubscript{50} value for A and B is then plotted on the y- and x-intercept, respectively, and the straight line connecting these two points is termed an isobole. The isobole represents a theoretical range of A-B dosage combinations that would result in 50% of the maximal effect, given there is a simple additivity between A and B. In other words, given there is no interaction between these agonists. Experimental dosage combinations can be compared to this isobole; points plotted below the isobole denote a synergistic effect between agonists (Tallarida 2011). Though the isobolographic study carried out by Hill et al. (2013) revealed no supra-additive effect, or synergy between CBD and CBDV, the simple additive effect may still be clinically relevant if it comes to light that one or the other phytocannabinoid has a worse adverse event profile. In this case, the ratio of compound concentrations may be adjusted to reduce side-effects, without compromising on therapeutic benefits. In any case, the Hill et al. (2013) study only tested the phytocannabinoid combination on one particular endpoint, clonic seizure incidence, in one seizure model. There is thus a possibility that a mixture of the compounds have a synergistic effect on other endpoints. To this end, it would be interesting to assess the modulation of I\textsubscript{GABA} by a combination of CBD and CBDV.

### 4.7 Study limitations

In this study I investigated the direct effects of CBD and CBDV on inhibitory receptors normally expressed in the CNS. An \textit{in vitro} approach was taken whereby GABA\textsubscript{A} or GlyRs were transiently transfected in HEK293 cells. These cells contain the biochemical machinery necessary for post-translational processing and expression of functional exogenous mammalian proteins (Thomas and Smart 2005). Use of such a non-neuronal heterologous expression system minimises the presence and interference of other ion channels otherwise present in neurones, and simplifies the study of one particular receptor subtype. Moreover, the ease of maintenance, high transfection efficiency and relatively straightforward patching of HEK293 cells makes these amenable to functional receptor studies and evaluation of pharmacological effects on receptors of interest. However, HEK293 cells remain an artificial system
that overexpress transfected plasmids. This high expression of exogenously introduced proteins, though ideal for out-competing and masking the presence of similar endogenous receptors, does not faithfully mimic tightly regulated protein expression in vivo. Moreover, receptor pharmacology studies in isolated cells will lack the feedback circuits and other signalling processes that occur naturally in vivo, presenting an incomplete picture of drug activity. On the other hand, experiments carried out on ex vivo hippocampal slices are likely to mirror in vivo responses more closely, with the caveat that the direct action of compounds on specific receptor subtypes may be confounded by the presence of other native receptors in the cellular milieu. Thus, both methodologies present limitations and data should be interpreted accordingly.

Another limitation of this study was the poor solubility of CBD and CBDV in the perfusion solution. Concentrations higher than 10 µM could not be tested and as such, dose-response curves could not be generated. Consequently, I was unable to compare potencies of the phytocannabinoids at different receptor subtypes. Moreover, Bakas et al. (2017) showed a higher efficacy of CBD at α2-containing GABA\textsubscript{A}Rs at concentrations above 10 µM. Indeed, such a distinction amongst receptors with different α subunits could not be made at the 10 µM concentration used in the present study. Additionally, an agonist dose-response relation was not carried out at the start of each recording due to the particular method of agonist application. A Picospritzer was used to pressure eject GABA or glycine from a patch pipette positioned near the voltage-clamped cell. Though this method provides rapid and reproducible pressure pulses, only one concentration of agonist can be applied within a single experiment. This is in contrast to other methods of compound application such as the U-tube, so called because of the shape of the glass capillary used to apply compounds of interest. The inlet of the U-tube is attached to silicon tubing that can be switched between reservoirs containing different solutions, enabling the application of multiple agonist concentrations on one cell (Thomas and Smart 2012). Establishing an agonist dose-response relationship for each cell allows a sub-saturating concentration of agonist (EC\textsubscript{20} or lower) to be determined and used for subsequent pharmacological investigation.
This both reduces variability between data obtained from different cells, and ensures that any maximal current potentiation induced by a test compound is limited to the efficacy of the compound and not hindered by receptor saturation. Though a high agonist concentration was used in the present study (1 mM), the short pulse duration and small pipette opening (2-3 µm versus 200 µm at the apex of the U-tube) ensured that a sub-saturating concentration of agonist reached the cell. Indeed, throughout experiments, larger current responses could be obtained by increasing agonist pulse duration or puff pressure, indicating that receptors were not saturated. Nonetheless, the effective concentration of GABA or glycine used in these experiments was not known and may have been higher than an EC20 concentration. A number of studies have shown that cannabinoids elicit a stronger enhancement of I_{GABA/gly} at lower doses of agonist (Hejazi et al. 2006, Sigel et al. 2011, Bakas et al. 2017). This may explain the lower magnitude of I_{GABA} and I_{gly} potentiation induced by CBD in this study compared with published findings (Xiong et al. 2012a, 2012b, Bakas et al. 2017). Due to the limitation imposed by the method of agonist application, it is likely that the data presented here are an underestimate of the actual maximal current potentiation that can be induced by CBD and CBDV. However, this lower magnitude of current enhancement may correspond more closely to what occurs at the synapse, where presynaptic release of neurotransmitter leads to a transient saturating concentration of agonist in the synaptic cleft.

The same method of agonist application was used in experiments with hippocampal slices as the objective was to study the direct action of the phytocannabinoids on postsynaptic GABA_ARs. Again, the same limitation presented itself in that the exact GABA concentration reaching the target cell was unknown. This issue was likely intensified by the presence of any cellular tissue between puff pipette and target neurone which would have hindered the flux of GABA and thus binding to receptors. Nonetheless, puff application of GABA gave rise to measurable currents in pyramidal neurones and a trend for CBDV to depress these currents was observed.
4.8 Conclusions and perspectives

Public interest in cannabis-derived compounds has greatly increased in the last decade, particularly within the last couple of years. This has partly been fuelled by anecdotal evidence and clinical trials highlighting a myriad of therapeutic benefits that range from pain management in oncology to seizure control in refractory epilepsy syndromes. Indeed, both CBD and CBDV have shown promising anticonvulsant properties in preclinical animal models of epilepsy (Jones et al. 2010, Hill et al. 2012a, Jones et al. 2012, Kaplan et al. 2017). With the United Kingdom government announcing that specialist doctors are now able to legally prescribe cannabis-derived products for medicinal use in situations where there is an unmet clinical need (Home Office and The Rt Hon Sajid Javid MP 2018) and with CBD being the first FDA-approved drug to be extracted from cannabis (US Food & Drug Administration 2018), it appears cannabis-based medicines are here to stay. It has thus become all the more necessary to decipher the mechanisms of action and identify the particular targets of these phytocannabinoids. Understanding these finer details will provide greater insight into the phytocannabinoids themselves, but also into the biological processes and disorders in which they exert beneficial effects. This in turn will enable the design of improved therapeutic treatments.

The study detailed herein examined the effects of CBD and CBDV on inhibitory GABA\textsubscript{A} and GlyRs. The hypothesis was that these compounds act directly at inhibitory receptors, thereby mediating the anticonvulsant effects reported in various seizure models (Jones et al. 2010, Hill et al. 2012a, Jones et al. 2012, Kaplan et al. 2017). This was based on observations from other cannabinoids that have been shown to act at these receptors (Table 2 and 3), as well as the recognition that a number of existing anti-seizure treatments specifically target GABA\textsubscript{A}Rs. Several inhibitory receptor subtypes were investigated in the hopes of identifying a subunit selectivity that could hint at potential phytocannabinoid binding sites. However, both CBD and CBDV were able to potentiate currents from all GABA\textsubscript{A} and GlyR subtypes examined. This suggests that the CBD or CBDV binding sites are likely to be located within conserved regions of the receptors, such as the TMDs. A rather
unexpected finding was that phytocannabinoid-induced potentiation of $I_{\text{GABA}}$ in HEK293 cells was transient, suggesting that prolonged action of 10 µM CBD and CBDV leads to GABA$_A$R desensitisation. However, phytocannabinoid concentrations of 3 µM and lower induced a more gradual and sustained current potentiation, indicating that lower concentrations should be favoured for future GABA$_A$R studies. Single channel analysis might shed light on distinct behaviours. Additionally, several GABA$_A$R subtypes still need to be explored, particularly $\alpha_5$-containing receptors which were shown to mediate the tonic current in hippocampal pyramidal neurones (Caraiscos et al. 2004). CBD has been found to potentiate currents from $\alpha_5\beta_2\gamma_2$ receptors (Bakas et al. 2017), however, it would be interesting to examine the effect of a prolonged application of the phytocannabinoid and determine whether this potentiation is sustained. A steady-state increase in tonic inhibitory conductance in pyramidal neurones via $\alpha_5$-containing GABA$_A$Rs would amount to reduced neuronal output (Farrant and Nusser 2005).

In contrast to GABA$_A$Rs, 10 µM CBD or CBDV enhanced $I_{\text{gly}}$ in a sustained manner from all homomeric GlyRs. As previously discussed, there is significant expression of GlyRs in the CNS and these provide a non-trivial source of inhibitory transmission that can regulate excitatory circuits (Chattipakorn and McMahon 2003, Brackmann et al. 2004, Song et al. 2006, Aroeira et al. 2011). As such, phytocannabinoid activity at these receptors could very well contribute to their anticonvulsant actions. Future studies would need to investigate the effect of CBD and CBDV on glycinergic inhibition in ex vivo brain slices and assess whether these compounds can dampen hyperexcitability in epilepsy models. The regulatory role of GlyRs in nociceptive signalling has steered clinical research towards identifying GlyR modulators for the treatment of chronic pain (Imlach 2017). In line with this, CBD and its modified derivatives have been shown to suppress chronic inflammatory and neuropathic pain in a GlyR-dependent manner in rodent models (Xiong et al. 2012b, Lu et al. 2018). In contrast, few studies have explored the role and potential benefits of modulating glycinergic neurotransmission in epilepsy disorders despite findings that GlyR-mediated inhibition is effective at depressing hyperexcitable circuits (Chattipakorn and McMahon 2003, Zhang et al. 2008). Though no seizure
medication targeting GlyRs currently exists, it is possible for such a mechanism of action to bring added value as an adjunct treatment in various epilepsy disorders.

The data from this study confirm that CBD, and for the first time CBDV, have direct effects on GABA_A and GlyRs, thus promoting the idea that action at inhibitory receptors underpin the anticonvulsant properties of these phytocannabinoids. However, the poly-pharmacological profile of these compounds should not be overlooked. As touched upon in section 1.2.1, CBD is likely to play a role in intracellular Ca^{2+} regulation, and thus neuronal excitability, by targeting T-type voltage-gated Ca^{2+} channels (Ross et al. 2008), the mitochondrial Na^+/Ca^{2+}-exchanger (Ryan et al. 2009) and GPR55 (Sylantyev et al. 2013). These studies used physiologically relevant concentrations of 1 μM CBD. Activation and rapid desensitisation of cation-permeable TRP channels by CBD could also regulate neuronal excitability (De Petrocellis et al. 2011, Iannotti et al. 2014). However, there is limited evidence suggesting a direct involvement of TRP channels in epilepsy and CBD potency at these receptors varies across studies (Ibeas Bih et al. 2015). In addition to these targets, CBD has also been shown to inhibit degradation of AEA by FAAH (Watanabe et al. 1996, Bisogno et al. 2001, De Petrocellis et al. 2011). AEA is an endogenous CB_1 receptor ligand and a CBD-induced elevation of this endocannabinoid would result in suppression of excitatory neurotransmitter release via activation of presynaptic cannabinoid receptors (Ohno-Shosaku et al. 2002). It should be noted, however, that in vitro effects of CBD on FAAH activity occurred at high concentrations of the phytocannabinoid (> 20 μM) that might not otherwise be physiologically achievable. In patients treated with both CLB and CBD, yet another indirect modulation of excitatory signalling could result from a CBD-mediated increase in the benzodiazepine and its active metabolite, N-desmethylclobazam. Indeed, CBD is a potent inhibitor of cytochrome P450 enzymes, CYP3A4 and CYP2C19 (Yamaori et al. 2011, Jiang et al. 2013), both responsible for the metabolism of CLB and its metabolite (Giraud et al. 2004). CBDV, by structural homology, is anticipated to have a similar myriad of effects and indeed this phytocannabinoid has already been shown to activate and rapidly desensitise various TRP channels (Iannotti et al. 2014), as well as inhibit GPR55 (Anavi-Goffer et
al. 2012) and CYP2C19 (Jiang et al. 2013). Future studies are likely to uncover additional targets that can contribute to the anticonvulsant properties of both CBD and CBDV. For instance, the effect of these phytocannabinoids on neurotransmitter transporters remains to be elucidated. Early studies demonstrated that high concentrations of CBD inhibit GABA uptake in synaptosomal preparations (Banerjee et al. 1975, Hershkowitz et al. 1977). It would be interesting to assess whether physiologically relevant concentrations of CBD and CBDV influence the activity of GABA, glycine or glutamate transporters, which would have significant consequences on neuronal excitability. Indeed, blockade of GABA uptake has been shown to result in a measurable tonic GABAergic current in CA1 pyramidal cells, which was masked under normal GABA uptake conditions (Semyanov et al. 2003). Similarly, inhibition of glycine transporter GlyT1 was shown to induce a reduction in EPSP-spike coupling in CA1 pyramidal neurones (Zhang et al. 2008). Thus, both GABA and glycine transporters play an important role in regulating ambient concentrations of these neurotransmitters and in the fine-tuning of the resulting tonic inhibitory currents, making these proteins potential targets of phytocannabinoids.

The multimodal actions of CBD and CBDV uncovered thus far, most of which impact neuronal excitability, suggest that the anticonvulsant effects produced by these phytocannabinoids are the result of several cannabinoid receptor-independent mechanisms. The direct potentiation of \( I_{GABA} \) and \( I_{gly} \) by CBD and CBDV shown in this study is likely to be an important component underlying their mechanisms of action, particularly considering that physiologically relevant low micromolar concentrations of the compounds had an effect on some of the inhibitory receptors investigated. Further studies would be required to confirm that these receptors represent phytocannabinoid target sites for mediating seizure alleviation. Conclusive findings, however, may not be reached without further information regarding CBD and CBDV binding at GABA\(_A\) and GlyRs, or without the assistance of an antagonist acting at the same site. For instance, knowledge of the benzodiazepine binding site on GABA\(_A\)Rs allowed the creation of genetically modified mice expressing a benzodiazepine-insensitive GABA\(_A\)R (Rudolph et al. 1999). DZP was shown to have reduced anticonvulsant activity against pentylenetetrazol-induced convulsions in these mice.
compared to wild-type mice. Moreover, any remaining anticonvulsant activity due to action at other GABA$_{A}$Rs was abolished by the benzodiazepine antagonist flumazenil (Rudolph et al. 1999). Studies such as these clearly demonstrate that the antiepileptic effect of benzodiazepines is due to GABA$_{A}$R activity and the enhancement of GABAergic inhibition. Thus, further work is required to identify possible binding sites of the phytocannabinoids and enable target validation in the context of epilepsy.

With 30% of seizure-sufferers not achieving seizure control with currently available AEDs (Kwan and Brodie 2000), it is critical to keep formulating alternative treatments. As clinical trials have shown, CBD has the potential to address unmet clinical needs of refractory epilepsy disorders (Devinsky et al. 2017, 2018a, Thiele et al. 2018). Further dissecting the pharmacologically complex profile of this phytocannabinoid and its structural homologue will be essential for identifying alternative molecules with similar, more selective, activity.
References


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Baur R., Gertsch J. and Sigel E. (2013b). "Do N-arachidonyl-glycine (NA-glycine) and 2-arachidonoyl glycerol (2-AG) share mode of action and the binding site on the β_2 subunit of GABA_A receptors?" PeerJ 1: e149.


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mutated α1S267I glycine receptors by cannabinoids." Naunyn Schmiedebergs Arch Pharmacol 381(5): 477-482.


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<table>
<thead>
<tr>
<th>Gene amplified</th>
<th>Restriction enzyme</th>
<th>Primer sequence (5’ – 3’)</th>
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**Table 1.** Oligonucleotide primer pairs used to amplify the specified gene sequence from human cerebellum cDNA. The restriction enzyme recognition site is highlighted in bold.
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</table>

*Table 2.* Site-directed mutagenesis oligonucleotide primer pairs with the mutated codon denoted in bold lowercase.