Maria del Carmen Claudio, Carisoprodol's Pharmacological Properties at $GABA_A$ Receptors, *in-vitro* and *in-silico* studies. Doctor of Philosophy (Biomedical Sciences), April 2021, 97 pp., 8 tables, 12 figures, bibliography, 72 titles.

Carisoprodol (CSP) is a centrally-acting prescription muscle relaxant that can directly activate, allosterically modulate and inhibit $GABA_A$ receptors. The $GABA_A$ receptor is a pentameric chloride ion channel in the cys-loop receptor family. The mechanism of $GABA_A$'s inhibitory role in the central nervous system lies in the resulting hyperpolarized state of the cell following chloride ion influx upon ligand binding. $GABA_A$ receptors are the target of many different clinically prescribed compounds because of the role they play in regulating the central nervous system. We used pharmacological and computational approaches to investigate the underlying mechanism mediating carisoprodols effects at $GABA_A$ receptors, with the ultimate goal of generating a new subunit selective compound related to the structure of carisoprodol and gaining a more thorough understanding of the molecular interaction governing carisoprodol's pharmacoglogical effects. Our evaluation of novel compounds related to the structure of carisoprodol did not yield promising leads, though the potential still remains for development of a novel carisoprodol-related compound with a unique selectivity profile. Probe for a binding site mediating carisoprodols postive modulatory effects and evaluation of additional novel compounds was eventually hindered by time. Our investigaiton into carisoprodol's direct gating effects involved a previously reported single amino acid residue, L415, located at the top of the fourth transmembrane domain (TM4) in the $\alpha 1$ subunit of the $GABA_A$ receptor that is critical to carisoprodol's direct gating. Whether the residue is involved in a carisoprodol binding site remained unsolved. In studies probing for a binding site for carisprodol's direct activation of

$GABA_A$ receptors, promising computaitonal docking data was not able to be validated with elecrophysiology and site-directed mutatgenesis studies, indicating that the residues revealed in docking studies do not form a binding pocket for carisprodol's direct activation effect. Our site directed mutagenesis, electrophysiology and molecular dynamic simulation studies to investigate carisoprodol's inhibitory effects at $GABA_A$ receptors revealed a binding site at the Cl^- channel pore, in a mechanism similar to picrotoxin, providing a mechanism of action for carisoprodol's inhibitory effects at $GABA_A$ receptors.

CARISOPRODOL'S PHARMACOLOGICAL PROPERTIES AT
$GABA_A$ RECEPTORS, *IN-VITRO* AND *IN-SILICO* STUDIES
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DISSERTATION

Presented to the Graduate Council of the Graduate School of Biomedical Sciences

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For the Degree of

DOCTOR OF PHILOSOPHY

By

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DEDICATION

This is dedicated to Reynaldo Miranda, who always supported my goals and would have been so proud of this achievement.

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GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
PTX	picrotoxin
$5\text{-}HT_3$	5-hydroxytryptamine type 3 receptor
CNS	central nervous system
TM1	first transmembrane domain
TM2	second transmembrane domain
TM3	third transmembrane domain
TM4	fourth transmembrane domain
FDA	Food and Drug Administration
nACh	nicotinic acetylcholine receptor
HEK	human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid
DMSO	dimethyl sulfoxide
Cryo-EM	cryogenic-electron microscopy

CHAPTER I
INTRODUCTION

GABA & $GABA_A$ Receptors

γ-aminobutyric acid (GABA) (Fig. 1) is the major fast inhibitory neurotransmitter of the adult central nervous system (CNS) and acts at two major classes of structurally distinct receptors: $GABA_A$ and $GABA_B$ receptors. $GABA_B$ receptors are a class of G-protein coupled receptors while $GABA_A$ receptors are ligand-gated ion channels. The focus of this dissertation is $GABA_A$ receptors.

When synaptic vesicles fuse with the presynaptic membrane as a result of action potential arrival at a GABAergic neuron, GABA is released into the synaptic cleft. Activation of $GABA_A$ receptors occurs when GABA binds extracellularly at the GABA binding site, triggering a conformational change resulting in chloride ions (Cl^-) to flow through the ion channel. In development, Cl^- concentrations inside cells are higher than extracellular concentrations, in contrast to mature neurons. $GABA_A$ receptor activation in early neuronal development results in depolarization as Cl^- follows its electrochemical gradient, rather than hyperpolarization seen in mature neurons. In mature neurons, however, the K^+Cl^- cotransporter type 2 (KCC2) maintains low intracellular Cl^- concentrations and activation of $GABA_A$ receptors results in Cl^- influx, hyperpolarization and reduction in membrane excitability. Relative Cl^- concentrations dictate the direction of flow of ions through the channel pore (Macdonald and Olsen, 1994; Whiting, 2003; Huang, Gonzales and Dillon, 2006).

$GABA_A$ receptors belong to the cys-loop receptor superfamily, which is comprised of pentameric ligand gated ion channels mediating fast synaptic transmission; a disulfide bridge

linking cysteines forms a loop at the extra-cellular domain of these receptors. The receptors in this family are further classified by the neurotransmitter which binds and activates. Other cys-loop receptors include nicotinic acetylcholine (nACh), 5-hydroxytryptamine type 3 ($5HT_3$) and glycine receptors (Miller and Smart, 2010). Common structural features of receptor subunits are a large amino-terminal domain containing the cys-loop and four transmembrane domains (TM1-4). TM2 lines channel pore while TM3 and TM4 form a large intracellular loop where phosphorylation plays a role in regulation. (Fig. 2) (Huang, Gonzales and Dillon, 2006; Miller and Smart, 2010; Sigel and Steinmann, 2012)

$GABA_A$ receptors are pentameric membrane proteins comprising eight different classes of subunits, many with multiple variants (α1-6, β1-3, γ1-3, ρ1-3, δ, ε, π and θ). (Barnard *et al.*, 1998; Whiting *et al.*, 1999). These receptors are expressed throughout the brain and the spinal cord (Waldvogel, Baer and Faull, 2010). About 70% sequence homology exists within the $GABA_A$ receptor subunit family. Further receptor diversity arises from alternative splicing and RNA editing (Sigel and Steinmann, 2012) Within the $GABA_A$ receptor family, the combination of α1β2γ2 (2α:2β:1γ subunit stoichiometry) is the most abundantly expressed receptor configuration in the brain, although many different combination of subunit isoforms can come together to make a functional receptor (Fritschy and Brünig, 2003). Subunit composition and arrangement of a given receptor configuration contributes to receptor trafficking, kinetics, and pharmacology. (Castellano, Shepard and Lu, 2021) Activation of $GABA_A$ receptors results in the opening of a Cl^- ion channel. The magnitude of influx or efflux of chloride ions is driven by relative chloride ion concentration across the membrane of the cells. (Sigel and Steinmann 2012). $GABA_A$ receptors can be found both at the synapse and extrasynaptically. Synaptic $GABA_A$ receptors mediate phasic inhibition, where there is rapid transmission of pre-synaptic

activity to post-synaptic signals. In phasic inhibition, GABA-release into the synaptic cleft binds to post-synaptic receptors to mediate $GABA_A$ receptor channel opening in fast inhibitory neurotransmission. Tonic inhibition refers a slower form of signaling, mediated by extra-synaptic $GABA_A$ receptors located on the cell body, resulting in a more random and constant activation of receptors compared to phasic inhibition. (Farrant and Nusser, 2005).

Specific $GABA_A$ receptor subunit distribution

Most commonly found at the synapse are α, β, and γ subunit receptors, while δ subunits are commonly associated with extra-synaptic receptors. (Whiting, 2003) The α1 subunit is the most widely available subunit in the human brain and can commonly be found associated with β2, β3 and γ2 subunits. Results from human mRNA probes and immunohistochemical staining of post-mortem human brains show that α1, β2, and β3 are the most ubiquitously expressed throughout the central nervous system (CNS) and most densely found in the cerebral cortex, basal ganglia, thalamus, hippocampus, brain stem, and spinal cord. Human antibodies revealed expression of α1-3, β2-3, and γ2 subunits at the spinal cord; α1-3 and β2-3 subunits were found in particularly high levels at the sensory region of the spinal cord. Other subunits have a much more restricted distribution, such as α6 subunits which has restricted expression to only granule cells of the cerebellum. The ρ subunits are mostly expressed in the retina and have limited expression in other areas of the CNS. The δ, ε, π and θ subunits are thought to be less widely available than other subunits, but studies have been limited by the lack of reliable antibodies for these subunits to study their distribution in the human brain. (Sigel and Steinmann, 2012).

Subunit composition and stoichiometry are responsible for different pharmacological properties of $GABA_A$ receptor subtypes.

Compounds acting at $GABA_A$ receptors and their specific binding sites

Because of GABA's widespread role in the brain it is the target of many different compounds to treat epilepsy, insomnia, anxiety, and certain types of pain (Rudolph, Crestani and Möhler, 2001; Rudolph and Möhler, 2006; Zeilhofer, Witschi and Hösl, 2009). In the following section I discuss specific compounds and their sites of action at $GABA_A$ receptors to provide insight to the physiological functions and diverse receptor pharmacology associated with $GABA_A$ receptors.

The GABA binding site is located at interface of α and β receptor subunits. Recent cryo-electron microscopy structures provide detailed binding information about several different chemicals that can bind to $GABA_A$ receptors, including the endogenous neurotransmitter, GABA. These structures provided insight to two GABA binding pockets at the extracellular domain of the α1-β2 interfaces of a 2-α1: 2-β2:1-γ2 receptor configuration. GABA binding was found to be mediated by interactions with aromatic residues and complementary electrostatic interactions at both the anionic and cationic ends of GABA's structure.(Miller and Aricescu, 2014; Zhu *et al.*, 2018)

Benzodiazepines are a class of $GABA_A$ receptors positive modulators that enhance GABA activated currents. These drugs are commonly used as anxiolytics and anticonvulsants, but can also be addictive. The benzodiazepine binding site is an allosteric site without a naturally occurring ligand (D'Hulst, Atack and Kooy, 2009; Castellano, Shepard and Lu, 2021). This binding site is located at the interface of the α and γ subunits; specific arginine residues at

the α2 and α6 subunit render α2/6 $GABA_A$ receptors insensitive to benzodiazepines such as diazepam (Fig. 1), whereas the other four subunits contain a histidine residue at the same position and retain sensitivity to benzodiazepines. These examples demonstrate the highly specific nature of $GABA_A$ receptor pharmacology. (Möhler, Crestani and Rudolph, 2001; Tan *et al.*, 2010) Recent cryo-electron microscopy structures revealed detailed interactions between diazepam and $GABA_A$ receptors at the extracellular domain of the α1-γ2 interface of a 2-α1: 2-β2:1-γ2 receptor configuration. This binding site was further confirmed with cryo-electron microscopy structures detailing interactions of the competitive antagonist flumazenil and the benzodiazepine site. Flumazenil is a drug that can be used clinically to reverse benzodiazepine overdose. Histidine 102 at α1-3 and α5 subunits was confirmed to render $GABA_A$ receptors sensitive to benzodiazepines at the benzodiazepine binding site, whereas insensitivity of α2 and α6 subunit receptors to benzodiazepines was shown to be attributed to an arginine residue at this same position. This is explained by the lack of ability of arginine to form an essential hydrogen bond with drugs able to bind to the benzodiazepine binding site. While diazepam and flumazenil are two similar compounds binding at the benzodiazepine site, it is interesting to note that diazepam is a positive modulator of activated GABA currents while flumazenil has no effect on GABA activated current and also blocks diazepam's action. Even with these new highly detailed structures, there is still much to be studied about drug interaction at the benzodiazepine binding site (Sieghart *et al.*, 1999; Zhu *et al.*, 2018; Masiulis *et al.*, 2019) .

Barbiturates are one of the oldest classes of anesthetics and it has been shown that their pharmacological effects are dependent on different $GABA_A$ receptor subunits, particularly the α subunit. Barbiturates are also able to directly gate these receptors and α6 receptors have the

highest potency for barbiturate action, while β subunits have the most impact on barbiturate efficacy. In addition to being able to potentiate $GABA_A$ receptor currents, at high concentrations a rebound current indicated the occupation of a low-affinity inhibitory binding site. (Huang, Gonzales and Dillon, 2006) More recent studies indicated a barbiturate binding site at the TM1 region of α subunits and the TM3 of β subunits in an inter-subunit binding site; both of these subunits have been indicated to influence barbiturate activity in previous studies. (Li *et al.,* 2006)

Neurosteroids are another class of compounds acting at the $GABA_A$ receptor and have been indicated in anxiolytic, sedative, anticonvulsant and analgesic effects. These compounds have a broad action at both synaptic and extra-synaptic $GABA_A$ receptors. At low concentrations, neurosteroids such as allopregnanolone have a robust modulatory effect at δ subunit receptors. Notably, GABA is not a strong agonist at these receptor subtypes and neurosteroids are thought to increase the likelihood of GABA to open this receptor subtype. (Castellano, Shepard and Lu, 2021) Recently, the neurosteroid brexanolone (also known as allopregnanolone) was approved by the FDA to treat post-partum depression, a condition during which there may be loss of normal GABAergic inhibition that can be restored by tonic activation of $GABA_A$ receptors. It is suspected that in a compensatory mechanism in response to high levels of neurosteroids in the brain during pregnancy, there is down regulation of δ $GABA_A$ receptors, resulting in decreased GABAergic inhibition, leading to post-partum depression. (Lambert *et al.*, 2003; Brickley and Mody, 2012; Zorumski *et al.*, 2019; Belelli *et al.*, 2020)

Picrotoxin is a pro-convulsant plant alkaloid that acts as a $GABA_A$ receptor channel blocker that irreversibly inhibits $GABA_A$ receptors; picrotoxin is made up of an equimolar mixture of the compounds picrotoxinin and picrotin. (Fig. 1) Recent cryo-electron microscopy structures reveal specific binding of picrotoxin to a region of transmembrane domain 2 (TM2) of

the $GABA_A$ receptor channel. Picrotoxin structures revealed sequestration into the channel pore between the 2' and 9' TM2 rings, which had been previously demonstrated by site-directed mutagenesis and electrophysiology studies. Irreversible inhibition was demonstrated as picrotoxin became trapped within the channel pore between the TM2 2' and 9' pore lining residues (Macdonald and Olsen, 1994; Huang, Gonzales and Dillon, 2006; Gonzales *et al.*, 2008; Masiulis *et al.*, 2019).

Each of these well-studied compounds have distinct mechanisms of action and demonstrate the highly specific nature of $GABA_A$ receptor pharmacology. In the following sections, I will expand on the importance of subunit-specific pharmacology in $GABA_A$ receptor drug development and implications for treatment of pain in regard to our drug of interest: carisoprodol, (Fig. 1) a clinically prescribed muscle relaxant used to treat low-back pain, which modulates $GABA_A$ receptor activity.

Targeting specific subunits to select for drug effects

Therapeutic effects associated with $GABA_A$ receptor activation include anxiolysis, muscle relaxation, anticonvulsant properties and pain modulation. Because of subunit specific receptor pharmacology observed with different compounds that act at $GABA_A$ receptors, there is a way to selectively target certain effects associated with modulation of GABA-activated currents, while potentially minimizing negative side effects such as sedation, tolerance, and addiction (Olsen and Tobin, 1990; Korpi, Gründer and Lüddens, 2002; Davies, 2003; Nutt, 2006).

Studies show that specific therapeutic and unwanted side effects are associated with certain $GABA_A$ receptor subunits, particularly the α subunit. In studies on benzodiazepine-based

drugs, it was found that sedative properties are associated with α1 receptors, anxiolytic properties are associated with α2 receptors, muscle relaxant effects are associated with α2, α3, and α5 receptors, anti-convulsive effects are associated with α1 receptors, amnesia is associated with α1 and α5 receptors, and addictive properties are associated with α1 receptors (Table 1) (Griebel *et al.*, 2001; Tan, Rudolph and Lüscher, 2011; Munro, Hansen and Mirza, 2013; B. D. Fischer *et al.*, 2017).

One of the most dangerous and unwanted side effects associated with $GABA_A$ drugs is addiction. Addictive properties of these drugs are thought to be associated with specific subunit distribution in mesolimbic dopaminergic pathways where there is high expression of α1 subunits on GABAergic neurons of the ventral tegmental area (VTA). These GABAergic neurons at the VTA form synapses with dopaminergic neurons that contain high concentrations of α3 subunits. Preferred activation of α1 receptors on GABAergic neurons in the VTA results in reduced GABA release, thereby decreasing inhibitory control on dopaminergic neurons, leading to increased dopamine release at the nucleus accumbens by dopaminergic neurons due to disinhibition from increased GABA activity. Repeated increased dopamine activity in this area promotes the formation of addiction. Alternatively, selective activation of α3 receptors on dopaminergic neurons of the VTA would decrease dopamine release in the nucleus accumbens, possibly reducing abuse potential of a given compound (Rudolph and Knoflach, 2011; Tan, Rudolph and Lüscher, 2011; Kumar and Dillon, 2015).

The search for a subunit selective positive modulator of $GABA_A$ receptors to minimize unwanted side-effects has been extensively studied in benzodiazepines and benzodiazepine-related compounds. Benzodiazepines are used clinically to treat sleep-disorders, anxiety

disorders, and seizures (Rudolph, Crestani and Möhler, 2001; Licata *et al.*, 2005). Use of benzodiazepines can induce tolerance, dependence, and withdrawal symptoms (Toki *et al.*, 1996). The addictive and sedative properties of benzodiazepines have been shown to be associated with the α1 subunit of the $GABA_A$ receptors and this may be true for other drugs acting at this receptor subtype.(Tan, Rudolph and Lüscher, 2011). Other subunits, such as α2, α3 and possibly α5 subunits, of the $GABA_A$ receptor have been shown to be essential to mediating therapeutic properties of $GABA_A$ receptor targeting drugs (Rudolph and Knoflach, 2011). Subunit selective compounds like the α2 and α3 receptor preferring drug MP-III-024, which acts at the benzodiazepine site has pain-relieving properties while lacking sedative and abuse-related behavioral response (B. Fischer *et al.*, 2017). Unfortunately, so far this type of benzodiazepine research has not been translatable to clinical use. After promising pre-clinical data, these compounds often posed issues of drug toxicity and other concerns with drug metabolism (Ralvenius *et al.*, 2015).

Another example of a recently discovered highly selective, nonbenzodiazepine $GABA_A$ receptor targeting drug is SL651498, synthesized by Licata et al. in 2005. SL651498 is a positive allosteric modulator of the $GABA_A$ receptor that has the ability to act as a full agonist at receptors that contain the α2 or α3 subunits and as partial agonists at receptors containing α1 or α5 subunits. This study used behavioral assays in monkeys to assess anxiolytic, motor and subjective effects of this compound. SL651498 was able to deliver anxiolytic effects comparable to classic benzodiazepines. Additionally, the compound did not exhibit the degree of adverse effects seen in $GABA_A$ positive allosteric modulators that activate α1 or α5 subunits, which was attributed to its low efficacy at α1 or α5 subunits. (Licata *et al.*, 2005).

The failure of benzodiazepine-based drug development calls for new strategy and direction in the search for subtype-selective compounds acting at $GABA_A$ receptors. It may be time to move away from benzodiazepines in the search for subunit selective compounds and carisoprodol provides a new avenue to explore the capacity to target the $GABA_A$ receptor for the development of therapeutics to treat pain conditions.

Role of the $GABA_A$ receptor in pain & associated pathologies

Less well understood than other clinical applications of GABAergic drugs is the use of these compounds in the treatment of pain and pathological pain conditions. GABA interneurons play a role in pain circuits at the brain and spinal cord. $\alpha 2$ and $\alpha 3$ subunits have proven to be essential in the usefulness of $GABA_A$ receptor positive allosteric modulators in treating different types of pain (Table 1) (Rudolph and Knoflach, 2011; Paul *et al.*, 2014).

Many studies have been carried out in an effort to develop a GABAergic drug that is effective in treating pain, but so far, no new drugs have made it to market. A major limitation to the development of analgesic compounds targeting the $GABA_A$ receptor is that these compounds cannot alleviate pain much of the time and when they can, the therapeutic potential does not measure up to other drug classes like opioids. Often times, GABAergic compounds cannot be used long-term, as a tolerance will develop. Still, these types of drugs show potential in treating pain disorders, while having a limited capacity to deliver analgesic effects (McCarson and Enna, 2014). Between 1999 and 2014, a 400% increase in overdose deaths has been observed in the U.S, with nearly two-thirds of these cases linked to opioids (Serecigni, 2018). A total of 351,630 opioid overdose deaths have occurred in the U.S. from 1999 to 2016 (Seth *et al.*, 2018). In the midst of a public health crisis like the recent opioid epidemic, there is a clear need for effective

non-opioid prescription of pain-management drugs with minimal abuse potential. Positive allosteric modulators of the $GABA_A$ receptor have the potential to serve as a treatment for acute and neuropathic pain symptoms (Rudolph and Knoflach, 2011).

In pathological pain, there is reduced activity of normal GABA-meditated inhibition in pain circuits. This dampened inhibition is believed to result in enhanced pain signaling, often in the absence of any stimulus.(Zeilhofer, Möhler and Di Lio, 2009; McCarson and Enna, 2014) For this reason, it is important that we continue to search for drugs capable of restoring this inhibition, in an effort to treat neuropathic pain conditions as well as attempt to treat non-pathological pain through this receptor (McCarson and Enna, 2014). A loss of GABAergic inhibition in pain circuits of the central nervous system may lead to the development of pain pathologies due to sensitization of normal pain responses. In addition, GABAergic drugs often have limitations to their clinical use because of unwanted side effects, as mentioned in the previous sections, one the most serious side effects of non-selective GABAergic drugs commonly being addiction. The development of subunit selective compounds to treat pain conditions holds promise when selecting for therapeutics effects of a compound. One idea is that these compounds may also be used to prevent the development of conditions such as chronic pain, by preventing the loss of GABAergic inhibition seen in these conditions. Most promising seems to be the use of these drugs as anti-hyperalgesics, to avoid enhanced pain response, as these compounds have limited use as analgesics. Hyperalgesia is abnormal sensitivity to pain seen in pathological pain conditions (Mirza *et al.*, 2008).

L-838,417 is a $\alpha2$, $\alpha3$ and $\alpha5$ partial-agonist and an antagonist of $\alpha1$ $GABA_A$ receptor subunits. This drug is able to act at $\alpha2$, $\alpha3$ and $\alpha5$ receptors at the spinal cord, which is thought to contribute to its anti-hyperalgesic properties. Additionally, functional magnetic resonance

imaging showed that this drug reduced activity in areas of the brain associated with the emotional components of pain, showing promise for the development of GABA drugs to treat pain (Rudolph and Knoflach, 2011; Paul *et al.*, 2014).

Another example of a recently developed subunit selective $GABA_A$ receptor positive allosteric modulator is NS11394, which is able to differentially bind α1, α2, α3, and α5 subunits with respective increasing affinity. This compound proved to be a very effective as an anxiolytic in rodents and had lowered incidence of unwanted side effects when compared with benzodiazepines in a battery of behavioral assays. Additionally, this drug was shown to be an effective anti-hyperalgesic in rodent models of inflammatory and neuropathic pain. The observed behavioral profile is attributed to low affinity for receptors containing α1 subunits. This supports the idea that the α1 subunit is responsible for mediating much of the unwanted side effects associated with benzodiazepines. Positive modulation of α5 subunit receptors was associated with impairment of memory, shown in this NS11394 study. This again shows the potential for modulating pain with $GABA_A$ receptor subtype selective drugs (Mirza *et al.*, 2008; Rudolph and Knoflach, 2011; Paul *et al.*, 2014)

Carisoprodol and beyond

Much of the research into subunit selective $GABA_A$ receptor drugs has been focused on the structure of benzodiazepines, but other classes of compounds are able to modulate the activity of the $GABA_A$ receptor. Carisoprodol, a carbamate compound, is a positive allosteric modulator of the $GABA_A$ receptor clinically used for the treatment of low-back pain. This drug is also able to directly activate the $GABA_A$ receptors in the absence of GABA, in addition to inhibiting receptors at high concentrations of drug. Unlike some of the potential GABA pain

modulating drugs mentioned previously, carisoprodol is already shown to be clinically effective in treating pain. Carisoprodol is metabolized into a less selective and less potent metabolite, meprobamate by the cytochrome P450 enzyme 2C19. While conversion meprobamate is thought to be responsible for many of the pharmacological effects associated with carisoprodol, signs of drug toxicity seen are seen in early overdose before there is significant metabolism of carisoprodol to meprobamate, demonstrating a clear distinction of carisoprodol effects from meprobamate (L. Gonzalez *et al.*, 2009).

Interesting about this compound is that, like many other GABAergic drugs, there is a difference of effects across receptor subtypes. Carisoprodol is able to directly modulate the $GABA_A$ receptor in a limited capacity through a distinct site of action than where its allosteric effects are mediated, although the location of the specific binding sites remains unresolved. Carisoprodol has been shown to have abuse liability and it has been suspected that this abuse liability is due to the fact that carisoprodol most strongly potentiates the $\alpha 1$ subunit of the $GABA_A$ receptor relative to other α subunits. There have been many documented cases of precipitated withdrawal when carisoprodol is abused (L. A. Gonzalez *et al.*, 2009; L. Gonzalez *et al.*, 2009; Reeves and Burke, 2010; Kumar, González and Dillon, 2015; Kumar *et al.*, 2017)

Carisoprodol was first approved by the Food and Drug Administration (FDA) in 1959 as a skeletal muscle relaxant, prescribed to treat acute muscle-spasms and associated pain. The discovery of carisoprodol resulted from an attempt to isolate sedative effects from myorelaxant effects in regard to the drug meprobamate. The best candidate arising from this study was the now clinically utilized compound carisoprodol. (Ludwig, Powell and Berger, 1969)

In 2010, 5,000 seizures were induced by carisoprodol as a result of drug abuse, demonstrating that while clinically useful, it still has serious consequences when misused. After

years of review, in 2010, carisoprodol was scheduled as a schedule IV-controlled substance by the FDA. In a study of ten healthy adults, an oral 700mg dose of carisoprodol had a half-life of 99 minutes ± 46 minutes in regard to nine participants, with extensive conversion to meprobamate. Notably in this same study, one individual displayed a half-life of 376 minutes an much lower levels of meprobamate conversion, due to altered metabolic activity. (Olsen *et al.*, 1994) Tolerance, dependence and withdrawal symptoms are often precipitated from carisoprodol abuse. (Fass, 2010; Reeves and Burke, 2010; Kumar and Dillon, 2015)

Subunit-dependent pharmacological effects of carisoprodol

Recall that the $\alpha1\beta2\gamma2$ subunit configuration is the most likely $GABA_A$ receptor configuration is the most common found in the brain. In receptor configurations containing the γ subunit, the γ subunit was found to have no influence on the direct or allosteric effects of carisoprodol at $GABA_A$ receptors. $\beta1$ receptors were found to have the highest efficacy relating to carisoprodol's direct gating effect, while $\beta2$ receptors had the highest efficacy relating to allosteric modulatory effects. At $\alpha1$ receptors, carisoprodol is most efficacious at positively modulating GABA-activated currents, while no specific subunit preference is observed with α subunits and carisoprodol's ability to directly gate $GABA_A$ receptors. As previously discussed, the $\alpha1$ subunit is often associated with unwanted side effects of other $GABA_A$ receptor drugs; carisoprodols enhanced efficacy at $\alpha1$ receptors, in regards to its positive modulatory effect, may explain the abuse liability that is associated with the abuse of carisoprodol (Kumar, González and Dillon, 2015).

Positive modulation of $GABA_A$ receptors by carisoprodol

As mentioned previously, carisoprodol is able to act as a positive modulator of $GABA_A$ receptors. Carisoprodol differentially modulates $GABA_A$ receptor subtypes. Previous studies demonstrate that carisoprodol's allosteric modulatory effects are dependent on α subunits of the $GABA_A$ receptor, with the strongest potentiation of GABA currents at α1-containing receptors (Kumar, González and Dillon, 2015). Carisoprodol as a drug template could provide the potential for development of an α1-sparing $GABA_A$ receptor compound by modifying carisoprodol's structure to retain its anti-hyperalgesic and muscle relaxant effects with a reduced abuse potential, by altering the original subunit selective basis for carisoprodol's pharmacological effects. Similar studies have been carried out with benzodiazepines to select for subunit specific effects. Unlike with the direct activation and inhibitory effects of carisoprodol, a lead for a binding site mediating positive modulatory effects of carisoprodol has not been established.

Direct Activation of $GABA_A$ receptors by carisoprodol

Carisoprodol has been shown to have differential direct gating properties at α3 receptors, with minimal direct gating is observed with α3 subunits (Kumar, González and Dillon, 2015). It has been reported that a single amino acid residue, L415, located at the top of the fourth transmembrane domain (TM4) in the α1- subunit of the $GABA_A$ receptor is critical to carisoprodol's direct gating effect. Whether this residue is involved in carisoprodol binding remains unsolved. It is worth noting that compounds with the ability to directly gate receptors are more likely to be lead to death when abused than those that only positively modulate receptor activity, offering an area for improvement in regards to carisoprodol's pharmacological action.

(Kumar, González and Dillon, 2015; Kumar *et al.*, 2017).

Inhibition of $GABA_A$ receptors by carisoprodol

There remains a lack of complete understanding of the mechanism underlying carisoprodol's inhibition at the $GABA_A$ receptors, although a channel-blocking mechanism has been previously studied in a limited capacity, specifically at the TM2 6' region of the Cl^- channel pore (Kumar and Dillon, 2014). Notably, a pore-lining threonine residue (T6') at the second transmembrane domain (TM2) which is highly conserved among all $GABA_A$ receptor subunits, has been identified to confer the antagonistic effect of $GABA_A$ receptors by picrotoxin(Gurley *et al.*, 1995; Zhang *et al.*, 1995), pentylenetetrazole (Dibas and Dillon, 2000), ethanol (Johnson *et al.*, 2012) and 4′-ethynyl-4-n-propylbicycloorthobenzoate (EBOB) (Hisano *et al.*, 2007). Recent cryo-electron microscopy studies show specific binding of picrotoxin at the 2', 6', and 9' TM2 region of the $GABA_A$ channel pore. Exocyclic oxygens in picrotoxin's structure form hydrogen bonds with threonine 256 at the 6' position, critical to picrotoxin binding in the $GABA_A$ receptor channel pore and inhibition of the receptor by picrotoxin. Carisoprodol has the potential to inhibit $GABA_A$ receptors by blocking the channel pore in a similar manner to picrotoxin, specifically interested in the involvement of the threonine 256 at the 6' position, which lies at the center of critical residues at the 2' and 9' position of TM2 along the channel pore (Masiulis *et al.*, 2019).

Objectives of the Project

This project aimed to develop a selective $GABA_A$ receptor positive allosteric modulator with low-abuse potential based on the structure of carisoprodol (CSP), a clinically effective

prescription compound with proven abuse liability used for the treatment of low-back pain. Additionally, we aimed to establish an understanding for underlying mechanism for carisoprodol's positive modulatory, direct activation and inhibitory effects at $GABA_A$ receptors.

We modified carisoprodol's structure in an effort to shift its subunit preference from putative α1 to α2/α3 and in turn maximize carisoprodol's clinical utility with lower abuse liability. Our initial goal was to determine the key elements of carisoprodol's molecular structures that confer α subunit-dependence of carisoprodol allosteric modulation on $GABA_A$ receptors and other pharmacological properties of carisoprodol. Furthermore, drug synthesis aimed to develop a compound that is not only selective for to α2/α3 receptors, but possesses more robust pharmacological effects than carisoprodol and prevent metabolism of a given compound to meprobamate. At the completion of this project, we expected to have identified the structure and function relationship that underlies subunit-dependence of carisoprodol allosteric modulation of $GABA_A$ receptors. We further expected to determine behavioral consequences associated with carisoprodol derivatives selective for α2/α3-containing $GABA_A$ receptors. These results are expected to have an important positive impact because they will provide a strong evidence-based framework for the continued development of pharmacological modulators of $GABA_A$ receptor function as non-opioid therapeutics for pain treatment and management.

In addition to investigating the effects of changes to carisoprodol's molecular structure, we also probed for a molecular basis for the pharmacological effects of carisoprodol at $GABA_A$ receptors utilizing site-directed mutagenesis and *in-silico* receptor models to investigate particular amino acid residues of interest for carisoprodol's three different pharmacological effects.

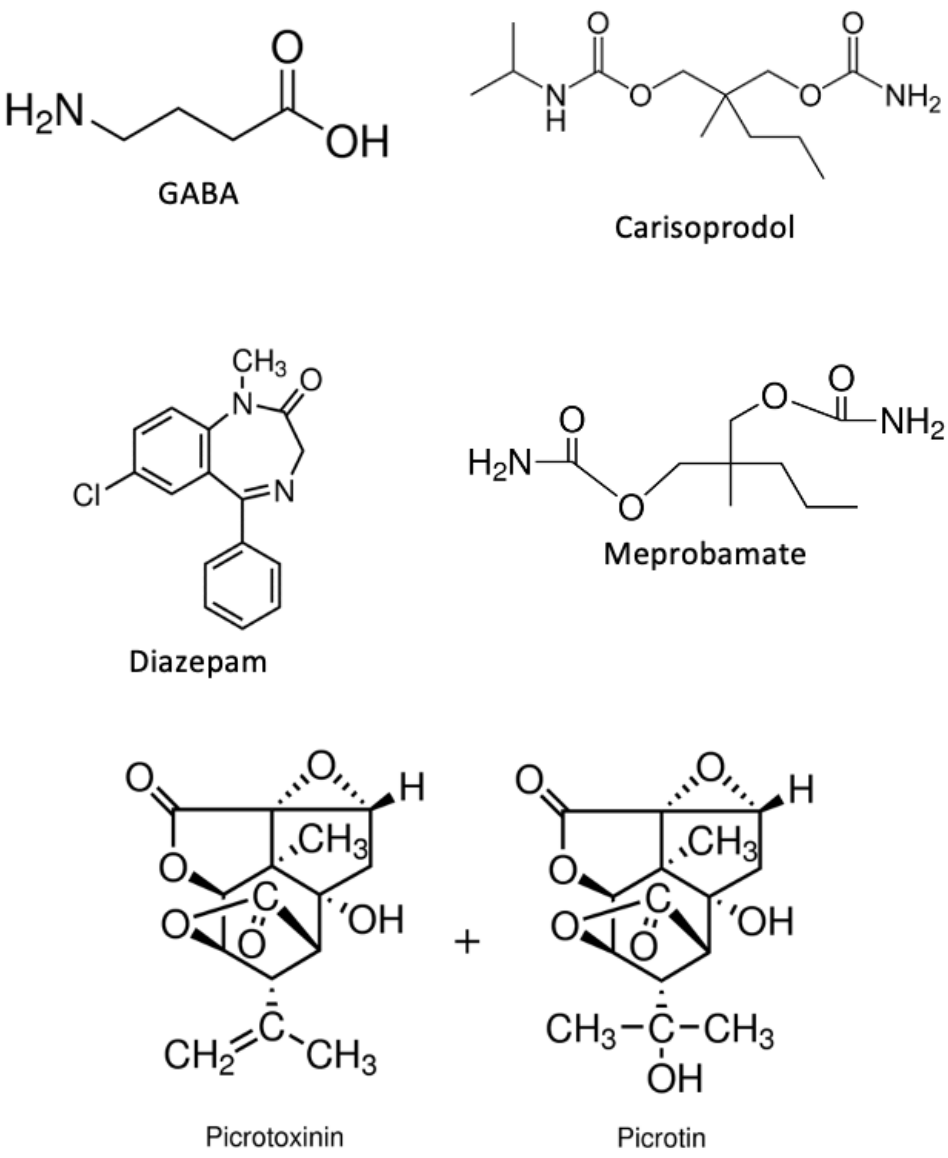


Figure 1. Chemical structures of $GABA_A$ receptor agonists, allosteric modulators, and inhibitors. γ-aminobutyric acid (GABA) is an endogenous agonist for $GABA_A$ receptors. Diazepam belongs to the benzodiazepine drug class and is a positive modulator. Carisoprodol and meprobamate are propanediol dicarbamates that can act as agonists and positive modulators. Picrotin and picrotoxinin exist in an equimolar mixture to comprise the channel blocking drug picrotoxin.

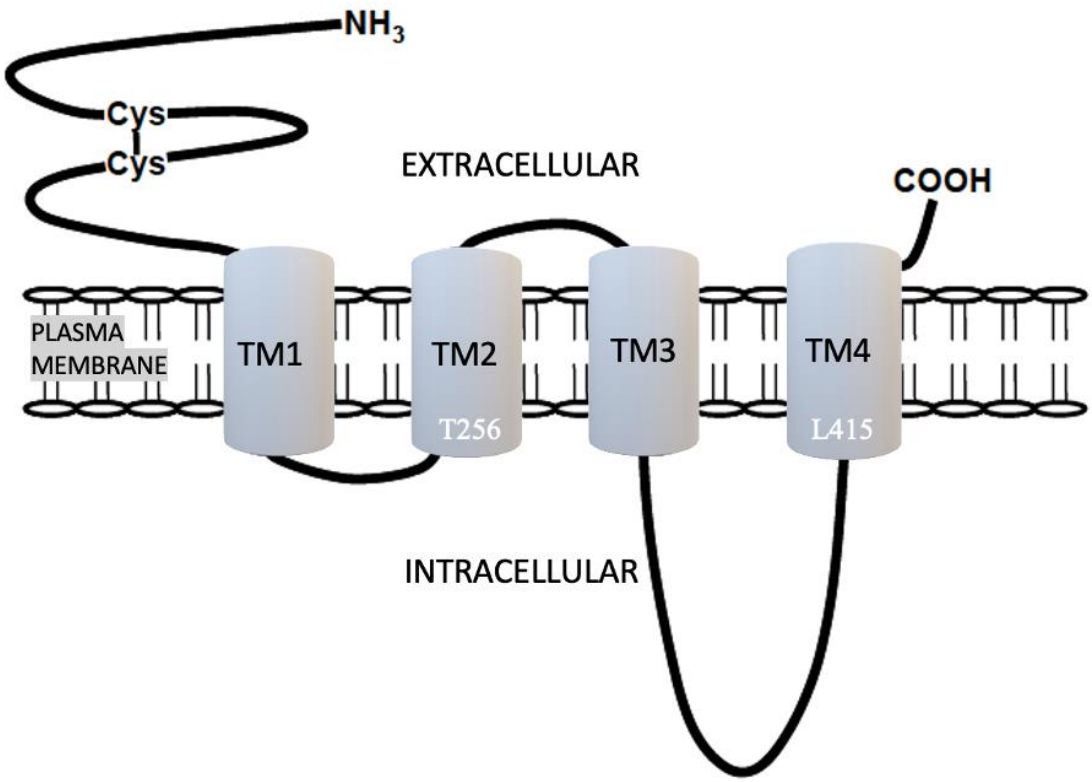
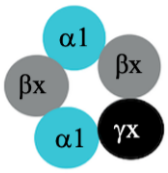
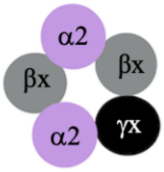
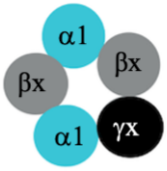
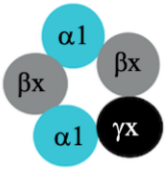
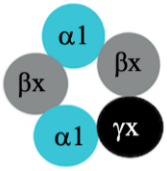
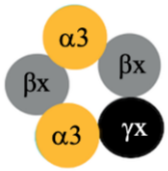
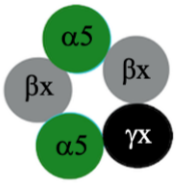
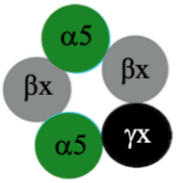


Figure 2. Subunit structure of $GABA_A$ receptors. Subunits contain a large amino-terminal domain containing the cys-loop and four transmembrane domains (TM1-4). TM2 lines channel pore while TM3 and TM4 form a large intracellular loop where phosphorylation plays a role in regulation. Subunit structure is conserved in different subunit subtypes. Indicated in white are residues T256 and L415,which are important to CSP's inhibitory effect and direct activation at $GABA_A$ receptors, respectively.

Table 1. $GABA_A$ receptor subtypes and associated specific pharmacological effects of positive modulators

SEDATION	ANXIOLYSIS	MUSCLE RELAXATION	ANTI-CONVULSIVE	AMNESIA	ADDICTION
					
					
					

CHAPTER II
MATERIALS & METHODS

1. Summary of the three studies comprising the overall project

Study 1 was set to investigate the positive modulatory effects of carisoprodol derivatives with anti-hyperalgesic potential.

Study 2 was set to investigate the structural basis of the direct activation of carisoprodol at $GABA_A$ receptors.

Study 3 was set to investigate the mechanism underlying the inhibitory action of carisoprodol.

Each study used a combination of the following methods that are described in details below.

2. Plasmids & site-directed mutagenesis

2.1. Carisoprodol Direct Activation

Human cDNA plasmids encoding wild-type or mutant α1, β2, and γ2 $GABA_A$ receptor subunits were used. Mutations in α1 cDNA plasmids were created using Stratagene's Quick Change II site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA.) and were sequenced to confirm mutations. Mutations at the α1 subunit included methionine 148 at the cys-loop region mutated to alanine. At the pre-TM1 region of the α1 subunit, lysine 220 was mutated to cystine, isoleucine 223 was mutated to alanine. Each of these residues was indicated in in-silico docking experiments to be important to carisoprodol binding.

2.2. Carisoprodol Inhibitory Effect

Rat isoforms of the $GABA_A$ receptor subunits α1, β2, and γ2 provided by Cynthia Czajkowski (Madison, WI) were subcloned into the pCDNA3.1 vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Threonine at the TM2 6' position of each subunit was mutated to phenylalanine using Stratagene's Quikchange site-directed mutagenesis kit (Agilent Technologies, La Jolla, CA.) Mutations were confirmed by sequencing by the Core Facility at Texas Tech University (Lubbock, TX.) For additional details, refer to previous studies. (Gonzales *et al.*, 2008)

3. **Cell culture and transfection**

Human embryonic kidney 293 (HEK293) cells stably or transiently expressing rat or human $GABA_A$ receptors were used. For stably expressed receptors, cells were used 24-48 hours after plating. PolyJet Transfection reagent (SignaGen Laboratories, Rockville, MD) was used for transiently expressed receptors. Cells were transfected once the necessary density (70-90%) was reached and used for recording 24-48 h later. Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes. Cells were incubated and maintained at 35-37°C in a humidified incubator with an atmosphere of 5% CO2.

3.1. Carisoprodol Positive Allosteric Modulation

All drug screening was performed using HEK293 cells stably expressing human $GABA_A$ receptors. Receptor configurations included α1β2γ2 and α2β2γ2.

3.2. Carisoprodol Direct Activation

For screening of novel carisoprodol-derived compounds, human $GABA_A$ receptors were used. Receptor configurations included $\alpha1\beta2\gamma2$ and $\alpha2\beta2\gamma2$. For probe of direct activation binding site, each of the three mutant $\alpha1$ subunits were co-expressed with wild-type $\beta2$ and $\gamma2$ subunits at a 1:1:3 ratio, for a $\alpha1\beta2\gamma2$ receptor configuration. The presence of the $\gamma2$ subunit was assessed pharmacologically by co-application of 1μM diazepam and relative EC_{20} GABA.

3.3. Carisoprodol Inhibitory Effect

For $\alpha1\beta2$ $GABA_A$ receptor configuration, HEK293 cells were transfected with rat $GABA_A$ $\alpha1$ and rat $\beta1$ cDNA in a 1:1 ratio. For $\alpha1\beta2\gamma2$ $GABA_A$ receptor configuration, HEK293 cells were transfected with $GABA_A$ rat $\alpha1$; rat $\beta1$; and rat $\gamma2s$ (short isoform) cDNA in a 1:1:3 ratio. The presence of the $\gamma2$ subunit was assessed pharmacologically by co-application of 1μM diazepam and relative EC_{30} GABA.

4. **Whole-Cell Patch-Clamp Electrophysiology**

Electrophysiology experiments were conducted at room temperature with the membrane potential clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Company, Novato, CA) to a tip resistance of 5-9 mΩ. Patch pipettes were filled with a solution consisting of internal solution (140 mM CsCl, 10 mM EGTA-Na^+, 10 mM HEPES, and 4 mM Mg^{2+}-ATP). Coverslips containing cultured cells were placed in the recording

chamber on the stage of an inverted light microscope and super-fused continuously with an external solution (125 mM NaCl, 10mM HEPES, 3 mM CaCl2, 5.5 mM KCl, 0.8 mM $MgCl_2$, and 10mM glucose, pH 7.3). Drug-induced Cl-currents were obtained with an Axopatch 200B amplifier with a rate of 50 samples per second (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU headstage. Currents were low pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder**,** and stored on a computer using a data acquisition system and analyzed later (pCLAMP 10.6, Molecular Devices LLC, San Jose, CA).

4.1. Carisoprodol Positive Allosteric Modulation

Whole-cell patch clamp electrophysiology was used to assess GABA-, diazepam-, and carisoprodol-, and novel compound (NM-XXX)-mediated Cl-currents.

4.2. Carisoprodol Direct Activation

Whole-cell patch clamp electrophysiology was used to assess GABA-, NM-XXX and carisoprodol- mediated Cl-currents.

4.3. Carisoprodol Inhibitory Effect

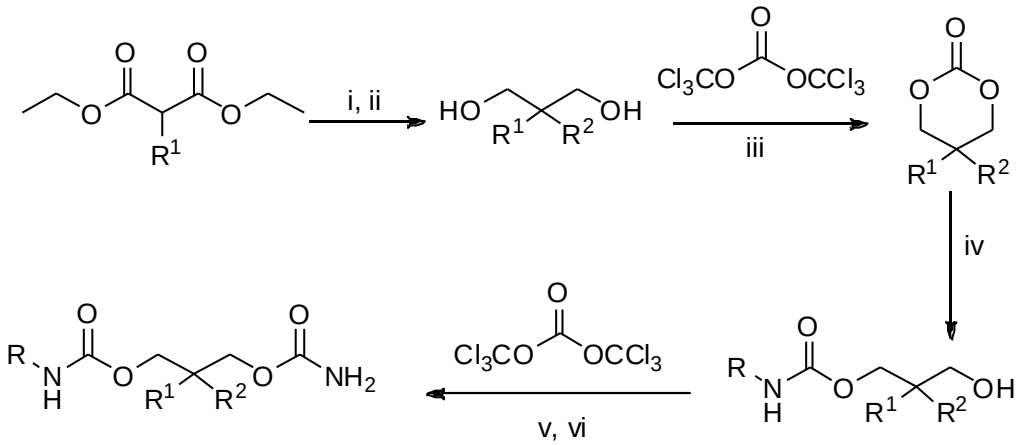
Whole-cell patch clamp electrophysiology was used to assess GABA-, picrotoxin-, diazepam, or carisoprodol-activated Cl- currents.

5. **Chemicals and solutions**

GABA stock solution was prepared in ultra-pure water and all other stocks were prepared in DMSO. DMSO stocks were diluted into external solutions for a final concentrations of < .05% DMSO. All drugs with the exception of novel compounds were purchased from Sigma Aldrich (Minneapolis, MN).

5.1. Carisoprodol Positive Allosteric Modulation and carisoprodol Direct Activation

Carisoprodol derivatives from Ludwig et al. (1969) + novel compounds, synthesized by Dr. Emmitte's Lab, based on the parent compound carisoprodol. For initial drug synthesis, a matrix approach was used to modify the parent structure carisoprodol. Novel compounds will be referred to as NM-XXX. Secondary synthesis of compounds focused on targeting specific modifications and drug metabolism. Reagents and conditions for drug synthesis were as follows: i) R2Br, NaOEt in EtOH, 40°C, 10h; ii) LiAlH4, THF, 0°C to rt; iii) TEA, THF, -78°C to rt, 8-10h; iv) RNH2, 60°C, 8h; v) TEA, 0°C, 1h; vi) NH_3 in Diethyl ether, -70°C to rt, overnight



6. Experimental protocol for electrophysiology studies

Solutions were applied to each cell by Harvard apparatus rapid application and perfusion fast-step system adjacent to the cell (Warner Instruments LLC, Holliston, MA).

6.1. Carisoprodol Positive Allosteric Modulation and Direct Activation

GABA, carisoprodol (with or without GABA), NM-XXX (with or without GABA), diazepam (with GABA) was prepared in external saline solution from stock solutions. Carisoprodol- or NM-XXX-activated currents were normalized to currents elicited by saturating GABA currents (1 mM GABA). Currents elicited by GABA co-applied with either diazepam (positive allosteric modulator study only), carisoprodol, or NM-XXX were normalized to relative 10 µM or 18 µM EC_{20} GABA control. Each drug was applied for 10 seconds, with a total of 30 seconds recorded. The equation ($I/Imax = [carisoprodol]^n/([carisoprodol]^n + EC^n_{50}$) was used for concentration response curves, where I is the normalized current amplitude at a given concentration of carisoprodol, Imax is the maximum current induced by carisoprodol, EC_{50} is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient.

6.2. Carisoprodol Inhibitory Effect

GABA, picrotoxin (with GABA), diazepam (with GABA) and carisoprodol (with or without GABA) were prepared in external saline solution from stock solutions Carisoprodol-activated currents were normalized to currents elicited by saturating GABA currents, either 500 mM or 1 mM GABA. Currents elicited by GABA co-applied with either picrotoxin, diazepam, or carisoprodol were normalized to relative 1 µM or 10 µM

GABA control, representative of about an EC_{20}-EC_{30} GABA control. Each drug was applied for 10-15 seconds, with a total of 30 seconds recorded.

7. **Data Analysis:** For electrophysiology experiments: All data are presented as mean values ± S.E.M. Statistical significance ($p < 0.05$) between control and test conditions was determined using Student's t-test (paired or unpaired) or one-way analysis of variance. $GABA_A$ receptor-mediated currents were analyzed with pClamp 10.6 and Origin 6.0 software.

7.1. Carisoprodol Positive Allosteric Modulation

For this study, EC_{20} GABA-currents were used as a relative control for carisoprodol/NM-XXX and co-applied GABA- mediated currents.

7.2. Carisoprodol Direct Activation

For this study, carisoprodol- and NM-XXX-mediated currents were normalized to 1 mM saturating GABA currents.

7.3. Carisoprodol Inhibitory Effect

To quantify the inhibitory factor of each receptor configuration, the equation *[1-(I1/I2)]x100,* where *I1* refers to the current measured at maximum inhibition and *I2* refers to the maximum peak current, at any time of testing. Correlation assessments were performed using linear fit in Origin 6.0 software using linear fit. (OriginLab Corp., Northhampton, MA.)

8. In-silico docking experiments for carisoprodol Direct Activation Study

Cryo-electron microscopy structure (3.92 Å) of human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Zhu et al., ***Nature***, 559:67,2018, PDB file: 6D6U). *In-silico* docking performed with Autodock tools and Autodock Vina.

9. Computational simulations for carisoprodol Inhibitory Study

9.1. Docking

We used the CryoEM structure of full length human $\alpha_1\beta_3\gamma_2$ $GABA_A$ (PDB ID: 6HUG) as the structure source for all computational studies including our docking setup. We removed the ligand Picrotoxin (PTX) from the receptor and prepared it as well as the receptor with Schrodinger package 2019. Then we generated a docking box that would encompass the surface inside the transmembrane helices with setting all hydroxyl groups that point inward the pocket flexible. We docked back the picrotoxin with glide module to assess our docking procedure. All docking experiments were performed with XP mode in glide and setting all ligand rotatable bonds flexible. The resulting pose of picrotoxin perfectly matched with its crystallographic pose. We prepared double α^{T261F} receptor mutant and prepared carisprodol (CSP) for docking.

9.2. Molecular Dynamics simulations: Preparation of picrotoxin and carisoprodol for simulations

To prepare the ligands for simulations, we clustered 20 ns of molecular dynamics simulation of the ligands with AM1-BCC partial atomic charges in implicit solvent to get the two most populated clusters. The structures of the representative conformations

were then optimized using 6-31G* basis set and Gaussian 09. The optimized conformations were used to generate high quality RESP partial atomic charges via REDIII program. These RESP charges along with the GAFF force field were used to parameterize the picrotoxin and carisoprodol for the MD simulations.

9.3. Preparation of the receptor and receptor-ligand complexes for simulations

We used the CryoEM structure of the $\alpha_1\beta_3\gamma_2$ $GABA_A$ (PDB ID: 6HUG) that is already prepared and solvated in membrane and water by Sansom group and available in http://memprotmd.bioch.ox.ac.uk/_ref/PDB/6hug/ for molecular dynamic simulations. The atom and residue names were translated to CHARMM format manually and then to AMBER format by charmmlipid2amber.py program in AmberTools 17. Disulfide bonds were created between SH groups of cysteine residues closer than 2.5 Å. Topology and coordinate files were saved using tLEaP program. FF14SB, LIPID14 and GAFF force fields were used to parameterize the protein, lipids and the ligands, respectively. Also Joung-Cheatham ion parameters were used to describe the monovalent ions. Parmed program was used to perform Hydrogen Mass Repartitioning on solute molecules in the topology files. Three systems including free receptor and receptor bound to either carisoprodol or picrotoxin were created in this way. Picrotoxin was positioned according to the CryoEM structure and carisoprodol was positioned according to the docking pose. Two independent copies of each simulation were prepared using random positioning of ions within the simulation box.

9.4. Equilibration, running and analysis of the simulations

A nine-step equilibration protocol including energy minimizations and short molecular dynamic simulations discussed elsewhere (Braun *et al.,* 2019) was used to prepare the systems before production. The production simulations were performed for 500 ns per system copy with a time step of 4 fs, using pmemd.cuda of Amber17 under constant pressure situation at 298.15 K. The temperature was regulated using Langevine thermostat and the pressure was fixed using Monte Carlo barostat with 5 ps relaxation rate. SHAKE was applied to constrain bonds between heavy atoms and hydrogens. Particle Mesh Ewald (PME) [was used with 9.0 Å cutoff for calculating the long-range interactions. The coordinates were written to the trajectories every 10 ps. Trajectories were analyzed using CPPTRAJ program of AmberTools17.

CHAPTER III
RESULTS

It is known that carisoprodol is able to positively modulate, directly activate, and inhibit $GABA_A$ receptors. The overarching goal of this project was to investigate the structural basis at governing carisoprodol's pharmacological effects in an effort develop an improved compound based on carisoprodol's structure.

1. Study 1: Development of $GABA_A$ Receptor Anti-hyperalgesics with Low Abuse Potential

   Many prescription compounds possess unwanted side effects, one of the most serious being abuse liability. The recent opioid abuse epidemic has demonstrated the need to develop drugs that are clinically effective with minimal serious side effects like abuse liability. In this study, we aimed to develop an α1-sparing and α2-selective $GABA_A$ receptor compound by modifying the structure of carisoprodol to retain its anti-hyperalgesic and muscle relaxant effects with a reduced abuse potential. We predicted that specific structural modification of carisoprodol will shift its subunit preference from putative α1 to α2 and in turn maximizes the clinical utility of carisoprodol by lowering its abuse liability. We tested our hypothesis with synthesis of carisoprodol (carisoprodol) analogs and whole-cell patch clamp electrophysiology.

1.1. Synthesis of new compounds based on the structure on carisoprodol

In 1968, Ludwig et al. developed a series of carbamate derivatives structurally related to carisoprodol and its active metabolite meprobamate, although meprobamate lab synthesis preceded that of carisoprodol. These compounds were evaluated for their paralytic muscle effect, anticonvulsant activity, and toxicity (Ludwig, Powell and

Berger, 1969). Based on this study and previous carisoprodol research done by our own lab, collaborators in the Emmitte Lab in the UNTHSC School of Pharmacy generated a library of carisoprodol derivatives by a matrix approach in initiation of our search for a subunit selective $GABA_A$ receptor positive allosteric modulators (positive allosteric modulators).

As shown in Fig. 3, carisoprodol's metabolism to meprobamate involves the removal of an isopropyl group and results in two distinct pharmacological profiles for each compound (Kumar, González and Dillon, 2015; Kumar and Dillon, 2016). Carisoprodol is a more potent and efficacious compound than meprobamate (Gonzalez et al. 2009). In addition to their differences in potency and efficacy, carisoprodol accumulates in the brain more rapidly than meprobamate and reaches higher concentrations in the nucleus accumbens, an area of the brain associated with the abuse liability of drugs (Prokai *et al.*, 2016). These studies indicate that there is an essential structural component of carisoprodol that contributes to its pharmacological profile.

The resulting library of compounds contains 14 structures that have been previously published by Ludwig et al. and ten novel compounds (Table 2). Each compound includes distinct modifications at two different positions of our parent compound, an N-substitution and a substitution to the central quaternary carbon, referred to as side and center modifications respectively. Due to issues with solubility, availability, or minimal receptor activity, only seven compounds were selected from our library for further screening, detailed in Fig. 3.

1.2. Screening of novel compounds and carisoprodol at 300μM to establish activity at α1β2γ2 and α2β2γ2 receptors

The criteria used to select the ideal candidate (lead) for compounds selective for α2- containing receptors are: 1) a significantly greater potentiation in α2- than α1-receptors; 2) α1: α2 ratio of efficacy ≤1; 3) more potent (EC_{50} ≤carisoprodol).

As shown in Fig. 4 and Table 3, carisoprodol had significantly greater potentiation on $h\alpha_1\beta_2\gamma_2$ than on $h\alpha_2\beta_2\gamma_2$ with an α1:α2 ratio of 1.59; carisoprodol is therefore selective for α_1-receptors (at 300μM carisoprodol co-applied with GABA, screening α_1-receptors mean=285.3, SE=11.6 and α_2 -receptors mean=216.7, SE=15.56; n=6-11). Modification of carisoprodol's structure was able to shift its original subunit selectivity at 300 μM for allosteric modulatory effects. Some of the synthesized carisoprodol analogs have shown relatively high potentiation on α2-containing receptors compared to carisoprodol; such a tendency is seen with compounds NM-234, NM-265, NM-243, and NM-278 (respective α1:α2 ratios were 0.91, 0.98, 0.66, 0.71).

In this study to establish which key structural components of carisoprodol contribute to its pharmacological profile, we determined that none of our newly synthesized compounds were able to shift carisoprodol's positive modulatory selectivity profile in a manner that met our criteria: 1) a significantly greater potentiation in α2 than α1; 2) α1: α2 ratio of efficacy ≤1; 3) more potent (EC_{50} ≤carisoprodol)). Compounds that were able to shift the α1:α2 ratio were not selective for α2- receptors, which is inferred from a significant difference between activities of a given compound at α1- compared with α2-receptors, screened at 300μM carisoprodol co-applied with GABA. (for α2-preferring

compounds NM-234, NM-265, NM-243, and NM-278 p=0.70, p=0.94, p=0.88, and p=0.16 respectively). However, our initial screen indicated important positions at carisoprodol's structure for future synthesis of novel compounds. For compounds containing an N-substitution, it was more likely to see a shift in α1:α2 receptor preference, from α1-preferring to α2-preferring. (Fig. 3 and Fig. 4, Table 3) Data represented as mean ± S.E.M., $p \leq 0.05$, unpaired t-test. Ratio of α1: α2 is defined as mean positive modulatory effect at α1-receptors divided by mean positive modulatory effect at α2-receptors.

1.3. Design of new NM-XXX compounds based on previous studies

Based on the results from the previous studies and the differing pharmacological profiles of carisoprodol compared to its metabolite meprobamate, a new library of compounds was generated in an effort to develop an α2-selective compound that would meet our criteria specified in the earlier studies. New compounds were designed to potentially prevent the metabolism of carisoprodol-derivatives to meprobamate and to be selective for α2-$GABA_A$ receptors, however these new compounds remain untested due to lack of time and funding (Table 4).

2. Study 2: Investigation of the mechanism underlying carisoprodol's direct activation of $GABA_A$ receptors

It has been reported that a single amino acid residue, L415 (labeled L345 in PDB: 6D6U), located at the top of the fourth transmembrane domain (TM4) in the α1- subunit of

the $GABA_A$ receptor is critical to carisoprodol's direct gating effect. Whether the residue is involved in carisoprodol binding remains unknown. The purpose of this study was to explore the binding site mediating carisoprodol's direct action with *in-silico* docking, site-directed mutagenesis and whole-cell electrophysiology. Initial *in-silico* docking of carisoprodol at the $GABA_A$ receptor suggested that the carisoprodol binding pocket may be formed by residues from the TM4, pre-TM1 and cys-loop regions of the α-subunits.

2.1. Synthesized derivatives of carisoprodol have strong direct activation at α1β2γ2 $GABA_A$ receptors

From our previous study to develop of $GABA_A$ receptor anti-hyperalgesics with low abuse potential which focused on shifting carisoprodol's positive modulatory effect, we found that some of our synthesized carisoprodol derivatives possessed a strong direct gating effect at $GABA_A$ receptors in a manner that differed significantly compare to carisoprodol. This indicates certain properties of carisoprodol can be modified to affect its direct activation properties at $GABA_A$ receptors. (Table 5 and Fig. 5) While less clinically relevant than carisoprodol's therapeutic positive modulatory effect, carisoprodol's ability to directly gate $GABA_A$ receptors is important as drugs able directly gate this receptor are more likely to result in death when misused than drugs that only positively modulate receptor activity, because of their ability to act in the absence of neurotransmitter. (Fass, 2010; Kumar, González and Dillon, 2015) The most significant shift in direct activation when compared with carisoprodol (2.6 ± 0.5, n=11) was observed with compounds NM-244 (23±1.6, n=3) and NM-243 (19.7±2.8, n=8). (All data represented as Mean ± S.E.M., $p \leq 0.05$, unpaired t-test) (Table 5).

Concentration response profiles were generated to further compare NM-243 and NM-244 with carisoprodol, at concentration 30µM-3mM. In concentration-response studies, NM-244 differed significantly from carisoprodol at 300µM (carisoprodol 2.9±0.7, n=5; NM-244 16.4±1.8, n=4), 1mM (carisoprodol 15±5.1, n=5; NM-244 44.2±4.1, n=4) and 3mM (carisoprodol 18.6±6.3, n=5; NM-244 44.8±6.3, n=4).

The equation ($I/Imax = [carisoprodol]^n/([carisoprodol]^n + EC^n_{50}$) was used for concentration response curves, where I is the normalized current amplitude at a given concentration of carisoprodol, Imax is the maximum current induced by carisoprodol, EC_{50} is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient. (All data represented as Mean ± S.E.M., $p \leq 0.05$, unpaired t-test) Results indicate that key components of carisoprodol's structure are responsible for the ability to directly gate $GABA_A$ receptors (Table 5 and Fig. 5).

2.2. Investigation of potential binding pocket using docking experiments near L415 at TM4 of the α1-subunit

Leucine 415 at the TM4 region of the α1 $GABA_A$ receptor subunit has been shown to be essential to carisoprodol's ability to directly activate the receptor channel (Kumar *et al.*, 2017). In order to investigate the involvement of L415 in a potential binding site for carisoprodol, *in-silico* docking experiments were performed using recent cryo-electron microscopy structure (PDB: 6D6U) of human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Zhu *et al.*, 2018). Carisoprodol was prepared and docked on a small searching box centered close to L415, which is labeled L345 in the *in-silico* strucrures due to the removal of a signal peptide (Fig. 6). These docking experiments indicated interaction of

methionine 148 at the cys-loop region, as well as lysine 220 and isoleucine 223 at the pre-TM1 region in a potential binding pocket involving the critical leucine L415 residue at TM4 of the α1-subunit (Fig. 6). Carisoprodol has hydrophobic interacts with L415(L345) which is in agreement with previous mutation studies, where L345G and L345S have the worst effect on binding as they distort the pocket hydrophobicity more than many other mutations (Kumar *et al.*, 2017). In docking experiments, carisoprodol hydrogen bonds with I223 and K220, indicating that mutation of K220 and I223 to amino acids with different chemical properties would adversely affect binding of carisoprodol. Methionine 148 at the cys-loop region was also indicated in docking studies and further evaluated in site-directed mutagenesis studies (Fig. 6 and 7). Mutations to either an alanine or a cysteine at M148, I223, and K220 were chosen because of their relatively small size compared to wild-type residues. Mutation of these residues was expected to negatively impact the ability of carisoprodol to directly activate the receptor if they are involved in a true binding pocket.

For further investigation, carisoprodol, NM-243, and NM-244 were docked at this potential binding site. Docking scores (kcal/mol) were used to compare the relative binding affinity of carisoprodol and two analogs, NM-243 and NM-244- which have enhanced direct gating properties compared to carisoprodol. (Table 5 and Fig. 5).Docking scores aligned with the ability of the compounds to directly activate the channel; NM-244 (-3.55 kcal/mol) which has enhanced direct gating properties had a higher docking score than NM-243(-2.15 kcal/mol) and carisoprodol (-1.96 kcal/mol), aligning with electrophysiology data. (Table 6) These results indicate that the suggested binding pocket of the α1-subunit may be involved in a direct gating for carisoprodol.

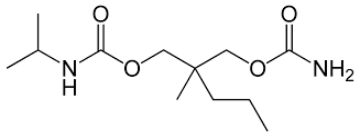
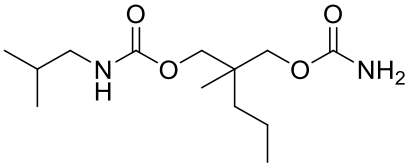
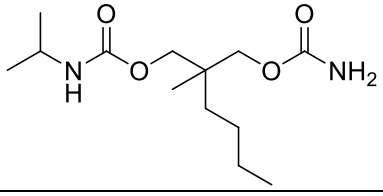
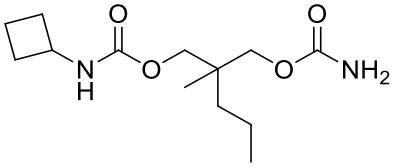
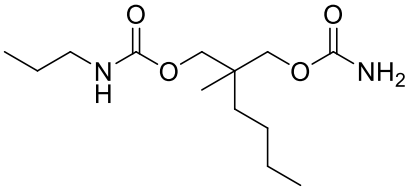
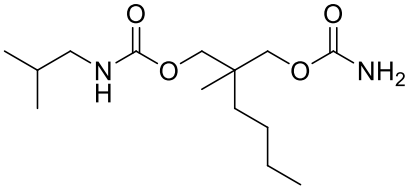
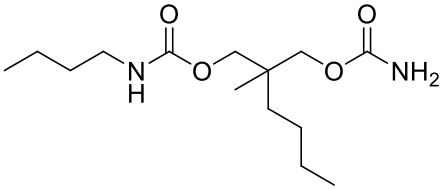
2.3. Site-directed mutagenesis to investigate residues forming potential binding pocket for carisoprodol direct gating effects

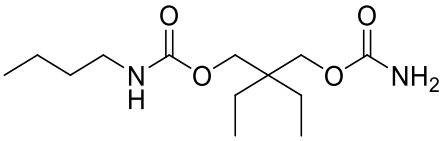
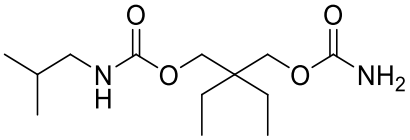
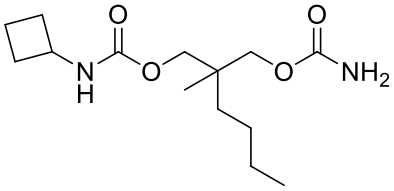
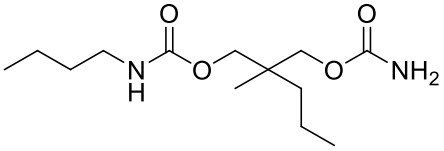
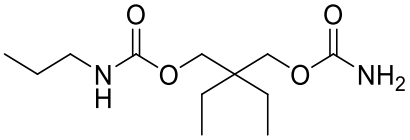
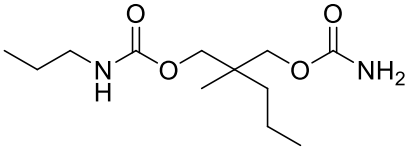
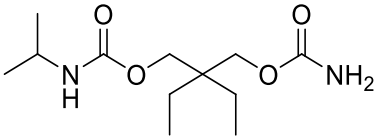
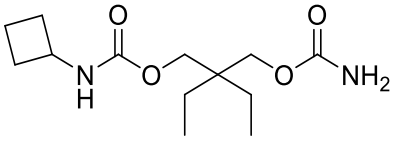
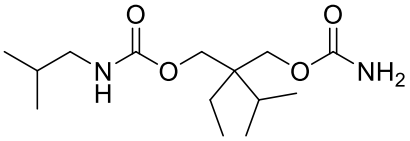
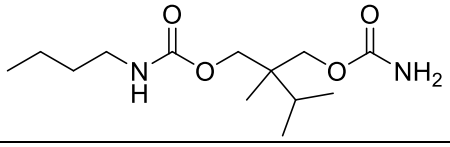
To investigate whether methionine 148 at the cys-loop region, lysine 220 and isoleucine 223 at the pre-TM1 region are involved in a potential binding pocket involving the critical leucine residue at TM4 of the α1-subunit, we used site-directed mutagenesis to alter the potential binding pocket in a way that would interrupt/decrease carisoprodol's binding affinity and therefore direct gating effects. These mutations were then evaluated using whole-cell patch clamp electrophysiology.

At concentrations 100μM, 300μM, 1mM and 3mM, no mutations negatively affected the binding of carisoprodol to the $GABA_A$ receptor as predicted in computational docking studies (Fig. 6 and Fig. 7). A significant increase in the ability of carisoprodol to directly gate $GABA_A$ receptors was observed at a concentration of 300μM for the α1(I223A)β2γ2 mutant receptor (15.0 ± 6.2) when compared to wild-type(3.1 ± 2.7). All data represented as Mean ± S.E.M., $p \leq 0.05$, unpaired t-test, n=3-5. Since the mutation of the three lead residues of interest (methionine 148, lysine 220, and isoleucine 223) either enhanced the direct activation of $GABA_A$ receptors by carisoprodol or had no effect, indicating that the L415 residue is likely not part of a binding pocket for carisoprodol's direct gating effects, as indicated on docking studies.

Figures and Tables for Studies 1 and 2

Table 2. Novel compounds synthesized by matrix approach based on carisoprodol structures and their respective molecular weight (g/mol)

Compound	Structure	Molecular Weight (g/mol)
Carisoprodol		260.33
NM-234		274.189
NM-243		274.189
NM-244		272.173
NM-251		274.1892
NM-252*		288.2049
NM-253		288.2049

NM-254		274.1892
NM-255*		274.1892
NM-262*		286.1892
NM-263		274.1892
NM-264		260.1736
NM-265		260.1736
NM-270		260.1736
NM-271*		272.1736
NM-272*		288.2049
NM-273		274.1892

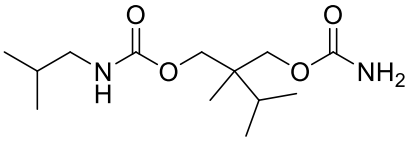
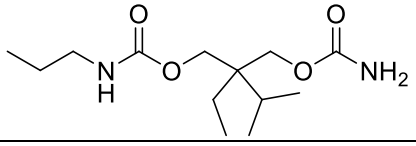
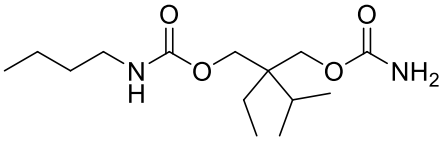
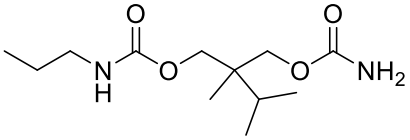
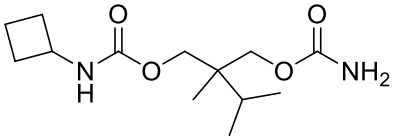
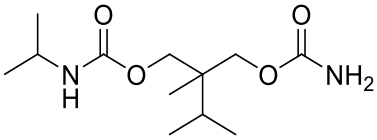
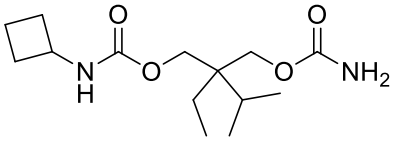
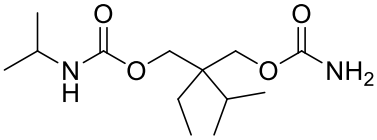
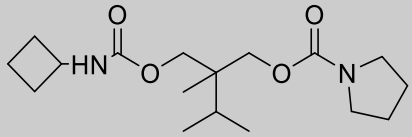
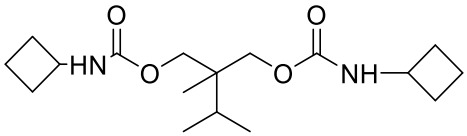
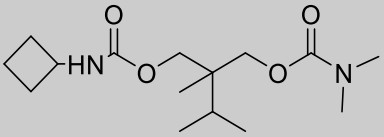
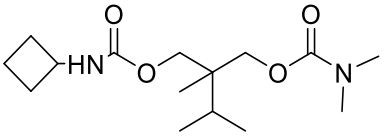
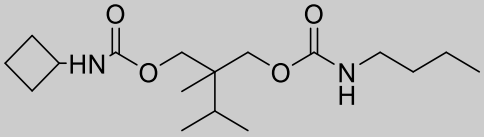
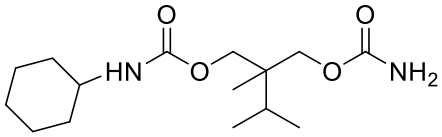
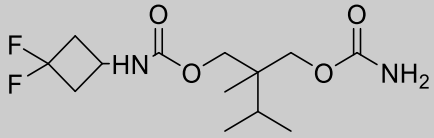
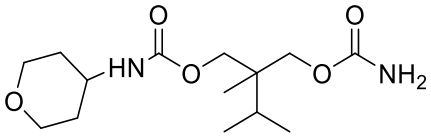
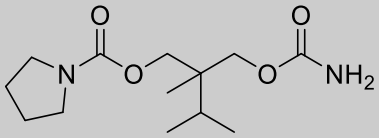
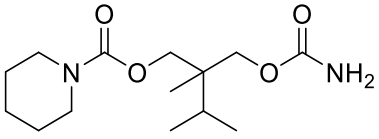
NM-274*		274.1892
NM-275*		274.1892
NM-276*		288.2049
NM-277		260.1736
NM-278*		272.1736
NM-279		260.1736
NM-280*		286.1892
NM-281		274.1892

Table 3. Comparison of α1:α2 effects

Compound	$h\alpha_1\beta_2\gamma_2$, % Potentiation ± S.E.M (n)	$h\alpha_2\beta_2\gamma_2$, % Potentiation ± S.E.M (n)	Ratio (α_1: α_2)
carisoprodol	185.3±11.6 (11)	116.6±15.6 (6)	1.59
NM-234	91.0±28.2 (6)	109.3±28.7 (3)	0.83
NM-243	174.9±32.0 (7)	183.7±51.0 (6)	0.95
NM-244	235.3±107.6 (3)	228.2±77.4 (6)	1.03
NM-265	163.7±44.6 (3)	168.3±45 (3)	0.97
NM-270	105.3±39.8 (3)	96.3±38.9 (3)	1.09
NM-278	172.2±57.0 (5)	358.0±95.0 (3)	0.48
NM-281	147.0±24.8 (3)	107.3±56.0 (3)	1.37

Table 4: New carisoprodol derivatives

Compound	Structure	Molecular Weight (g/mol)
NM-990		326.44
NM-992		326.44
NM-995		286.37
NM-996		300.40
NM-998		328.45
NM-999		300.40
NM-1006		308.33
NM-1007		302.37
NM-1014		272.35
NM-1015		286.37

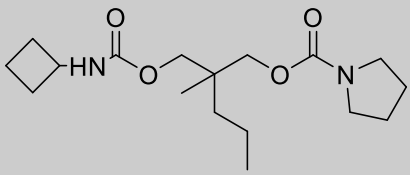
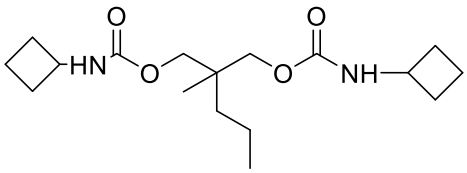
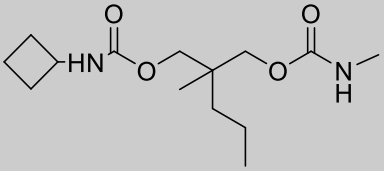
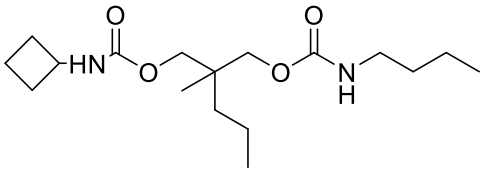
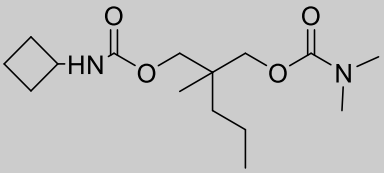
NM-1028		326.44
NM-1029		326.44
NM-1032		286.37
NM-1033		328.45
NM-1034		300.40

Table 5. Direct Activation of NM-XXX compounds compared with carisoprodol at 300µM

Ranked compounds	**% max. current ± S.E.M (n)**	**p-value (Compared to carisoprodol)**
NM-244	23 ± 1.6 (3)	1.05×10^{-9}
NM-243	19.7± 2.8(8)	0.0001
NM-278	14.2± 56.9(5)	3.31×10^{-5}
NM-252	6.4± 4.4(7)	0.0168
NM-234	6 ± 1.6 (6)	0.0196
NM-281	5.4 ± 1.7 (3)	0.0385
NM-270	4.5 ± 0.6 (3)	0.0802
NM-255*	3.1± 1.5 (3)	0.6554
NM-265	2.7 ± 1.6 (3)	0.9024
carisoprodol	2.6 ± 0.5 (11)	--------

Table 6. Docking scores from suggested binding pocket

Compound	Docking Score (kcal/mol)	EC50 (μM)	Efficacy (% 1mM GABA)
Carisoprodol	-1.96	770.96 (± 444)	18.6 (±6.3)
NM-243	-2.15	499.42 (± 248)	22.0 (± 2.9)
NM-244	-3.55	173.57 (± 64.7) **	39.8 (± 4.9) *

Figure 3

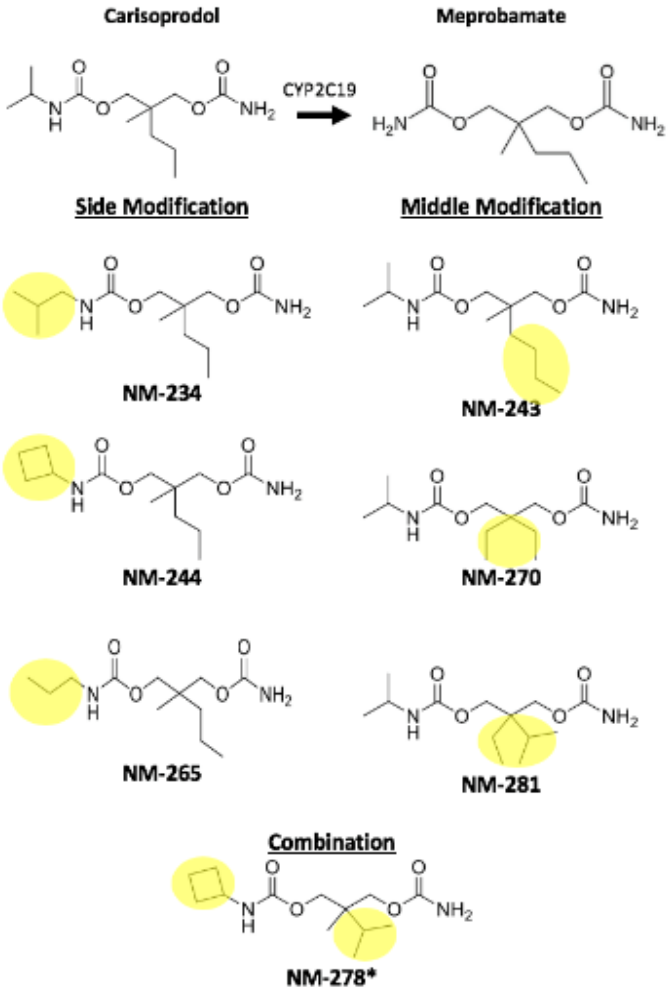


Figure 3. Carisoprodol, its active metabolite meprobamate and seven structurally distinct derivatives selected for further screening. Structural modifications to the original structure of carisoprodol are highlighted in yellow.

Figure 4

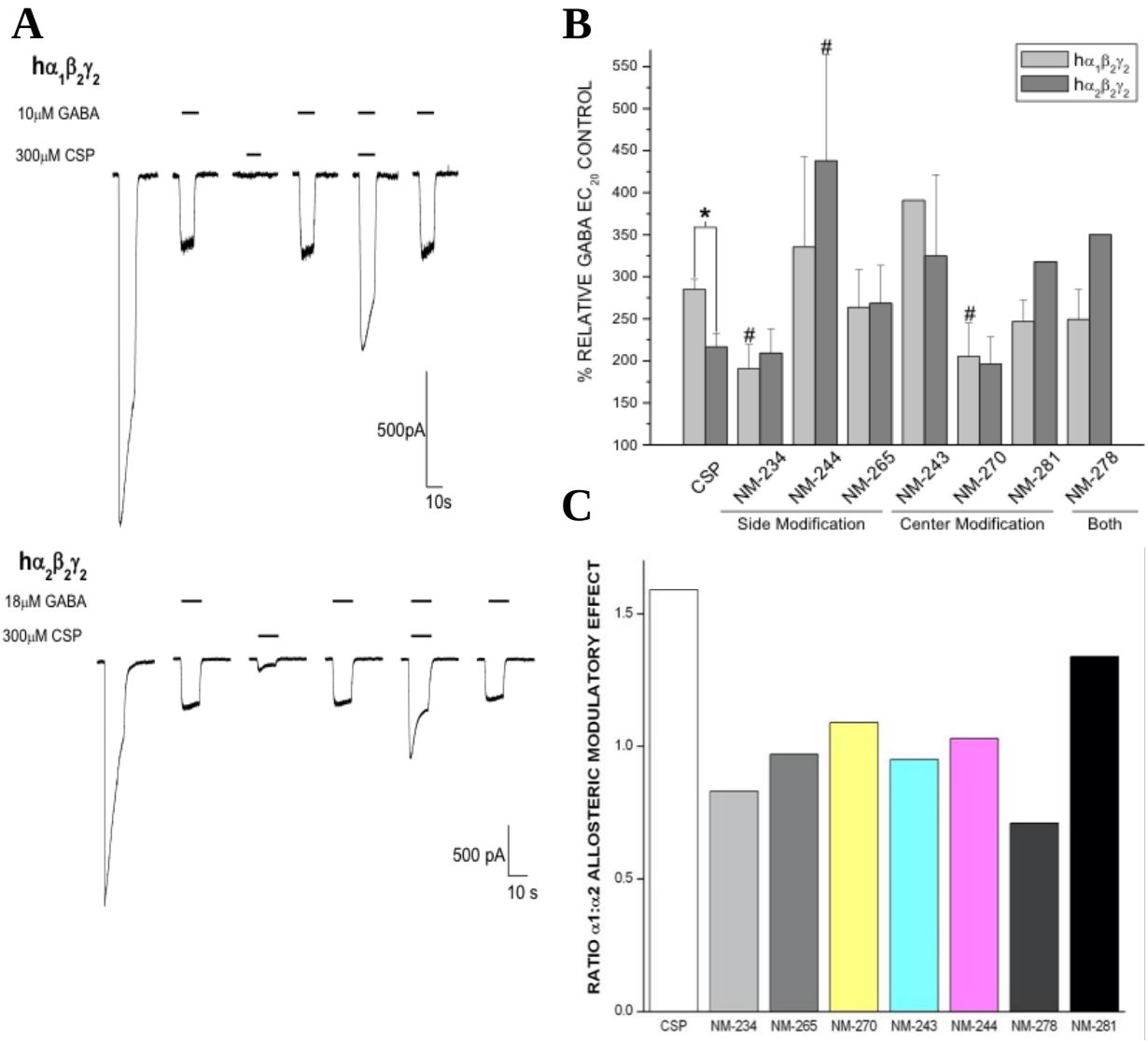


Figure 4. **Summary of Allosteric Modulatory Effects**. (A) Representative traces for 300μM carisoprodol at HEK cells stably expressing α1β2γ2 or α2β2γ2 $GABA_A$ receptors. (B) Summary of allosteric modulatory effects of seven compounds and carisoprodol. #= significant difference compared to carisoprodol at respective receptor, *= significant difference when compared between a1 and a2 receptors. (C) Ratio of α1: α2 allosteric modulatory effect.

Figure 5

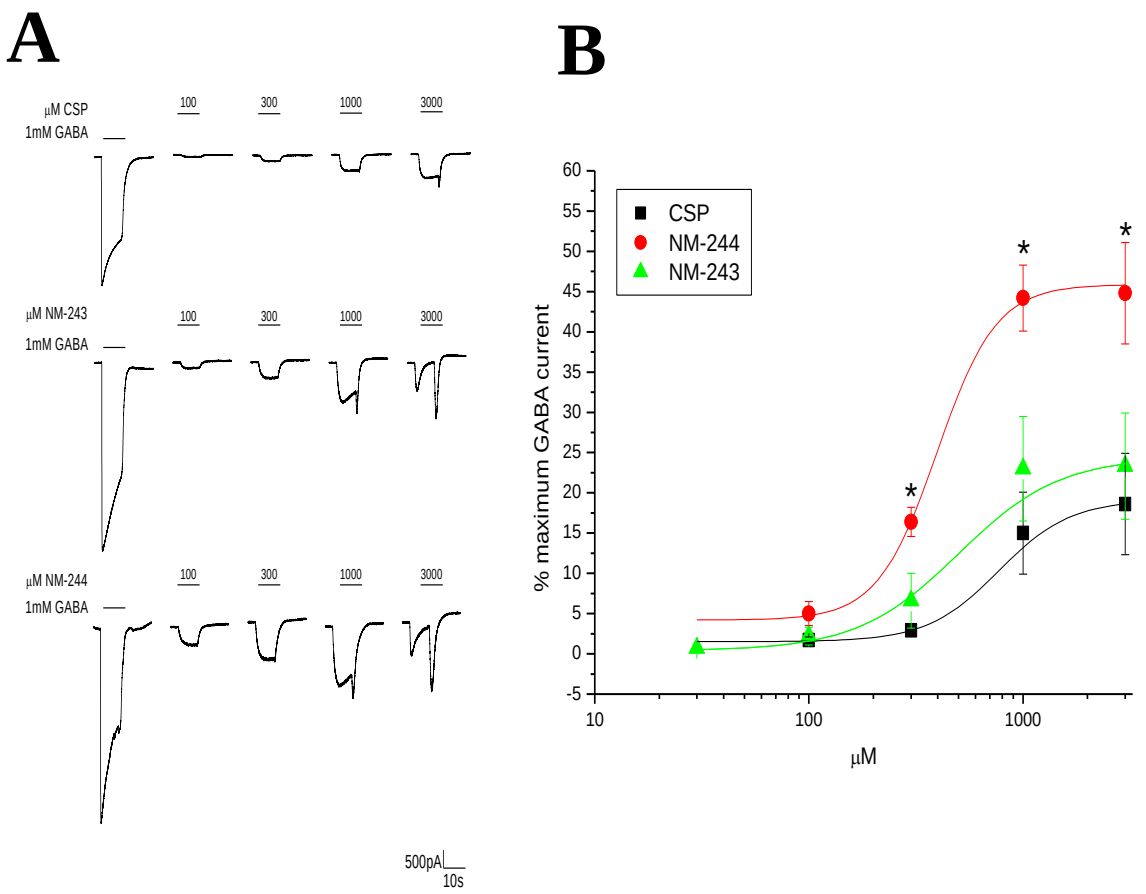


Figure 5. Direct activation of carisoprodol compared with NM-243 and NM-244 compounds at human α1β2γ2. (A) Representative traces of carisoprodol, NM-243, and NM-244 direct activation currents (100μM-3mM) and relative 1mM GABA current. (B) Concentration response profile for carisoprodol, NM-243, and NM-244. Each data point represents the mean ± S.E.M. of a minimum of three cells. *, $p \leq 0.05$ when compared with carisoprodol direct activation.

Figure 6

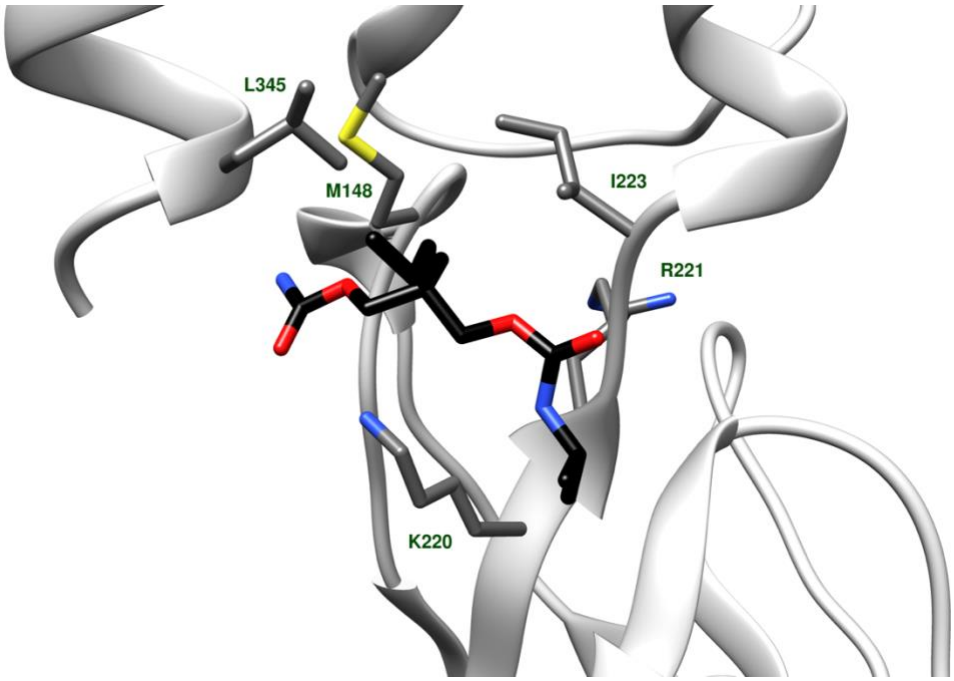


Figure 6. Proposed carisoprodol binding site from in-silico docking experiments at human α1β2γ2 GABA$_A$ receptors. Model of the docking of carisoprodol near residue L415 (L345 in docking structure), which has been shown to be critical to the binding of carisoprodol. Suggested interactions with methionine 148, lysine 220, and isoleucine 223 may play an essential role in stabilizing the binding of carisoprodol to the α-subunit of the GABA$_A$ receptor.

Figure 7

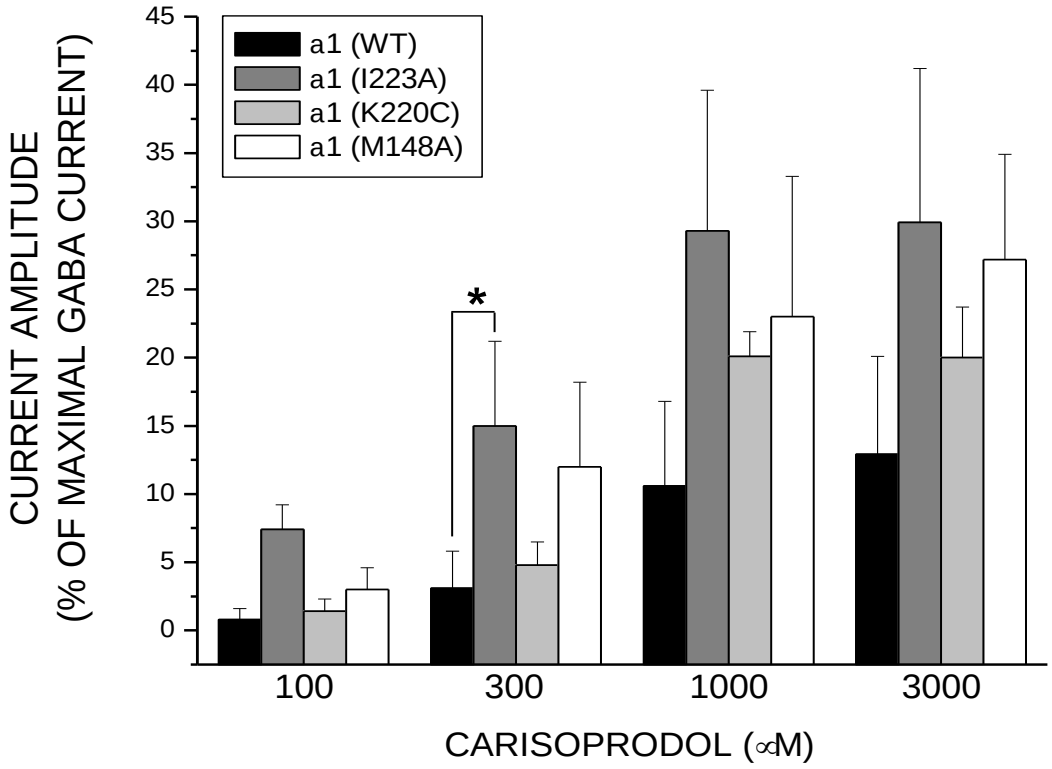


Figure 7. Summary of direct activation of WT or mutant α1β2γ2 $GABA_A$ by carisoprodol. Direct activation effects are expressed as a percentage of 1mM maximal GABA current. All mutations had no effect across all concentrations, with the exception of α1(I223A) β2γ2 at 300μM carisoprodol, when compared to WT α1β2γ2 $GABA_A$**.** *, $p \leq 0.05$, n=3-5

Study 3: Carisoprodol inhibits $GABA_A$ receptors at the picrotoxin binding site

Our previous studies have shown that carisoprodol differentially potentiates $GABA_A$ receptor subtypes via allosteric modulation and direct activation, while little is known about carisoprodol's mechanism of channel inhibition. The purpose of this study was to explore whether the picrotoxin (PTX) binding site, particularly a pore-lining threonine residue at the 6' position at the TM2 region, may play a role in carisoprodol's inhibitory action. This residue has been shown to be essential to a binding site mediating the inhibitory action of picrotoxin at $GABA_A$ receptors (Masiulis *et al.*, 2019). We tested our hypothesis using molecular dynamic simulations, site-directed mutagenesis and whole-cell electrophysiology.

3.1 Concentration-response profiles for the $GABA_A$ receptor configurations were assessed in the present studies (Table 7). The approximate EC_{30} agonist (GABA) concentrations were calculated and used for each configuration in subsequent investigation of potential carisoprodol effects.

3.2 **TM2 (T6'F) mutation influences the ability of carisoprodol to inhibit $GABA_A$ receptors**

Previous studies report a decrease in current and/or rebound current present at high concentrations of carisoprodol when applied to $GABA_A$ receptors. This rebound current is understood to be due to carisoprodol release from a low-affinity site responsible for inhibition of $GABA_A$ receptors. (Kumar, González and Dillon, 2015) Interest in the T6'F TM2 mutation is due to an established relationship between the mutation and imparting resistance to the channel-blocking compound, picrotoxin

(PTX). Other compounds are understood to also inhibit the GABA-receptor at the pore, such as pentylenetetrazole (Dibas and Dillon, 2000), ethanol (Johnson *et al.*, 2012) and 4′-ethynyl-4-n-propylbicycloorthobenzoate (EBOB)(Hisano *et al.*, 2007). We decided to test a single, double and triple TM2 6' mutation by introducing a mutation γ, α, or β respectively and testing either α1β2 or α1β2γ2 receptor configurations. For α1β2 receptors, when cells are transfected with a 1α:1β cDNA ratio, the receptor configuration 2α:3β is favored. For α1β2γ2 receptors, when transfected in a ratio from 1:1:1 to 1:1:4, a 2α:2β:1γ configuration is reliably expressed. (Tretter *et al.*, 1997) In this study, we assessed 1mM and 3mM carisoprodol with co-applied approximate EC_{30} GABA (1μM for α1β2 receptors and 10μM for α1β2γ2 receptors) to study carisoprodol's inhibitory action and the influence of the TM2 threonine 6' to phenylalanine mutation (T6'F) at each α1,β2, and γ2 $GABA_A$ receptor subunit. This concentration was chosen as to allow for adequate receptor modulation. For each mutant subunit there was co-expression with wild-type subunits. For assessment of α1 or β2 mutant subunits, the receptor conformation α1β2 was used, as to allow for the maximum number of α1 and β2 subunits. For assessment of γ2 mutant subunit, α1β2γ2 receptor conformation was used. Inhibitory action of carisoprodol was quantified by an inhibitory factor (IF), which is defined as *[1-(I1/I2)]x100*, where *I1* refers to the current measured at maximum inhibition and *I2* refers to the peak current. The greatest difference in inhibitory action was observed with the β2 (T6'F) mutant subunit carisoprodol (IF=6.87 ± 5.5, n=5 and IF=10.7 ± 10.7, n=3 respectively) when compared to wild-

type α1β2 receptors at 1mM and 3mM carisoprodol (IF=24.04 ± 4.4, n=4 and IF=46.41 ± 7.7, n=4 respectively). (Fig. 8A and 8B). While a difference was also observed at the α1(T6'F) mutant subunit, a significant difference was observed only at 3mM carisoprodol (IF=21.47 ± 3.2, n=6; at 1mM carisoprodol IF=14.53 ± 2.8, n=6) when compared to wild-type α1β2 receptor configuration at 3mM carisoprodol (Fig. 8A and 8B). No significant difference was observed at either 1mM or 3mM carisoprodol for γ2 (T6'F) mutant subunits (IF=41.61 ± 13.5, n=3 at 1mM carisoprodol, IF=45.97 ± 0.4 at 3mM carisoprodol, n=3), when compared to wild-type α1β2γ2 receptors(IF=43.41 ± 9.6, n=4 at 1mM carisoprodol, IF=68.87 ± 10.1, n=4 at 3mM carisoprodol) (Fig. 8C and 8D). For each of these receptor configurations, sensitivity to 100 μM picrotoxin co-applied with approximate EC_{30} GABA was also screened on each day of testing, not shown as the relationship between picrotoxin and the TM2 T6'F mutation has been clearly demonstrated (Gonzales *et al.*, 2008; Masiulis *et al.*, 2019). Statistical significance was determined by a p-value ≤ 0.05 and unpaired t-test. Our results indicate that carisoprodol is able to bind to the channel pore of $GABA_A$ receptors at the same region as picrotoxin.

3.3 **Influence of (T6'F) mutation on direct activation of α1β2 $GABA_A$ receptors**

We assessed the influence of the (T6'F) mutation on carisoprodol's ability to directly activate α1β2 $GABA_A$ receptors. A shift in carisoprodol's ability to directly activate mutant receptors was observed. At α1(T6'F) mutants (12 ± 2.4, n=5 at 1mM and 27 ± 6.5, n=5 at 3mM), when compared with wild-type α1β2 receptors (39.36 ± 8.2, n=3

at 1mM and 47.9 ± 3.9, n=3 at 3mM), a decrease was seen at both 1mM and 3mM carisoprodol. A decrease in direct activation was also present at β2 (T6'F) receptors, with a significant decrease at 1mM (22 ± 4.5, n=5 at 1mM and 29 ± 9.3, n=3 at 3mM). (Fig. 9A and 9B) Direct activation of $GABA_A$ receptors by carisoprodol was only assessed at α1β2 receptors, as there is relatively minimal inhibition of receptors observed with direct activation compared with positive modulatory effects. Statistical significance was determined by a p-value ≤ 0.05 and unpaired t-test. Previous studies determined that the γ subunit is not essential in carisoprodol's action at $GABA_A$ receptors, observing no significant difference in direct activation or modulatory effects of carisoprodol at α1β2 and α1β2γ2 receptors. (Kumar, González and Dillon, 2015) Additionally, there is relatively low inhibition of $GABA_A$ receptors when directly activated, compared with modulatory effects. Therefore, we only investigated the TM2 (T6'F) mutation at α1β2 receptors. A shift in direct activation of the receptor when the TM2 (T6'F) mutation is introduce may indicate a role of the TM2 6' region in carisoprodol's direct effects.

3.4 **Influence of (T6'F) mutation on positive modulation of $GABA_A$ receptors**

Next, we assessed the influence of the (T6'F) mutation on α1β2 and α1β2γ2 receptors. At 1mM and 3mM, a significant increase in positive modulatory effects was observed at both α1(T6'F) (676 ± 234.9, n=6 at 1mM and 789 ± 260.7, n=6 at 3mM) and β2(T6'F) receptors (871.38 ± 166.2, n=5 at 1mM and 743 ± 156.3, n=3 at 3mM) when compared to wild-type(193.9 ± 50.8, n=3 at 1mM and 239.3 ± 64.7, n=3

at 3mM). (Fig. 9C) For the wild-type α1β2γ2 receptor configuration (104.33 ± 13.9, n=4 at 1mM and 84.32 ± 13.7, n=4 at 3mM), no statistical difference was observed when compared with γ2(T6'F) receptors (100.20 ± 11.9, n=3 at 1mM and 125.04 ± 613.7, n=3 at 3mM). (Fig. 9D) Significance was determined by a p-value ≤ 0.05 and unpaired t-test. Our results indicate that the (T6'F) mutation shifts the sensitivity of the receptor to carisoprodol's positive modulatory effects at α1β2 receptors.

3.5 **Stoichiometric effects of T6'F mutation of pore lining residues**

For this study, we assessed the relationship between the number of pore-lining TM2 (T6'F) mutations and the degree of channel inhibition for carisoprodol's positive modulatory effect. For α1β2γ2 receptors transfected at a 1:1:3 ratio and α1β2 receptors transfected at a 1:1 ratio, α1β2γ2 and α1:3β. A negative correlation was observed at both 1mM and 3mM carisoprodol and co-applied GABA. At 1mM carisoprodol, R = -0.95 (P=0.054, n=4) and at 3mM R = -0.99 (p=0.0126, n=4) , indicating a negative correlation between the number of TM2 (T6'F) mutations present at the channel pore and the degree to which carisoprodol inhibits the $GABA_A$ receptor. γ2 (T6'F) receptors possess one mutation along the channel pore, α1(T6'F) receptors possess two mutations, and β2(T6'F receptors possess three mutations, respective of the number of mutant subunits incorporated into the α1β2γ2 receptor configuration. (Fig. 10) (*Inhibitory factor = [1-(I1/I2)]x100*). From the relationship observed, we can infer that the degree to which carisoprodol inhibits $GABA_A$ receptors is positively correlated with the number of threonines assumed to be present

at the TM2 6’ position in a given receptor configuration.

3.6 Docking studies validate picrotoxin, but not carisoprodol binding at TM2 6’ site

To validate our docking protocol with this system, we re-docked picrotoxin to its crystallographic position on $GABA_A$ using XP precision of Glide. The resulting docking pose perfectly matched with the crystallographic pose. We also docked carisoprodol onto the same binding site. The docked carisoprodol interacts with the key threonine residues on the receptor. We inserted two α1 T261F mutations and re-docked both picrotoxin and carisoprodol. In accordance with the experimental observations, no docking pose was generated for picrotoxin. However, carisoprodol was docked in the same pocket with comparable score (Table 8 and Fig. 11), implying that unlike the electrophysiology results, the binding of carisoprodol is not inhibited by the mutations, this might be due to change of the structure and dynamics of the pockets as a result of the mutations, which was not considered in docking.

3.7 Molecular dynamic simulations studies indicate interaction of picrotoxin and carisoprodol at the same binding pocket at $GABA_A$ receptors

To see how the mutations affect the pocket and binding of the ligands to it, we set up molecular dynamic simulations of free $GABA_A$ and $GABA_A$ bound to picrotoxin and carisoprodol in 3 copies, which were extended to 500 ns each (Totally 4500 ns). We measured the Root Mean Square Deviation (RMSD) of the residues in the binding site (Fig. 12) to see how its structure deviates from the crystal conformation in different conditions. When having no ligand in the binding site, both pockets with and

without mutations deviate significantly from the crystal conformation (Fig. 12, Top panel). Adding picrotoxin to the wild-type pocket (crystal structure condition), retains the crystal conformation of the pocket (Black line in Fig. 12, middle panel). This observation agrees with the experimental structure and can be considered as validation of our method. Adding picrotoxin to the mutated pocket does not shift its conformation to the crystal conditions. Rather, it causes population of different conformations of the pocket in the simulation ensemble (Red line in Fig. 12, middle panel). These results demonstrate that the consideration of the pocket dynamics is necessary to capture the changes mutations potentially cause in $GABA_A$ Rs. The similar trend in binding energies and pose changes for carisoprodol and picrotoxin in molecular dynamic simulation upon mutation validates our hypothesis that the two molecules share the same binding pocket on $GABA_A$ Rs. These data provide further information on how carisoprodol may interact with the receptors

Tables and Figures for Study 3

Table 7: GABA sensitivity of different $GABA_A$ receptor configurations

Receptor	EC_{50} (μM)	Hill Coefficient	n	EC_{30} (μM)
WT α1β2γ2	34 ± 4.9	1.08 ± 0.15	8	10
WT α1β2	1.6 ± 0.2	1.3 ± 0.10	4	0.5
α1 (T6'F)β2	1.7 ± 0.3	0.9 ± 0.10	4	0.5
α1β2(T6'F)	3.6 ± 0.5	1.1 ± 0.10	4	1.2
α1β2γ2(T6'F)	9.4 ± 1.4	0.8 ± 0.10	≥ 3	N/A

Figure 8

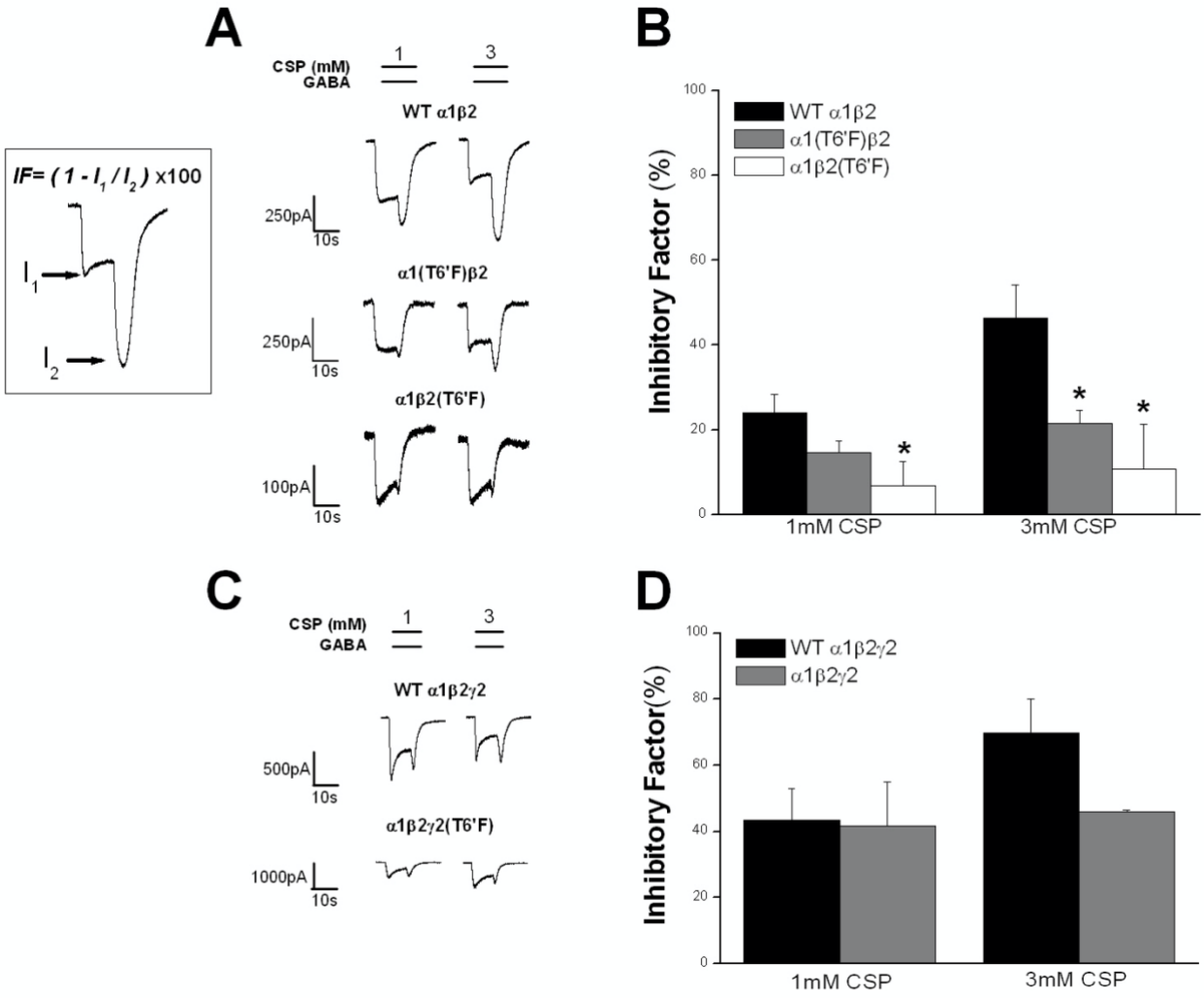


Figure 8. Influence of TM2 (T6'F) mutation on inhibitory action of carisoprodol. (A) Representative traces of WT α1β2, α1(T6'F)β2, and α1β2(T6'F) receptors and respective inhibitory action associated with positive modulation of approximate EC_{30} GABA current. (B)Bar graph representing inhibitory factor mean ± S.E.M. for respective receptor configuration. A significant difference was observed at 1mM carisoprodol for α1(T6'F)β2 receptors and at 3mM for both α1(T6'F)β2 and α1β2(T6'F) when compared to WT α1β2 receptors (C) Representative traces of WT α1β2γ2 and α1β2γ2 (T6'F) receptors and respective inhibitory action associated with positive modulation of approximate EC_{30} GABA current. (D) Bar graph representing inhibitory factor mean ± S.E.M. for respective receptor configuration. No significant difference observed between WT α1β2γ2 and α1β2γ2 (T6'F) receptors. All mean ± S.E.M. are representative of a minimum of three cells. *, $p \leq 0.05$.

Figure 9

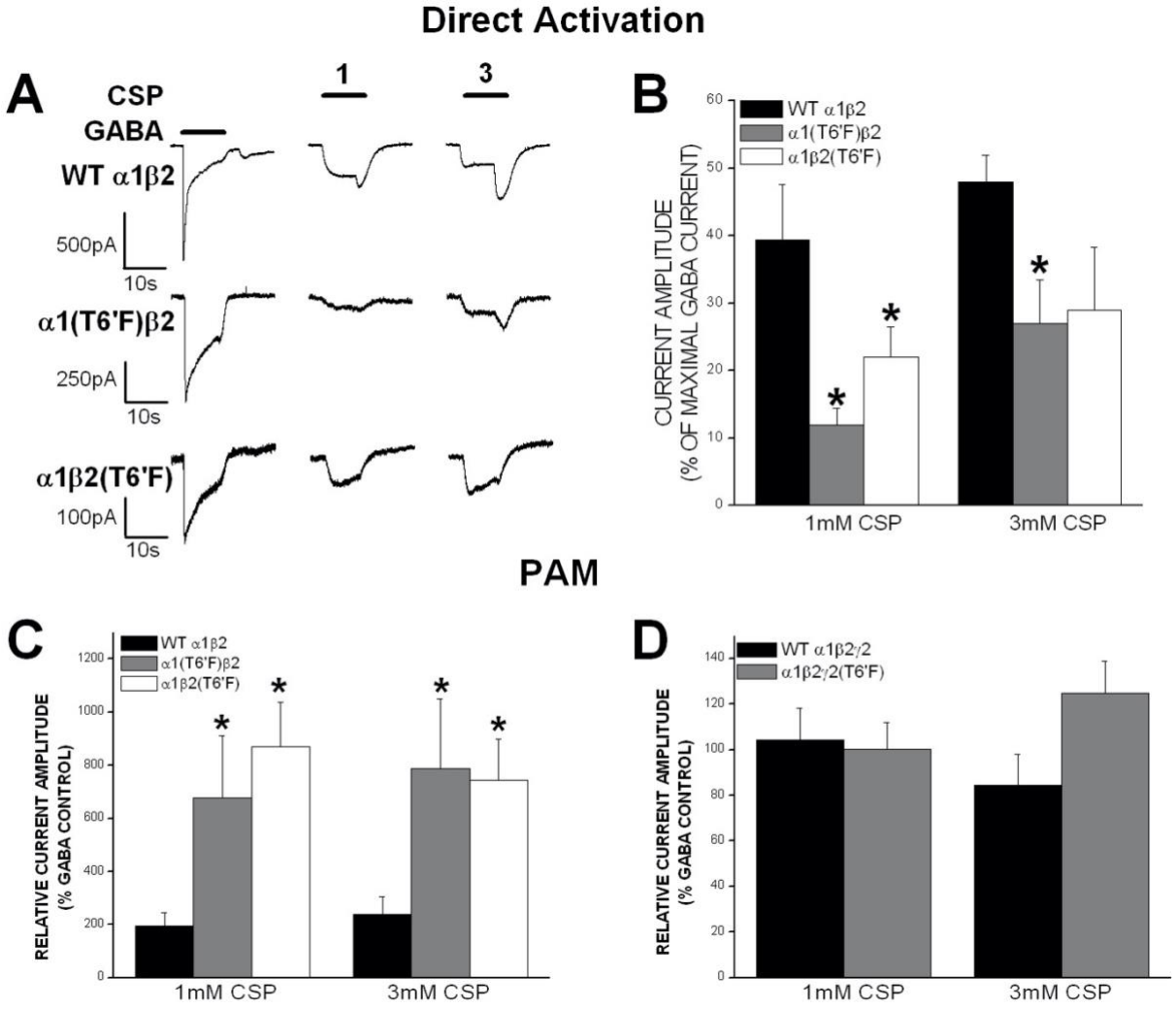


Figure 9. Influence of TM2 (T6'F) mutation on direct activation and positive allosteric modulation of $GABA_A$ receptors by carisoprodol. (A) Representative traces of WT α1β2, α1(T6'F)β2, and α1β2(T6'F) receptors and respective direct activation of $GABA_A$ receptors, normalized to 1mM GABA current. (B)Bar graph representing direct activation mean ± S.E.M. for respective receptor configuration. A significant difference was observed at 1mM carisoprodol for both α1(T6'F)β2 and α1β2(T6'F) receptors and at 3mM for both α1(T6'F)β2 and α1β2(T6'F) when compared to WT α1β2 receptors (C) Bar graph representing positive allosteric modulatory effect mean ± S.E.M. for respective receptor configuration. A significant difference was observed at 1mM carisoprodol for both α1(T6'F)β2 and α1β2(T6'F) receptors and at 3mM for both α1(T6'F)β2 and α1β2(T6'F) when compared to WT α1β2 receptors (D) Bar graph representing positive allosteric modulatory effect mean ± S.E.M. for respective receptor configuration. No significant difference observed between WT α1β2γ2 and α1β2γ2 (T6'F) receptors. All mean ± S.E.M. are representative of a minimum of three cells. *, $p \leq 0.05$.

Figure 10

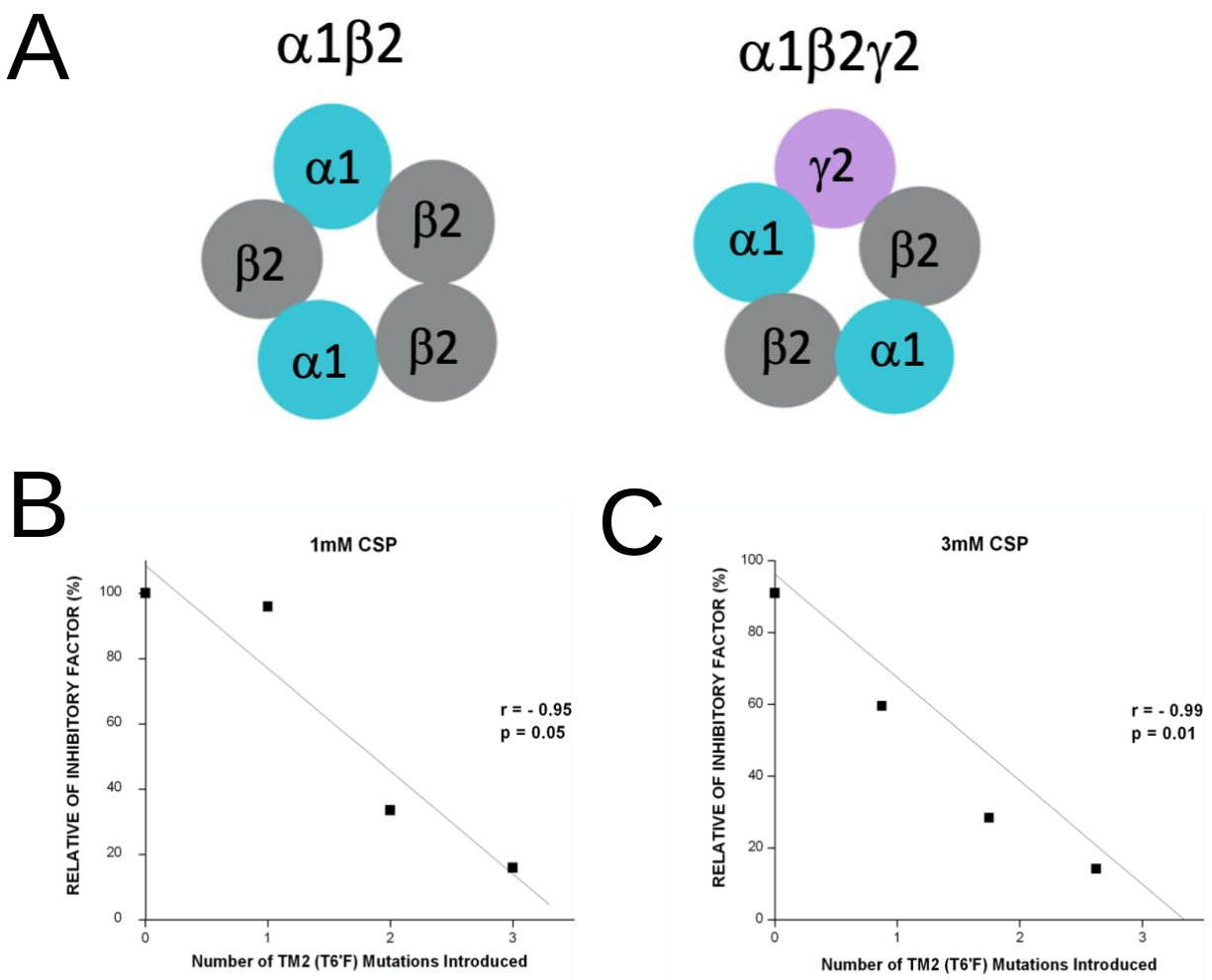


Figure 10. Representative receptor configurations and correlation analysis for relative inhibitory factors. (A) Schematic representation of the number of respective subunits for α1β2 and α1β2γ2 $GABA_A$ receptor configurations (B) Correlation analysis of WT α1β2γ2, α1β2γ2(T6'F), α1(T6'F)β2, and α1β2(T6'F) receptors representing 0, 1, 2, and 3 TM2 (T6'F) mutation(s) respectively at 1mM carisoprodol with co-applied approximate EC_{30} GABA. (C) Correlation analysis of WT α1β2γ2, α1β2γ2(T6'F), α1(T6'F)β2, and α1β2(T6'F) receptors representing 0, 1, 2, and 3 TM2 (T6'F) mutation(s) respectively at 3mM carisoprodol with co-applied approximate EC_{30} GABA. Data points are representative of mean inhibitory factor for at least three cells.

Figure 11

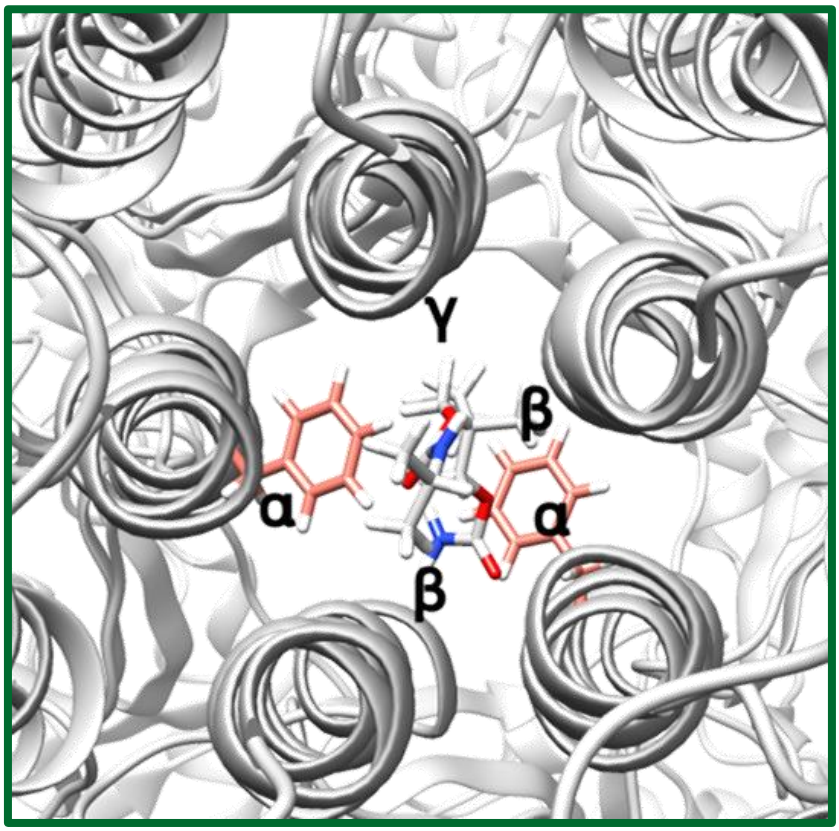


Figure 11. Docking pose of carisoprodol at the picrotoxin pocket with two α1 TM2 (T6'F) mutations. Under these conditions, picrotoxin was not able to dock at the TM2 6' region of the channel pore, generating no pose or docking score. Carisoprodol was able to dock in these experimental conditions, for a relative docking score of -4.96 kcal/mol.

Table 8. Glide XP docking scores for picrotoxin and carisoprodol on wild type and mutant $GABA_A$ receptor.

Compound	Score (kcal/mol) with wild type $GABA_A$ receptors	Score (kcal/mol) with double T261F $GABA_A$ receptors
Picrotoxin	-7.91	No pose generated
Carisoprodol	-4.41	-4.96

Figure 12

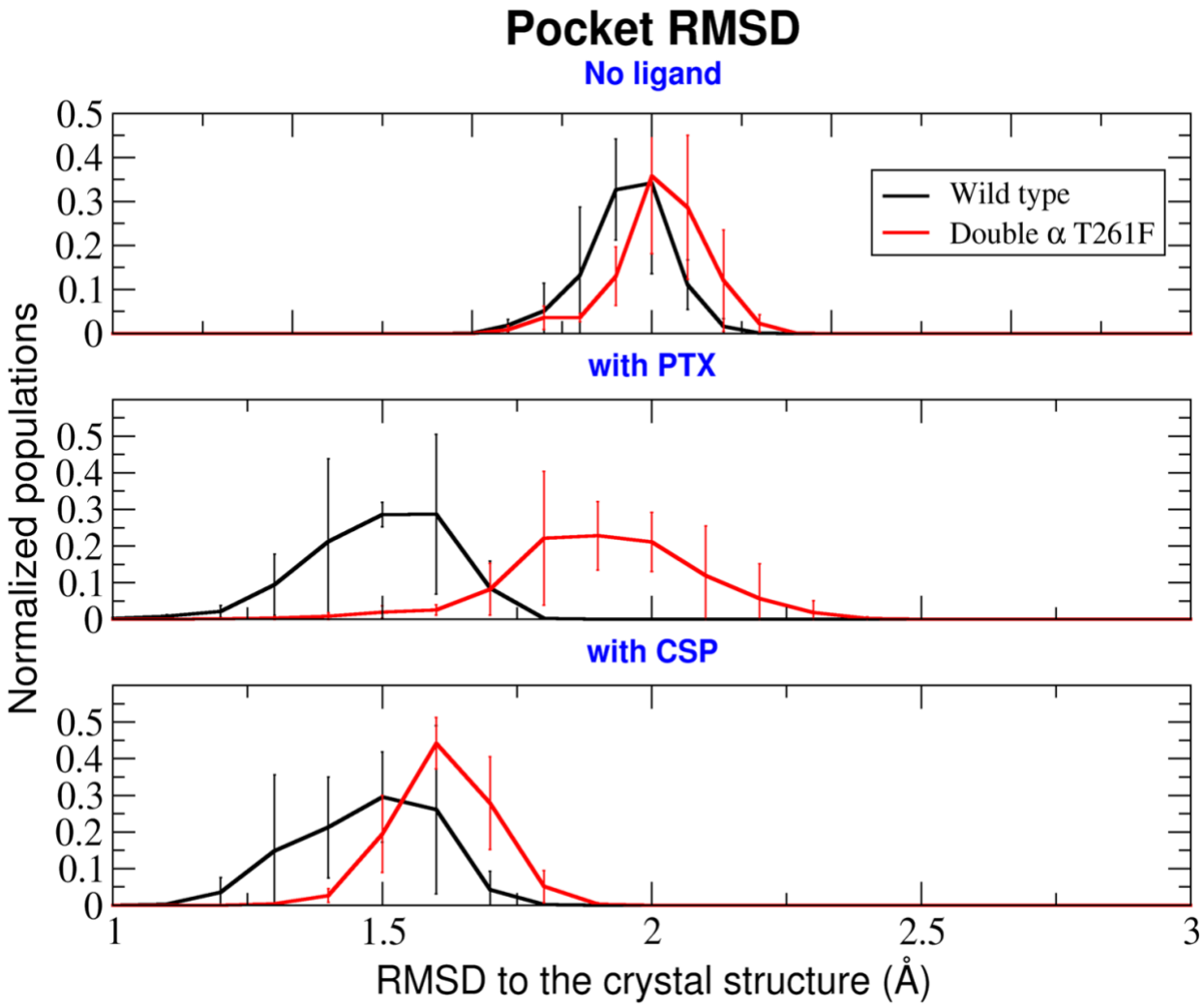


Figure 12. Root Mean Square Deviation (RMSD) graph representative of ligand-free, picrotoxin, and carisoprodol at the TM2 6' binding site.

CHAPTER IV
SUMMARY and DISCUSSION

At the conclusion of the three studies discussed in this dissertation, we have a better understanding of carisoprodol's pharmacological properties at $GABA_A$ receptors.

Carisoprodol's positive allosteric modulatory effect

Our results indicate that specific structural modifications to carisoprodol's structure alter the drug's selectivity profile in a manner that shifts the original selectivity profile from α1-preferring to α2-preferring, shown most strongly with compound NM-278. Unfortunately, none of the compounds from our first-round of drug synthesis met our criteria for further evaluation in concentration response profiles and eventual behavior analysis. However, we applied what we learned in this initial study to generate a new library of drugs structurally similar to carisoprodol and our best leads from this first study (NM-234 and 278). Additionally, we took into account issues that may be posed with drug metabolism to carisoprodol's primary metabolite meprobamate, generating drug structures that may prevent the metabolism of our novel NM-XXX compounds to meprobamate. (These structures can be found in Table 4 of Study 1 and 2 results section.) Due to issues with funding and time, these compounds have yet to be tested.

The idea of a subunit selective positive allosteric modulator of the $GABA_A$ receptor to minimize unwanted side-effects has been extensively studied in benzodiazepine compounds. Benzodiazepines are a popular class of prescription $GABA_A$ receptor positive allosteric modulators that are used clinically to treat sleep-disorders, anxiety disorders, and seizures (Rudolph, Crestani and Möhler, 2001; Licata *et al.*, 2005). Like carisoprodol, use of benzodiazepines can induce tolerance, dependence, and withdrawal symptoms (Toki *et al.*,

1996). The addictive properties of benzodiazepines have been shown to be associated with the α1 subunit of the $GABA_A$ receptors (Tan, Rudolph and Lüscher, 2011). α2, α3 and possibly α5 subunits of the $GABA_A$ receptor have been shown to be essential to mediating the anti-hyperalgesic properties of many $GABA_A$ receptor targeting drugs (Rudolph and Knoflach, 2011). This has been demonstrated with selective compounds like the α2 and α3 receptor preferring drug MP-III-024, which acts at the benzodiazepine site (B. Fischer *et al.*, 2017). This provides a rationale for targeting non-α1 $GABA_A$ receptor subunits for the development of selective compounds to treat pain and associated conditions. However, so far benzodiazepine research targeting pain treatment has not been translatable to clinic, after promising pre-clinical data, often posing issues of drug toxicity and other issues with drug metabolism (Ralvenius *et al.*, 2015). The historic failure of benzodiazepine-based drug development to treat pain is what initiated our carisoprodol drug-discovery project, as carisoprodol is already clinically utilized as a pain treatment.

Our research is innovative because carisoprodol provides a unique template and a site of action distinct from benzodiazepines. (Gonzalez et al. 2009). As far as the therapeutic myorelaxation effects associated with $GABA_A$ positive allosteric modulators, carisoprodol has proven more effective than the classic benzodiazepine, diazepam (Chou, Peterson and Helfand, 2004). Therefore, the results from our research have the potential to lead to new therapeutics that may be more effective than benzodiazepines and potentially avoid the limitations and issues seen with benzodiazepine-based drugs and provide an alternative to opioid drugs to treat pain.

Carisoprodol's direct activation effects

In this study, *in-silico* docking experiments were utilized to probe for a possible binding pocket near L415 at the α1 $GABA_A$ receptor subunit. This L415 residue has been shown to be essential to carisoprodol's ability to directly gate $GABA_A$ receptors, but it's potential role in a binding pocket was unresolved (Kumar *et al.*, 2017). Results from site directed-mutagenesis and electrophysiology studies indicate that at concentrations 100μM, 300μM, 1mM and 3mM, no mutations negatively affected the binding of carisoprodol to the $GABA_A$ receptor as predicted in docking studies. These results were surprising, as relative docking scores for carisoprodol, NM-243, and NM-244 at this proposed binding site correlated with increased direct activation of receptors seen in electrophysiology experiments, in respective increasing order. Mutations of the three lead residues of interest (M148A, K220C, and I223A) either enhanced the direct activation of $GABA_A$ receptors by carisoprodol or had no effect; docking studies indicated that these mutations would have negatively impacted carisoprodol's binding if these residues had a role in a binding site. These results indicate that the L415 residue is likely not part of a binding pocket for carisoprodol's direct gating effects, but still plays a role in carisoprodol's direct activation properties. Direct activation of $GABA_A$ receptors by carisoprodol makes it more likely to lead to death than drugs that only positively modulate receptor activity, highlighting the importance of understanding carisoprodol's mechanism of direct activation, especially when our synthesized drugs have altered direct-activation profiles(Kumar, González and Dillon, 2015). Enhanced direct activation by some of our NM-XXX compounds indicates modification to carisoprodol's structure are able to alter its direct activation profile. Further probing for site of action for carisoprodol's direct activation is needed to better understand the mechanism by which carisoprodol is able to directly gate $GABA_A$ receptors.

Carisoprodol's inhibitory action

Although carisoprodol has been reported to inhibit $GABA_A$ receptors via a low affinity site, the mechanism and action stie is unclear. In the present studies, our data suggest that carisoprodol inhibits $GABA_A$ receptors at the Cl^- channel pore, in a manner similar yet distinct to picrotoxin near the threonine TM2 6' residue.

Carisoprodol inhibition of GABA response can be greatly attenuated by the T6'F mutation at the TM2 of either α1 or β2 subunits. Furthermore, stoichiometry analysis indicates that carisoprodol's blocking effects is dependence of the number of Fs introduced to TM2 6' position. This is in contrast to picrotoxin whose inhibitory action is completely abolished by one T6'F mutation at (Erkkila, Sedelnikova and Weiss, 2008; Gonzales *et al.*, 2008; Masiulis *et al.*, 2019)A logical interpretation for this difference may be due to the difference between the structure of picrotoxin and carisoprodol. Picrotoxin contains a mixture of two large poly-cyclical structures (picrotoxinin and picrotonin). Carisoprodol is a relatively much smaller, non-cyclical structure that may be less sterically hindered by specific interaction with pore-lining residues; picrotoxin has a molecular weight of 602.6 g/mol, while carisoprodol has a molecular weight of 260.3 g/mol. To gain insight of gain insight of how carisoprodol and picrotoxin intact with $GABA_A$ receptors at the TM2 region, we performed computational simulations in wild type α1β2γ2 and mutant α1(T6'F)β2γ2 receptors. Docking studies indicate that both picrotoxin and carisoprodol interact with the threonine TM2 6'residue. picrotoxin generates no pose with the introduced Fs at TM2 6' position of α1 subunit. This is consistent with previous reports using electrophysiology and high-resolution cryo-electron microscopy (Erkkila, Sedelnikova and Weiss, 2008; Gonzales *et al.*, 2008; Masiulis *et al.*, 2019). Unlike picrotoxin, however, carisoprodol docking score was not altered by

of a phenylalanine substitution at the α1 TM2 6' position, which appears inconsistent with a significant reduction of carisoprodol inhibition of GABA response observed in α1(T6'F)β2 receptors. Additionally, in our experimental protocol involving a screening of 100μM picrotoxin to test for sensitivity, picrotoxin inhibition took longer than carisoprodol inhibition to reverse.

Inconsistency between electrophysiology studies and docking studies may be explained in part by the variation in receptor configurations used in these experiments. In electrophysiology studies, in an effort to establish the relationship between the number of mutated 6' TM2 residues and the degree of channel inhibition, mutant α1 subunits were only introduced in the α1β2 binary receptor configuration, while docking studies were carried out with mutant α1 subunits in the α1βγ2 receptor configuration. This may contribute the observed differences in the two studies. This observed difference may also be due to change of the structure and dynamics of the pockets as a result of the mutations, which was not considered in docking, but addressed in the molecular dynamic simulations that followed.

Recent cryo-EM $GABA_A$ structures revealed sequestration of picrotoxin into the channel pore between the 2' and 9' TM2 pore-lining residues, which had been previously demonstrated by site-directed mutagenesis and electrophysiology studies(Macdonald and Olsen, 1994; Huang, Gonzales and Dillon, 2006; Gonzales *et al.*, 2008; Masiulis *et al.*, 2019). Cryo-EM structures established by Masiulis *et al.* highlight specific interactions with the 9' leucine ring (hydrophobic) and hydrogen bonds with 6' threonine ring for the more active picrotoxin component picrotoxinin; these residues are conserved in the α1, β3, and γ2 subunits involved in the receptor conformation used to generate these structures.

Picrotoxin's channel-blocking actions result in a complete shutdown of GABA activated currents, unlike with carisoprodol (Masiulis *et al.*, 2019). At very high concentrations of carisoprodol (up to 10mM), while a dose dependent increase in rebound current and assumed channel inhibition is observed, complete shutdown of GABA-activated current is not observed, highlighting different interaction of carisoprodol and picrotoxin with $GABA_A$ receptors. (Kumar, González and Dillon, 2015) The role of this mutation in the inhibitory activation of carisoprodol was reported previously by our research group in abstract form in homomeric β3 $GABA_A$ receptors, though an effort to fully quantitate the inhibitory action was not established due to the nominal role in carisoprodol's inhibitory action. Consistent with our current findings is the observation that the T6'F TM2 mutation in homomeric β3 receptors had an increased effect compared to the α1β2 receptor configuration. (Kumar and Dillon, 2014). In our studies, we were able to more thoroughly define and quantitate the inhibitory action of carisoprodol at both the α1β2 receptors and the more common and physiologically relevant α1β2γ3 $GABA_A$ receptor configuration.

The significance of studying this inhibitory effect of carisoprodol is that a strong inhibitory effect has the potential to diminish the positive modulatory action of carisoprodol associated with its therapeutic effectiveness. This inhibitory action would be most physiologically relevant at relatively high concentrations of carisoprodol, which would more likely be present in cases of carisoprodol abuse. (Olsen *et al.*, 1994; L. Gonzalez *et al.*, 2009; Fass, 2010; Reeves and Burke, 2010; Gatch *et al.*, 2012; Kumar and Dillon, 2015) All-together, out results offer evidence of a mechanism for carisoprodol's ability to inhibit $GABA_A$ receptors. These findings are significant because $GABA_A$ receptor positive modulators such as carisoprodol remain some of the most popular clinically prescribed compounds to treat conditions like

anxiety, insomnia, and post-partum depression. (Tan *et al.*, 2010; Munro, Hansen and Mirza, 2013; Schwienteck *et al.*, 2017) Additionally, in data from our lab not shown here, newly-synthesized compounds based on carisoprodol's structure possess varying degrees of inhibitory activity at $GABA_A$ receptors, highlighting the importance of understanding the pharmacological effects associated with carisoprodol at $GABA_A$ receptors in the development of new drugs.

CONCLUSION & FUTURE DIRECTIONS

At the conclusion of these studies, while we have learned much about carisoprodol's pharmacological actions, many questions remain. Our results, particularly for drug discovery studies, were initially limited by funding and eventually time. An essential future study would be to evaluate our second round of newly synthesized compounds, for which there was not time to test. A strong lead that presents a significant $\alpha 2$ subunit preference and will likely not be metabolized to meprobamate would be further evaluated in behavioral paradigms for pain tolerance and abuse liability. This lead would potentially be able to serve as a novel pain treatment with reduced abuse liability compared with carisoprodol and provide a non-opioid alternative to pain treatment in the midst of an opioid abuse epidemic.

Previous studies show that carisoprodol has the greatest efficacy at $GABA_A$ receptors containing the $\alpha 1$ subunit, for both positive modulatory and direct activation effects (Kumar, González and Dillon, 2015). Because the $\alpha 1$ subunit has been associated with negative side-effects of GABAergic compounds, like addiction and sedation which are both observed with carisoprodol, further research into the molecular basis for this subunit selectivity is an important future direction for this research. (Wafford, 2005; Munro *et al.*, 2008; Munro, Hansen and Mirza,

2013) Although not reported in our studies presented in this dissertation because residues of interest went untested, we generated a series of α1 subunit mutants in an effort to probe for residues that may be important to carisoprodol's ability to positively modulate GABA-activated currents at an allosteric site. In an effort to elucidate specific structural elements on the α1 subunit which confer greater carisoprodol positive allosteric modulation than other α-containing receptors, we searched for amino acid residues that were unique between the sequences of α1 $GABA_A$ receptor subunit and α2 subunit. We utilized site-directed mutagenesis, where point mutations were applied to 12 residues unique to the N-terminus of the α1 subunit of the $GABA_A$ receptor to identify amino acid residues that are important to carisoprodol's subunit selectivity profile. These mutants include H56T, T82N, R95W, T122Q, E123D, R136Q, R173Y, E174N, A176S, R177D, D199G, and Q204K which are all unique to the α1 compared to the α2 subunit and may play a role in the enhanced positive modulatory effects observed at α1 $GABA_A$ receptors. An important future direction of this research is to evaluate carisoprodol's pharmacological activity at these mutant α1 subunits. Regarding carisoprodol's direct activation of $GABA_A$ receptors, further research is needed to establish why we saw a general enhancement of direct activation effects with mutant subunits in our studies. It is likely that the mutations imparted in our direct activation studies may have indirectly enhanced the binding of carisoprodol at a further direct activation site; cysteine and alanine mutations are able to stabilize receptor conformations because of their size and chemical properties. Alanine residues specifically stabilize any alpha helices or beta sheets they are involved in. Substitution to alanine residues eliminates side-chain interactions but does not alter main-chain conformation or introduce steric or electrostatic effects, preserving native protein structure (Weiss *et al.*, 2000).

Future studies with different combination of mutations are needed to further rule out the indicated M148, K220, and I223 residues involvement in a binding pocket.

Additionally, much of our focus throughout this dissertation was centered on the role of α subunits because of specific subunit association with certain therapeutic or side effects, in addition to observed difference in carisoprodol's action at different α receptor subunits. Future studies should further evaluate the role of β subunits in carisoprodol's action, specifically in suspected binding sites for direct activation and positive modulatory effects. Previous studies show the greater efficacy of carisoprodol at β1 receptors than β2 receptors in regard to direct activation. In contrast, greater potency and efficacy of carisoprodol was seen in β2 receptors compared with β1 in regards to positive modulatory effects. (Kumar, González and Dillon, 2015) As the γ subunit was previously found to not be essential to direct or allosteric effects of carisoprodol at $GABA_A$ receptors, focus may remain on the role of different α and β subunits in the interaction of carisoprodol at these receptors for future studies. (Kumar, González and Dillon, 2015; Kumar *et al.*, 2017) Efforts to further study carisoprodol's pharmacological actions at $GABA_A$ receptors will provide a better understanding of a clinically utilized drug that serves as a promising target for future drug development and possibly reduce its abuse liability.

This project provides important insight into carisoprodol's pharmacological activity at $GABA_A$ receptors. PAM studies indicate that carisoprodol's structure is able to be altered in a manner that is able to shift its selectivity profile, offering promise for the further development of a carisoprodol-based drug with low abuse-liability while remaining therapeutically effective. Direct activation studies also indicate structural modifications are able alter carisoprodol's direct activation effects, which is an important consideration as drugs that can act in the absence of neurotransmitter are more dangerous than those that rely on neurotransmitter activation.

Inhibitory studies show a novel binding site for carisoprodol at the $GABA_A$ receptor, where carisoprodol is able to inhibit $GABA_A$ receptors at high concentrations by blocking the chloride ion channel pore. All-together, this project provides a better understanding of the complex interactions of carisoprodol and $GABA_A$ receptors.

CHAPTER V
REFERENCES

1. Barnard, E. A. *et al.* (1998) 'International Union of Pharmacology. XV. Subtypes of g-Aminobutyric AcidA Receptors: Classification on the Basis of Subunit Structure and Receptor Function', *Pharmacological Reviews*, 60(3), pp. 243–260. doi: 10.1124/pr.108.00505.
2. Belelli, D. *et al.* (2020) 'Realising the therapeutic potential of neuroactive steroid modulators of the GABAA receptor', *Neurobiology of Stress*. Elsevier, 12(December 2019). doi: 10.1016/j.ynstr.2019.100207.
3. Braun, E. *et al.* (2019) 'Best Practices for Foundations in Molecular Simulations [Article v1.0]', *Living Journal of Computational Molecular Science*, 1(1), pp. 1–28. doi: 10.33011/livecoms.1.1.5957.
4. Brickley, S. G. and Mody, I. (2012) 'Extrasynaptic $GABA_A$ Receptors: Their Function in the CNS and Implications for Disease', *Neuron*. Elsevier Inc., 73(1), pp. 23–34. doi: 10.1016/j.neuron.2011.12.012.
5. Castellano, D., Shepard, R. D. and Lu, W. (2021) 'Looking for Novelty in an "Old" Receptor: Recent Advances Toward Our Understanding of GABAARs and Their Implications in Receptor Pharmacology', *Frontiers in Neuroscience*, 14(January), pp. 1–15. doi: 10.3389/fnins.2020.616298.
6. Chou, R., Peterson, K. and Helfand, M. (2004) 'Comparative efficacy and safety of skeletal muscle relaxants for spasticity and musculoskeletal conditions: A systematic review', *Journal of Pain and Symptom Management*, 28(2), pp. 140–175. doi: 10.1016/j.jpainsymman.2004.05.002.

7. D'Hulst, C., Atack, J. R. and Kooy, R. F. (2009) 'The complexity of the GABAA receptor shapes unique pharmacological profiles', *Drug Discovery Today*, 14(17–18), pp. 866–875. doi: 10.1016/j.drudis.2009.06.009.
8. Davies, M. (2003) 'The role of GABAA receptors in mediating the effects of alcohol in the central nervous system', *Journal of psychiatry & neuroscience*, 28(4), pp. 263–274. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=165791&tool=pmcentrez&rendertype=abstract.
9. Dibas, M. I. and Dillon, G. H. (2000) 'The central nervous system convulsant pentylenetetrazole stimulates gamma-aminobutyric acid (GABA)-activated current in picrotoxin-resistant GABA(A) receptors in HEK293 cells.', *Neuroscience letters*. Ireland, 285(3), pp. 193–196. doi: 10.1016/s0304-3940(00)01064-8.
10. Erkkila, B. E., Sedelnikova, A. V. and Weiss, D. S. (2008) 'Stoichiometric pore mutations of the GABAAR reveal a pattern of hydrogen bonding with picrotoxin', *Biophysical Journal*. Elsevier, 94(11), pp. 4299–4306. doi: 10.1529/biophysj.107.118455.
11. Farrant, M. and Nusser, Z. (2005) 'Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors.', *Nature reviews. Neuroscience*, 6(3), pp. 215–229. doi: 10.1038/nrn1625.
12. Fass, J. A. (2010) 'Carisoprodol legal status and patterns of abuse', *Annals of Pharmacotherapy*, 44(12), pp. 1962–1967. doi: 10.1345/aph.1P339.
13. Fischer, B. *et al.* (2017) 'Pharmacological and antihyperalgesic properties of the novel α2/3 preferring GABAAreceptor ligand MP-III-024', *Brain Research Bulletin*. Elsevier

Inc., 131, pp. 62–69. doi: 10.1016/j.brainresbull.2017.03.001.

14. Fischer, B. D. *et al.* (2017) ‘Pharmacological and antihyperalgesic properties of the novel alpha 2/3 preferring GABA(A) receptor ligand MP-III-024’, *Brain Research Bulletin*. Elsevier Inc., 131, pp. 62–69. doi: 10.1016/j.brainresbull.2017.03.001.
15. Fritschy, J. M. and Brünig, I. (2003) ‘Formation and plasticity of GABAergic synapses: Physiological mechanisms and pathophysiological implications’, *Pharmacology and Therapeutics*, 98(3), pp. 299–323. doi: 10.1016/S0163-7258(03)00037-8.
16. Gatch, M. B. *et al.* (2012) ‘Carisoprodol tolerance and precipitated withdrawal’, *Drug and Alcohol Dependence*. Elsevier Ireland Ltd, 123(1–3), pp. 29–34. doi: 10.1016/j.drugalcdep.2011.10.010.
17. Gonzales, E. B. *et al.* (2008) ‘Stoichiometric analysis of the TM2 6′ phenylalanine mutation on desensitization in α1β2 and α1β2γ2 GABAA receptors’, *Neuroscience Letters*, 431(2), pp. 184–189. doi: 10.1016/j.neulet.2007.11.039.
18. Gonzalez, L. *et al.* (2009) ‘Abuse potential of Soma: the GABA-A receptor as Target’, *Mol Cell Pharmacol*, 1(4), pp. 108–186. doi: 10.1016/j.biotechadv.2011.08.021.Secreted.
19. Gonzalez, L. A. *et al.* (2009) ‘Carisoprodol-Mediated Modulation of GABA A Receptors : In Vitro and in Vivo Studies’, *Pharmacology*, 329(2), pp. 827–837. doi: 10.1124/jpet.109.151142.soprodol.
20. Griebel, G. *et al.* (2001) ‘SL651498: an anxioselective compound with functional selectivity for alpha2- and alpha3-containing gamma-aminobutyric acid(A) (GABA(A)) receptors.’, *The Journal of pharmacology and experimental therapeutics*, 298(2), pp. 753–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11454940.
21. Gurley, D. *et al.* (1995) ‘Point mutations in the M2 region of the alpha, beta, or gamma

subunit of the GABAA channel that abolish block by picrotoxin.', *Receptors & channels*. England, 3(1), pp. 13–20.

22. Hisano, K. *et al.* (2007) 'The channel-lining 6′ amino acid in the second membrane-spanning region of ionotropic GABA receptors has more profound effects on 4′-ethynyl-4-n-propylbicycloorthobenzoate binding than the 2′ amino acid', *Invertebrate Neuroscience*, 7(1), pp. 39–46. doi: 10.1007/s10158-006-0035-x.
23. Huang, R., Gonzales, E. B. and Dillon, G. H. (2006) 'GABAA receptors: Structure, function and modulation', *Biological and Biophysical Aspects of Ligand-Gated Ion Channel Receptor Superfamilies*.
24. Johnson, W. D. 2nd *et al.* (2012) 'The TM2 6' position of GABA(A) receptors mediates alcohol inhibition.', *The Journal of pharmacology and experimental therapeutics*, 340(2), pp. 445–456. doi: 10.1124/jpet.111.188037.
25. Korpi, E. R., Gründer, G. and Lüddens, H. (2002) 'Drug interactions at GABAA receptors', *Progress in Neurobiology*, 67(2), pp. 113–159. doi: 10.1016/S0301-0082(02)00013-8.
26. Kumar, M. and Dillon, G. H. (2014) 'Distinct amino acid domains confer direct gating, allosteric modulation and blocking properties of carisoprodol on GABA(A) receptors (Abstract)', *Neuroscience Meeting Planner. Washington DC: Society for Neuroscience*, Program 12. doi: 10.15036/arerugi.44.821_2.
27. Kumar, M. and Dillon, G. H. (2015) 'Carisoprodol: Update on abuse potential and mechanism of action', *Molecular and Cellular Pharmacology*, 7(1), pp. 1–10. doi: 10.4255/mcpharmacol.15.01.
28. Kumar, M. and Dillon, G. H. (2016) 'Assessment of direct gating and allosteric

modulatory effects of meprobamate in recombinant GABAAreceptors', *European Journal of Pharmacology*. Elsevier, 775, pp. 149–158. doi: 10.1016/j.ejphar.2016.02.031.

29. Kumar, M., González, L. A. and Dillon, G. H. (2015) 'Assessment of subunit-dependent direct gating and allosteric modulatory effects of carisoprodol at GABAAreceptors', *Neuropharmacology*, 97, pp. 414–425. doi: 10.1016/j.neuropharm.2015.04.007.
30. Kumar, Manoj *et al.* (2017) 'A Single Amino Acid Residue at Transmembrane Domain 4 of the a Subunit Influences Carisoprodol Direct Gating Efficacy at GABA A Receptors', *THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS J Pharmacol Exp Ther*, 362(September), pp. 395–404. doi: 10.1124/jpet.117.242156.
31. Lambert, J. J. *et al.* (2003) 'Neurosteroid modulation of GABAA receptors', *Progress in Neurobiology*, 71(1), pp. 67–80. doi: 10.1016/j.pneurobio.2003.09.001.
32. Li, G. D. *et al.* (2006) 'Identification of a GABAA receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog', *Journal of Neuroscience*, 26(45), pp. 11599–11605. doi: 10.1523/JNEUROSCI.3467-06.2006.
33. Licata, S. C. *et al.* (2005) 'Contribution of GABA A Receptor Subtypes to the Anxiolytic- Like , Motor , and Discriminative Stimulus Effects of Benzodiazepines : Studies with the Functionally Selective yl-carbonyl)-2 ,9-dihydro-1H-pyridol [3,4-b] indol-1-one]', *The Journal of Pharmacology and Experimental Therapeutics*, 313(3), pp. 1118–1125. doi: 10.1124/jpet.104.081612.rently.
34. Ludwig, B. J., Powell, L. S. and Berger, F. M. (1969) 'Carbamate Derivatives Related to Meprobamate', *Journal of Medicinal Chemistry*, 12(3), pp. 462–472. doi: 10.1021/jm00303a029.
35. Macdonald, R. L. and Olsen, R. W. (1994) 'GABAA receptor channels.', *Annual Review*

of Neuroscience, 17, pp. 569–602. doi: 10.1146/annurev.ne.17.030194.003033.

36. Masiulis, S. *et al.* (2019) 'GABAA receptor signalling mechanisms revealed by structural pharmacology', *Nature*, 565(7740), pp. 454–459. doi: 10.1038/s41586-018-0832-5.
37. McCarson, K. E. and Enna, S. J. (2014) 'GABA Pharmacology: The Search for Analgesics', *Neurochemical Research*, 39(10), pp. 1948–1963. doi: 10.1007/s11064-014-1254-x.
38. Miller, P. S. and Aricescu, A. R. (2014) 'Crystal structure of a human GABAA receptor', *Nature*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 512, p. 270. Available at: http://dx.doi.org/10.1038/nature13293.
39. Miller, P. S. and Smart, T. G. (2010) 'Binding, activation and modulation of Cys-loop receptors', *Trends in Pharmacological Sciences*. Elsevier Ltd, 31(4), pp. 161–174. doi: 10.1016/j.tips.2009.12.005.
40. Mirza, N. R. *et al.* (2008) 'yl] -biphenyl-2-carbonitrile], a Unique Subtype-Selective GABA A Receptor Positive Allosteric Modulator : In Vitro Actions , Pharmacokinetic Properties and in Vivo Anxiolytic Efficacy', 327(3), pp. 954–968. doi: 10.1124/jpet.108.138859.TPA023.
41. Möhler, H., Crestani, F. and Rudolph, U. (2001) 'GABAA-receptor subtypes: A new pharmacology', *Current Opinion in Pharmacology*, 1(1), pp. 22–25. doi: 10.1016/S1471-4892(01)00008-X.
42. Munro, G. *et al.* (2008) 'Comparison of the Novel Subtype-Selective GABA A Receptor-Positive Allosteric Modulator NS11394 [3J- [5- (1-Hydroxy-1- in Rat Models of Inflammatory and Neuropathic Pain', *Pharmacology*, 327(3), pp. 969–981. doi: 10.1124/jpet.108.144568.although.

43. Munro, G., Hansen, R. R. and Mirza, N. R. (2013) ‘GABAareceptor modulation: Potential to deliver novel pain medicines?’, *European Journal of Pharmacology*, 716(1–3), pp. 17–23. doi: 10.1016/j.ejphar.2013.01.070.

44. Nutt, D. (2006) ‘GABA A Receptors : Subtypes , Regional Distribution , and Function’, *Journal of Clinical Sleep Medicine*, 2, pp. S7–S11.

45. Olsen, H. *et al.* (1994) ‘Carisoprodol elimination in humans.’, *Therapeutic drug monitoring*. United States, 16(4), pp. 337–340. doi: 10.1097/00007691-199408000-00001.

46. Olsen, R. W. and Tobin, a J. (1990) ‘Molecular biology of GABAA receptors.’, *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 4, pp. 1469–1480. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2155149.

47. Paul, J. *et al.* (2014) ‘Antihyperalgesia by 2-GABA A receptors occurs via a genuine spinal action and does not involve supraspinal sites’, *Neuropsychopharmacology*, 39(2), pp. 477–487. doi: 10.1038/npp.2013.221.

48. Prokai, L. *et al.* (2016) ‘Mass spectrometric analysis of carisoprodol and meprobamate in rat brain microdialysates’, 51(10), pp. 900–907. doi: 10.1002/jmri.24785.Free-Breathing.

49. Ralvenius, W. T. *et al.* (2015) ‘Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABA<inf>A</inf> receptor subtype’, *Nature Communications*, 6. doi: 10.1038/ncomms7803.

50. Reeves, R. R. and Burke, R. S. (2010) ‘Carisoprodol: abuse potential and withdrawal syndrome.’, *Current drug abuse reviews*, 3(1), pp. 33–38. doi: 10.2174/1874473711003010033.

51. Rudolph, U., Crestani, F. and Möhler, H. (2001) ‘GABAA receptor subtypes: Dissecting their pharmacological functions’, *Trends in Pharmacological Sciences*, 22(4), pp. 188–194. doi: 10.1016/S0165-6147(00)01646-1.

52. Rudolph, U. and Knoflach, F. (2011) ‘Beyond classical benzodiazepines: Novel therapeutic potential of GABAA receptor subtypes’, *Nature Reviews Drug Discovery*, 10(9), pp. 685–697. doi: 10.1038/nrd3502.Beyond.

53. Rudolph, U. and Möhler, H. (2006) ‘GABA-based therapeutic approaches: GABAA receptor subtype functions’, *Current Opinion in Pharmacology*, 6(1 SPEC. ISS.), pp. 18–23. doi: 10.1016/j.coph.2005.10.003.

54. Schwienteck, K. L. *et al.* (2017) ‘Abuse-related effects of subtype-selective GABAAreceptor positive allosteric modulators in an assay of intracranial self-stimulation in rats’, *Psychopharmacology*, 234(14), pp. 2091–2101. doi: 10.1007/s00213-017-4615-8.

55. Serecigni, J. G. (2018) ‘Overdose epidemic linked to the prescription of opioid analgesics in the United States’, 30(2), pp. 87–92. doi: 10.20882/adicciones.936.

56. Seth, P. *et al.* (2018) ‘Overdose deaths involving opioids, cocaine, and psychostimulants — United States, 2015–2016’, *American Journal of Transplantation*, 18(6), pp. 1556–1568. doi: 10.1111/ajt.14905.

57. Sieghart, W. *et al.* (1999) ‘Structure and subunit composition of GABA(A) receptors’, *Neurochemistry International*, 34(5), pp. 379–385. doi: 10.1016/S0197-0186(99)00045-5.

58. Sigel, E. and Steinmann, M. E. (2012) ‘Structure, function, and modulation of GABAA receptors’, *Journal of Biological Chemistry*, 287(48), pp. 40224–40231. doi:

10.1074/jbc.R112.386664.

59. Tan, K. R. *et al.* (2010) ‘Neural bases for addictive properties of benzodiazepines’, *Nature*, 463(7282), pp. 769–774. doi: 10.1038/nature08758.
60. Tan, K. R., Rudolph, U. and Lüscher, C. (2011) ‘Hooked on benzodiazepines: GABAAreceptor subtypes and addiction’, *Trends in Neurosciences*, 34(4), pp. 188–197. doi: 10.1016/j.tins.2011.01.004.
61. Toki, S. *et al.* (1996) ‘Diazepam physical dependence and withdrawal in rats is associated with alteration in GABA(A) receptor function’, *Life Sciences*, 59(19), pp. 1631–1641. doi: 10.1016/0024-3205(96)00494-8.
62. Tretter, V. *et al.* (1997) ‘Stoichiometry and assembly of a recombinant GABA(A) receptor subtype’, *Journal of Neuroscience*, 17(8), pp. 2728–2737. doi: 10.1523/jneurosci.17-08-02728.1997.
63. Wafford, K. A. (2005) ‘GABAAreceptor subtypes: Any clues to the mechanism of benzodiazepine dependence?’, *Current Opinion in Pharmacology*, 5(1), pp. 47–52. doi: 10.1016/j.coph.2004.08.006.
64. Waldvogel, H. J., Baer, K. and Faull, R. L. M. (2010) ‘Distribution of GABAA Receptor Subunits in the Human Brain’, in *GABA and Sleep*, pp. 73–93.
65. Weiss, G. A. *et al.* (2000) ‘Rapid mapping of protein functional epitopes by combinatorial alanine scanning’, *Proceedings of the National Academy of Sciences of the United States of America*, 97(16), pp. 8950–8954. doi: 10.1073/pnas.160252097.
66. Whiting, P. J. *et al.* (1999) ‘Molecular and functional diversity of the expanding GABA-A receptor gene family.’, *Annals of the New York Academy of Sciences*, 868, pp. 645–653. doi: 10.1111/j.1749-6632.1999.tb11341.x.

67. Whiting, P. J. (2003) ‘GABA-A receptor subtypes in the brain: A paradigm for CNS drug discovery?’, *Drug Discovery Today*, 8(10), pp. 445–450. doi: 10.1016/S1359-6446(03)02703-X.

68. Zeilhofer, H. U., Möhler, H. and Di Lio, A. (2009) ‘GABAergic analgesia: new insights from mutant mice and subtype-selective agonists’, *Trends in Pharmacological Sciences*, 30(8), pp. 397–402. doi: 10.1016/j.tips.2009.05.007.

69. Zeilhofer, H. U., Witschi, R. and Hösl, K. (2009) ‘Subtype-selective GABAA receptor mimetics—novel antihyperalgesic agents?’, *Journal of Molecular Medicine*, 87(5), pp. 465–469. doi: 10.1007/s00109-009-0454-3.

70. Zhang, D. *et al.* (1995) ‘Cloning of a γ-aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block’, *Proceedings of the National Academy of Sciences of the United States of America*, 92(25), pp. 11756–11760. doi: 10.1073/pnas.92.25.11756.

71. Zhu, S. *et al.* (2018) ‘Structure of a human synaptic GABAA receptor’, *Nature*, 559(7712), pp. 67–72. doi: 10.1038/s41586-018-0255-3.

72. Zorumski, C. F. *et al.* (2019) ‘Neurosteroids as novel antidepressants and anxiolytics: GABA-A receptors and beyond’, *Neurobiology of Stress*. Elsevier, 11(September). doi: 10.1016/j.ynstr.2019.100196.