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**ASSESSMENT OF MOLECULAR ACTION OF DIRECT GATING AND
ALLOSTERIC MODULATORY EFFECTS OF MEPROBAMATE (MILTOWN®)
ON GABA_A RECEPTORS**

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**Dissertation submitted to the School of Pharmacy at
West Virginia University in partial fulfillment of
Requirements for the degree of
DOCTOR OF PHILOSOPHY**

IN

PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

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ABSTRACT

ASSESSMENT OF MOLECULAR ACTION OF DIRECT GATING AND ALLOSTERIC MODULATORY EFFECTS OF MEPROBAMATE (MILTOWN®) ON GABAA RECEPTORS

Manish Kumar

Meprobamate (tradenames Miltown, Equanil) is a schedule IV anxiolytic and the primary metabolite of the muscle relaxant carisoprodol. Meprobamate modulates GABA_A (γ -aminobutyric acid type A) receptors, and has been described as having barbiturate-like activity. A thorough understanding of its mechanism remains ill-defined, however. To gain insight into its actions, we have conducted a series of studies using recombinant GABA_A receptors. In $\alpha x \beta z \gamma 2$ GABA_A receptors (where $x = 1-6$ and $z = 1-3$), the ability to enhance GABA-mediated current was evident for all α subunit isoforms, with the largest effect observed in $\alpha 5$ -expressing receptors. Direct gating was comparable with all α subunits, although the effect was attenuated in $\alpha 3$ -expressing receptors. Allosteric potentiating and direct gating effects were comparable in $\alpha 1 \beta 1 \gamma 2$ and $\alpha 1 \beta 2 \gamma 2$ receptors, whereas allosteric effects were enhanced in $\alpha 1 \beta 2$ compared to $\alpha 1 \beta 2 \gamma 2$ receptors. In “extrasynaptic” GABA_A receptors ($\alpha 1 \beta 3 \delta$ and $\alpha 4 \beta 3 \delta$ receptors), meprobamate allosterically enhanced both EC₂₀ and saturating GABA currents, and directly activated these receptors with an efficacy comparable to that of GABA. Bemegride, which antagonizes effects of pentobarbital, attenuated direct gating effects of meprobamate. Whereas pentobarbital directly gated homomeric $\beta 3$ receptors, meprobamate did not, and instead blocked the spontaneously open current present in these receptors. In wild type homomeric $\rho 1$

receptors, both pentobarbital and meprobamate were ineffective in direct gating; a mutation (W328M) previously shown to confer sensitivity to pentobarbital, did not confer sensitivity to meprobamate. Our results provide additional insight into the actions of meprobamate and parent therapeutic agents such as Carisoprodol as well as possible domains involved in the direct gating actions of the ligands including transmembrane domains TM4. Further, using site-directed-mutagenesis and whole cell patch clamp electrophysiology in transiently transfected HEK293 cells, we examined the role of GABAA receptor α -subunit transmembrane domain 4 (TM4) and transmembrane domain 2 (TM2) amino acids in direct gating, and inhibitory actions of meprobamate. Mutation of $\alpha 3$ valine at position 440 to leucine (present in the equivalent position in the $\alpha 1$ subunit) increased the direct gating potency and efficacy of meprobamate. In R- $\alpha 1\beta 2$ heteromeric GABAA receptor, both pentobarbital and carisoprodol had inhibitory effect for allosteric modulatory action at higher concentrations. Meprobamate did not have an inhibitory effect at the concentrations tested. T6'F mutation of the $\beta 2$ -subunit abolished the inhibition of carisoprodol but not of pentobarbital. Thus, our studies provide further insight into actions of meprobamate, its parent drug carisoprodol and their comparison to barbiturate pentobarbital.

DEDICATION

I dedicate my dissertation to my mother **Mrs. Manju Kumari** whose tireless patience and encouragement helped me in toughest of the times. My Grand Father, Late **Mr. Awadh Kishore Das**. My aunt **Bindu Kumari**, whose first words when I joined medical school was, “win a Nobel prize in medicine”. My father, **Mr. Sachchidanand Roy**, who stood behind me through thick and thin and last but not the least, my mentor **Dr. Glenn H. Dillon**.

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LIST OF ABBREVIATIONS

GABA	γ -aminobutyric acid
GABAAR	γ -aminobutyric acid type A receptor
LGIC	Ligand-gated ion channel
CSP	Carisoprodol
BZD	Benzodiazepines
PTX	Picrotoxin
PB	Pentobarbital
CYP2C19	Cytochrome P450 2C19 enzyme
TM	Transmembrane
HEK	Human embryonic kidney
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis (β -aminoethyl ether)
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid N, N, N', N'-tetra acetic acid

CHAPTER I

INTRODUCTION

MEPROBAMATE

History

Meprobamate was the first drug to be marketed specifically as an anxiolytic drug, that is, capable of allaying anxiety and related conditions, without inducing sedation or sleep. Development of meprobamate is attributed to some serendipitous observations during the course of development of new preservatives, after discovery of penicillin. During the course of a search for compound to prevent enzymatic destruction of penicillin by penicillinase producing gram-negative bacteria, normally present in the air, a compound mephesisin was found to have reversible tranquilizing and muscle relaxing properties (Berger *et al.*, 1946). Although later a powder form of penicillin made obsolete the use of preservatives, some interesting properties observed with these α -substituted esters of glycerol, created a lot of attention. As stated earlier, mephesisin possessed tranquilizing and at high doses, sedative and muscle relaxant properties. Initial excitatory effect, associated with barbiturate use was absent, showing a lack of central action. For the muscle relaxant properties, diaphragm muscle was last to get affected, causing the respiratory paralysis to appear last as a lethal side effect and at a very high dose. There was a large gap between dose for paralysis of other muscles and diaphragmatic muscle (Berger, 1947). However, curare, a widely used muscle relaxant of the day as an adjuvant to anesthetics, had respiratory paralysis as a major side effect (Delahanty *et al.*, 2011; Chen *et al.*, 2014). In addition, mephesisin possessed a unique anticonvulsant action. Strychnine-induced convulsions (convulsions induced via actions at spinal cord) were inhibited by tranquilizing doses of mephesisin while hexobarbitone, a barbiturate, was unable to prevent these. Leptazol induced convulsions (convulsions induced via actions on midbrain), were

inhibited by hexobarbitone, but required a very high dose of mephenesin. Above effects show the effect of mephenesin at spinal cord level rather than at the midbrain level. This also explained the initial missing euphoric and exciting action associated with the use of mephenesin (associated with a central action), present with the use of barbiturates. However, its use as such was limited by its three major drawbacks—mephenesin was quickly metabolized to inactive products leading to a very short duration of action, greater effect on the spinal cord than on supra-spinal structures and a weak action, so large doses were required (Berger, 1947). Mephenesin was quickly metabolized via oxidation of its terminal hydroxyl group leading to the formation of inactive metabolites. Blocking the oxidation of the terminal hydroxyl group would be a major mechanism for extended action of mephenesin. After some trial and error, carbamate esters of 1, 3-propane diols were prepared which had longer duration of action and stronger muscle relaxant properties. By the late 1950s meprobamate was the most widely prescribed drug in the United States and in many other countries. It retained its lead until diazepam, the second drug from the benzodiazepine series, was introduced into clinical use in late 1960 (Greenblatt *et al.*, 1974).

Within 2 years of introduction of meprobamate, cases of its abuse and withdrawal after long term use were reported (Boyd *et al.*, 1958). By 1970 it was listed as a controlled substance, in USA market, after it was discovered to cause physical and psychological dependence. While meprobamate was made a controlled substance, drugs metabolized to meprobamate are widely used and abused and are associated with serious side effects. One such drug carisoprodol, would be discussed in the later sections of this dissertation.

Fig 1. Chemical structure of meprobamate and mephenesin

Mephenesin

Meprobamate

Chemistry and Physiological Effects of Meprobamate

Meprobamate (Miltown by wallace laboratories and Equanil by Wyeth Laboratories), chemically, is 2-methyl-2-npropyl- 1, 3-propanediol dicarbamate. First synthesized in 1950 by Ludwig and Piech (1951), meprobamate has the following physical properties: It is a white crystalline powder with a characteristic bitter taste and faint odor. It melts without decomposition at 104-106°C. It is soluble in water to the extent of 0.34 per cent at 20°C and 0.79 per cent at 37°C, but is readily soluble in most organic solvents. It is unaffected by dilute acid and alkali and thus is not broken down in gastric or intestinal juices (Berger, 1954).

Meprobamate when applied to mice, lead to a sedative action accompanied by loss of righting reflex. Unlike barbiturates, loss of righting reflex associated with the use of meprobamate is unaccompanied by any sign of excitability. Common signs of excitability like aimless running, arching and stretching of back and hind limbs, licking of paws, are present during induction of barbiturates, while absent even at paralyzing doses of meprobamate. Recovery is spontaneous and complete. Although mephensin too had similar anesthetic action, meprobamate had longer periods of induction and duration and is also effective orally (Berger, 1954).

Monkeys exposed to 200 mg/kg meprobamate, calmed down and became easier to control. They became easier to pet and easily had their food. There were no signs of agitation or excitement. This stage lasted for 5-6 hours. At 400 mg/kg dose, there was a complete flaccid paralysis that lasted for 7 hours. At higher doses there was longer duration of action. Even at these higher doses respiration and heart beat were normal and the monkeys recovered almost completely without any immediate or later ill effects (Berger, 1954).

Dogs exposed to high dose of meprobamate for a long duration (sub-acute, 60-75 days), did not show any hematological abnormality or abnormalities in kidney function tests. Autopsy reports also did not show ill effects in viscera of kidney, liver, stomach, small intestine, bladder or adrenal glands. Mice exposed to very high doses for a very long time period (chronic, 12-15 months) also did not show signs of toxicity, except for decreased weight gain (Berger, 1954).

Meprobamate was also effective in preventing pentylenetetrazole, strychnine and electroshock mediated convulsions and death in mice (Berger, 1954).

A search for separation of muscle relaxant properties of meprobamate with its tranquilizing properties led to invention of N-isopropyl-2-methyl-npropyl-1,3-propanediol dicarbamate or carisoprodol which differed from meprobamate in having an iso-propyl group in place of hydrogen atom in one of the carbonyl nitrogens (Berger 1959). Although minor, the difference in structure produced surprisingly different pharmacological and functional properties in carisoprodol.

In electro encephalic recordings in cats and rabbits, meprobamate did not have any effect in brain wave patterns (Gangloff, 1959). Carisoprodol in similar doses, produced an increase in amplitude and a decrease in frequency in the cortical and sub cortical regions (Berger *et al.*, 1960). The characteristic spindling of electro encephalic recordings of barbiturates was also missing with that of both carisoprodol and meprobamate. Larger doses of carisoprodol caused a progressive slowing of frequency and an increase in amplitude until 40-60mg/kg dose. Still higher doses caused greatly reduced activity and longer stretches of electric silence (Berger *et al.*, 1960). A remarkable property of carisoprodol, at lower doses (5 – 10 mg/kg), as we see above is the changes in the brain wave pattern, unaccompanied by

behavioral changes. In this respect carisoprodol resembled atropine, the anti-cholinergic agent, which also made changes in the brain wave pattern unaccompanied by behavioral changes (Longo, 1956; Bradley *et al.*, 1957). However at higher doses, while carisoprodol caused muscle relaxation, paralysis and a generalized central nervous system depression, atropine lead to an excitement. On account of the above similarity, when carisoprodol was explored for peripheral anticholinergic actions, it was found to have none. Convulsions by convulsive discharges set up in limbic system by electric stimulation, were prevented by meprobamate, mephenesin and barbiturates, but not by carisoprodol. This was a strange finding considering more similarity in the structure of meprobamate and carisoprodol compared to meprobamate and mephenesin. This lack of action of carisoprodol in the limbic system fits with clinical finding of lack of tranquilising action with carisorpodol (Berger *et al.*, 1960).

Felbamate

Felabamte (2-phenyl-1,3-propanediol dicarbamate) was prescribed as an anti-epileptic drug prescribed in adult (Absence seizure), and childhood epilepsy (Lenaux-Gastaut syndrome). However, fatal cases of aplastic anemia and hepatotoxicity has precluded its use as an anti-epileptic agent. Thus its use has been confined to refractory cased of epilepsy and

Lenaux-gastaut syndrome. However recent clinical trials have shown it to be safe, well tolerated and effective in various types of epilepsy syndromes (Shah *et al.*, 2016).

Felbamate has been known to act via activation of GABA_A receptor and inhibition of NMDA receptors. The dual mechanism of action has been responsible for the broad spectrum anti-epileptic action (Rho *et al.*, 1994). Meprobamate has also been known to have similar action on GABA_A and NMDA receptors. But while meprobamate both directly gate and allosterically modulate GABA_A receptors, felbamate has just direct gating action on GABA_A receptor (Rho *et al.*, 1997). Clinically, while felbamate is mildly sedating, broad spectrum antiepileptic agent, meprobamate has been shown to have a strong tranquilizing, sedating but weak anti-epileptic effect (Rho *et al.*, 1997). A detailed subunit dependent effect of felbamate may explain the molecular basis for the differences in the clinical effects of the two drugs.

Most severe side effects of felbamate include aplastic anaemia and severe hepatotoxicity. Others include nausea, vomiting, insomnia, anorexia, dizziness and headache.

Pharmacology and Metabolism of Meprobamate

Meprobamate is used either alone or in combination with other common over-the-counter drugs like, diphenhydramine, acetylsalicylic acid or codeine. Meprobamate pharmacokinetics are unaffected by these commonly used combinations. However, diphenhydramine metabolism is affected by meprobamate. Bioavailability is similar for oral or rectal application (Gilbert *et al.*, 1984).

After oral ingestion of a therapeutic dose, most of meprobamate is absorbed via GI tract and peak plasma levels are reached within 1 hour to 3 hours. Less than 10 % of

meprobamate was found in the feces (showing maximum absorption via GI tract). In studies using carbon labelled meprobamate, meprobamate started appearing in the urine within 30 minutes and by 24 hours most of it was excreted from the body (Walkeinstein et al 1958). Protein binding of meprobamate is negligibly small (14%–24%) and the volume of distribution (Vd) was reported to be 0.70 L/kg and is not significantly altered in overdose (Verpooten *et al.*, 1982). 50% of amount of drug is unchanged meprobamate. Meprobamate is primarily metabolized to inactive metabolites by the liver, primary metabolite being hydroxyl-meprobamate and 10%–20% of the parent compound is excreted by the kidneys (Bismuth *et al.*, 1985). Elimination can be described by first-order pharmacokinetics and reported elimination half-lives range between 6.4 hours and 16.6 hours in therapeutic dose and were similar in overdose (Hollister *et al.*, 1964; Maddock *et al.*, 1967).

Meprobamate Side effects, Abuse and Withdrawal

Meprobamate was the first block-buster drug, selling billions of pills. It was supposed to be free of toxic side effects, abuse and dependence potential. A common side effect of meprobamate associated with regular use is allergic skin rashes, which appear in the form of urticaria or erythema. They have been found in as high as 1-2% of patients taking the drug (Hollister, 1957). Allergic rashes respond to drug withdrawal or concomitant use of anti-histaminic medication. More severe form of skin reactions, like pustular skin lesions and purpura, have also been reported (Friedman *et al.*, 1956; Holoubek *et al.*, 1957). These promptly respond to discontinuation of the drug and corticosteroid therapy (Stroud, 1957). Other common side effects associated with meprobamate use include nausea, dizziness, drowsiness and fainting spells especially found when initial dose is more than 400 mg. An alcoholic patient with long term use of high dose of meprobamate showed a grand mal epilepsy

upon withdrawal (Pennington, 1957). A case of death with aplastic anemia has also been reported with meprobamate use (Meyer *et al.*, 1957).

Meprobamate was initially advertised as a non-addictive drug. Its good tranquilizing effect along with relatively good safety profile prompted it to be sold without a prescription. But many cases characteristic of a typical habit-forming drug were reported in the initial few years of use of the drug. Addiction or a habit formation to a drug, has the following characteristics: (1) an intense craving for the drug based on its euphoric effects, (2) tolerance build up, requiring increasingly larger doses to produce the same effect, and (3) withdrawal symptoms associated with sudden discontinuation of the drug (Lemere, 1956). An example include a patient with a history of alcohol and barbiturate abuse and suffering excessive anxiety at work, was prescribed meprobamate 400 mg 3 times a day. He had a good response, but had to consume increasing doses of the drug to sustain the same response. Sometimes it was 50-70 tablets a day. An attempt at quitting led to anxiety and tremor and a grand mal seizure after 20 hours. Upon admission he was stuporous, confused and anxious and suffered another grand mal seizure. He recovered after gradual weaning of the drug (Mohr *et al.*, 1958). In another study, patients did not show dependence (physical or psychic) or withdrawal even after prolonged use of clinically effective dose (Boyd *et al.*, 1958). So, other factors have been associated with the development of meprobamate addiction and dependence, which include amount of drug intake, previous history of drug abuse and personality of the patient. At the peak of its use, meprobamate had been implicated in 7% of self-poisoning cases (Allen *et al.*, 1977).

Commonly known side effects of a meprobamate overdose include central nervous system (CNS) depression, weakness, clonus and hyperactive reflexes, tachycardia, hypotension and respiratory depression. Meprobamate also produces CNS-depressing

symptoms (Bramness *et al.*, 2005). Meprobamate intoxication is often serious and sometimes fatal (5%), resulting from hemodynamic disturbance and circulatory collapse, secondary to severe acute cardiac failure (Charron *et al.*, 2005). In a case report, Lhoste *et al.*, 1977, suggested a predominant cardiogenic mechanism responsible for such hemodynamic failure. The mechanism of cardiac toxicity is unknown. Patients with meprobamate poisoning were shown to have a low cardiogenic index, ejection fraction, high right atrial pressure and pulmonary capillary wedge pressure, showing a cardiogenic shock and thus a direct cardiac effect (Lhoste *et al.*, 1977; Charron *et al.*, 2005). In a case of fatal meprobamate poisoning, maximum meprobamate accumulation was found in heart, showing a possible direct cardio toxic effect (Kintz *et al.*, 1988). Also, inotropic drugs have been useful in such cases, showing presence of a cardiac depression (Lhoste *et al.*, 1977; Taboulet *et al.*, 1994). A related compound, felbamate, is protective in cerebral ischemia and hypoxia (Wallis *et al.*, 1992) via a vasodilatory mechanism. Meprobamate, having a similar structure, may share some of this vasodilatory effect. In the case report of Lhoste *et al.* 1977, a patient with severe meprobamate poisoning had decreased peripheral resistance, unlikely in an isolated cardiogenic shock. Thus, meprobamate-mediated hypotension is due to dual mechanism of direct cardiac depression and peripheral vasodilatory effect.

An overdose of meprobamate also leads to an altered consciousness which corresponds to its plasma concentrations. Below 5 mg/100 ml, patients have been found to be awake. A non-serious coma occurs between 6-12 mg/100 ml and a deep coma is induced at concentrations greater than 10 mg/100 ml (Maddock *et al.*, 1967).

Recently, acute pancreatitis has also been reported as a possible effect of meprobamate poisoning (Fathallah *et al.*, 2011). Possible mechanism is stasis due to hypovolemia leading to increased viscosity and thrombosis or a direct cellular toxicity (Underwood *et al.*, 1993).

GABAA receptor

GABA is the primary inhibitory neurotransmitter in the CNS (Bormann, 2000) with glycine, being the primary inhibitory neurotransmitter in the spinal cord (Betz *et al.*, 1988; Moss *et al.*, 2001). Primary excitatory neurotransmitter in the CNS is the glutamate (Meldrum, 2000). GABA is synthesized from glutamate in a one step process by enzyme, L-glutamic acid decarboxylase (GAD). Thus along with itself working as a neurotransmitter and a co-factor for folic acid and hormone TSH (Thyrotropin releasing hormone), glutamate is used for the synthesis of GABA (Erlander *et al.*, 1991)(figure 1).

GABA receptors are of 3 types: GABA_A, GABA_B and GABA_C. Considering similar functional nature of GABA_C receptor as GABA_A, they have been nominated as a part of GABA_A receptor (Barnard *et al.*, 1998). But there has been an accumulating evidence of a distinct structure, function, pharmacology and genetics which makes the case of a separate nomenclature (Bormann, 2000).

GABA interacts with GABA_A receptors resulting in an opening of chloride channel leading to depolarization and inhibitory post synaptic potential (IPSP). In some cases GABA can be excitatory in nature. Example, in developing neurons, with high intracellular chloride concentrations, a GABA interaction leads to an outward chloride flow and generation of an excitatory post synaptic potential (EPSP) (Rivera *et al.*, 1999). This phenomenon has a profound effect on survival and differentiation during neuronal development (Ben-Ari *et al.*, 1997; Belhage *et al.*, 1998).

Figure 2: Features of a GABA Synapse.

GABA_A receptors are members of cys-loop ligand-gated ion channels. Cys-loop is a characteristic loop formed by a disulfide bond between two cysteine (Cys) residues which are 13 highly conserved amino acids apart near the N-terminal extracellular domain of the alpha subunit. Common members of this group are nicotinic acetylcholine receptors, Zn⁺⁺ receptors, type 3 serotonin (5HT₃) receptors, glycine and GABA_C receptors (Schofield *et al.*, 1987; Connolly *et al.*, 2004). GABA_A receptors are sensitive to bicuculline (Bormann, 1988; Bormann, 2000) GABA_A receptors are sites for many ligands like benzodiazepines, barbiturates, alcohol, anesthetics and muscle relaxants like carisoprodol and meprobamate (Akaike *et al.*, 1985; Orser *et al.*, 1994; Sigel *et al.*, 1997; Kumar *et al.*, 2015). GABA_B receptors are metabotropic G protein-coupled receptors sensitive to baclofen (Bormann, 1988; Bowery, 1989; Marshall *et al.*, 1999). They mediate their effect via signal transduction pathways (Couve *et al.*, 2000). Located on the presynaptic membrane, they inhibit the release of inhibitory GABA in synaptic terminal. So overall having an excitatory effect. GABA_B as a therapeutic target has been less exploited (Bowery, 1989; Marshall *et al.*, 1999). A different type of receptor not sensitive to bicuculline or baclofen, previously known as GABA_C receptors (Johnston, 1996), are sensitive to GABA and the GABA analogue, aminocaproic acid. They are also ligand-gated ion channel, composed of ρ -subunits, with the opening of chloride channel on activation by GABA (Bormann *et al.*, 1995; Johnston, 1996). However, they are more sensitive and have a very weak desensitization. GABA_C receptors are normally located in the bipolar cells of retina (Shimada *et al.*, 1992). As stated earlier, GABA_C has unique pharmacology compared to GABA_A receptor. It is insensitive to bicuculline and baclofen (Johnston, 1996). However $\rho 1$ -homomeric GABA_C receptors were inhibited by picrotoxin like

GABA_A, while the ρ 2-homomeric or ρ 1 ρ 2 were not inhibited by picrotoxin (Cutting *et al.*, 1991; Enz *et al.*, 1998; Feigenspan *et al.*, 1998; Enz *et al.*, 1999).

Structure of GABA_A receptor and subunits classes and roles

GABA_A receptor is a member of cys-loop ligand gated ion channels. Much has been learned about the structure of pentameric LGICs by solving of crystal structure of acetylcholine binding protein (AChBP) (Brejc *et al.*, 2001). Recently structure of GABA_A receptor itself has been solved (Miller *et al.*, 2014). Cys loop ligand-gated ion channels are pentameric in nature, i.e., each ion channel consists of 5 subunits (Toyoshima *et al.*, 1990; Nayeem *et al.*, 1994). Each subunit consists of long extracellular N terminal domain, a short extracellular c terminal domain, four trans-membrane (TM1-TM4) domains and connecting loops. Connecting loops are short intracellular TM1-TM2 and a long intracellular TM3-TM4. There is also a short extra-cellular TM2-TM3 connecting loop (Schofield *et al.*, 1987; Unwin, 1993). Five of these subunits come together to form pentameric ion channel. The TM2 domain of all the five subunit form the pore of the channel (Unwin, 1993; Xu *et al.*, 1996). Extracellular TM2-TM3 play an important part in channel gating, i.e., carrying information after binding of ligand, leading to channel opening (Miyazawa *et al.*, 2003; Thompson *et al.*, 2010).

Above receptors were isolated using benzodiazepine affinity chromatography leading to partial sequencing and cloning of two receptor genes. cDNAs for the two polypeptides formed receptors with GABA activated current in *Xenopus* oocytes and these were designated as α and β (Mohler *et al.*, 1980; Schoch *et al.*, 1985; Schofield *et al.*, 1987; Sieghart, 1995).

These were used as probes to identify other subunits. Those with maximum homology (70-80%) were assigned the same Greek letter and those with less homology (15-40%) were

assigned different Greek letter. Thus so far the following GABA_A receptor subunits have been identified. α 1-6, β 1-3, γ 1-3, δ , θ , ϵ , π and ρ 1-3 (Lynch *et al.*, 1995; Lynch *et al.*, 1997; Siegwart *et al.*, 2002). Some splice variants have also been identified. For example, the γ 2 has a short and a long form differing in the 8 amino acid insert in the intracellular (Whiting *et al.*, 1990). Similarly a short and a long form splice variant have also been identified for β 2-subunit (McKinley *et al.*, 1995) .

So the above subunits assemble together to form hetero-pentameric, chloride permeable channel. Out of the many possibilities of combinations, most common GABA_A receptor expressed in brain is 2 α , 2 β and one γ stoichiometry with the γ subunit, being able to be replaced by δ , θ , π or ϵ subunit (Im *et al.*, 1995; Chang *et al.*, 1996; Tretter *et al.*, 1997; Baumann *et al.*, 2001; Olsen *et al.*, 2008). Out of all possible combinations, α 1 β 2 γ 2 is most abundant with approximately 60 % of the total GABA_A receptor population. Distribution of other combinations are α 2 β 3 γ 2, 15-20%; α 3 β n γ 2, 10-15%; α 4 β n γ 2 or α 4 β n δ , 5%; α 5 β 2 γ 2, < 5%; α 6 β 2/3 γ 2, < 5% (Fritschy *et al.*, 1995). Some GABA_A receptor have two different α -subunits. In recombinant receptors, the α -subunit adjacent to the γ 2-subunit determines the sensitivity to the BZDs (Benke *et al.*, 2004). Binding of GABA occurs at the interface of the α and β -subunits (Baumann *et al.*, 2003) while benzodiazepines bind at the interface of α and γ -subunits (Sigel *et al.*, 1997), leading to influx of chloride ions and generation of IPSP.

Functional roles of various subunits have been assessed using histidine to arginine point mutations at a conserved residue. In α 1, α 2, α 3 or α 5 subunit, this mutation abolish the binding of diazepam, whereas action of GABA is preserved (Rudolph *et al.*, 1999; McKernan *et al.*, 2000; Crestani *et al.*, 2002; van Rijnsoever *et al.*, 2004). The positions are, α 1H101R, α 2H101R, α 3H126R, α 5H105R.

From the above mutation, it was assessed that $\alpha 1$ subunit-containing GABAA receptors mediate sedative, partly anti-convulsant and anterograde amnesic action (Rudolph *et al.*, 1999); $\alpha 2$ subunit containing GABAA receptors mediate anxiolytic and myorelaxant actions (large portion of it) (Low *et al.*, 2000; Crestani *et al.*, 2001); rest of myorelaxant action is mediated by $\alpha 3$ and $\alpha 5$ subunit containing GABA_A receptors (Low *et al.*, 2000; Crestani *et al.*, 2001; Crestani *et al.*, 2002); $\alpha 5$ -containing GABA_A receptor further mediate tolerance to sedative action (van Rijnsoever *et al.*, 2004); and addictive property $\alpha 1$ -containing GABA_A receptors (Tan *et al.*, 2010).

β -subunit also has important implications in health and disease. $\beta 3$ subunit has been associated with schizophrenia (Huang *et al.*, 2014), genetic epilepsies (Macdonald *et al.*, 2010), autism (Delahanty *et al.*, 2011), cleft palate (Inoue *et al.*, 2008) as well as substance abuse (Chen *et al.*, 2014). Angelman syndrome, associated with mental disorders and developmental delay and gait ataxia, is associated with the deletion of GABA_A receptor $\beta 3$ subunit (Williams *et al.*, 1995; DeLorey *et al.*, 1998). In PTSD, a heterogeneity of $\beta 3$ GABA_A receptor is associated with higher degree of anxiety insomnia and social dysfunction than a homozygosity (Feusner *et al.*, 2001).

Various modes of GABAA receptor activation

GABA_A receptor synaptic communication transmits a pre-synaptic signal. Depolarization following an action potential, lead to a calcium influx. Calcium causes synaptic vesicles to fuse with the presynaptic membrane. Each vesicle release thousands of GABA molecules, leading to a concentration in milli-molar range in the synaptic cleft. Some post-synaptic receptors exposed to GABA, have synchronous activation, leading to a phasic current (Edwards *et al.*, 1990; Mody *et al.*, 1994; Nusser *et al.*, 1997; Brickley *et al.*, 1999). Synaptic

GABA_A receptors that mediate phasic currents are made up of mostly $\alpha 1$ -3, β and γ subunits (Rudolph *et al.*, 2004). Phasic currents are characterized by fast activation, fast desensitization and fast deactivation. On the other hand there is a tonic current mediated by highly sensitive extrasynaptic-GABA_A receptors, characterized by slow desensitization which can also open spontaneously in absence of GABA (Brown *et al.*, 2002; Tang *et al.*, 2010; Wlodarczyk *et al.*, 2013). Extrasynaptic GABA_A receptors are δ -containing (with mostly $\alpha 1$, $\alpha 4$ or $\alpha 6$) or γ subunit with $\alpha 5$ (Fritschy *et al.*, 1998; Brunig *et al.*, 2002). δ subunit expressing receptors are characterized by many remarkable properties. As stated earlier, they generate a tonic current which are important component of CNS inhibition (Maguire *et al.*, 2009; Bonin *et al.*, 2011; Sarkar *et al.*, 2011). They regulate behaviors like memory and anxiety (Whissell *et al.*, 2013; Cushman *et al.*, 2014; Lee *et al.*, 2014; Paydar *et al.*, 2014). They have a remarkable property of different expression levels under different physiological and pathological conditions. Neurosteroids mediate a stress-induced increase in expression of $\alpha 4\beta\delta$ GABA_A receptors (Maguire *et al.*, 2005; Shen *et al.*, 2005). Since these receptors constrain memory, their upregulation in hippocampus may lead to stress-induced memory impairment. During pregnancy, stimulation by steroid hormones cause upregulation of δ subunit (Smith *et al.*, 2006; Maguire *et al.*, 2008). A partial or complete rebound following pregnancy may be responsible for post-partum depression and abnormal maternal behavior which responds to pharmacologically increasing delta activity. Expression of δ subunit and $\alpha 4$ subunit is reduced in pre-frontal cortex (of suicide victims) in depression (Merali *et al.*, 2004). δ -Expression is reduced in schizophrenia (Hashimoto *et al.*, 2008b; Hashimoto *et al.*, 2008a; Maldonado-Aviles *et al.*, 2009) and an upregulation is observed in cases of stroke and traumatic brain injury (Clarkson *et al.*, 2010; Mtchedlishvili *et al.*, 2010; Kharlamov *et al.*, 2011). An expression of δ subunit in various brain regions and its functional role is stated in figure 4. So δ subunit expressing receptors have important physiological and pathological implications.

Figure 3: Representation of GABAA receptors. **A**, GABAA receptor subunit consists of long N-terminal domain, short C-terminal domain, 4 transmembrane (TM1-TM4) domain and long intracellular TM3-TM4 loop. **B**, 5 of the subunits assemble to form a pentameric cys loop ligand gated ion channel with GABA binding site between α and β -subunit and benzodiazepine binding site between α and γ -subunit. Note, in recombinant receptors, the α -subunit adjacent to the γ -subunit determine the sensitivity to the benzodiazepines. **C**, synaptic and extra-synaptic GABAA receptors composed of $\alpha 1-3$, β , γ and $\alpha 4-6$, β , δ respectively. $\alpha 5 \beta \gamma$ also form extrasynaptic GABAA receptors.

Figure 4: Delta GABAA expression and function in CNS. Red show a high expression level while orange a medium and yellow low level of expression. Probable functions are stated in boxes.

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Pharmacology of GABAA receptors

Ligands have various modes of action on GABA_A receptors. These include direct activation, allosteric modulation, inverse agonism and antagonism. Direct activation involves activation of receptor in the absence of GABA via the GABA site or a different site. Common ligands capable of direct activation are barbiturates, common tranquilizers and muscle relaxants like carisoprodol (Kumar *et al.*, 2015) and meprobamate (Kumar *et al.* 2015, accepted pending revision). Ligands capable of direct activation at all the α subunits are commonly associated with fatal complications (Rho *et al.*, 1997; Fass, 2010). Allosteric modulation is effect of a ligand on GABA mediated current via a different binding site. Common ligands capable of allosteric modulation are benzodiazepines and barbiturates (Hunkeler *et al.*, 1981). Barbiturates, carisoprodol and meprobamate are capable of both direct action and allosteric modulation whereas benzodiazepines do not have a direct gating effect at GABA_A receptors. An inverse agonist is a ligand having an effect opposite to that of an agonist (Braestrup *et al.*, 1982; Bonetti *et al.*, 1988). Antagonists do not have effects on their own on the receptor but block current by both the agonists and the inverse agonists. Common antagonists at GABA_A receptors are picrotoxin and bicuculline (Bormann, 2000). Common antagonist at the benzodiazepine site on GABA_A receptor is flumazenil (Hunkeler *et al.*, 1981).

Aims of the study

So, the main aim of this study is to understand a detailed mechanism of action of meprobamate on the various subunits of GABA_A receptors. To understand whether meprobamate has a direct gating, allosteric modulatory or inhibitory effects on GABA_A receptors. Meprobamate, though earlier was widely prescribed and later controlled, is the major metabolite of currently widely prescribed muscle relaxant, carisoprodol. Carisoprodol has direct gating, allosteric modulatory and inhibitory actions on GABA_A receptor subunits. We wanted to test the hypothesis that meprobamate also has similar actions on GABA_A receptors and the difference attributed to the difference in the structure of the two ligands. Carisoprodol has an isopropyl group that is absent in meprobamate.

Mechanism of action of meprobamate, known so far, is its action on GABA_A receptor in a barbiturate like manner. So, we also wanted to test how similar and different is the action of meprobamate to barbiturates, represented in our studies by pentobarbital.

We further set out to explore the specific amino acids and domains responsible for the various modes of action of meprobamate on GABA_A receptors. To summarize, the aims of study are as follows:

AIM 1: To determine the degree to which the lack of the isopropyl group may alter potency, efficacy and subunit preferring effect of meprobamate compared to CSP

AIM 2: To determine the extent to which actions overlap between meprobamate and barbiturate pentobarb

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CHAPTER II

ASSESSMENT OF DIRECT GATING AND ALLOSTERIC MODULATORY EFFECTS OF
MEPROBAMATE
IN RECOMBINANT GABA_A RECEPTORS

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Abstract

Meprobamate is a schedule IV anxiolytic and the primary metabolite of the muscle relaxant carisoprodol. Meprobamate modulates GABA_A (γ -aminobutyric acid type A) receptors, and has barbiturate-like activity. To gain insight into its actions, we have conducted a series of studies using recombinant GABA_A receptors. In $\alpha x \beta z \gamma 2$ GABA_A receptors (where $x = 1-6$ and $z = 1-3$), the ability to enhance GABA-mediated current was evident for all α subunit isoforms, with the largest effect observed in $\alpha 5$ -expressing receptors. Direct gating was present with all α subunits, although attenuated in $\alpha 3$ -expressing receptors. Allosteric and direct effects were comparable in $\alpha 1 \beta 1 \gamma 2$ and $\alpha 1 \beta 2 \gamma 2$ receptors, whereas allosteric effects were enhanced in $\alpha 1 \beta 2$ compared to $\alpha 1 \beta 2 \gamma 2$ receptors. In “extrasynaptic” ($\alpha 1 \beta 3 \delta$ and $\alpha 4 \beta 3 \delta$) receptors, meprobamate enhanced EC₂₀ and saturating GABA currents, and directly activated these receptors. The barbiturate antagonist bemegride attenuated direct effects of meprobamate. Whereas pentobarbital directly gated homomeric $\beta 3$ receptors, meprobamate did not, and instead blocked the spontaneously open current present in these receptors. In wild type homomeric $\rho 1$ receptors, pentobarbital and meprobamate were ineffective in direct gating; a mutation known to confer sensitivity to pentobarbital did not confer sensitivity to meprobamate. Our results provide insight into the actions of meprobamate and parent therapeutic agents such as carisoprodol. Whereas, in general, actions of meprobamate were comparable to those of carisoprodol, differential effects of meprobamate at some receptor subtypes suggest potential advantages of meprobamate may be exploited. A re-assessment of previously synthesized meprobamate-related carbamate molecules for myorelaxant and other therapeutic indications is warranted.

Key words: GABA_A receptor; meprobamate; carisoprodol; muscle relaxant; drug abuse

1. Introduction

Meprobamate, a propanediol carbamate, was the first drug to be used as an anxiolytic agent, and was also prescribed as an anticonvulsant and sedative/hypnotic (Hendley et al., 1954). By the late 1950s, meprobamate was the most widely prescribed drug in the United States and in many other countries. It retained this position until diazepam the benzodiazepine diazepam was introduced into clinical use in late 1960 (Berger, 1964; Greenblatt *et al.*, 1974). Within two years of introduction of meprobamate, cases of its abuse and withdrawal after long term use were reported (Ewing *et al.*, 1957), and it was relatively soon thereafter listed as a controlled substance.

While meprobamate itself is no longer widely used, drugs metabolized to meprobamate are widely available, and misuse of these drugs is associated with serious side effects. One such drug is the centrally acting skeletal muscle relaxant carisoprodol, which remains highly prescribed for low back pain (Toth *et al.*, 2004; Sullivan, 2015). Use of carisoprodol is associated with abuse and dependence, and related dangers such as psychomotor impairment (Elder, 1991; Rust et al., 1993; Zacny et al., 2011, 2012). Dependence and severe withdrawal may lead to seizures and death (Adams *et al.*, 1975; Elder, 1991; Littrell *et al.*, 1993; Rust *et al.*, 1993; Reeves *et al.*, 2003; Zacny *et al.*, 2011; Zacny *et al.*, 2012). Although carisoprodol has its own independent effects (Gonzalez et al., 2009b), many of its therapeutic and abuse-dependent effects are likely due to its metabolite meprobamate, which differs from carisoprodol by the absence of an isopropyl group (Fig. 1). Indeed, the half-life of meprobamate far exceeds that of carisoprodol ($t_{1/2}$ for carisoprodol of 1-2 hours (Olsen et al., 1994) and $t_{1/2}$ for meprobamate between 6.4 hours and 16.6 hours (Hollister *et al.*, 1964; Maddock *et al.*, 1967).

Meprobamate has been shown to have barbiturate-like activity at neuronal GABA_A receptors (Rho et al., 1997), the predominant inhibitory neurotransmitter receptor in the central nervous system and the target of many therapeutics. Carisoprodol has similarly been suggested to have barbiturate-like actions (Gonzalez et al., 2009a). A detailed understanding of meprobamate's interaction with GABA_A receptors is lacking, however. For instance, the extent to which its

interaction with GABA_A receptors may be subunit-dependent is unknown. As the extensive array of GABA_A receptor configurations that exist throughout the CNS contribute to specific physiological and pharmacological responses of GABA and modulatory agents (Olsen *et al.*, 2008), an understanding of meprobamate's subunit-dependent actions could prove enlightening regarding therapeutic and adverse effects of both it and parent therapeutics that are metabolized to meprobamate, such as carisoprodol. We have thus assessed the potential subunit-dependent interaction of meprobamate at these receptors and have further explored potential commonality and differences of action with barbiturates.

2. Materials and Methods

2.1 Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells, stably or transiently expressing varying GABA_A receptor subunits, were used in the present study. For transient expression, cells were transfected with human GABA_A α 1- α 6; human β 1-2; and human γ 2s (short isoform) cDNA in a 1:1:5 ratio (2 μ g total cDNA) using PolyJetTM in vitro transfection reagent (SignaGen Laboratories, Jamesville, MD). The γ 2s subunit will be referred to as γ 2 from here forward. For studies assessing meprobamate effects in a model of extrasynaptic receptors (rat α 1 β 3 δ and α 4 β 3 δ subunits), a total of 3 μ g of cDNA in a transfection ratio of 2:1:0.25 for α : β : δ plasmids was used (Wagoner *et al.*, 2010). For studies on wild type β 3 homomeric receptors, wild type ρ 1 subunits, and barbiturate-sensitive ρ 1 (W328M) subunits (generated previously in our lab, Gonzalez et al, 2009b), 2 μ g of cDNA was transfected. The rat GABA_A α 4 subunit cDNA was purchased from Genescript (Piscataway, New Jersey). Human GABA_A α 1 subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). The wild-type human GABA ρ 1 subunit was generously provided by David Weiss (University of Texas Health Science Center at San Antonio). HEK293 cells stably expressing human α 2 β 2 γ 2 receptors were also used. A complete description of the preparation and maintenance of these stable cell lines has been published previously (Hawkinson *et al.*, 1996). Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes, and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂. In all cases, cells were used for recording 24-72 hr after transfection.

2.2 Electrophysiology

Whole-cell patch clamp electrophysiology was used to assess GABA-, meprobamate-, or pentobarbital-activated Cl⁻ currents. All electrophysiology experiments were conducted at room

temperature (22 - 25 °C) 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 4–6M Ω . Patch pipettes were filled with a solution consisting of 140 mM CsCl, 10 mM EGTA-Na⁺, 10 mM HEPES-Na⁺, and 4 mM Mg²⁺-ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl₂, 5.5 mM KCl, 0.8 mM MgCl₂, and 10 mM glucose, pH 7.3. Agonist-induced Cl⁻ currents were obtained with an Axopatch 200B amplifier (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 (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

2.3 Chemicals and solutions

Meprobamate, carisoprodol, pentobarbital, diazepam, THIP (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol), salts and buffers were purchased from Sigma Aldrich (St. Louis, MO). GABA was obtained from Acros Organics (New Jersey, US). Bemegride was obtained from Pfaltz & Bauer, Inc. (Waterbury, CT). Stock solutions of meprobamate, pentobarbital and carisoprodol were made using dimethylsulfoxide (DMSO). Drugs were diluted in normal saline, so that the final DMSO concentration (vol/vol) of the test solutions was $\leq 0.3\%$. GABA, diazepam and bemegride stock solutions were prepared using de-ionized H₂O. All stock solutions were stored at -20 °C. On experimental days, drug-containing solutions were prepared from stock by serial dilution into external solution.

2.4 Experimental Protocol

GABA (with or without modulatory ligand), meprobamate, carisoprodol or pentobarbital were prepared in external solution on the day of use and applied via a 16-barrel rapid perfusion system in which all barrels (200 μ m outer diameter quartz tubes; ALA Scientific, Folsom, CA) emptied via a common tip positioned adjacent to the cell under study. Flow through each barrel was pressure fed and regulated by solenoid or pinch valves operated by a programmable microprocessor-based controller. Only one valve was open at a time, and the buffer solution was applied continuously between drug applications via gravity or positive pressure. As per the manufacturer specifications, solution flow rate as configured in the present studies is approximately 3 μ l/second, and exchange rate is approximately 5 msec. For some experiments, ligands were applied via gravity flow using a Y-tube placed adjacent to the cell.

The modulatory effects of meprobamate on GABA-gated currents were assessed using an EC₂₀ gating concentration of GABA as the control. This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of meprobamate. To ensure the gating concentration was approximately an EC₂₀, control responses were compared to the maximal GABA-gated current for each individual cell. On a cell by cell basis, control GABA currents were deemed acceptable for assessment of allosteric modulatory effects of meprobamate if they were within a 15-25 % range of maximal current for that particular cell. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than $\pm$ 10%. In our analyses of the modulatory effects of meprobamate, peak current amplitude was defined as the maximum current elicited by meprobamate. For studies investigating meprobamate-mediated currents, meprobamate was dissolved in external solution and applied in the manner described above. In all studies assessing direct gating effects of meprobamate, the magnitude of the response was expressed relative to the maximal effect of GABA (designated 100%). In studies on β 3 homomeric receptors, the magnitude of meprobamate-mediated block of spontaneously open channels was measured relative to the maximal blocking effect of picrotoxin

(designated 100%). For $\alpha\beta\gamma$ configurations, GABA-gated control currents were recorded in the presence of diazepam to confirm incorporation of the $\gamma 2$ subunit. Presence of the δ subunit in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors was confirmed by loss of inhibition to 1 μM Zn^{2+} .

2.5 Data Analysis

To ensure equipotent concentrations were used for gating, GABA concentration-response data were collected for all synaptic and extrasynaptic GABA_A receptors tested, (Table 1). From these data, EC_{20} and saturating GABA concentrations were calculated for each configuration and used in subsequent investigations of the allosteric and direct effects of meprobamate, respectively. For studies assessing allosteric actions of meprobamate, a correction was applied to subtract underlying direct gating effects, which were present at higher meprobamate concentrations. All data are presented as mean $\pm$ S.E.M. For experiments in which three or more datasets were statistically analyzed, one-way analysis of variance was conducted, followed by a Tukey-Kramer post hoc test as indicated. For those experiments in which two datasets were analyzed, Student's t-tests (paired or unpaired) were conducted. In all cases, statistical significance was designated at a p value of 0.05 or less.

3. Results

3.1 α subunit isoform influence on direct and allosteric actions of meprobamate

Direct effects of meprobamate were assessed in $\alpha x \beta 2 \gamma 2$ GABA_A receptors, where $x = \alpha$ subunit 1-6. We initiated direct gating studies using concentrations of meprobamate as low as 100 μ M; as direct gating effects were not generally observed below 1 mM, we report here direct gating effects of 1, 3 and 10 mM meprobamate. Meprobamate directly gated each of the configurations tested at high concentrations, evoking inward currents in the absence of GABA (Fig. 2). The relatively low apparent affinity of meprobamate combined with limits on its solubility precluded definitive calculation of maximal meprobamate-gated current. At 10 mM, meprobamate-gated current was of similar magnitude in $\alpha 1$ -, $\alpha 2$ -, $\alpha 4$ -, $\alpha 5$ - and $\alpha 6 \beta 2 \gamma 2$ s GABA_A receptors (20-36% of maximal GABA current), and significantly less (7%) in $\alpha 3$ -expressing receptors (Fig. 2).

We also assessed α subunit isoform effects on allosteric modulatory actions of meprobamate. Meprobamate positively modulated the effects of GABA EC₂₀ concentration in all configurations tested (Fig. 3). 3 mM meprobamate on average roughly doubled the amplitude of the GABA EC₂₀ current for most α subunit isoforms. We observed a somewhat enhanced effect in receptors expressing the $\alpha 5$ subunit, as 3 mM meprobamate elicited a potentiating effect of more than 3-fold (Fig. 3).

3.2 β subunit isoform influence on direct and allosteric actions of meprobamate

To assess whether the β subunit isoform affected the actions of meprobamate, we assessed both direct and allosteric effects of it in $\alpha 1 \beta 2 \gamma 2$ and $\alpha 1 \beta 1 \gamma 2$ receptors. We observed no significant difference in the magnitude of current elicited by meprobamate alone in these receptors, as 10 mM meprobamate elicited currents roughly 1/3 the amplitude of peak GABA current in both $\alpha 1 \beta 2 \gamma 2$ and $\alpha 1 \beta 1 \gamma 2$ receptors (Fig. 2C).

With regard to β subunit effects on allosteric actions of meprobamate, we found receptors expressing either $\beta 1$ or $\beta 2$ subunits (in combination with $\alpha 1$ and $\gamma 2$) showed similar allosteric modulation by meprobamate. In both receptors, 3 mM meprobamate effectively doubled the amplitude of GABA-gated current (Fig. 3C).

3.3 γ subunit isoform influence on direct and allosteric actions of meprobamate

Presence of a γ subunit is critical for the allosteric actions of benzodiazepines (Pritchett et al., 1989), but not for direct or allosteric effects of other ligands such as barbiturates (Horne et al., 1993) or neurosteroids (Hosie et al., 2006). To assess the extent to which the γ subunit impacts actions of meprobamate, we tested effects of it in receptors lacking the $\gamma 2$ subunit. In comparison to $\alpha 1\beta 2\gamma 2$ receptor, direct gating in $\alpha 1\beta 2$ receptor was generally comparable. There was a statistically smaller direct gating response at 1 mM meprobamate in $\alpha 1\beta 2$ receptor compared to $\alpha 1\beta 2\gamma 2$ receptor, but this difference was modest and not present at other concentrations tested (Fig. 2D).

The $\gamma 2$ subunit had a more prominent effect on allosteric modulation by meprobamate. Its presence significantly attenuated the ability of meprobamate to allosterically an EC_{20} concentration of GABA at all concentrations of MEP above 100 μM . At 1 and 3 mM MEP, the magnitude of enhancement in $\alpha 1\beta 2$ receptors was more than twice that observed in $\alpha 1\beta 2\gamma 2$ receptors (Fig 3D).

3.4 Effects of meprobamate on δ -subunit containing “extrasynaptic” receptors

GABA and ligands that regulate its receptors may act at synaptic or extrasynaptic sites. To assess the influence of meprobamate extrasynaptically, we studied its effect in recombinant $\alpha 4\beta 3\delta$ and $\alpha 1\beta 3\delta$ receptors, models of extrasynaptic GABA_A receptors. As reported by others (Brown et al., 2002), sensitivity of $\alpha 4\beta 3\delta$ receptors to GABA was high, with an EC_{50} of $0.04 \pm 0.02 \mu M$. As shown in Figure 4, meprobamate directly gated $\alpha 4\beta 3\delta$ receptors with an efficacy comparable to

that of GABA itself. This direct gating effect was concentration-dependent, and saturated at 3 mM meprobamate. We found very similar effects for the ability of meprobamate to directly gate $\alpha 1\beta 3\delta$ receptors. Sensitivity to the direct gating effects in extrasynaptic receptors was comparable to that observed in synaptic receptors. Current gated by meprobamate rapidly decayed following activation, and we noted a prominent rebound effect following termination of ligand application (Fig 4A, arrow). GABA is not a full agonist in δ -expressing receptors (Brown et al., 2002); we observed the same effect, as the agonist THIP elicited a current $151 \pm 12\%$ of that elicited by saturating GABA.

We also assessed the extent to which meprobamate might allosterically potentiate extrasynaptic GABA_A receptors. As illustrated in Fig. 5, meprobamate enhanced GABA-gated (EC₂₀) current, with a threshold effect at 100 μ M, and a maximal effect observed at 1 mM. The sensitivity and magnitude of the potentiating effect is comparable to that observed in synaptic receptors.

Pentobarbital and carisoprodol, the parent molecule to meprobamate, have been reported to enhance maximal GABA-gated current in δ -subunit containing receptor (Feng et al., 2004; Kumar et al., 2015). We thus assessed for this capability with meprobamate. Co-application of 1 mM meprobamate with 1 mM GABA (EC₉₅₊) to $\alpha 4\beta 3\delta$ receptors resulted in a current $183 \pm 17\%$ of that seen with maximal GABA alone. As anticipated, 100 μ M pentobarbital, which was used as a positive control, showed enhancement of maximal GABA-gated current (to $223 \pm 38\%$ of maximal GABA, Fig. 6B). No enhancement was observed in $\alpha 1\beta 3\gamma$ or $\alpha 1\beta 3$ subunit containing receptors with meprobamate or pentobarbital (Fig. 6B).

3.5 Further assessment of the barbiturate-like effects of meprobamate

Based on a mechanistic assessment of its effects in GABA_A receptors recorded from cultured rat hippocampal neurons, others have concluded meprobamate has “barbiturate-like”

activity (Rho et al., 1997). Our results are broadly consistent with that suggestion. We thus conducted additional studies to test further commonality of action between meprobamate and pentobarbital.

Bemegride is known to antagonize pentobarbital stimulus effect in animal models (Schechter, 1984) and phenobarbital direct gating effects in mouse spinal neurons (Mistry *et al.*, 1990). We thus assessed the influence of bemegride on the direct gating effects of meprobamate. As shown in Figure 7, meprobamate-mediated direct gating current was significantly and reversibly attenuated by bemegride. The ability of bemegride to antagonize meprobamate-activate current was greater when tested against a lower concentration of meprobamate, demonstrating a concentration-dependent effect.

$\rho 1$ homomeric GABA receptors are anion-selective cys loop ligand-gated channels, with a pharmacology distinct from that of heteromeric GABA_A receptors (Amin *et al.*, 1994). They are found in very high concentration in retina and other visual areas (Enz *et al.*, 1995; Boue-Grabot *et al.*, 1998). Barbiturates cannot directly activate wild type $\rho 1$ homomeric GABA receptors, but can when tryptophan at position 328 is mutated to methionine (W328M) (Amin, 1999). We assessed whether meprobamate can gate wild type or W328M $\rho 1$ homomeric GABA receptors. In wild type homomeric $\rho 1$ receptors, both pentobarbital and meprobamate were ineffective in direct gating (Fig. 7A, 7B). As originally reported (Amin, 1999), we observed that the W328M mutation in the $\rho 1$ subunit conferred sensitivity to direct gating effects of pentobarbital. In contrast, the W328M mutation did not confer on meprobamate the ability to directly gate the channel (Fig. 7A, 7B).

Homomeric $\beta 3$ receptors do not appear to form natively, but they can form a functional receptor in vitro, and thus are a useful tool for studying drug action (Davies *et al.*, 1997; Chen *et al.*, 2012). Homomeric $\beta 3$ receptors are spontaneously open, and they can be directly activated by barbiturates (Davies et al., 1997). We evaluated whether meprobamate had direct gating effects in these receptors. Meprobamate, in contrast to pentobarbital, could not directly gate homomeric $\beta 3$

receptors. Instead, meprobamate, blocked the spontaneously open current present in homomeric $\beta 3$ receptors (Fig. 7A, 7B). This effect was concentration-dependent, and approached the efficacy of the GABA receptor antagonist picrotoxin in blocking the spontaneously open channel (Fig. 7B).

4. Discussion

This is the first study to assess subunit-dependent effects of meprobamate at its major target, GABA_A receptors. Drugs metabolized to meprobamate, such as carisoprodol, have remained widely utilized. Carisoprodol was actually synthesized as an alternative to meprobamate, and early studies suggested it had a profile that was preferable to meprobamate (Berger et al., 1960). Early metabolism studies in dogs indicated the primary metabolite of carisoprodol was hydroxyl-carisoprodol (Douglas et al., 1962). Toxicology studies some years later demonstrated that in humans, carisoprodol was in fact metabolized primarily to meprobamate, which has a half-life that is several-fold longer than that of carisoprodol (Bramness et al., 2004; Olsen et al., 1994). Whereas the recent scheduling of carisoprodol will likely result in a reduction in its use (Bramness et al., 2012), it remains a key therapeutic for treatment of acute low back pain (Witenko et al., 2014). Even with its recent scheduling, a quick internet search reveals a number of pharmacies noting the ready availability of carisoprodol without a prescription. Thus the potential for continuing abuse of carisoprodol remains. Considering a significant proportion of its effect are likely attributable to its metabolite meprobamate, the focus of the present study was thus to gain greater insight into meprobamate modulation of its major therapeutic target, the GABA_A receptor.

Our assessment of meprobamate's ability to directly gate and allosterically modulate the GABA_A receptor is comparable to that reported in neurons (Rho et al., 1997), in that both effects are of relatively low potency. Rho et al. (1997) further categorized its actions as "barbiturate-like", and carisoprodol also displays barbiturate-like effects both in vivo and in vitro (Gonzalez et al., 2009b). We conducted a number of experiments to better understand the extent to which effects of meprobamate mimic those of the barbiturate pentobarbital. The results demonstrate some commonality of effect between the two ligands, but in general do not support a common site of action for meprobamate and barbiturates. The barbiturate antagonist bemegride reversibly and significantly inhibited meprobamate-mediated direct gating current in a concentration-dependent manner, consistent with a competitive nature of inhibition. However, in homomeric $\rho 1$ receptors,

a mutation (W328M) that conferred sensitivity to pentobarbital (Amin, 1999) did not confer sensitivity to meprobamate. In addition, whereas pentobarbital could directly gate homomeric $\beta 3$ receptors, this effect was not observed with meprobamate. Thus, whereas a number of *in vivo* and *in vitro* actions of meprobamate are in fact similar to those observed with pentobarbital, the comparison cannot be extended to indicate a common binding domain for these ligands. In this regard, meprobamate is comparable to carisoprodol (Gonzalez et al., 2009b). Recent photoaffinity labeling studies have provided considerable evidence that barbiturates bind to inter-subunit interfaces in GABA_A (Chiara et al., 2013) and nicotinic (Hamouda et al., 2014) receptors. With regard to GABA_A receptors, a photoreactive barbiturate binds to a number of residues at both the α - β and γ - β interfaces in the transmembrane domain (Chiara et al., 2013). Our current results suggest it unlikely that meprobamate would interact with those residues.

It has become increasingly clear in recent years that many therapeutic and adverse actions of GABAergic drugs associate predominantly with particular subunits of the GABA_A receptor (Low *et al.*, 2000; Crestani *et al.*, 2001; Crestani *et al.*, 2002; van Rijnsoever *et al.*, 2004; Licata *et al.*, 2008; Tan *et al.*, 2010; Rudolph *et al.*, 2014). Of particular relevance to the current study is the fact that $\alpha 2$ - and $\alpha 3$ expressing receptors are involved in both anxiolytic (Low *et al.*, 2000; Crestani *et al.*, 2001; Licata *et al.*, 2008) and muscle-relaxing (Crestani *et al.*, 2001; Griebel *et al.*, 2003) effects of GABAergic ligands, while abuse potential associates with the $\alpha 1$ subunit (Licata *et al.*, 2008; Tan *et al.*, 2010). Our recent report (Kumar *et al.*, 2015) shed some light on the subunit-dependent effects of carisoprodol itself and how they may relate to its therapeutic and adverse effects. To fully understand actions of carisoprodol, it is necessary to also understand potential subunit-dependent effects of the primary and comparatively long-lived metabolite, meprobamate. At the maximal concentration assessed, meprobamate-gated current was of similar magnitude in $\alpha 1$ -, $\alpha 2$ -, $\alpha 4$ -, $\alpha 5$ - and $\alpha 6\beta 2\gamma 2s$ GABA_ARs, and significantly less in $\alpha 3$ -expressing receptors. The parent drug carisoprodol has a similar profile, with roughly three-fold higher potency (Kumar *et*

al., 2015). With regard to allosteric modulation, carisoprodol displayed considerably greater efficacy in $\alpha 1$ -expressing receptors (Kumar *et al.*, 2015), whereas meprobamate displayed generally similar effects regardless of α subunit, with some enhanced efficacy in $\alpha 5$ -expressing receptors.

The lack of effect of β subunit isoform on direct gating or allosteric effects of meprobamate contrasts with carisoprodol, in which direct gating was most efficacious in $\beta 1$ -expressing receptors, and allosteric modulation was greatest in $\beta 2$ -expressing receptors (Kumar *et al.*, 2015). In addition, whereas the γ subunit did not have an effect on direct gating or allosteric modulation of carisoprodol (Kumar *et al.*, 2015), its presence quite markedly attenuated the modulatory capacity of meprobamate. This effect may not be therapeutically significant as few native receptors express only α and β subunits, but it may prove useful in identifying critical domains for effects of meprobamate.

Ligands that influence the actions of GABA may also act at extrasynaptic GABA_A receptors, which include $\alpha 4\beta 3\delta$ and $\alpha 1\beta 3\delta$ subtypes; these receptors have been implicated in diseases such as schizophrenia, depression and epilepsy (Brickley *et al.*, 2012). Meprobamate directly gated these receptors with an efficacy comparable to that of GABA, which is a partial agonist in these receptors (Bianchi *et al.*, 2003). We also observed that GABA's efficacy was about 2/3 that of the full agonist THIP in δ subunit-expressing receptors. Barbiturates (Feng *et al.*, 2004), neurosteroids (Wohlfarth *et al.*, 2002) and the parent drug carisoprodol (Kumar *et al.*, 2015) all have the ability to enhance the actions of a saturating concentration of GABA in extrasynaptic receptors. We observed a similar effect of meprobamate in the present investigation. Whereas a portion of the peak GABA current enhancement could be attributable to some direct action of meprobamate, the majority of the enhancement may relate to shifting of GABA from a low efficacy to high efficacy ligand (Bianchi *et al.*, 2003).

In considering where on the receptor meprobamate acts, key questions include: 1) is the site(s) where meprobamate acts the same as that for carisoprodol?; and 2) does meprobamate acts

at distinct sites for gating and allosteric effects? When initiating these studies, we hypothesized that the two ligands act at the same site for both effects. As noted, experimental evidence has shown that both carisoprodol and meprobamate allosterically modulate, directly gate and inhibit the GABA_A receptor. Both ligands also display similar actions at δ -expressing receptors. Specifically with regard to question 1 above, we consider here both direct and allosteric effects. Results for direct gating studies demonstrate the subunit-dependent profile for the two ligands are quite similar, and the main difference between the two ligands is potency. These data are consistent with a single overlapping site of action. The isopropyl group of carisoprodol may enhance hydrophobic interactions that result in its enhanced potency. In considering allosteric modulatory effects of the two ligands, the subunit-dependent profile showed significantly more variability than that observed for direct gating effects. The influence of α , β and γ subunits was different for meprobamate compared to carisoprodol. Although a definitive conclusion is not warranted, the data are consistent with only modestly overlapping or possibly distinct sites for allosteric effects of meprobamate, compared to carisoprodol. Further studies are required to more definitively address this issue.

In considering whether the direct and allosteric effects of meprobamate are mediated via one or two sites, the possibility of two sites is likely. With regard to carisoprodol, separate sites for direct gating and allosteric modulatory effects have been proposed (Kumar and Dillon, 2015). As with carisoprodol, the subunit-dependent profiles for direct and allosteric effects of meprobamate are distinct. Additional evidence suggestive of distinct sites comes from recent mutagenesis studies. We have reported in abstract form (Kumar et al., 2013) the fact that amino acid mutations in transmembrane domain IV of the α subunit impact direct gating of carisoprodol, but have no significant effect on its allosteric actions. Unpublished results of effects of these mutations on the actions of meprobamate suggest a similar effect. Whether the amino acid residues in question form part of the binding site for these ligands or are critical for transduction is not

known. Nevertheless, the fact that the mutations had effects only on direct gating is consistent with the conclusion that distinct sites exist for direct and allosteric modulatory effects for both meprobamate and carisoprodol.

Our results provide additional insight regarding the therapeutic and abuse-facilitating effects of carisoprodol. Both carisoprodol and meprobamate display direct gating and allosteric modulatory effects on GABA_A receptors across the spectrum of α -expressing receptors, which supports physical dependence and abuse potential (Licata *et al.*, 2008). Both ligands effectively modulate $\alpha 2$ -, $\alpha 3$ - and $\alpha 5$ -expressing receptors, which provides an effective muscle relaxant profile (Crestani *et al.*, 2001; Griebel *et al.*, 2003). Drawing further from an understanding of benzodiazepines, the considerably higher efficacy (and potency) of carisoprodol for allosteric enhancement of $\alpha 1$ -expressing receptors would seem to be a key contributor for its abuse and addictive potential (Licata and Rowlett, 2008; Tan *et al.*, 2010). In addition, in a study of impaired and unimpaired drivers, blood concentration of carisoprodol, but not meprobamate, correlated with driver impairment (Bramness *et al.*, 2004). The authors concluded that carisoprodol itself has impairing effects. As sedative effects are strongly associated with activity at $\alpha 1$ -expressing receptors, our finding that meprobamate has considerably reduced effects at these receptors, compared to carisoprodol, provides a molecular basis in support of the conclusion of Bramness *et al.* (2004). Meprobamate itself has important limitations as a therapeutic; hence its classification as a scheduled drug decades ago. However, as meprobamate strongly modulates $\alpha 5$ -expressing receptors (consistent with muscle relaxant effects), and has comparatively inefficient ability to modulate $\alpha 1$ -expressing receptors (associated with dependence and abuse potential), a re-evaluation of meprobamate for possible modification is warranted.

During the time period both meprobamate and carisoprodol were being widely prescribed, an extensive series of derivatives of them was generated (Ludwig *et al.*, 1969). Many of these displayed enhanced potency for muscle relaxation (as measured via paralyzing effects that leads to

loss of righting reflex) compared to both carisoprodol and meprobamate, as well as an enhanced safety margin. It appears none of these molecules made it to market. Whether they failed for safety or other reasons, or whether the decision to abandon this series of compounds was made in light of the subsequent scheduling of meprobamate is not known. Considering the availability of improved assays and our current understanding of molecular pharmacology associated with therapeutic effects, it might prove worthwhile to re-assess a number of these meprobamate-related carbamate molecules for myorelaxant and other indications.

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Table 1. GABA sensitivity of the GABA_A receptor subunit configurations studied. EC₅₀ values for each receptor are expressed as mean $\pm$ S.E.M.

Receptor Configuration	EC₅₀ (μM)	n
α1β2	16.4 ± 2.39	5
α1β2γ2	28.6 ± 2.75	8
α2β2γ2	32.8 ± 1.81	5
α3β2γ2	34.8 ± 2.09	8
α4β2γ2	5.9 ± 0.16	5
α5β2γ2	0.8 ± 0.23	4
α6β2γ2	1.7 ± 0.24	4
α1β1γ2	15.7 ± 0.15	7
α4β3δ	0.04 ± 0.02	4

Figure 1. Structure of meprobamate and a prominent parent molecule, carisoprodol.

The molecules differ by an isopropyl present on one of carisoprodol's carbamyl nitrogens; the isopropyl is removed by CYP2C19 to form meprobamate.

Figure 2. Assessment of subunit-dependent effects of meprobamate on direct activation of GABA_A receptors. **A**, Representative traces demonstrating direct activation by meprobamate in $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, $\alpha 1\beta 2$ and $\alpha 1\beta 1\gamma 2$ receptors. **B, C and D**, summary data of meprobamate mediated direct-gating effects associated with varying α , γ and β subunits, respectively. Direct activation capability was diminished in $\alpha 3\beta 2\gamma 2$ compared to the other configurations, whereas varying the β subunit or deleting the $\gamma 2$ subunit had negligible effects. Each data point represents the mean $\pm$ S.E.M. of a minimum of 4 cells. *, $p < 0.05$; **, $p < 0.01$.

A

CSP (mM)
MEP (mM)
GABA (μ M)

 $\alpha 1\beta 2\gamma 2$

CSP (mM)
MEP (mM)
GABA (μ M)

 $\alpha 3\beta 2\gamma 2$

MEP (mM)
GABA (μ M)

 $\alpha 1\beta 2$

MEP (mM)
GABA (μ M)

 $\alpha 1\beta 1\gamma 2$ **B** **C** **D**

Figure 3. Assessment of subunit-dependent effects of allosteric modulatory effects of meprobamate on GABA_A receptors. **A**, Representative traces demonstrating direct allosteric potentiation by meprobamate of GABA-gated (EC₂₀) currents in $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, $\alpha 1\beta 2$ and $\alpha 1\beta 1\gamma 2$ receptors. **B, C and D**, summary data of allosteric effects of meprobamate associated with varying α , γ and β subunits, respectively. Allosteric potentiation was present in all $\alpha\beta\gamma$ receptors, but greatest in those expressing the $\alpha 5$ subunit; absence of the γ subunit also resulted in enhanced potentiation. Note for this and other figures assessing allosteric modulation, underlying direct gating effects were subtracted out for calculation of summary data. Each data point represents the mean $\pm$ S.E.M. of a minimum of 4 cells. *, $p < 0.05$; ***, $p < 0.001$.

A **B** **C** **D**

Figure 4. Assessment of direct gating effects of meprobamate in extrasynaptic GABA_A receptors. **A**, representative traces demonstrating meprobamate direct gating action on $\alpha 4\beta 3\delta$ and $\alpha 1\beta 3\delta$ GABA_A receptors, in comparison to a saturating concentration of GABA. The maximal efficacy of meprobamate was comparable to that of GABA. **B**, Summary data illustrating the efficacy of meprobamate compared to GABA in $\alpha 1\beta 2$, $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_A receptor. Data for $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ receptors are re-plotted from Figure 1 for comparison. Efficacy of the full agonist THIP was also assessed; it was significantly more efficacious than either GABA or meprobamate on δ -containing GABA_A receptors. Each bar represents the mean $\pm$ S.E.M. of a minimum of 3 cells for δ -containing receptors, and mean $\pm$ S.E.M. of a minimum of 4 for all others. *, $p < 0.05$; **, $p < 0.01$.

Figure 5. Assessment of allosteric effects of meprobamate in extrasynaptic receptors. A, representative traces demonstrating the potentiation by meprobamate of GABA-gated (EC_{20}) currents in $\alpha 4\beta 3\delta$ GABA_A receptors. **B,** Bar graph summarizing the concentration-response profile for the allosteric modulatory effects of meprobamate on extrasynaptic $\alpha 4\beta 3\delta$ GABA_A receptors. Each bar represents the mean $\pm$ S.E.M. of a minimum of 3 cells.

Figure 6. Potentiation of maximal GABA by meprobamate in extrasynaptic receptors.

A, Illustrative traces showing that in both $\alpha 4\beta 3\delta$ and $\alpha 1\beta 3\delta$ extrasynaptic GABA_A receptors, 1 mM meprobamate significantly enhanced the magnitude of a saturating concentration of GABA alone, whereas this potentiation was not observed in $\alpha 1\beta 3$ or $\alpha 1\beta 2\gamma 2$ receptors. **B**, Summary data illustrating potentiation of maximal GABA current by both pentobarbital and meprobamate in δ -subunit expressing receptors, compared to those not expressing the δ subunit. Data are expressed relative to the peak current amplitude elicited by saturating concentration of GABA. Each bar represents the mean $\pm$ S.E.M. of a minimum of 3 cells for δ -containing receptors, and mean $\pm$ S.E.M. of a minimum of 4 for all others. **, $p < 0.01$.

A **B**

Figure 7. Assessment of “barbiturate-like” actions of meprobamate. **A, top,** representative traces demonstrating meprobamate-activated currents are reversibly reduced in the presence of the barbiturate antagonist bemegride in stably expressed human $\alpha 1\beta 2\gamma 2$ receptors. **A, middle,** Neither pentobarbital nor meprobamate could directly gate wild type homomeric $\rho 1$ receptors; the W328M mutation conferred sensitivity to pentobarbital, but not meprobamate. **A, bottom,** Pentobarbital directly gated homomeric $\beta 3$ receptors, whereas meprobamate blocked the spontaneously open current present in these channels. **B,** Summary results for experiments presented in panel A. Each data point represents the mean $\pm$ S.E.M. of a minimum of 4 cells for all data sets. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; #, $p < 0.05$ compared to the 3 mM MEP effect, illustrating a concentration-dependent effect of bemegride.

A

B

CHAPTER III

ASSESSMENT OF GABA_A RECEPTORS TM4 AND TM2 AMINO ACID RESIDUES IN DIRECT
GATING AND INHIBITORY EFFECTS OF CARISOPRODOL AND MEPROBAMATE

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Abstract

Meprobamate (tradenames Miltown, Equanil) is a schedule IV anxiolytic and the primary metabolite of the muscle relaxant carisoprodol. Though it used to be a widely prescribed tranquilizer, a thorough understanding of its direct-gating, allosteric-modulation and inhibitory effects remains ill-defined. Meprobamate modulates GABA_A (γ -aminobutyric acid type A) receptors, and has been described as having a barbiturate-like activity. In previous reports, we found that in comparison to $\alpha 1\beta 2\gamma 2$ receptors, $\alpha 3\beta 2\gamma 2$ receptors had attenuated direct gating effects for both meprobamate and carisoprodol. Here, using site-directed-mutagenesis and whole cell patch clamp electrophysiology in transiently transfected HEK293 cells, we examined the role of GABA_A receptor α -subunit transmembrane domain 4 (TM4) amino acids in direct gating actions of meprobamate, and transmembrane domain 2 (TM2) amino acids in inhibitory actions of meprobamate and carisoprodol. Mutation of $\alpha 3$ valine at position t440 to leucine (present in the equivalent position in the $\alpha 1$ subunit) increased the direct gating potency and efficacy of meprobamate. In $\alpha 1\beta 2$ heteromeric GABA_A receptors, both pentobarbital and carisoprodol had inhibitory effect for allosteric modulatory action at higher concentrations. Meprobamate did not have an inhibitory effect at the concentrations tested. T6'F mutation of the $\beta 2$ -subunit abolished the inhibition of carisoprodol but not of pentobarbital. Thus, our studies provide further insight into molecular determinants of actions of meprobamate, its parent drug carisoprodol and their comparison to barbiturate pentobarbital.

Key words: GABA_A receptor; meprobamate; carisoprodol; muscle relaxant; drug abuse

Introduction

GABA_A receptors are members of cys-loop ligand-gated ion channel superfamily. Other members of this group are glycine, acetylcholine receptor, GABA_C receptors, serotonin (5HT-3) receptors (Sine *et al.*, 2006). GABA_A receptors are the primary inhibitory receptors of the CNS (Bormann, 2000). Upon activation, opening of chloride channels and chloride ion entry, lead to the development of inhibitory post-synaptic potential (IPSP) (Bormann, 2000).

GABA_A receptors are pentameric in nature. Each of the subunits are composed of a long extracellular N-terminal domain, a short C-terminal domain, 4 transmembrane domains (TM1-TM4), an extracellular TM2-TM3 loop and two intracellular loops, a large TM3-TM4 and a short TM1-TM2 loop (Cockcroft *et al.*, 1995). TM2 of all the 5 subunits make the pore of the channel (Xu *et al.*, 1996; Miyazawa *et al.*, 2003). Some inhibitors of GABA_A receptors interact with amino acids constituting TM2 loop (Gurley *et al.*, 1995). GABA_A receptors are seats of action of many inhibitory ligands like benzodiazepines, barbiturates, neuro-steroids and muscle relaxants like carisoprodol and meprobamate (Gonzalez *et al.*, 2009b; Rudolph *et al.*, 2011; Kumar *et al.*, 2015)

Meprobamate, used to be a widely prescribed tranquilizer, anti-convulsant and muscle relaxant (Hendley *et al.*, 1954; DeLong *et al.*, 1985). Within few years of its introduction, cases of abuse and physical and psychological dependence started piling up (Ewing *et al.*, 1957). So it was made a controlled substance (schedule IV) at the federal level. While meprobamate was made a controlled substance, drugs metabolized to meprobamate are widely available. One such drug is the muscle relaxant carisoprodol whose easy availability due to internet pharmacies, has made it a widely abused drug (Toth *et al.*, 2004; Sullivan, 2015). There have been many cases of abuse, dependence and in some cases even death due to carisoprodol toxicity (Adams *et al.*, 1975; Elder, 1991; Littrell *et al.*, 1993; Rust *et al.*, 1993; Reeves *et al.*,

2003; Zacny *et al.*, 2011; Zacny *et al.*, 2012). Although carisoprodol can have independent action (Gonzalez *et al.*, 2009b), a significant portion of its action and abuse is due to meprobamate. As much of the research done on meprobamate has been in the years prior and during its major use, its detailed mechanism of action is not clear. It was shown to act on GABA_A receptors in a barbiturate like manner (Rho *et al.*, 1997). Our lab has shown meprobamate to have both direct-gating and allosteric modulatory effect on all synaptic and extra-synaptic GABA_A receptors subunit combinations. For direct-gating, meprobamate and carisoprodol, had maximum effect on $\alpha 1$ containing receptor while $\alpha 3$ had the least effect (Kumar *et al.*, 2015)(Kumar *et al.* 2016, in press). TM1-TM3 are fully conserved between $\alpha 1$ and $\alpha 3$ subunits. However, amino acids I419, I423 and V440 of TM4 of $\alpha 3$ are different from that of $\alpha 1$ subunit (Barnard *et al.*, 1998; Bergmann *et al.*, 2013). Here we tested the hypothesis, how this difference in the amino-acid compositions between $\alpha 3$ and $\alpha 1$ is responsible for the difference in the direct gating actions on the two subunits.

High concentrations of carisoprodol and pentobarbital had inhibitory effects for allosteric modulatory action in GABA_A receptor. TM2 T6' residue of the β -subunit of glycine receptor, has been implicated in the blocking action of picrotoxin (Pribilla *et al.*, 1992). Later it was also shown to be critical in the picrotoxin blocking action in GABA_A (Gurley *et al.*, 1995) and $\rho 1$ GABA_C receptors (Zhang *et al.*, 1995). T6'F mutation has been known to cause a significant reduction in picrotoxin inhibitory effect without abolishing it completely (Shan *et al.*, 2001; Sedelnikova *et al.*, 2006). This residue has been shown to be involved in blocking effects of other ligands of GABA_A receptors. We tested the hypothesis that T6' residue is also implicated in the blocking action of carisoprodol and pentobarbital.

2. Materials and Methods

2.1 Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells transiently expressing varying GABA_A receptor subunits, were used in the present study. For transient transfection, cells were transfected with human GABAA α ; human β ; and human γ 2s (short isoform) cDNA in a 1:1:5 ratio (2 μ g total cDNA) using PolyJetTM in vitro transfection reagent (SignaGen Laboratories, Jamesville, MD). The γ 2s subunit will be referred to as γ 2 from here forth. Human GABAA α 1 subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). Rat α 1 and β 2 subunits were also used. Transfection using just the α and β subunits were made using the plasmids in ratio 1:1 with a total of 2 μ g of cDNA. PolyJetTM in vitro transfection reagent was used 3 μ l/ μ g of DNA. Cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes, and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂. In all cases, cells were used for recording 24-72 hours after transfection.

2.2 Electrophysiology

Whole-cell patch clamp electrophysiology was used to assess GABA-, meprobamate- or pentobarbital-activated Cl⁻ currents. All electrophysiology experiments were conducted at room temperature (22-25 °C) 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 4–6 M Ω . Patch pipettes were filled with a solution consisting of 140 mM CsCl, 10 mM EGTA-Na⁺, 10 mM HEPES-Na⁺, and 4 mM Mg²⁺-ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and

superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl₂, 5.5 mM KCl, 0.8 mM MgCl₂, and 10 mM glucose, pH 7.3. Agonist-induced Cl⁻ currents were obtained with an Axopatch 200B amplifier (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 (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

2.3 Chemicals and solutions

Meprobamate, carisoprodol, pentobarbital, diazepam, salts and buffers were purchased from Sigma Aldrich (St. Louis, MO). GABA was obtained from Acros Organics (New Jersey, US). Stock solutions of meprobamate, pentobarbital and carisoprodol were made using dimethylsulfoxide (DMSO). Drugs were diluted in normal saline, so that the final DMSO concentration (vol/vol) of the test solutions was $\leq 0.3\%$. GABA and diazepam stock solutions were prepared using de-ionized H₂O. All stock solutions were stored at -20 °C. On experimental days, drug-containing solutions were prepared from stock by serial dilution into external solution.

2.4 Experimental Protocol

GABA (with or without modulatory ligand), meprobamate, carisoprodol or pentobarbital were prepared in external solution on the day of use and applied via a 16-barrel rapid perfusion system in which all barrels (200 μ m outer diameter quartz tubes; ALA Scientific, Folsom, CA) emptied via a common tip positioned adjacent to the cell under study. Flow through each barrel was pressure fed and regulated by solenoid or pinch valves operated by a programmable microprocessor-based controller. Only one valve was open at a time, and

the buffer solution was applied continuously between drug applications via gravity or positive pressure. For some experiments, ligands were applied via gravity flow using a Y-tube placed adjacent to the cell.

The modulatory effects of meprobamate, pentobarbital or carisoprodol on GABA-gated currents were assessed using an EC₂₀ gating concentration of GABA as the control. This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of meprobamate. To ensure the gating concentration was approximately an EC₂₀, control responses were compared to the maximal GABA-gated current for each individual cell. On a cell by cell basis, control GABA currents were deemed acceptable for assessment of allosteric modulatory effects of meprobamate if they were within a 15-25 % range of maximal current for that particular cell. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than $\pm 10\%$. In our analyses of the modulatory effects of meprobamate, peak current amplitude was defined as the maximum current elicited by GABA. In all studies assessing direct gating effects, the magnitude of the response was expressed relative to the maximal effect of GABA (designated 100%). For $\alpha\beta\gamma$ configurations, GABA-gated control currents were recorded in the presence of diazepam to confirm incorporation of the $\gamma 2$ subunit.

2.5 Data Analysis

To ensure equipotent concentrations were used for gating, GABA concentration-response data were collected. From these data, EC₂₀ and saturating GABA concentrations were calculated for each configuration and used in subsequent investigations of the allosteric and direct effects, respectively. For studies assessing allosteric actions, a correction was applied to subtract underlying direct gating effects. All data are presented as mean values $\pm$ S.E. Statistical significance ($p < 0.05$) between control and test conditions was determined using

Student's *t*-test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer *post hoc* test for multiple comparisons was performed as needed.

2.6 Plasmids and site-directed-mutagenesis

Human cDNA plasmids encoding $\alpha 1$, $\alpha 3$, $\beta 2$ and $\gamma 2$ GABAA receptor subunits were used in the present study. Individual and combined mutations in $\alpha 3$ cDNA plasmids were created using Stratagene's Quik Change II ® site-directed-mutagenesis kit (Agilent Technologies; La Jolla, CA) and were sequenced to confirm mutations at West Virginia University's Genomics Core Facility.

3. Results

3.1 TM4 amino acid mutations $\alpha 3$ to corresponding $\alpha 1$, lead to an increased meprobamate mediated direct gating effect

On testing for the action of meprobamate on various subunit compositions of GABA_A receptors, we found it to have maximum direct-gating effect on $\alpha 1$ and a significantly attenuated effect on $\alpha 3$ containing GABA_A receptors. Further we saw the effect of meprobamate to be consistent with its action in the transmembrane region (Hosie *et al.*, 2006). When we looked for difference in structure, amino-acid compositions of TM1-TM3 regions is conserved between $\alpha 1$ and $\alpha 3$ subunit isoforms. TM4 region is also largely conserved, except for, I420, I424 and V441 of $\alpha 3$, which differs from $\alpha 1$, which are L420, A424 and L441 (Barnard *et al.*, 1998; Bergmann *et al.*, 2013) (Fig.1). To test the roles of these amino acids unique to $\alpha 3$ in the attenuated direct gating effect, successive mutations were made to convert $\alpha 3$ to $\alpha 1$ either singly or in combination, using site directed mutagenesis. Mutations tested were, $\alpha 3$ (V441L), $\alpha 3$ (I420L/I424A) and $\alpha 3$ (I420L/I424A/ V441L). In order to assess the receptor function, GABA concentration response was done on each mutants. In general, the shift in GABA EC₅₀ was modest (Table 1). We observed an increase in direct gating efficacy for meprobamate in mutant receptors compared to WT $\alpha 3$ receptors, suggesting the role of above amino acids in different direct gating efficacy of $\alpha 3$ and $\alpha 1$ receptors.

3.2 Carisoprodol and pentobarbital, but not meprobamate show an inhibition and rebound at high concentrations in WT $\alpha 1\beta 2$ GABA_A receptors

At lower concentrations ($< 100 \mu\text{M}$), pentobarb, meprobamate and carisoprodol enhanced GABA mediated current in a concentration-dependent manner. At higher concentrations ($> 100 \mu\text{M}$), there was successive attenuation of GABA-mediated current by both pentobarb and carisoprodol. At 3 mM a rebound or offshoot was observed on drug application termination (Fig 3A and 4A). Neither an attenuation nor rebound was observed with meprobamate at the concentrations tested (till 3 mM) (Fig. 5A). We may observe a similar effect for meprobamate at higher concentrations as it has shown approximately 3 times less sensitivity than carisoprodol in our previous reports.

3.3 T6'F mutation in $\beta 2$ subunit, attenuates inhibition and rebound (offshoot) currents in carisoprodol, but not in pentobarital

Lower concentration of both pentobarb and carisoprodol enhanced GABA-mediated currents in WT $\alpha 1\beta 2$ GABA_A receptor. At concentrations $> 100\mu\text{M}$, there was progressive inhibition and a rebound and offshoot was observed upon drug termination at 3 mM with both the ligands. A T6' amino-acid in the $\beta 2$ subunit has previously been implicated in inhibitory effects of other inhibitory ligands. TM2 T6' residue of the β -subunit of glycine receptor, has been implicated in the blocking action of picrotoxin (Pribilla *et al.*, 1992). Later it was also shown to be critical in the picrotoxin blocking action in GABA_A receptor (Gurley *et al.*, 1995) and $\rho 1$ GABA_C receptor (Zhang *et al.*, 1995). T6'F mutation was tested since it has been known to cause a significant reduction in picrotoxin sensitivity without abolishing it completely (Shan *et al.*, 2001; Sedelnikova *et al.*, 2006). We tested the hypothesis that T6' residue is implicated in the above blocking actions. TM2 T6' residue, when mutated to T6'F, both inhibition and rebound mediated by carisoprodol, was attenuated. No effect was observed on pentobarb

mediated inhibition or rebound. Thus, pentobarbital may have different binding site or functional domain for this particular action. Pentobarbital had a direct gating effect in $\beta 3$ homomeric receptors. Both meprobamate and carisoprodol, like picrotoxin, blocked spontaneously open current in the $\beta 3$ homomer. This MEP and CSP mediated blockage was abolished in homomer with T6'F mutation, unmasking their direct gating effect. Homomer with T6'F mutation in $\beta 3$ subunit, did not affect pentobarbital mediated direct gating current, again suggesting a different binding site or a functional domain for this particular action.

4. Discussion

Meprobamate was the first widely prescribed tranquilizer agent (DeLong *et al.*, 1985). Initially found to be free of side effects in animal studies and in populations using regular therapeutic doses (Boyd *et al.*, 1958), many cases of abuse and dependence were observed in patients having history of misuse of other drugs and alcohol abuse (Mohr *et al.*, 1958). Also, after long term use, cases of physical and psychological dependence started to appear (Lemere, 1956; Ewing *et al.*, 1957). Thus, it was made a controlled substance and its use started to dwindle after the introduction of benzodiazepines (Berger, 1964; Greenblatt *et al.*, 1974). Although meprobamate was made a controlled substance, drugs metabolized to meprobamate are widely available. One such drug is the centrally acting muscle relaxant carisoprodol (Toth *et al.*, 2004; Sullivan, 2015). It is easily available and widely used, especially after the advent of internet pharmacies. Many serious cases of abuse, dependence and withdrawal have also been reported for carisoprodol (Adams *et al.*, 1975; Elder, 1991; Littrell *et al.*, 1993; Rust *et al.*, 1993; Reeves *et al.*, 2003; Zacny *et al.*, 2011; Zacny *et al.*, 2012). Carisoprodol was known to act via its metabolite meprobamate, already a controlled substance and without a known mechanism of action. Our previous reports have shown carisoprodol itself, independent of meprobamate, and meprobamate act on synaptic and extra-synaptic GABA_A receptor in a subunit dependent manner (Gonzalez *et al.*, 2009b; Kumar *et al.*, 2015). For direct-gating actions, meprobamate potency and potentially efficacy, was much higher for $\alpha 1$ expressing GABA_A receptor compared to $\alpha 3$ expressing GABA_A receptor. In this respect, action of meprobamate is qualitatively similar to its parent drug carisoprodol, however, the potency of this effect is lot less for meprobamate compared to carisoprodol (Gonzalez *et al.*, 2009b; Kumar *et al.*, 2015) (Kumar *et al.* 2015, in press).

Activity of meprobamate and carisoprodol suggest a transmembrane-domain specific action (Hosie *et al.*, 2006). Upon alignment, there is similar amino-acid compositions in the TM1-TM3 region, while TM4 region has 3 different amino-acid composition between $\alpha 1$ and $\alpha 3$ subunits. These were I419, I423 and V440 (Barnard *et al.*, 1998; Bergmann *et al.*, 2013). To test their role in attenuated direct gating effects of meprobamate at $\alpha 3$, we tested successive mutations from $\alpha 3$ to $\alpha 1$ subunit. An increase in direct gating efficacy was observed, suggesting the role of these amino acids for the direct-gating actions of meprobamate. A similar gain in function was also observed for carisoprodol, though of a much higher magnitude. This shows similar domains may be responsible for the direct-gating actions of carisoprodol and meprobamate.

Further, meprobamate and carisoprodol actions have been described as barbiturate like (Rho *et al.*, 1997; Gonzalez *et al.*, 2009b). In our previous reports, we have seen several points of similarities and differences in the actions of meprobamate, carisoprodol and barbiturates. Supra-potentiation of GABA saturation current seen in δ -containing GABA_A receptors was also observed with carisoprodol and meprobamate. Mice trained to identify carisoprodol also identified meprobamate and pentobarbital. Barbiturate-antagonist, bemegride, reversibly inhibited meprobamate and carisoprodol mediated currents (Gonzalez *et al.*, 2009b)(Kumar *et al.*, 2015, in press). Also in $\rho 1$ -homomeric receptor, natively found in retina, meprobamate, carisoprodol and barbiturate pentobarbital, did not have any direct effect. However, a W328M mutation in $\rho 1$, salvaged pentobarbital mediated current, but not that of carisoprodol and meprobamate (Gonzalez *et al.*, 2009b)(Kumar *et al.*, 2015, in press). Also in $\beta 3$ homomeric receptors pentobarbital had a direct-gating action whereas meprobamate had an inhibitory action similar to picrotoxin.

In this study we used $\alpha 1\beta 2$ hetero-pentamer to test for inhibitory actions of carisoprodol, meprobamate and pentobarbital. At lower concentrations ($< 100 \mu\text{M}$), all three enhanced GABA mediated current. However at higher concentrations, an inhibition was observed with pentobarbital and carisoprodol. At 3 mM, a rebound current was observed upon termination of both pentobarbital and carisoprodol. Meprobamate continued to enhance GABA mediated current at concentrations tested (3mM). One reason could be the lower sensitivity of meprobamate compared to carisoprodol (approximately one third of carisoprodol). We may see a similar phenomenon at higher concentrations. T6' of $\beta 2$ is found in the TM2 region (pore region) and has been implicated in the inhibitory effect of picrotoxin (Pribilla *et al.*, 1992; Gurley *et al.*, 1995; Zhang *et al.*, 1995). We tested whether this amino acid is responsible for the inhibitory effects observed with the two ligands as T6'F mutation has been shown to abolish picrotoxin mediated inhibition (Shan *et al.*, 2001; Sedelnikova *et al.*, 2006). T6', mutated to T6'F, abolished the inhibitory and rebound effects of carisoprodol but not of pentobarbital. This suggests T6' residue responsible for the inhibitory effect of carisoprodol but not of pentobarbital. At the concentrations tested, meprobamate did not show an inhibitory effect. Taken together these findings suggest a distinct sight for inhibitory actions for barbiturates compared to carisoprodol and meprobamate.

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Table III-1. Influence of GABAA receptor $\alpha 3$ TM4 amino acids mutation to corresponding $\alpha 1$ amino acids on GABA EC50 values Each data point represents the mean $\pm$ S.E.M of n cells. *, p < 0.05; **, p < 0.01 relative to wild type $\alpha 3\beta 2\gamma 2$ GABAA receptors.

Receptor Configuration	GABA EC₅₀ (μM)	n	MEP gating (% of GABA max)	n
α3 WT	34.8 ± 2.1	6	7.25 ± 2.8	5
α3(V440L)	07.5 ± 0.9 **	7	13.25 ± 1.89	3
α3(I419L/I423A)	18.1 ± 2.2 *	9	12 ± 4	3
α3(I419L/I423A/V440L)	15.8 ± 5.0 *	6	8 ± 2.65	3
α1 WT	28.6 ± 2.75	8	32 ± 8.1	5

Figure 1. Alignment of $\alpha 1$ and $\alpha 3$ domains of TM4 showing non-identical (red) and mutated (*) amino acids. Conserved (black).

Figure 2. Influence of the alpha3 subunit TM4 mutations on direct activation by meprobamate. **A**, representative traces demonstrating carisoprodol and meprobamate activate human $\alpha 3(V441L)$ $\beta 2\gamma 2$ GABA_ARs. **B**, bar graphs summarizing meprobamate direct gating currents for human $\alpha 3$ -, $\alpha 3(V441L)$ -, $\alpha 3(I420L/I424A/V441L)$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. **, $p < 0.01$, *, $p < 0.05$. **MT1** - $\alpha 3(V441L)$; **MT2** - $\alpha 3(I420L/I424A)$ and **MT3** - $\alpha 3(I420L/I424A/V441L)$.

Figure 3. Influence of β -subunit TM2 T6'F Mutation on inhibitory effects of carisoprodol.

A) Representative traces from R- $\alpha 1\beta 2$ GABAA receptor demonstrating modulation of GABA-gated currents by carisoprodol. At high concentrations, currents are inhibited and offshoot currents are observed upon termination of drug application. This phenomenon is attenuated with $\alpha 1\beta 2$ (T6'F) GABAA receptor. **B)** Low concentration CSP potentiated the GABA gated current of both R- $\alpha 1\beta 2$ GABAA receptor and R- $\alpha 1\beta 2$ (T6'F) GABAA receptor. High concentration of CSP inhibit R- $\alpha 1\beta 2$ receptors but in R- $\alpha 1\beta 2$ (T6'F) receptors, inhibitory effect was attenuated. Each bar represents the mean $\pm$ S.E. of the 7-9 cells.

Figure 4. Influence of β -subunit TM2 T6'F Mutation on inhibitory effect by pentobarbital. **A)** Representative traces from R- $\alpha 1\beta 2$ GABAA receptor demonstrating modulation of GABA-gated currents by pentobarbital. At high concentrations, currents are inhibited and offshoot currents are observed upon termination of drug application. A similar phenomenon is observed with $\alpha 1\beta 2$ (T6'F) GABAA receptor. **B)** Low concentration PB potentiated the GABA gated current of both R- $\alpha 1\beta 2$ GABAA receptor and R- $\alpha 1\beta 2$ (T6'F) GABAA receptor. High concentration of PB inhibit R- $\alpha 1\beta 2$ receptors but in R- $\alpha 1\beta 2$ (T6'F) receptors, inhibitory effect was not attenuated unlike CSP. Each bar represents the mean $\pm$ S.E. of the 7-8 cells.

Figure 5. Influence of β -subunit TM2 T6'F Mutation on allosteric modulation by MEP.

A) Representative traces from R- $\alpha 1\beta 2$ GABAA receptor demonstrating modulation of GABA-gated currents by MEP. At high concentrations, no offshoot currents are observed upon termination of drug application and currents are not inhibited as seen with CSP or PB. A similar phenomenon is observed with $\alpha 1\beta 2$ (T6'F) GABAA receptor. **B)** Low concentration MEP potentiated the GABA gated current of both R- $\alpha 1\beta 2$ GABAA receptor and R- $\alpha 1\beta 2$ (T6'F) GABAA receptor. High concentrations of MEP do not inhibit R- $\alpha 1\beta 2$ or R- $\alpha 1\beta 2$ (T6'F) receptors. Each bar represents the mean $\pm$ S.E. of the 7-8 cells.

CHAPTER IV

SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

Meprobamate was the first drug to be prescribed purely as a tranquillizer, that is, capable of allaying anxiety without inducing sedation or sleep (DeLong *et al.*, 1985). It was a wonder drug when introduced and sold billions of pills. With therapeutic doses, it did not produce addiction or dependence, even after prolonged use (Boyd *et al.*, 1958). Higher than normal dose, in patients with history of other drugs or alcohol abuse, led to all the characteristics of a habit forming drug. These include an intense craving for the drug based on its euphoric effect, tolerance build up, requiring increasingly larger doses to produce the same effect, and withdrawal symptoms associated with sudden discontinuation of the drug (Lemere, 1956; Mohr *et al.*, 1958). Considering increasing number of cases of physical and psychological dependence, meprobamate was made a controlled substance (Greenblatt *et al.*, 1971; Hollister, 1983). While meprobamate is a controlled substance, drugs metabolized to meprobamate are widely and easily available, and abused. One such drug, carisoprodol, is a widely prescribed centrally acting skeletal muscle relaxant (Toth *et al.*, 2004; Sullivan, 2015). It is most commonly used for short term allaying of skeletal muscle pain and discomfort. Carisoprodol use has been characterized by quick development of tolerance, leading to use of increasing doses to produce the desired effect. After long term use of high dose of carisoprodol, strong withdrawal symptoms have been observed. These include delusion, seizure and even death (Adams *et al.*, 1975; Elder, 1991; Littrell *et al.*, 1993; Rust *et al.*, 1993; Reeves *et al.*, 2003; Zacny *et al.*, 2011; Zacny *et al.*, 2012). Considering the dangers posed by the abuse of carisoprodol, it was made a scheduled 4 controlled substance at the federal level in January 2012 by the United States Drug Enforcement Administration. Considering present and potential danger posed by carisoprodol use and abuse, it was of utmost importance to know its detailed mechanism of action. For long it was known to act via its metabolite meprobamate,

which lacks the isopropyl group found in carisoprodol. Meprobamate is already a controlled substance, but its detailed mechanism of action is also unknown.

It has been shown that the therapeutic and adverse effects of GABA ligands depend upon their interactions with the GABA_A receptor subunits (Ito *et al.*, 1996; Ator, 2005; Licata *et al.*, 2005; Wafford, 2005; Olsen *et al.*, 2008). $\alpha 2$ containing GABA_A receptors have the anxiolytic and, to a large extent, myorelaxant actions; and the $\alpha 3$ - and $\alpha 5$ -containing GABA_A receptors, have the myorelaxant actions. Moreover, tolerance to the sedative action has been linked to $\alpha 5$ -containing GABA_A receptors, and their addictive properties to $\alpha 1$ -containing GABA_A receptors (Low *et al.*, 2000; McKernan *et al.*, 2000; Crestani *et al.*, 2001; Crestani *et al.*, 2002; Rudolph *et al.*, 2004; van Rijnsoever *et al.*, 2004; Tan *et al.*, 2010). In addition, physical dependence is more likely to develop with drugs that interact with a broader collection of GABA_A receptor subtypes and misuse of agents that directly gate the receptor is more likely to result in fatal complications than those that only allosterically enhance receptor activity (Licata *et al.*, 2008; Fass, 2010). So we tested for detailed subunit dependent action of carisoprodol on GABA_A receptor, but in order to understand a complete pharmacological profile of this widely used and abused drug, we also set out to understand the complete subunit dependent action of its metabolite, meprobamate. The only knowledge regarding the action of meprobamate was its interaction in a barbiturate like manner with GABA_A receptors. So, we also set out to chalk out the difference between the action of meprobamate and barbiturate pentobarbital.

We found meprobamate to have direct gating, allosteric modulatory and inhibitory actions on various synaptic and extra-synaptic GABA_A receptors. For direct gating, $\alpha 1$ had significantly more efficacy than $\alpha 3$ containing receptors. To understand the molecular basis for the difference, amino-acid compositions of the $\alpha 3$ and $\alpha 1$ -subunits were aligned. We identified

several amino-acids of difference between the two subunits in the TM4 region. Using site directed mutagenesis, amino-acids from $\alpha 3$ were converted to those in $\alpha 1$ either singly or in combination. We found a gain in function, suggesting these amino-acids to be responsible for attenuated $\alpha 3$ direct gating action. A similar gain in function was also observed for carisoprodol, however of a much higher magnitude. Structural difference between carisoprodol and meprobamate is minimal. Both are propanediol bicarbamate, with carisoprodol having an isopropyl group, absent in meprobamate.

For allosteric modulatory action of meprobamate, $\alpha 5$ subunit was somewhat more efficacious compared to other subunits. In carisoprodol, $\alpha 1$ subunit was significantly more potent and efficacious than other subunits. Also, carisoprodol potency on all the subunits, was more than meprobamate. So for allosteric-modulatory actions, a difference in potency and subunit preferring effect was observed.

Effects of β and γ subunits were also tested for direct gating and allosteric modulatory effects. γ subunit did not have any influence on both these effects of carisoprodol. While for meprobamate, γ subunit did not influence the direct gating but had a significant inhibitory effect for allosteric-modulation. β subunit had an effect on both the direct gating and allosteric modulation of carisoprodol. $\beta 1$ subunit had significantly more direct-gating effect compared to $\beta 2$ while $\beta 2$ had significantly more allosteric-modulatory effect compared to $\beta 1$. β -subunit did not influence either meprobamate direct gating or allosteric modulation. The above differences may be exploited to know about the critical domains responsible for the action of meprobamate and carisoprodol.

Extra-synaptic GABA_A receptors represented by $\alpha 4\beta 3\delta$ and $\alpha 1\beta 3\delta$ were also tested. Both meprobamate and carisoprodol had a supra-potentiation effect on GABA saturation. In

this respect, actions of both carisoprodol and meprobamate were barbiturate like. For direct gating action on the extra-synaptic receptors, both carisoprodol and meprobamate were almost as much efficacious as GABA. Both meprobamate and carisoprodol also had allosteric-modulatory effects on the extra-synaptic receptors.

Further comparison with the barbiturates was done using $\rho 1$ homomeric receptors found natively in retinal ganglion cells and $\beta 3$ homomeric receptors, not found natively. All three, meprobamate, carisoprodol and pentobarbital did not have a direct gating action on $\rho 1$ -homomeric receptors. A mutation W328M, however, salvaged the direct gating action of pentobarbital, but not of carisoprodol or meprobamate. For $\beta 3$ -homomeric GABA_A receptors, barbiturate pentobarbital had an agonist effect while meprobamate, blocked the spontaneously open current, a mechanism followed by picrotoxin, a known GABA inhibitor. A T6'F mutation in $\beta 3$ subunit abolished the meprobamate mediated inhibition, without affecting the pentobarbital mediated direct gating.

In receptors formed by $\alpha 1\beta 2$, allosteric actions of meprobamate, carisoprodol and pentobarbital were tested. Meprobamate, at tested concentrations (maximum 3 mM), continuously potentiated GABA mediated current in a concentration dependent manner. At lower concentrations (< 100 μ M), carisoprodol and pentobarbital potentiated GABA gated currents in a concentration dependent manner but at higher doses, showed an inhibition and a rebound at 3mM. T6' residue in β -subunit TM2 region (pore forming region) when mutated to T6'F, attenuates the inhibition and abolishes the rebound in carisoprodol, but not pentobarbital. Thus 6' residue seems to be involved in the inhibitory actions of carisoprodol, but not pentobarbital. At concentrations tested, meprobamate did not show an inhibitory action in $\alpha 1\beta 2$ receptors. Meprobamate may show an inhibitory and rebound effect on testing at higher concentration as it has shown to be approximately 3 times less sensitive than carisoprodol, in

our previous reports. In $\beta 3$ homomeric receptor both meprobamate and carisoprodol block spontaneously open current. A T6'F mutation abolishes this action for both carisoprodol and meprobamate and unmask their direct gating effect. So the $\beta 3$ actions represent a similarity of action of meprobamate and carisoprodol, representing a similar binding site/or functional domain for this particular action, between carisoprodol and meprobamate. Pentobarb has an agonist action at $\beta 3$ homomeric receptors representing a different binding site/functional domain than both meprobamate and carisoprodol.

In an effort towards drug development, we need to further explore the mechanism of actions of meprobamate. We saw a gain in function for the direct action from $\alpha 3$ to $\alpha 1$ conversion mutations in the TM4 region. We need to test the same for the reverse mutations from $\alpha 1$ to $\alpha 3$ and observe whether there is a loss of function. A similar study conducted for carisoprodol, showed leucine residue at position 415 of $\alpha 1$ subunit to be responsible for its direct gating action. The combination of the above two studies would help us to identify the amino acids responsible for the direct gating actions of the meprobamate.

An important study would be to identify the property of the amino-acids responsible for the direct gating action. For this, amino-acid identified for the direct gating action shall be mutated to various other amino acids with varying size, charge, hydrophobicity or polarity. A correlation observed between the magnitude of the current and a particular property would help us identify that particular property to be responsible for the direct gating. For carisoprodol, at position 415, volume and polarity of the amino acids have been shown to be associated with its direct gating efficacy. A similar study shall be undertaken to find the binding domain and property of the amino acids responsible for the allosteric modulation. Above amino acids did not seem to have an effect on the allosteric modulatory effect of carisoprodol. For further

understanding of the domains responsible for the binding pocket for direct gating and allosteric modulatory action, we shall also take resort to molecular modeling techniques.

Further, allosteric modulatory actions of general anesthetics etomidate, propofol and isoflurane have shown voltage dependence (O'Toole *et al.*, 2012). Parent drug of meprobamate, carisoprodol has also shown voltage dependent allosteric modulatory actions but not direct gating action (Kumar *et al.*, 2015). Again showing a different binding sites for carisoprodol for direct gating and allosteric modulatory effects and thus multiple binding sites for carisoprodol at GABA_A receptor. It would be interesting to see whether meprobamate mediated actions also show voltage dependence.

We are still not sure whether the amino acid identified via the mutation studies participate in the direct binding or carry the wave of conformational changes, a mechanism called coupling. Substituted cysteine accessibility method would help us to differentiate between the two. The substituted-cysteine accessibility method (SCAM) provides an approach for identifying the amino acid residues that line channels, transporters, or binding-site crevices in membrane-spanning proteins (Chen *et al.*, 1997; Javitch *et al.*, 1998; Dodd *et al.*, 2005). SCAM can also be used to investigate structural alterations in membrane-spanning segments in different functional states of proteins. Sulfhydryl group of native or engineered cysteine of a protein can be at water accessible surface, lipid accessible surface or a protein interior. Rate of reaction of a hydrophilic reagent would be much faster with an ionized sulfhydryl group (at water accessible region) than a non ionized sulfhydryl group. Measuring the rate of change of reaction of an engineered cysteine with a hydrophilic reagent in presence or absence of a ligand can help us conclude the movement of the particular amino acid (and thus the loop) in presence of the ligand and thus its participation in the gating process of a ligand.

It has become increasingly clear in recent years that many therapeutic and adverse actions of GABAergic drugs associate predominantly with particular subunits of the GABA_A receptor. This has been studied extensively in animal models in which a particular subunit is deleted and resultant absence of a particular effect is attributed to the deleted subunit. This technique has been used to show the subunits responsible for alcohol tolerance, dependence, consumption and taste aversion (Barnard *et al.*, 1998; Jacob *et al.*, 2008; Miller *et al.*, 2014; Whissell *et al.*, 2015). A role for extrasynaptic receptors have also been attributed for alcohol consumption using similar techniques (Feusner *et al.*, 2001). Knockout mice have also been used for assessing therapeutic and abuse potentials for benzodiazepines (Rudolph *et al.*, 2011). Our study demonstrates that meprobamate action in-vitro depends upon GABA_A receptor subtype isoforms. A further testing of our findings in in-vivo model in the knockout mice would further confirm actions of meprobamate on the tested subunits and help us better predict subunit dependent effects of meprobamate.

Meprobamate has both tranquilizing and muscle relaxing properties. An initial effort was made by Berger for preparation of compounds for the separation of the above two activities. In this effort many compounds chemically related to meprobamate were prepared and subjected to pharmacological evaluation (Berger *et al.*, 1960). From the chemical formula of meprobamate, 2-methyl-2-propyl-1, 3-propanediol dicarbamate, the N-substituted and N-nonsubstituted compounds were tested for muscle relaxant, anti-convulsant and toxic actions. N-isopropyl-2-methyl-2-propyl-1, 3-propanediol dicarbamate, or carisoprodol showed a promising muscle relaxant action alienated from tranquilizing action of meprobamate (Berger *et al.*, 1960). Carisoprodol was further tested and made available for medical use. One reason CSP was further evaluated was its proximity to the structure of meprobamate which was widely used at that time and thus had a proven safety profile. Also with the cost of further evaluation,

not many compounds could have been tested at that time. However many compounds generated showed a promising muscle relaxant and anti-convulsant profile. Chemical groups commonly tested were 3-propanediols; 1, 3-propanediol bicarbamates and 3-hydroxypropyl carbamates (Figure 1) (Ludwig *et al.*, 1969).

Compounds of interest were 2-substituted 1, 3-propanediol bicarbamates. A total of 5-7 carbon atoms at the 2-position had the greatest muscle relaxing properties. An aryl group reduces muscle relaxing properties, while a methyl and a phenyl group has an enhanced anti-convulsant activity and reduced muscle relaxing properties. N-Substituted compounds have also shown interesting properties. Replacement of one of N-hydrogen of the carbamate has yielded a number of compounds with interesting muscle relaxing properties. A prominent example is widely prescribed muscle relaxant carisoprodol or N-isopropyl-2-methyl-2-propyl-1, 3-propanediol dicarbamate (Ludwig *et al.*, 1969).

2-Substituted and N-substituted 3-hydroxypropyl carbamate, also show prominent muscle relaxing and anti-convulsant properties. 4-5 alkyl groups at position 2 gave good muscle relaxing property while an aromatic group at this position had good anti-convulsant property and attenuated muscle relaxant property. N-Substituted compounds also had prominent muscle relaxant and anti-convulsant compounds (Ludwig *et al.*, 1969).

Our lab provides low cost methods for further evaluation of these drugs. A testing at GABA_A subunits would enable us to have a quick prediction of therapeutic and abuse potentials of these drugs. We would start by generation of as many of the above promising ligands as possible. Each of the ligand would be tested for direct gating and allosteric modulatory effects at various α and β subunit combinations of the GABA_A receptors. Thus a pharmacologic profile

of each ligand would be generated, along with a note on possible therapeutic as well as abuse potentials of each ligand. Promising ligands would be further tested in animal models.

Figure 1: Chemical structure of meprobamate; 1,3 propanediol; 3-hydroxypropyl carbamate

Meprobamate

1,3-propanediol

3-hydroxypropyl carbamate

In between carisoprodol and meprobamate, they have global effect on various subunit combinations of synaptic and extra-synaptic GABA_A receptors. In addition to having effects on GABA mediated currents, they also have a direct-gating actions on all these native receptors. Meprobamate have also been shown to have partial inhibitory effect on NMDA receptors (Rho *et al.*, 1997). In our lab and our colleague's, we have shown carisoprodol also to have partial inhibitory effect on NMDA receptors, both in vitro in HEK-t transfected cells, as well as in animals trained to identify carisoprodol. Further clinical effects of carisoprodol also seem to affect serotonergic receptors (Bramness *et al.*, 2005). Both the actions of carisoprodol and meprobamate on NMDA and 5 HT-3 receptors need to be further explored. Our knowledge of qualitative and quantitative aspects of effects of meprobamate and carisoprodol on various subunits of synaptic and extra-synaptic GABA_A receptors, have enabled us to explain molecular basis of various clinical and toxic effects observed with the use and abuse of these drugs. Further research on the molecular mechanism of actions of these drugs (further exploration on GABA_A receptors, NMDA and 5HT-3 receptors), would not only explain the clinical effects observed, but may also enable us to bring these drugs for new uses in select group of patients with specific needs. Our studies in lab could also prove to be important for drug development, as molecules with induced structural changes to have desired properties, can be quickly tested in cellular models, rather than time taking and costly animal models used in the initial development of these drugs. MEP and CSP would prove to be important drugs for blanket testing of reagents for a particularly useful action at an unknown potential therapeutic site of action or interaction with useful enzymes. Like amantadine, an anti-influenza drug also used as an anti-parkinsonism drug, their global action on receptors with varying physiological actions may prove to be an important property to test for actions on unknown useful sites.

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