Snell, Heather D., Characterization of the interactions of guanidine compounds with the human GABA-A ρ1 receptor

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This dissertation investigates the activity of guanidine compounds GMQ, and amiloride and its derivatives on the human GABA-A ρ1 receptor, compounds classified as antagonists for the heteromeric GABA-A αβγ receptor.

The GABA-A ρ receptor possesses many differences in kinetics, expression, and pharmacology from the heteromeric GABA-A αβγ receptors. Many GABA-A αβγ receptors ligands interact differently, or fail to interact with, the GABA-A ρ receptor. Thus the activity of these guanidine compounds on the GABA-A ρ1 receptor remains unknown. Based on the differential pharmacology displayed by the GABA-A ρ receptors, we propose that GMQ and amiloride would interact with the GABA-A ρ1 receptor as agonists, different from their activity on the heteromeric GABA-A αβγ receptors.

Importantly, our data demonstrates GMQ and amiloride interacts with the GABA-A ρ receptors as negative and positive allosteric modulators, respectively. The 15' residue of the second transmembrane domain of the GABA-A ρ1 receptor is important in the positive allosteric modulatory mechanism, and the accessibility of the guanidine group on the guanidine compound is integral in the positive allosteric modulation mechanisms of amiloride and its derivative 5-(N,N-Hexamethylene) amiloride (HMA).

The investigation of novel compounds that interact with the GABA-A ρ receptor differently from GABA-A αβγ receptor would contribute to a better understanding of the

GABA-A ρ receptor structure and the production of novel therapeutics specific for the GABA-A ρ receptor. Particularly, the GABA-A ρ receptor is implicated in retinal hypoxic disorders such as diabetic retinopathy. These guanidine compounds could be utilized as a back-bone for the production of compounds that could alleviate the pathologies caused by advanced stages of diabetic retinopathy.

CHARACTERIZATION OF THE INTERACTIONS OF GUANIDINE COMPOUNDS WITH THE HUMAN GABA-A ρ1 RECEPTOR

Heather D. Snell

APPROVED:

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Major Professor

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Committee Member

_____

Committee Member

_____

Committee Member

_____

University Member

_____

Chair, Department of Pharmacology and Neuroscience

_____

Dean, Graduate School of Biomedical Sciences

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LIST OF ABBREVIATIONS

GABA: γ-aminobutyric acid, **GAD**: L-glutamic acid decarboxylase, **IPSC**: inhibitory postsynaptic current, **TPMPA**: (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid, **P4MPA**: piperidin-4-yl)methylphosphinic acid, **GAT-1**: GABA transporter 1, **OPL**: outer plexiform layer, **IPL**: inner plexiform layer, **BC**: bipolar cell, **mRNA**: messenger RNA, **5-methyl-I4AA**: 5-methyl-imidazole-4-acetic acid, **TM**: transmembrane domain, Amil: amiloride, **ASIC**: acid-sensing ion channel, **GMQ**: 2-guanidine-4-methylquinazoline, **VIAAT**: vesicular inhibitory amino transporter, **THIP**: 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol, **TACA**: trans-4-Aminocrotonic acid, **P4S**: piperidine-4-sulphonic, **CACA**: cis-4-aminocrotonic acid, **EGTA**: ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, **HEPES**: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, **TEA-Cl**: tetraethylammonium chloride, **DMSO**: dimethylsulfoxide, **GLIC**: *Gloeobacter violaceus* pentameric ligand-gated ion channel, **PAM**: positive allosteric modulation, **HMA**: 5-(N,N-Hexamethylene) amiloride, **Amil**: amiloride, **BRB**: blood-retinal barrier

I. INTRODUCTION

THE HISTORY OF GABA

Gamma-aminobutyric acid, more commonly known as GABA, is an amino acid, but it is not referred to as such because it doesn't qualify as an alpha amino acid and fails to be incorporated into proteins (Bown and Shelp, 1997). It was first synthesized in 1883, and thought only to be present in plants and microbes as a metabolic product (Roth *et al*., 2003). GABA is well established as the major inhibitory neurotransmitter in the vertebrate brain, but in the mid 1900's, it wasn't yet classified as a neurotransmitter. Its presence in biological tissue was established as early as 1910, initially discovered as a substance in tissue of the central nervous system of vertebrates by a paper chromatographic screen analyzing free amino acids present in normal and neoplastic tissues (Roberts and Frankel, 1950).

In this experiment, researchers utilized the chemical ninhydrin, which is used to detect ammonia or primary and secondary amino acids in proteins, producing a deep blue or purple color upon reacting with these substances (vlab.amirita.edu.,). An unknown ninhydrin-reactive material was found in abundance in extracts of brains from various animals, such as; rabbit, rat, guinea pig and human, while little was found in the blood or urine of these same samples (Roberts and Frankel, 1950). A more in-depth analysis of the properties of this substance revealed it to be gamma-aminobutyric acid or GABA (Roberts and Frankel, 1950). Having established that GABA is a substance present in the body of many animals, its neurophysiological localization and biochemical properties remained unknown.

Researchers suggested that GABA could exhibit inhibitory activity in the central and peripheral nervous systems, validated through topical application of GABA, which exhibited inhibitory effects on brain activity (Hayashi and Nagai, 1956). Interest shifted from investigating its importance in neuronal activity in the vertebrate brain, to utilizing the crustacean as a model to study excitatory/inhibitory control mechanisms (Kuffler 1954, Florey 1955). Despite the evidence, there were still many scientists that doubted the validity of GABA being important in neuronal activity. Many studies failed to reproduce the results or resulted in contrasting findings of GABA's inhibitory activity in neurons. One finding stated that GABA could not be a neurotransmitter because it did not have rapid inactivation (Hayashi, 1958, Elliott and Van Gelder, 1958).

In 1959, Florey and McLennan extracted "Factor I," which they attributed to the inhibitory neuronal activity of GABA, and suggested this substance could be a neurotransmitter. This conclusion led to experiments detailing the properties of GABA to determine if it met the many criteria for classification of neurotransmitter, which include; (1) synthesized or present in neurons, (2) released from active neuron and produces response in a target, (3) the same response can be elicited when the chemical is experimentally applied on the target, and (4) a mechanism must be present for removing the chemical from its site of activity (University, S. Marc Breedlove, 2013). These criteria were mostly met in GABA research conducted in crustaceans (Kravitz *et al*., 1963; Otsuka *et al*., 1966). GABA was finally categorized as an inhibitory neurotransmitter in cerebral cortical neurons, which were blocked by bicuculline, a natural alkaloid (Krnjevic and Schwartz, 1967). Endogenous GABA cannot penetrate the blood brain barrier and thus GABA needed for brain function is synthesized in the brain from glutamate utilizing the enzyme L-glutamic acid decarboxylase (GAD), and converted back to glutamate by

the “GABA shunt” (Petroff, 2002) (Figure 1).

Due to its ubiquitous localization throughout the central nervous system, early GABAergic compounds had a generalized effect, thus it was difficult to discern GABA receptor types and subtypes (Florey 1991). It became easier to differentiate between GABAergic receptors with the development of more specific compounds. Initially, there were two types of GABA receptors; the ionotropic GABA-A receptor, and the metabotropic GABA-B receptor (Nistri and Constant, 1979). In the 1980’s a new ionotropic GABA receptor was discovered and termed GABA-C due to its insensitivity to many classical GABA-A and GABA-B receptor ligands, such as, bicuculline and baclofen (Drew *et al*., 1984). Due to its structural and functional similarities with the ionotropic GABA-A receptor, it was later renamed as a subtype of the GABA-A receptor family; the GABA-A ρ receptor (Barnard *et al*., 1998).

GABA RECEPTORS- FROM A to C

GABA receptors can be separated into two classifications: ionotropic and metabotropic. The GABA-A receptor, GABA-B receptor, and more recently discovered GABA-C (GABA-A ρ) receptor vary in both their structural and pharmacological properties. Electrophysiological techniques have been utilized to differentiate and identify between the receptor types and receptor subtypes.

The GABA-B receptor is a G-protein coupled receptor that is coupled indirectly to potassium channels (Geng at al., 2013). Upon activation, these receptors decrease calcium conductance and inhibit cyclic AMP production by utilizing intracellular G proteins (Bowery,

1993). Unlike GABA-A receptors, which are primarily post-synaptic, GABA-B receptors are both pre-synaptic and post-synaptic. Pre-synaptically, GABA-B receptors cause a decrease in calcium influx, reducing the release of neurotransmitters such as glutamate (Ladera *et al*., 2008). While GABA-B receptors are integral in the slow inhibitory response, this dissertation will focus solely on GABA-A receptors, due to their contribution to the inhibitory postsynaptic current (IPSC), diverse functions in the central nervous system, and differential pharmacology.

The GABA-A receptors are classified as members of the Cys-loop ligand-gated ion channel family (Sine and Engel, 2006). This receptor family contains a disulfide bond between two cysteines in the extracellular binding domain (Figure 2). These transmembrane receptors open to allow the influx of ions in response to the binding of a chemical agent (the ligand) (Jansen et al., 2005). GABA binds to the GABA-A receptor, and promotes a conformational change, which opens the pore allowing chloride to pass down its electrochemical gradient (Figure 2) (Rissman *et al*., 2011; Beleli *et al*., 2009).

The direction of the flow of chloride ions determines the activity of the GABA-A receptor. In the neonatal stages of development, the external concentration of chloride is lower than the internal (Li and Xu, 2008). Thus, chloride ions flow out of the cell and GABA-A receptors are depolarizing (Li and Xu, 2008). As the brain develops and matures, the chloride concentration shifts to adult concentrations of promoting the flow of chloride ions into the cell. This causes hyperpolarization of the membrane potential, leading to inhibition and reduction of activity of the neuron in which it is expressed (Herbison and Moenter, 2012). When there is no change in flow of chloride ions, GABA exhibits shunting inhibition. This type of inhibition occurs when the membrane conductance is increased and does not have an effect on the membrane potential,

but instead reduces the probability of neurons firing (Song *et al*., 2011). GABA-A receptors mediate a fast inhibition in the vertebrate brain, while GABA-B receptors mediate a slow, sustained inhibitory response (Sigel *et al*., 2012; Kuffler *et al*., 1958; Hevers *et al*., 1998).

The hyperpolarizing inhibition induced by the GABA-A receptors allows for maintenance of the "I/E balance" (Rissman *et al*., 2011). This term is used to describe the balance or equilibrium state between the inhibitory and excitatory neurons (Rissman *et al*., 2011). If either the inhibitory neurons, such as those containing GABA receptors, or excitatory neurons, such as those containing excitatory glutamate receptors, are altered or modulated, then this balance can be disrupted, resulting in pathologies such as epilepsy, and seizures (Fitschy 2008). During development, the I/E balance establishes its equilibrium state, and thus there are changes in the expression of receptors. During aging, this balance may be disturbed due to subunit expression modulation of the GABA-A receptor.

Functional GABA-A receptors are pentamers; composed of five subunits (Sieghart and Sperk, 2002) (figure2). Each subunit is composed of four transmembrane domains, extracellular N terminal binding domain and C terminal domain, and extracellular and intracellular loops (Mohler 2011). The five subunits form a central pore through which chloride passes (Figure 2). The subtype composition of the GABA-A receptor varies depending on the neuron and tissue in which it is located (Sieghart and Sperk, 2002). These GABA-A receptor subtypes determine many pharmacological aspects of the receptor, such as conductance, chance of opening, and ligand affinity (Mohler, 2011). There have been at least 20 genes encoding GABA-A receptor subunits identified, and each subunit has unique expression and functional properties (Uusi-Oukari and Korpi, 2010) (Figure 3). In humans, there are six α subunits, three of each of the

GABA-A β, γ, and ρ subunits, and only one of each of the GABA-A δ, ε, θ, and π subunits (Olsen and Tobin, 1990) (Figure 3). There are also splice variants within some of the subunit families, but the exact function of these variants is not well understood (Olsen and Tobin, 1990). The receptor may be composed of a number of subunit combinations, but the most common is two α, two β and one γ (Sigel and Steinmann, 2012) (Figure 2). The GABA-A receptor contains many different binding sites within and between the subunits that compose it (Figure 2). Each subtype conveys its own pharmacological profile and is uniquely affected by biological processes.

GABA-A RECEPTOR SUBTYPES-EXPRESSION AND FUNCTION

GABA-A α (1-6)

A majority of the native GABA-A receptors are composed of αβγ subunits (Rissman and Mobley, 2011; Beleli *et al*., 2009) (Figure 2). Previous studies have determined that the α subunit is important in the affinity of many pharmacological compounds (Rissman and Mobley, 2011). There are 6 subtypes of the α subunit (α1-α6) (Korpi *et al*., 1995). Each subtype conveys a unique intrinsic activity to the GABA-A receptor. Studies have determined that the receptors containing the α6 subunit convey a 100- fold increase in selectivity for the diuretic furosemide (Korpi *et al*., 1995).

The benzodiazepine-binding site is located at the interface between the α and the γ subunits (Korpi *et al*., 1995; Loh and Ball, 2000) (Figure 2). This compound has been characterized as an allosteric modulator of GABA-A receptors containing these subunits (McKernan *et al*., 2001). It

increases the conductance of the channel, and only elicits its effects if GABA has already bound the receptor. Mice containing a knockout of the α6 subunit were sensitive to the motor-impairing action of benzodiazepines (Loh and Ball, 2000). These mice had changes in GABA-A receptor expression in the cerebellum, and had an up-regulation of potassium channels (Loh and Ball, 2000).

Point mutations (α1His101Arg) in the α1 subunit resulted in insensitivity of the receptor to benzodiazepines without affecting the GABA sensitivity (McKernan *et al*., 2001). Mice with this mutation lacked the sedative, amnesia and anticonvulsant effects of diazepam, but the anxiolytic and ethanol-potentiating effects were still present, indicating that these properties are conveyed by other α subunits (α2, α3, α5) (McKernan *et al*., 2001). The α5 subunit is highly expressed in the hippocampus and has been found to be involved in learning and memory (Collison *et al*., 2002; Dawson *et al*., 2006; Ballard *et al*., 2008) (Table 1). In fact, the α5 subunit is expressed in 25% of all hippocampal GABA-A receptors (Klausgerger, 2009; Olsen and Sieghart 2009). Mice deficient in the α5 subunit exhibited an increase in learning and memory (Collison *et al*., 2002). This could indicate that this subunit contributes to memory impairments, which increase with age, and are associated with diazepam use.

The α6 subunit was demonstrated to be integral in the effects of the barbiturate pentobarbital, conveying both a higher affinity and efficacy for the direct activation of the GABA-A receptor (Thompson *et al*., 1996). This complex pharmacology remains consistent with non-classical GABA-A ligands, including guanidine compounds. The guanidinoacetic acids are agonists in the presence of heteromeric GABA-A receptors (Neu *et al*., 2002), whereas the same compounds exhibit antagonistic effects on the GABA-A ρ1 receptors (Chebib *et al*., 2009). The

inhibitory effect of the diuretic amiloride, an acid-sensing ion channel antagonist, on GABA-A receptors was first reported in the frog sensory neurons (Inomata *et al*., 1988) and subsequent studies focused on amiloride in GABA-A αβγ receptors showed that the guanidine compound competitively antagonized the receptors with a 10-fold increased potency in GABA-A α6 containing receptors (Fisher, 2002)

GABA-A γ (1-3)

The GABA-A γ subunit is involved in the most common GABA-A receptor configuration (αβγ). There are three GABA-A γ subunits, and previous studies have analyzed the difference in benzodiazepine selectivity in the presence of each. The γ1 is mainly expressed in the substantia nigra, amygdala, the pallidum, the septum, and the thalamus (Korpi *et al*., 2002) (Table 1). Alternative splicing of the pre-mRNA of the γ2 subunit has resulted in γ2L (the long splice variant) and γ2S (the short splice variant) from the one gene (Sarlo *et al*., 2002). This receptor subunit subtype is ubiquitously expressed in the central nervous system, and studies have shown that if it is substituted by either one of the other γ subtypes the sensitivity of GABA-A for benzodiazepine is markedly altered (Hevers and Luddens, 1998). Receptors that express the γ2 subunit may be located either inside of the synaptic cleft, or extra-synaptically (Devor *et al*., 2001; Cariascos *et al*., 2004; Luscher and Keller, 2004). The combination of GABA-A α1β1γ1 receptor was compared to GABA-A α1β1γ2 configuration, and the presence of the γ1 was required for the enhancement of the GABA current by some benzodiazepine analogs, while others, such as flumazenil, which is a benzodiazepine site antagonist, was found to need the γ2 subunit (Khom *et al*., 2006). Electrophysiological studies have also shown that the γ3 subunit can also convey benzodiazepine sensitivity (Knoflash *et al*., 1991). From these studies we can

conclude that all of the γ subunit subtypes are involved in some way in the benzodiazepine sensitivity of the GABA-A receptor.

GABA-A β(1-3)

There are three isoforms of the GABA-A β subunit: β1, β2, and β3 (Chen and Olsen, 2007). The GABA-A β subunit is integral in the expression of the GABA-A receptor, playing a role in the ion selectivity (Jansen *et al*., 2002). Several intracellular proteins that regulate the trafficking and membrane insertion of the GABA-A receptor are capable of interacting with different β isoforms (Herd *et al*., 2008, Brandon *et al*., 2003). Also, the intracellular loop of the β subunit can be phosphorylated by kinases, which influences the overall function of the GABA-A receptor (Kilter and Moss, 2003, Houston *et al*., 2007, Saunders and Ho, 1990). GABA-A receptors composed of solely α and β subunits are said to contain two α and three β subunits (Herd *et al*., 2008). Electrophysiological studies have discovered that these GABA-A αβ receptors are not sensitive to the allosteric modulators such as benzodiazepines, which need the presence of the γ subunit to elicit its effects (Hevers and Luddens, 1998). However, these receptors are sensitive to another allosteric modulator: barbiturates (Sigel and Steinmann, 2012). The binding site for barbiturates is located on the β subunit (Sigel and Steinmann, 2012) (Figure 2).

At low concentrations, these allosteric modulators bind to the GABA-A receptor and potentiate the GABA-A inhibitory response by increasing the duration of opening of the receptor. At high concentrations, these allosteric modulators inhibit the GABA-A receptor (Houston *et al*., 2007). Clinically, barbiturates are utilized as mild sedatives, anxiolytics, hypnotics, and anticonvulsants (Houston *et al*., 2007). These compounds have been largely replaced with

benzodiazepines due to ease of overdose. The muscle relaxer carisoprodol is also an allosteric modulator of the GABA-A receptors and also prone to abuse. Different effects of the compound arise with the presence of different subunits: the GABA-A β1 subunit conveys maximum direct activation, while the β2 subunit conveyed the highest efficacy of allosteric modulation (Kumar *et al*., 2015).

Unlike the subunits mentioned previously, the β subunit is able to form functional homo-oligomeric channels. These receptors are not found endogenously, but exhibit a unique pharmacological profile and are important in understanding the β subunit structure and function (Saunders and Ho, 1990). These homo-oligomeric channels composed solely of the β3 subunit are constitutively open, and require an antagonist, such as picrotoxin, to be applied beforehand to elicit a GABA response (Saunders and Ho, 1990). In 2014, the structure of the homomeric human GABA-A β3 receptor was solved, the first x-ray crystal structure of the GABA-A receptor family (Miller and Aricescu, 2014). This discovery could lead to a better understanding of the GABA-A receptor function, and open the door for the crystallization and better understanding of other GABA-A receptor subunit compositions.

GABA-A $\delta,\varepsilon,\theta,\pi$

GABA-A receptors containing the δ subunit exhibit many common characteristics. These receptors, most commonly a combination of αβδ, are not located in the post-synaptic terminal, most prominently located extra synaptically or pre-synaptically. Due to their location and the fact they these receptors are highly sensitive to GABA, they are proposed to interact with "spill over" GABA, GABA that failed to interact with the receptors on the post-synaptic cleft and thus is lose

in the synapse. The GABA-A receptors containing the δ subunit also show little desensitization, and convey a tonic inhibition (Zheleznova *et al*., 2009).

The GABA-A ε and θ subunits are expressed in very specific nuclei, including the bilateral brainstem nucleus locus coeruleus (Sinkkonen *et al*., 2000) (Table 1). These two genes are clustered with the GABA-A α3 subunit, suggesting common ancestry (Kopri *et al*., 2002; Russek, 1999) (Figure 3). Sequence homology has shown that the θ subunit is most closely homologous to the GABA-A β1 subunit (Sinkkonen *et al*., 2000). Little pharmacological and functional information is known about GABA-A θ subunit because combinations with other subunits have been difficult to determine and the combination of the αβθ subunit was insensitive to GABA (Bonnet *et al*., 1999). The GABA-A ε subunit is most closely related to the γ subunit. (Figure 3) GABA-A Receptors containing the GABA-A ε subunit are insensitive to benzodiazepines and display a reduced efficacy when exposed to anesthetics (Davies *et al*., 1997). They also have spontaneous channel opening and desensitize more rapidly than GABA-A receptors lacking the ε subunit (Wagner *et al*., 2005; Thompson *et al*., 2002).

The GABA-A π subunit was initially discovered in 1997, and shares 30-40% similarity to other members in the GABA-A receptor family (Russek, 1999). It has been found in many different tissues including the lung, thymus and prostate, and can co-localize with a variety of other GABA-A receptor subunits (Table 1). Most notably, it was detected in uterus, where it can combine with known GABA-A receptor subunits and decrease the sensitivity to modulatory agents such as neurosteroids (Hedblom and Kirkness, 1997).

GABA-A ρ (1-3)

There are three GABA-A ρ subunits: GABA-A ρ1, ρ2, and ρ3, named in order of discovery (Figure 3). The GABA-A ρ subunit is the most recently cloned GABA-A subunit, originally classified as the GABA-C receptor. While it is structurally similar to the other subunits, it has unique pharmacological profiles (Olsen and Sieghart, 2009). Like the β subunit, it forms functional homo-oligomers, but unlike the β subunit, the homo-oligomers are endogenously expressed (Olsen and Sieghart, 2009, Reyes-Haro *et al*., 2013, Qian, 2000). Each subunit only forms functional receptors with other ρ subunits or with itself as a homo-oligomer (Reyes-Haro *et al*., 2013, Qian, 2000). There is evidence that the GABA-A ρ1 subunit can form functional pentamers with the γ2 subunit (Qian and Ripps, 1999).

The GABA-A ρ receptor family has unique expression patterns. This subunit is mainly expressed on retinal bipolar and horizontal cells, but this receptor has been found in other regions of the body (Olsen and Sieghart 2009, Qian, 2000). Studies have shown that it is also expressed in the CA1 region of the hippocampus, and has some implications in learning and memory (Wegelius *et al.*, 1998) (Table 1). Research investigating the influence of GABA on the chick bead discrimination concluded that the GABA-A and the GABA-A ρ receptors had opposite effects on this process. Bicuculline, the GABA-A antagonist inhibited strongly reinforced memory, while TPMPA and P4MPA, selective GABA-A ρ antagonists, enhanced weakly reinforced memory, suggesting that these receptors have opposite effects on short-term memory (Gibbs and Johnston, 2005). Application of GABA-A ρ antagonist facilitated learning and memory in a mouse behavioral model, suggesting its activity prevents learning and memory (Chebib *et al*., 2009). Additionally, studies utilizing reverse transcriptase-polymerase chain reaction have determined all three GABA-A ρ receptor subunits are expressed in the

gastrointestinal tract of rats, and the effects of GABA on intrinsic sensory and the inhibition of motor neurons in this area are likely partly influenced by the GABA-A ρ receptor (Fletcher *et al*., 2001).

The unique desensitization properties of the GABA-A ρ receptor makes it a possibly important target for pharmaceutical intervention for pathologies in which it may play a role. For example, it has been found in the amygdala, more specifically the lateral part of the central nucleus and the lateral amygdala where it has been implicated in the mechanism of fear anxiety (Delaney and Sah, 1999, Cunha *et al*., 2010). In the lateral amygdala, inhibition of both GABA-A and GABA-B receptors resulted in reduction of evoked inhibition and increase in excitation, while activation of the GABA-A ρ receptor resulted in an increase in excitation, inhibition resulting in the opposite (Cunha *et al*., 2010). Similar to the activity of the GABA-A and GABA-A ρ receptors in short-term learning, the two receptor types play opposing roles in fear learning in the amygdala, thus GABA-A ρ receptor compounds could be utilized to treat anxiety (Cunha *et al*., 2010).

In addition to having different effects on brain processes, the GABA receptors have different pharmacology. One of the hallmarks of the GABA-A ρ subunit is insensitivity to many GABA-A and GABA-B receptor ligands, such as bicuculline and baclofen (Hevers and Luddens, 1998) (Table 2). It is 10-fold more sensitive to GABA binding, and is also not modulated by barbiturates, or benzodiazepines (Qian, 2000). Many of the classical GABA-A ligands are inactive at the GABA-A ρ1 receptor (Table 2). Barbiturates do not influence GABA-A ρ receptors in the, requiring a single point mutation to confer the sensitivity (Amin, 1999).

Neurosteroids have been reported to differentially modulate the GABA-A homomeric and heteromeric receptors (Morris *et al.*, 1999). Studies have shown that neurosteroids such as allopregnanolone interact with two separate sites on the GABA-A heteromeric receptors; a direct activation site formed by a cavity in α subunit transmembrane domains, where it elicits potentiation of the GABA evoked current, and a second site formed by the interface between the α and β subunits, where it elicits direct activation (Hosie *et al.*, 2006). Studies have also characterized the intrinsic activity of neurosteroids on the GABA-A ρ1 receptor. As seen in the heteromeric GABA-A receptors, allopregnanolone potentiates the GABA-evoked current, but also prolonged the current's decay time (Hosie *et al*., 2006).

Other neurosteroids, such as pregnanolone sulfate, inhibit the GABA-evoked current. This study hypothesized that the differential activity was conveyed by the position of the hydrogen atom attached to the fifth carbon of the neurosteroid (Morris *et al.*, 1999). Unlike the GABA-A heteromeric receptors, none of the neurosteroids directly activated the GABA-A ρ1 receptor (Morris *et al.*, 1999). Kinetic analysis of this receptor has indicated that unlike the fast acting GABA-A receptors, this receptor is slow to initiate and has a sustained response, much like that seen in the GABA-B receptors (Qian, 2000). This quality is utilized in the retina to regulate other GABA-A receptor activity.

GABA-A AND THE RETINA

In retinal bipolar cells, GABA-A ρ receptors help maintain axon terminal excitability (Matthews *et al*., 1994). In response to GABA, GABA-A ρ receptors mediate tonic inhibitory

current, which is controlled locally by GAT-1 transporters in the amacrine cells (Hull *et al*., 2006; Jones and Palmer, 2009) (Figure 4). Similar pharmacological profiles between endogenous GABA-A ρ receptors and transiently expressed ρ subunits suggest retinal GABA-A ρ receptors are composed of homomeric ρ1 subunits (Amin *et al*., 1994; Qian and Dowling, 1993). Subsequent studies, however, have revealed differences in homomeric GABA-A ρ1 receptor and retinal GABA-A ρ receptors, suggesting the subunit composition might be more complex than previously thought (Qian and Ripps, 1999). Rodent studies have suggested the subunit composition is mainly composed of a combination ρ1 and ρ2 subunits (Enz *et al*., 1995, Yeh at al., 1996).

There are two stages of inhibition in the retina. The first initial inhibitory synaptic interactions take place in the outer plexiform layer (OPL) and are mediated by horizontal cells. The second inhibition is mediated by amacrine cells and occurs in the inner plexiform layer (IPL), and this lateral inhibition is conveyed by GABA receptors (Cook and McReynolds, 1998) (Figure 4). All three GABA receptors are located in the IPL, but in the mammalian retina, only the ionotropic GABA-A receptors are located on retinal bipolar cells (Shen and Slaughter, 2001, Koulen *et al*., 1997) (Figure 5). Knockout studies have shown that without the GABA-A ρ receptor, ganglion light response, the inner retinal function, and light-evoked inhibitory synaptic inputs are all altered (Eggers and Lukaiewicz, 2004, McCall *et al*., 2002, McCall and Sagdullaev, 2003).

The subcellular localization of the ionotropic GABA-A receptors is also different. The GABA-A ρ receptors are found mainly in the axon terminal, while the GABA-A receptors are located on the dendrites and presynaptic terminals (McCall *et al*., 2002, Lukasiewicz *et al*.,

1994). The GABA-A αβγ receptors are also expressed at different synapses than the GABA-A ρ receptor (Koulen *et al*, 1998). In bipolar cells, the GABA-A ρ receptor expressed at cell axons is predominately located in the synapse, while GABA-A ρ receptors on the dendrites are mainly located extrasynaptically (Wassle *et al*., 1998). Studies have also shown the subcellular localization of the GABA-A αβγ receptors and the GABA-A ρ receptor changes depending of the illumination state of the retinal bipolar cells (Du and Yang, 2000). OFF bipolar cells (BC), which are active in dark environments possess a higher concentration of GABA-A ρ receptors at the axon terminal, whereas ON BCs have higher concentrations of GABA-A αβγ receptors and a lower concentration of GABA-A ρ receptors at the axon terminal (Figure 5). In light environments, there is a decrease in the release of GABA from horizontal and amacrine cells, and activation of the ON BCs. The presence of the GABA-A ρ receptor, which has a 10-fold increase in sensitivity to GABA as compared to the GABA-A αβγ receptor, leads to inhibition at low levels of GABA (Du and Yang, 2000). Retinal hypoxic disorders cause a change in the expression of the GABA-A receptors, resulting in deregulated inhibition in the retina.

Diabetic retinopathy is the most common diabetic eye disease and can result in complete vision loss in diabetic patients. As the disease progresses the retina turns hypoxic, resulting in a dysfunction of γ-aminobutyric acid (GABA) receptors. Studies have shown conflicting results regarding to the involvement of the GABA-A ρ receptor in the pathology of diabetic retinopathy. Studies have shown that even though rats with diabetes exhibit a decrease in overall expression of retinal GABAA-ρ receptors, there is an increase in the ratio of ρ1 to ρ2 subunit mRNA. This increase leads to increased sensitivity to GABA and an increase in vascular permeability, which may lead to retinal vascular leakage that causes edema in the macula and loss of vision (Ramsey

2008) Other studies have shown that completely removing the GABA-A ρ1 receptor through a subunit-knockout mouse resulted in hypoxia in the retina and severe vascular leakage (Zheng *et al*., 2010).

Several pharmacological interventions have been proposed in an attempt to treat the pathologies of diabetic retinopathy. The use of the compound 5-methyl-I4AA, a selective GABA-ρ agonist was proposed in an attempt to suppress the neural activity of the inner retina, and reestablish normal neurovascularization, thus relieving the ischemia induced by the onset of diabetic retinopathy (Qian and Ripps, 2011). If, as proposed, there is a difference in subunit composition upon ischemia, application of an agonist that interacts with all subtypes could be detrimental to the retina and further perturb the pathology. In 2013, the guanidine compound amiloride was proposed as a pharmacological intervention to relieve retinal degeneration in ischemic disorders such as diabetic retinopathy through the antagonism of retinal acid sensing ion channel (ASIC) receptors. They concluded that 100 μM amiloride reduced retinal degeneration. Inexplicably, amiloride at higher concentrations led to a worsening of retinal degeneration (Miyake at al., 2013).

GABA-A AND GUANIDINE COMPOUNDS

The observed mixed pharmacology extends to endogenous guanidine compounds, which have exhibited different modulatory effects on the heteromeric GABA-A receptors than GABA-A ρ1 receptor (Neu *et al*., 2002; Chebib *et al*., 2009). In the inherited disorder guanidinoacetate methyltransferase deficiency, there is a build-up of guanidinoacetate (Neu *et al*.,

2002). This is the immediate precursor to creatine and can directly activate the heteromeric GABA-A receptors (Neu *et al*., 2002). While guanidinoacetic acid acts as an agonist in the heteromeric GABA-A receptors, this compound antagonizes the GABA-A ρ1 receptor (Chebib *et al*., 2009). Furthermore, guanidine compounds that modulate the acid-sensing ion channel influence Cys-loop receptor activity. In the inhibitory glycine receptor, amiloride exhibited competitive antagonism in receptors expressed in rat spinal neurons and the inferior colliculus (Li *et al*., 2003a; Li *et al*., 2003b; Tang *et al*., 2006). Furthermore, a recent report outlined the antagonistic effects of the guanidine compound 2-guanidine-4-methylquinazoline (GMQ) on the heteromeric GABA-A αβγ receptors (Xiao *et al*., 2013). Many aspects of the guanidine compound interaction with the GABA-A receptors remain unexplored. These aspects include exploring the activity of amiloride and its derivatives, along with GMQ on the human GABA-A ρ1 receptor, where on the GABA-A ρ1 receptor these compounds are interacting, and whether the guanidine group is important in conveying this activity.

SPECIFIC AIMS

The effect of guanidine compounds such as amiloride and GMQ on the GABA-A ρ1 receptor still remains unknown. Amiloride is classified as an acid sensing ion channel blocker, but previous studies focusing on amiloride in GABA-A αβγ receptors showed the compound was a competitive antagonist with a 10-fold increased potency in the GABA-A α6 containing receptors (Fisher 2002). Additionally 2-guanidine-4-methylquinazoline (GMQ), an acid sensing

ion channel subtype 3 agonist, also has antagonistic effects on the GABA-A αβγ receptors (Xiao *et al*., 2013). While the activity of these compounds have been investigated in the GABA-A αβγ receptors, the differential pharmacology and unique properties of the GABA-A ρ1 receptor suggest that the activity of these compounds could be different on the GABA-A ρ receptor family (Table 2).

GABA-A ρ1 displays insensitivity to bicuculline/baclofin, heteromeric GABA-A antagonists, and is also insensitive to allosteric modulators for the GABA-A receptors such as benzodiazepines and barbiturates (Korpi *et al*., 2002) (Table 2). Selective antagonists such as TPMPA affect GABA-A ρ1 receptors, while having no effect on other GABA-A receptors. Picrotoxin and zinc can antagonize both the heteromeric and homomeric GABA-A receptors (Dong and Werblin, 1996). Key residues at the 6' and 15' positions in the second transmembrane domain of the GABA-A receptors have been shown to convey the antagonistic effect of picrotoxin, and the modulatory effect of barbiturates (Xu *et al*., 1995; Dibas *et al*., 2002). Where guanidinoacetic acid acts as an agonist in the heteromeric GABA-A receptor, this compound antagonizes the GABA-A ρ1 receptor (Chebib *et al*., 2009). Based on this differential pharmacology, we hypothesize that the guanidine compounds would act as agonists for the GABA-A ρ1 receptor. Furthermore, we hypothesize that two aspects of the receptor and compound are integral in production of this activity; specific non-conserved amino acids in the second transmembrane domain of the GABA-A ρ1 receptor, and accessibility to the guanidine group on the guanidine compound.

We chose to test this hypothesis with three specific aims:

1. Characterize the intrinsic activity of guanidine compounds such as amiloride and GMQ

on the GABA-A ρ1 receptor utilizing whole cell patch-clamp electrophysiology.

2. Determine the location of the modulatory site in the GABA-A ρ1 integral in the activity of the guanidine compounds receptor by utilizing site-directed mutagenesis of the second transmembrane domain.
3. Assess the importance of guanidine compound accessibility in the modulation by utilizing amiloride derivatives.

SIGNIFICANCE

According to the National Eye Institute, diabetic retinopathy is the most common diabetic eye disease and can result in complete vision loss in diabetic patients. As the disease progresses the retina turns hypoxic, resulting in a dysfunction of γ-aminobutyric acid (GABA) receptors. The progression of the disorder leads to the need for retinal laser surgery to attempt to restore vision, but there is no cure for this disease. The GABA-A ρ1 receptor is located on the axonal terminal of retinal bipolar cells, where it not only exhibits a tonic inhibitory current, but also regulates the GABA-A and other GABA-A ρ synaptic currents (Jones *et al*., 2009). Even though rats with diabetes exhibit a decrease in overall expression of retinal GABA-A ρ receptors, there is an increase in the ratio of ρ1 to ρ2 subunit composition. This increase leads to increased sensitivity to GABA and an increase in vascular permeability, which may lead to retinal vascular leakage that causes edema in the macula and loss of vision (Ramsey 2008).

In 2013, amiloride was proposed as a pharmacological intervention to relieve retinal degeneration in ischemic disorders such as diabetic retinopathy through the antagonism of retinal ASIC receptors. This study concluded that 100 μM amiloride reduced retinal degeneration. Inexplicably, amiloride at higher concentrations led to a worsening of retinal degeneration (Miyake at al., 2013). This worsening in retinal degeneration could be due to activation of the GABA-A ρ1 receptor, which as described earlier, is already overactive in this ischemic state.

These findings have implications in the GABA-A receptor field, as it will contribute to the development of novel therapeutics that influences the actions of the GABA-A ρ1 receptor. Furthermore, others in the scientific community would be interested as it would indicate another use for a classic drug (amiloride) and to make others aware that modulators of unrelated ion channels (e.g., ASICs) can have greater implications on other ion channel targets.

FIGURE LEGENDS AND CORRESPONDING FIGURES

Figure 1. Neuronal synthesis of γ-aminobutyric acid (GABA). In neurons, glutamine is converted into glutaminate utilizing the enzyme glutaminase. Glutamate is then converted to GABA, a reaction catalyzed by the enzyme glutamate-decarboxylase (GAD). GABA is then packaged into vesicles and released from the neuron via the vesicular inhibitory amino acid transporter (VIAAT), also termed the vesicular GABA transporter for VGAT. GABA can then cross the synaptic cleft and interact with ionotropic or metabotropic GABA receptors located on the post-synaptic cleft. The GABA transporter (GAT) allows for two possible re-uptake mechanisms: (1) GABA that does not interact with the post-synpaptic cleft can be taken up by the pre-synaptic neuron and repackaged into vesicles, or (2) GABA is taken up by glial cells and then converted into glutamine that can re-enter the neuron and be converted back to GABA. *Figure adapted from D'Hulst and Kooy, 2007.*

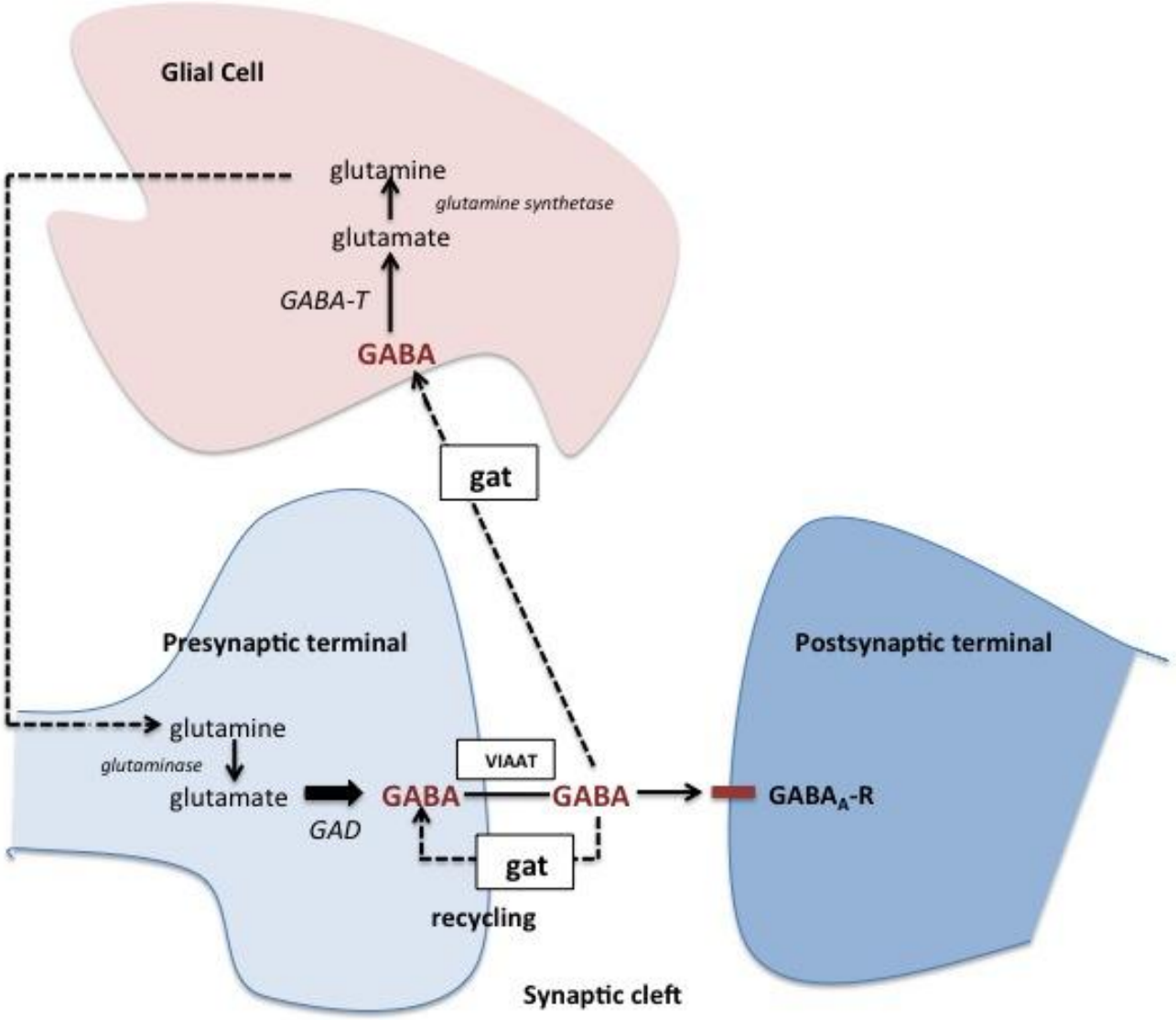

Figure 1. Neuronal synthesis of γ-aminobutyric acid (GABA). ***Figure adapted from D'Hulst and Kooy, 2007.***

Figure 2. GABA-A subunit and receptor composition, and binding sites of classical GABA-A αβγ receptor ligands. (A) Schematic structure of a GABA-A monomeric subunit imbedded in a lipid bilayer (black lines connected by blue circles). Each subunit is composed of four transmembrane α-helices (1-4 depicted as cylinders). All Cys-loop receptors contain a disulfide bond in the extracellular N-terminus (red line). (B) Five subunits, most prominently two α, two β, and one γ GABA-A subunit, compose a functional GABA-A receptor. These five subunits arrange symmetrically to form a central pore through which chloride ions pass. Ligand binding sites are depicted with colored circles. Picrotoxin binds inside the pore, occluding it and preventing the passage of chloride ions. *Figure adapted from Mohler, 2011*.

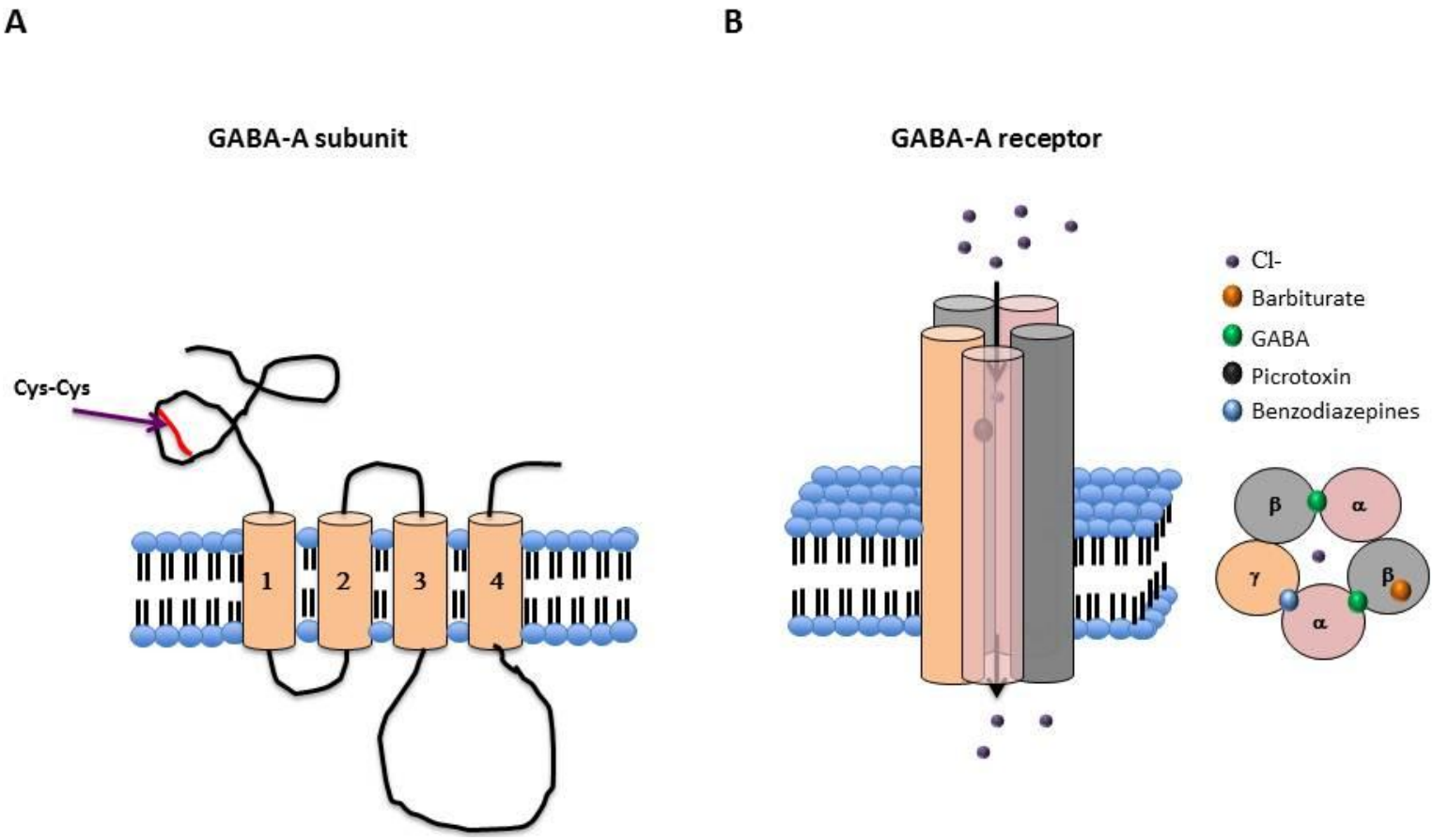

Figure 2. GABA-A subunit and receptor composition, and binding sites of classical GABA-A αβγ receptor ligands. ***Figure adapted from Mohler, 2011*****.**

Figure 3. Phylogenetic tree analysis of 19 genes coding for human GABA-A receptor subunits. Subunits with common chromosomes are color coded corresponding to the chromosome of origin. Note that subunits that share the same origin in the tree can come from different chromosomes. *Figure adapted from Sigel and Steinmann, 2012*

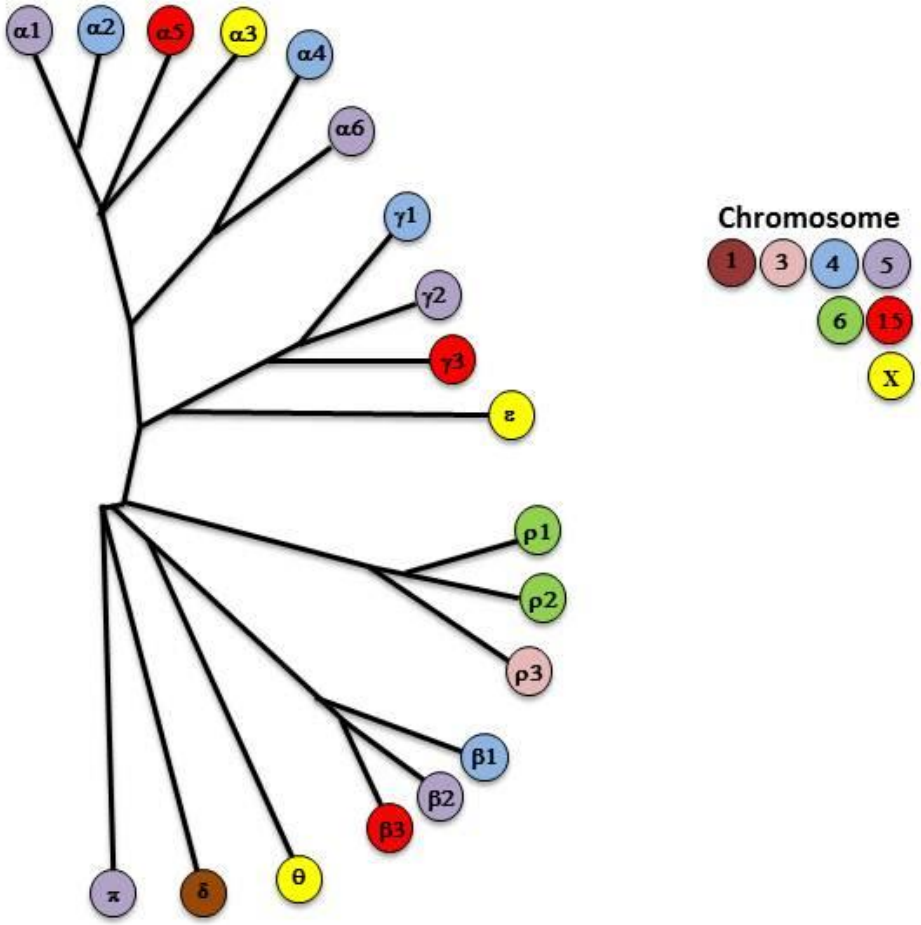

Figure 3. Phylogenetic tree analysis of 19 genes coding for human GABA-A receptor subunits. ***Figure adapted from Sigel and Steinmann, 2012***

Figure 4. Organization of retinal layers and cellular localization of GABAergic neurons in the retina. Retina is composed of three laminar layers: Outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). There are six different neuronal cell types and one glial cell type within these layers. Horizontal and amacrine cells are GABAergic neurons, whose cell bodies are predominantly located in the INL and dendrites project into the outer and inner plexiform layers. *Figure from Yang, 2004*.

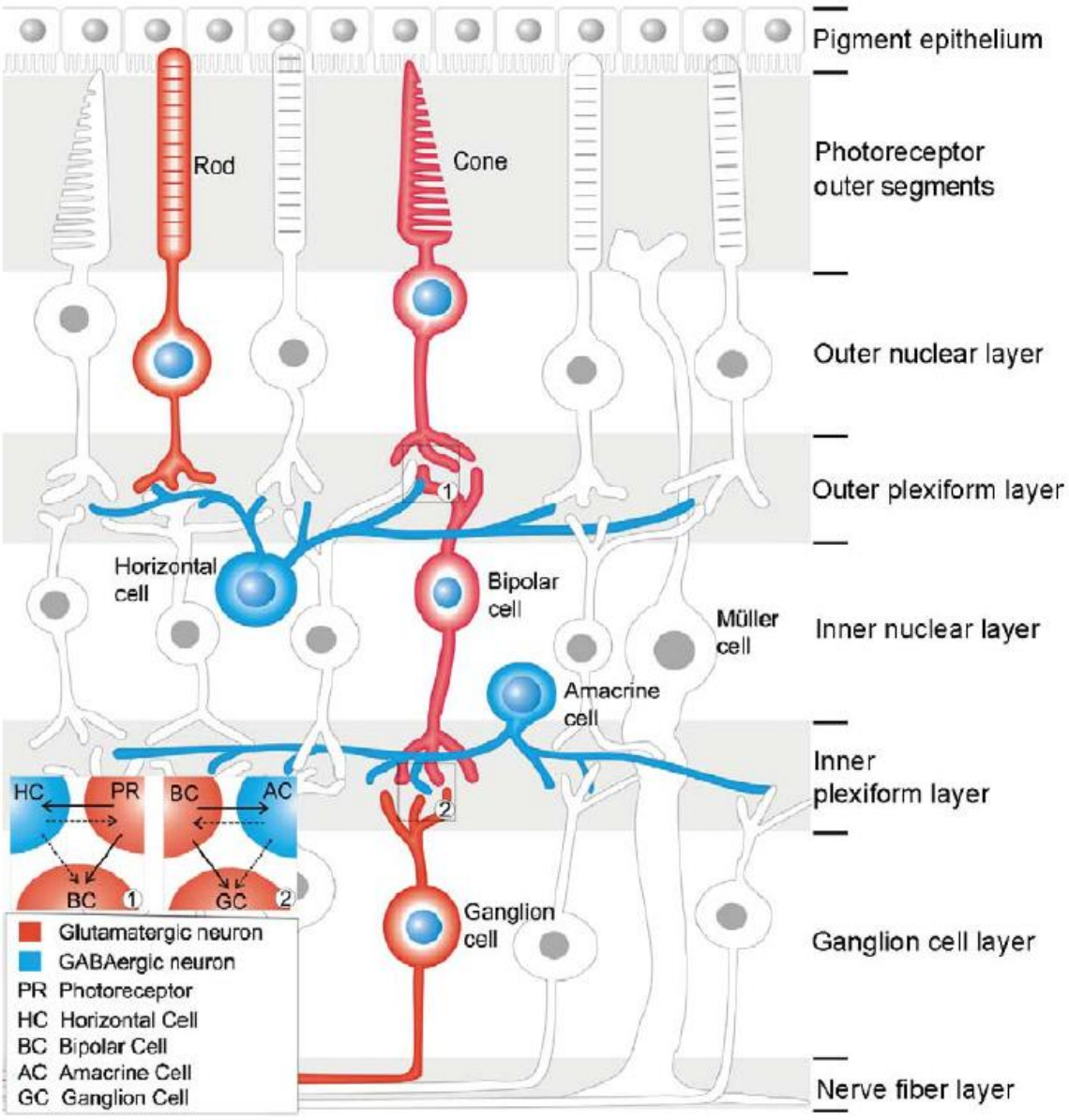

Figure 4. Organization of retinal layers and cellular localization of GABAergic neurons in the retina. ***Figure from Yang, 2004.***

Figure 5. Subcellular localization of GABA-A αβγ receptors and GABA-A ρ receptors in light and dark environments. Schematic of distribution of GABA-A receptors on ON and OFF biopolar cells (BC). OFF bipolar cells are activated in dark environments and states of low illumination. GABA release from horizontal and amacrine cells is decreased in the dark, thus there is a high concentrations of GABA-A ρ receptors, which have a 10-fold increase in GABA sensitivity. Therefore there is still inhibition at lower levels of GABA. *Figure adapted from Du and Yang, 2000.*

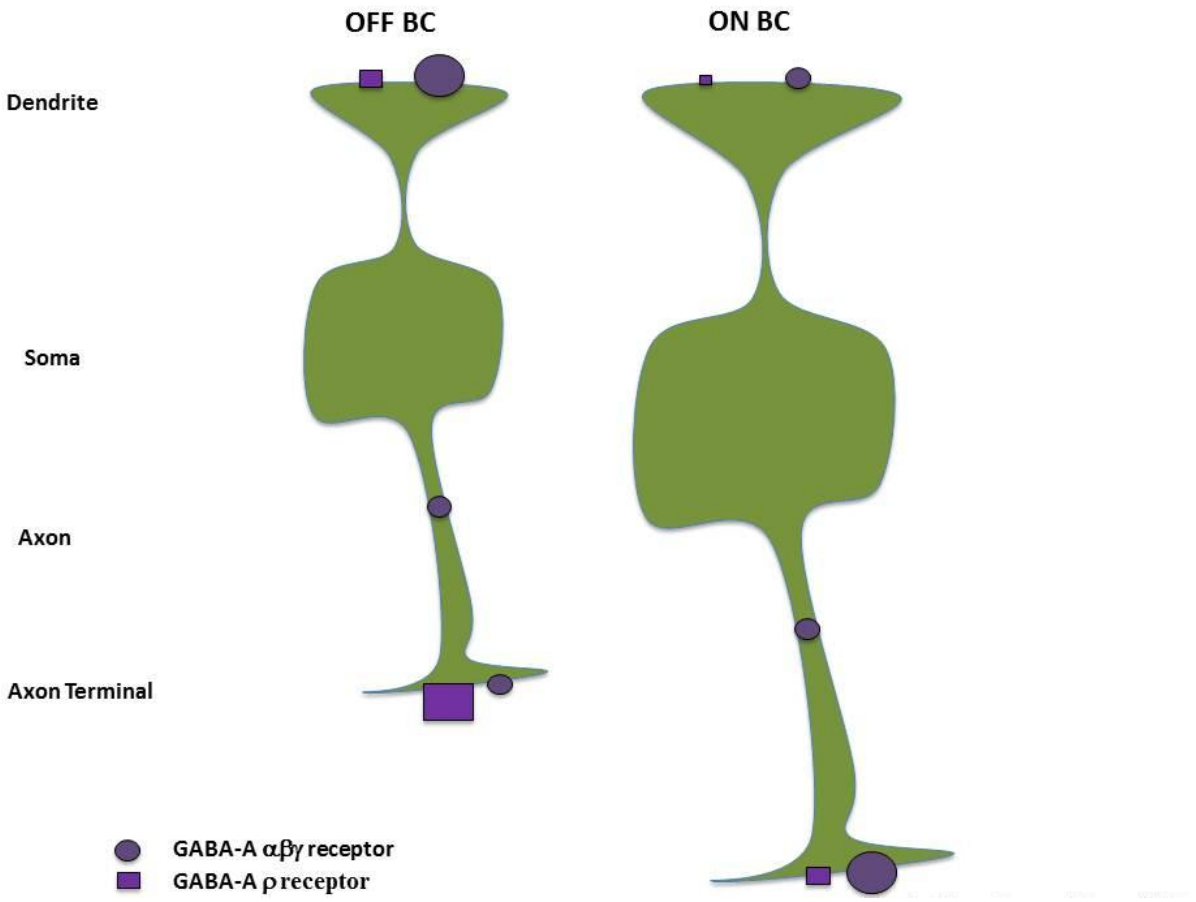

Figure 5. Subcellular localization of GABA-A αβγ receptors and GABA-A ρ receptors in light and dark environments. ***Figure adapted from Du and Yang, 2000.***

Table 1. Differential expression of the GABA-A receptor subunits and their implications in disorders throughout the human CNS.

Brain Region/Tissue	Disorder/Symptoms	GABA-A-receptor subunit
Hippocampus	Memory and learning disruption	α5, δ, ρ1
Hippocampus; amygdala	Anxiety and mood disruption	α2, α4, β3, δ, ρ1
Wide Spread	Fatigue, sedation, and exhaustion	α1
Hippocampus, amygdala,	Stressed induced depression	α1, α3, β1, β3, δ, γ2
Hypothalamus	Eating disorders	α3
Retina	Diabetic retinopathy	ρ1, ρ2, ρ3
Substantia Nigra	Huntington's disease	β2, β3, γ1
Nucleus locus coeruleus	Sleep disorders	ε, θ
Lung	Asthma, chronic obstructive lung disease,	π

Table 2. Differential modulation of the GABA-A ρ receptor and the heteromeric GABA-A αβγ receptors.

Compound	GABA-A αβγ	GABA-A ρ
GABA	Agonist	Agonist
Muscimol	Agonist	Partial Agonist
Isoguvacine	Agonist	Partial agonist
THIP	Agonist	Antagonist
TACA	Agonist	Agonist
P4S	Agonist	Antagonist
CACA	Inactive	Partial Agonist
Bicuculine	Antagonist	Inactive
Barbiturates	Allosteric modulator	Inactive
Benzodiazepines	Allosteric modulator	Inactive
TPMPA	Inactive	Antagonist
Picrotoxin	Blocker	Blocker
Guanidinoacetic acids	Agonist	Antagonist

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II. AMILORIDE AND GMQ ALLOSTERIC MODULATION OF THE GABA-A ρ1 RECEPTOR: INFLUENCES OF THE INTERSUBUNIT SITE.

Heather D. Snell and Eric B. Gonzales

Affiliations

Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Boulevard, Fort Worth, 76107, USA (H.D.S., E.B.G.); Institute for Aging and Alzheimer's Disease Research, University of North Texas Health Science Center, Fort Worth, 76107, USA (E.B.G); Cardiovascular Research Institute, University of North Texas Health Science Center, Fort Worth, 76107, USA (E.B.G).

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Corresponding Author:

Eric B. Gonzales, Ph.D.

Assistant Professor

Department of Pharmacology & Neuroscience

University of North Texas Health Science Center

3500 Camp Bowie Blvd.

Fort Worth, TX 76107

Phone: 817-735-2755

Fax: 817-735-0408

Email: eric.b.gonzales@unthsc.edu

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Nonstandard abbreviations:

GABA: γ-aminobutyric acid

TM: transmembrane domain

Amil: amiloride

ASIC: acid-sensing ion channel

GMQ: 2-guanidine-4-methylquinazoline

EGTA: ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

TEA-Cl: tetraethylammonium chloride

DMSO: dimethylsulfoxide

GLIC: *Gloeobacter violaceus* pentameric ligand-gated ion channel

PAM: positive allosteric modulation

ABSTRACT

Amiloride, a diuretic used in the treatment of hypertension and congestive heart failure, and 2-guanidine-4-methylquinazoline (GMQ), are guanidine compounds that modulate acid-sensing ion channels. Both compounds have demonstrated affinity for a variety of membrane proteins, including members of the Cys-loop family of ligand-gated ion channels, such as the heteromeric GABA-A αβγ receptors. The actions of these guanidine compounds on the homomeric GABA-A ρ1 receptor remains unclear, especially in light of how many GABA-A αβγ receptor modulators have different effects in the GABA-A ρ1 receptors. We sought to characterize the influence of amiloride and 2-guanidine-4-methyquinazoline (GMQ) on the human GABA-A ρ1 receptors using whole-cell patch clamp electrophysiology. The diuretic amiloride potentiated the human GABA-A ρ1 GABA-mediated current, while GMQ antagonized the receptor. Furthermore, a GABA-A second transmembrane domain site, the intersubunit site, responsible for allosteric modulation in the heteromeric GABA-A receptors, mediated amiloride's positive allosteric actions. In contrast, the mutation did not remove GMQ antagonism but only changed the guanidine compound's potency within the human GABA-A ρ1 receptor. Through modeling and introduction of point mutations, we propose that the GABA-A ρ1 intersubunit site plays a role in mediating the allosteric effects of amiloride and GMQ.

INTRODUCTION

γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain, and targets the ionotropic GABA-A receptors, members of the Cys-loop receptor superfamily (Olsen and Sieghart, 2008). GABA-A receptors can be found as homomeric or heteromeric pentamers, consisting of a combination of α, β, γ, δ, ε, π, ρ, and θ subunits. Of these, the GABA-A ρ1 receptor can form functional homomeric anion-selective ion channels found in the retina (Jones and Palmer, 2011; Ng *et al*., 2011). Furthermore, the GABA-A ρ1 receptor has a distinct pharmacological profile than the heteromeric GABA-A receptors that complicates studies that focus on characterizing the receptor's role in brain function. The GABA-A ρ1 receptor is insensitive to modulators of the heteromeric GABA-A receptor such as bicuculline, benzodiazepines, and barbiturates (Korpi *et al*., 2002) and the selective antagonist TPMPA inhibits GABA-A ρ1 receptors, which has no effect on heteromeric GABA-A receptors. Picrotoxin and zinc can antagonize both the heteromeric and homomeric GABA-A receptors (Dong and Werblin, 1996). Key residues at the 6' and 15' positions in the second transmembrane domain of the GABA-A receptors have been shown to convey the antagonistic effect of picrotoxin (Xu *et al*., 1995; Dibas *et al*., 2002).

The observed mixed pharmacology extends to endogenous guanidine compounds, which have exhibited different modulatory effects on the heteromeric GABA-A receptors than GABA-A ρ1 receptor (Neu *et al*., 2002; Chebib *et al*., 2009). In the inherited disorder guanidinoacetate methyltransferase deficiency, there is a build up of guanidinoacetate, the immediate precursor to creatine and can directly activate the heteromeric GABA-A receptors (Neu *et al*., 2002). Where guanidinoacetic acid acts as an agonist in the heteromeric GABA-A receptors, this compound antagonizes the GABA-A ρ1 receptor (Chebib *et al*., 2009). Furthermore, guanidine compounds

that modulate the acid-sensing ion channel influence Cys-loop receptor activity. The inhibitory effect of the diuretic amiloride, an acid-sensing ion channel antagonist, on GABA-A receptors was first reported in the frog sensory neurons (Inomata *et al*., 1988) and subsequent studies focused on amiloride in GABA-A αβγ receptors showed that the guanidine compound competitively antagonized the receptors with a 10-fold increased potency in GABA-A α6 containing receptors (Fisher, 2002). In the inhibitory glycine receptor, amiloride exhibited competitive antagonism in receptors expressed in rat spinal neurons and inferior colliculus (Li *et al*., 2003a; Li *et al*., 2003b; Tang *et al*., 2006). Furthermore, a recent report outlined the antagonistic effects of the guanidine compound 2-guanidine-4-methylquinazoline (GMQ) on the heteromeric GABA-A αβγ receptors (Xiao *et al*., 2013). The actions of both amiloride and GMQ in the human GABA-A ρ1 receptor remain unclear.

Here, we have characterized the activities of amiloride and GMQ on the human GABA-A ρ1 receptors transiently expressed in HEK293T cells using whole-cell patch-clamp electrophysiology. Amiloride potentiated GABA-mediated current in a concentration-dependent manner. Due to this potentiation and based on modeling amiloride interaction with a bacterial Cys-loop receptor, we introduced mutations within the channel's second transmembrane (TM) domain to probe which site, the TM2 domain 6', 15', and 19' residues, was responsible for amiloride's actions. A mutation at the 15' residue, within the receptor's intersubunit site, suggested that amiloride and the other guanidine compound, GMQ, interact with the receptor at this site. We propose that these guanidine compounds modulate GABA-A ρ1 receptor activity through the receptor's intersubunit site like other Cys-loop receptor allosteric modulators. This

suggests that these guanidine compounds can be used as the template in the design human GABA-A ρ1 receptor allosteric modulators.

Materials and Methods

Cell Culture and Expression of human GABA-A ρ receptors

Wild type and mutant hGABA-A ρ1 receptors cDNA was co-transfected in human embryonic kidney cells containing the SV40 T-antigen (HEK-293T, ATCC Manassas, Virginia). The HEK293T cell line was maintained in T25 flasks at 37° C in a 5% CO_2 water-jacketed incubator and cultured in Dubelcco's Modified Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (Phenix Research) and 1% penicillin-streptomycin (Cellgro, Manassas, Virginia). Upon reaching 80% confluency, HEK293T cells were plated on glass coverslips 2-4 hours in preparation for transfection. Cells were co-transfected with pNEGFP-EU (2 μg) and human GABA-A ρ1 cDNA (2 μg) were mixed with 5-6 μl TransIT-293 transfection reagent (Mirus Bio, LLC, Madison, Wisconsin), according manufacture's instructions. Whole cell patch-clamp electrophysiology was performed 18-24 hours following transfection.

Site-Directed Mutagenesis of human GABA-A ρ receptor

Human GABA-A ρ1 cDNA, subcloned in pCDNA3.1 was a generous gift from Glenn Dillon (West Virginia University). Enhanced green fluorescent protein (EGFP) cDNA (in the pNGFP-EU mammalian expression vector) was a kind gift from Eric Gouaux (Vollum Institute, Portland OR). QuikChange Lightning Site-Directed Mutagenesis kit (Agilent) was utilized to perform mutations at the 6', 15', and 19' residues of the GABA-A ρ1 receptor. After PCR,

mutated DNA was ligated into plasmid cDNA3.1 for expression in human embryonic kidney cells.

Electrophysiology:

Borosilicate glass capillary tubes (Sutter Instrument Company, Novato, CA) were pulled to a resistance of 8-12 MΩ using a pipette puller (P-87 pipette puller, Sutter Instrument Co, Novato, CA). Recording patch electrodes were filled with internal solution consisting of (in mM): CsCl (120), TEA-Cl (20), $CaCl_2$ (1), $MgCl_2$ (2) EGTA (11), HEPES (10) and adjusted to pH 7.2 using N-methyl-d-glucamine (Chebib *et al*., 2009). Cells were perfused with external solution containing (in mM): NaCl (137), KCl (5.4) $CaCl_2$ (1.8) $MgCl_2$ (1) HEPES (5), adjusted to pH 7.4 (Chebib *et al*., 2009). Amiloride hydrochloride hydrate, 2-guanidine-4-methylquinazoline (GMQ), picrotoxin, and γ-aminobutyric acid (GABA) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions of each compound were made and stored at -20°C. On the day of experimentation, ligand stock solutions were thawed and diluted to needed concentrations. For the high concentrations (1 mM) of GMQ, picrotoxin, and amiloride, stock solutions were made using dimethylsufoxide (DMSO). Solution exchange was achieved via an array of capillary tubes that are arranged perpendicularly on an inverted fluorescent microscope. Solution flow was controlled electronically using computer driven PTFE valves using a ValveLink8.2 controller (AutoMate Scientific). Recorded currents were obtained using an Axopatch 200B patch clamp amplifier (Axon Instruments), and were filtered and sampled at 5 and 10 kHz, respectively. Data was collected using pClamp 10.0 (Molecular Devices) software. Patch-clamped cells were held at a constant voltage of -70 mV during all experiments. Current

was analyzed online using Clampfit 10.0 and the resulting data were analyzed offline using Origin 8.1 (OriginLab).

Cells were bathed continuously in external solution in the absence of ligand. Upon successful establishment of the whole-cell patch clamp configuration, test solution was applied for 5 seconds. In between exposures, cells were washed with external solution for 1.5 minutes to allow for complete recovery from the previously test solution. In both amiloride and GMQ experiments, the GABA EC_{50} control was established (two consecutive exposures that differed by < 10% peak current amplitude) prior to assessing the guanidine compound effect on the receptor. If failure to re-establish the control peak current amplitude after exposure to the nonproton ligand occurred, the recording was aborted. Both GMQ and amiloride were applied in the absence of GABA to assess direct activation of the GABA-A ρ1 receptor. A positive response a guanidine compound was assigned only if the resulting peak current amplitude was greater than a pre-established cutoff of 20 pA. In the absence of GABA, amiloride exhibited minimal direct activation activity (Supplement Fig 1). In co-application studies, The amiloride potentiation was assessed using 10, 30, and 100 μM amiloride in the presence of increasing GABA concentrations and were normalized to GABA EC_{100} concentration on the wild type hGABA-A ρ1 receptor. Both amiloride and GMQ were co-applied in increasing concentrations with the control, EC_{50} GABA on all constructs. Concentration response profiles were fit to a dose-response function using OriginLab 8.1.

Amiloride docking:

An amiloride coordinate file was generated using the online serve PRODRG (Schuttelkopf and van Aalten, 2004). Modeling of amiloride docking with a Cys-loop receptor crystal structure was

produced using the molecular docking algorithm based on complementarity principles in PatchDock (Schneidman-Duhovny *et al*., 2005). The ethanol-bound GLIC protein crystal structure (PDB code 4HFE) (Sauguet *et al*., 2013) was used as the receptor molecule while the PRODRG generated amiloride coordinate file was used as the ligand. The clustering root mean square deviation (RMSD) was set to 1.5 angstrom while the complex type was set to the protein-small ligand option, as suggested. Of the resulting models, one result showed amiloride docking with the intersubunit site and was used as a starting point for the described studies. Model was presented graphically using Pymol (Schrodinger, 2010).

Data Analysis:

Maximum peak current amplitude in each whole cell electrophysiological experiment was obtained and normalized to the maximum peak current amplitude elicited by a GABA control. Data is presented as the mean ± standard error of the mean (SEM) of the indicated patch-clamped HEK293T cells. Statistical significance was determined using unpaired Student's *t*-test.

Results

Amiloride, the prototypical ASIC antagonist, acts as a competitive antagonist for the heteromeric GABA-A αβγ receptors (Inomata *et al*., 1988; Fisher, 2002; Liu *et al*., 2010). The activity of amiloride on the GABA-A ρ1 receptor remains unclear. Thus, we sought to determine the intrinsic activity of amiloride on the homomeric human GABA-A ρ1 receptors. We transiently transfected cDNA encoding the wild type human GABA-A ρ1 receptor in HEK293T cells. The wild type human GABA-A ρ1 receptor had a GABA EC_{50} of 9.4 ± 0.6 μM with a Hill coefficient of 1.1 ± 0.2 (Table 1). The described wild type GABA-A ρ1 receptor EC_{50} is within the range of reported GABA-A ρ1 receptor GABA EC_{50} values expressed in the HEK293T cell line (Kusama *et al*., 1993; Amin and Weiss, 1994; Greka *et al*., 2000; Harrison and Lummis, 2006; Chebib *et al*., 2009).

Initially, we utilized an amiloride concentration-response profile experiment using coapplication of 10 μM GABA as our control and increasing concentrations of amiloride (Figure 1A). Amiloride did not exhibit an inhibitory effect on the expressed human GABA-A ρ1 receptor (Figure 1A). When co-applied in increasing concentrations from 1 μM to 1,000 μM amiloride, there was a 52% increase in peak current amplitude at the maximum concentration of amiloride as compared to the control (Figure 1B). We calculated that the amiloride EC_{50} was 77.0 ± 6.3 μM and a Hill coefficient of 1.6 ± 0.2 ($n \geq 4$) (Figure 1B, Table 2). At the lower concentrations of amiloride (1, 3, and 10 μM) tested, we observed minimal increase in GABA-mediated peak current amplitude, but there were noticeable changes in the recordings profile (Figure 1A). Our results suggest that amiloride enhances GABA-mediated current generated by the human

GABA-A ρ1 receptor, which is in contrast with heteromeric GABA-A receptor where the ASIC nonproton ligand is a competitive antagonist.

Since amiloride exhibited positive allosteric modulation (PAM), we considered that amiloride may have directly activated the WT GABA-A ρ1 receptor. Direct activation of the receptor was observed at 1 mM amiloride, which resulted in a modest response (Supplemental Figure 1). In line with the activity indicative of a positive allosteric modulator, amiloride may influence the apparent GABA affinity of GABA-A ρ1 receptors. To assess this effect, we generated GABA concentration-response profiles in the presence of amiloride (30, 100, and 300 μM) (Figure 1C). The resulting GABA concentration-response profiles were compared to control GABA concentration-response profile for the wild type human GABA-A ρ1 receptor. Increasing amiloride concentrations revealed an overall leftward shift of the calculated GABA EC_{50} values, from the control value of 9.4 ± 0.1 μM with a Hill coefficient of 1.1 ± 0.2 to a GABA EC_{50} value of 5.9 ± 0.9 μM and Hill coefficient of 0.9 ± 0.1 in the presence of 300 μM amiloride ($n \geq 5$, Figure 1C and Table 1). These results suggest that amiloride acts as a positive allosteric modulator of the human GABA-A ρ1 receptor and could interact with another site within the receptor.

There are multiple sites within the Cys-loop receptors that could serve as the amiloride binding site. We focused our attention on the TM2 domains. A comparison of the second transmembrane domains of the human GABA-A (hGABA-A) α1, β3, ρ1, *C. elegans* glutamate

chloride (GluCl) α1 subunit, and GLIC subunit revealed multiple residues that could be responsible for amiloride allosteric modulation (Figure 2). To narrow our focus further, we generated a model of amiloride docked in a Cys-loop receptor crystal structure. We utilized the solved structure of the ethanol-bound GLIC protein structure as our model utilizing the online server PatchDock (Figure 2). One result revealed an amiloride molecule within the GLIC intersubunit cavity, between the transmembrane domains of neighboring subunits. Furthermore, two residues stood out within the intersubunit site: the TM2 15' and 19' residues, which had amino acid side chains that protrude into the intersubunit cavity. Furthermore, we focused on sites that are responsible for allosteric modulation. Thus, we included the TM2 T6'F mutation in our study, a mutation that influence channel gating and abolishes picrotoxin sensitivity. In GABA-A ρ1, the TM2 15' position is an isoleucine. The other mammalian subunits shown have a serine (GABA-A α1) or asparagine (GABA-A β3) at this position. Based on our modeling, both 15' and 19' residues extend into the intersubunit site, and we hypothesized that these residues could be integral in mediating amiloride and GMQ activity (Figure 2). We introduced a TM2 N19'D mutation in an effort to preserve the size of the residue. These allosteric modulator sites could play a role in mediating the effects of amiloride or GMQ on the human GABA-A ρ1 receptor and are suitable candidates for serving as the amiloride allosteric modulatory site.

To assess the role of allosteric sites on guanidine compound influence, we generated the hGABA-A ρ1 T6'F, the I15'N, and N19'D mutants. The latter mutations would address if the guanidinium group of amiloride interacts within the intersubunit site where the TM2 15', and 19' residues reside, as suggested in the model (Figure 1). We began our studies by establishing the

GABA EC_{50} for each of the mutant receptors. Using whole cell patch clamp electrophysiology, we applied increasing concentrations of GABA (1 to 1,000 μM) for 5 seconds followed by 2 minutes of an active wash (Supplemental Figure 2). Upon electrophysiological examination of the hGABA-A ρ1 (T6'F), (I15'N) and (N19'D) mutant receptors, each mutant affected the observed GABA EC_{50} values. The hGABA-A ρ1 (T6'F) mutant receptors places five phenylalanine residues within the channel lining domain. In heteromeric receptors, including five phenylalanine residues at the TM2 6' position failed to yield functional receptors (Gonzales *et al*., 2008). In contrast, we were able to generate GABA-mediated current and determined a GABA EC_{50} value for the mutant GABA-A ρ1 T6'F receptor. The hGABA-A ρ1 (T6'F) mutant receptor had a calculated GABA EC_{50} was 8.0 ± 1.7 μM and had a Hill coefficient of 0.6 ± 0.1, which were not significantly different from wild type GABA-A ρ1 receptors (Table 2). The hGABA-A ρ1 I15'N mutant receptor displayed significantly increased agonist potency, with a GABA EC_{50} value of 2.9 ± 0.6 μM and a Hill coefficient of 1.0 ± 0.2 (Table 2). There was less of an effect on the GABA EC_{50} value for the hGABA-A ρ1 (N19'D) mutant receptor. The GABA EC_{50} shifted to 7.4 ± 1.5 μM and a Hill coefficient of 1.0 ± 0.2 (Table 2).

Since amiloride potentiated GABA-mediated current in the wild type hGABA-A ρ1 receptor (Figure 1A, 1B), we examined amiloride's activity in mutant hGABA-A ρ1 receptors. Instead of the amiloride potentiating effect observed in the wild type hGABA-A ρ1 receptor, amiloride antagonized the hGABA-A ρ1 T6'F and N19'D mutant receptor's GABA-mediated current, with an IC_{50} of 277.3 ± 88.6 μM and a Hill coefficient of 1.2 ± 0.3, and an IC_{50} of 66.4 ± 9.4 with a Hill coefficient of 0.4 ± 0.02 respectively. (Figure 3A, 3D, Table 2). The hGABA-A

ρ1 T6'F mutation resulted in a 10-fold increase in amiloride potency compared to the reported amiloride potency in the heteromeric GABA-A receptor composed of α6β3γ2, while the hGABA-A ρ1 N19'D mutation resulted in a 2-fold decrease in potency (Drafts and Fisher, 2004). Furthermore, amiloride failed to exhibit potentiation or antagonism in the hGABA-A ρ1 I15'N mutant receptors (Figure 3B, 3D). The loss of both potentiation and antagonism activity observed in the wild type hGABA-A ρ1 receptor and the antagonistic intrinsic activity observed in both the hGABA-A ρ1 T6'F mutant receptor and the N19'D mutant receptor, suggests that the 15' residue might be the site of interaction for amiloride within the hGABA-A ρ1 receptor.

Due to amiloride's displayed potentiation of the GABA-mediated current, we considered that the other ASIC ligand that antagonized heteromeric GABA-A receptors, 2-guanidine-4-methylquinazoline (GMQ), may have similar activity in the GABA-A ρ1 receptor. Like amiloride, GMQ is a ringed molecule with a guanidine group (Figure 2). To assess GMQ activity in wild type hGABA-A ρ1 receptors, we generated concentration-response profiles of GMQ in the presence of the approximate GABA EC_{50} (10 μM). The guanidine compound GMQ antagonized the GABA-mediated response in a concentration-dependent manner with a determined GMQ IC_{50} value of 13.2 ± 0.6 and Hill coefficient of 1.4 ± 0.1 ($n \geq 4$) (Figure 4A, 4D) (Table 2). Upon co-application of 30 μM GMQ, we observed a rebound current at the end of the GABA-mediated current. This is similar to picrotoxin's activity on the perch GABA ρ1A receptor, where the plant alkaloid exhibited a rebound current (Qian *et al*., 2005). Similar to our amiloride studies, we generated GABA concentration-response profiles in the presence of 10 and 100 μM GMQ to asses its influence on GABA potency (Supplemental Figure 3). Despite the

inhibition in current exhibited by GMQ, the guanidine compound decreased the GABA EC_{50} values (increased potency) (Table 1). In the presence of 10 μM and 100 μM GMQ, the GABA EC_{50} was reduced to 7.2 ± 0.6 μM with a Hill coefficient of 1.9 ± 0.3 and 4.8 μM ± 0.9 with a Hill coefficient of 2.6 ± 0.7, respectively (Table 1). Unlike amiloride, however, the presence of GMQ decreased the efficacy of GABA on the hGABA-A ρ1 receptor (Supplemental Figure 3). This decrease in efficacy, along with the inhibition in GABA-induced current, suggests GMQ may act as a negative allosteric modulator.

We examined the effect of GMQ on the hGABA-A ρ1 (T6'F), (I15'N), and (N19'D) mutant receptors. First, we applied increasing concentrations of GMQ with the hGABA-A ρ1 (T6'F) GABA EC_{50} (Figure 4B, 4E). The guanidine compound GMQ had an IC_{50} of 0.4 ± 0.1 μM and a Hill coefficient of 0.8 ± 0.2, a 33-fold increase in potency compared to the wild type receptor (WT IC_{50} 13.2 μM) (Table 2). Furthermore, the IC_{50} reported here was similar to that obtained when assessing GMQ potency in the heteromeric GABA-A receptor (0.39 ± 0.05 μM) (Table 2) (Xiao *et al*., 2013). In contrast, the hGABA-A ρ1 I15'N receptor exhibited a significant decrease in the GMQ IC_{50} value (630.5 ± 58.4 μM) with a Hill coefficient of 1.5 ± 0.2 ($n \geq 5$) (Table 2, Figure 4C, 4E). The hGABA-A ρ1 (I15'N) GMQ IC_{50} value (was an approximate 48-fold reduction in potency (Figure 4E). The hGABA-A ρ1 N19'D mutant receptor also displayed a marked decrease in the potency of GMQ, with and estimated IC_{50} value greater than 1 mM (Figure 4D, 4E).

Both amiloride and GMQ activity were influenced by the TM2 mutations at the 6', 15', and 19' positions. Since the GABA-A ρ1 T6'F mutation resulted in changes in amiloride and GMQ

potency, we sought to characterize picrotoxin's actions on the hGABA-A ρ1 receptor mutations to as a comparison for these guanidine allosteric modulators. Exposure of the expressed wild type hGABA-A ρ1 receptors to a GABA EC_{50} concentration and increasing picrotoxin concentrations exhibited similar responses to those previously reported (Alakuijala *et al*., 2005) (Figure 5A). Picrotoxin had an IC_{50} concentration of 4.8 ± 0.2 μM and a Hill coefficient of 1.3 ± 0.1 for the wild type hGABA-A ρ1 (Table 2). The described IC_{50} value obtained here is similar to values reported in the literature (Abbott *et al*., 2012). Whole cell patch clamp assessment of the picrotoxin antagonism in the hGABA-A ρ1 T6'F mutant confirmed that the mutant receptor was insensitive to picroxtoxin, as increasing the concentration of picrotoxin was unable to antagonize the GABA evoked current (Figure 5B, 5D). In the hGABA-A ρ1 (I15'N) mutant receptor, there was a significant decrease in the picrotoxin potency with an IC_{50} of 63.4 ± 12.0 and a Hill coefficient of 0.6 ± 0.1 (Figure 5C, 5D, Table 2).

Discussion

Here we have demonstrated that mutations within the second transmembrane domain of the human GABA-A ρ1 receptor alter the allosteric modulatory properties of amiloride and GMQ. Additionally, a single residue in the GABA-A ρ1 receptor intersubunit site, the I15'N mutation, abolished positive allosteric modulation produced by amiloride. The compounds amiloride and GMQ have both been found to inhibit the heteromeric GABA-A receptors (Fisher, 2002; Xiao *et al*., 2013). Mutagenesis experiments conducted on the heteromeric GABA-A receptor affected the competitive inhibition of GMQ, but had no effect on the inhibition exhibited by amiloride, suggesting that these inhibitory actions are mediated through distinct sites (Xiao *et al*., 2013).

The observed amiloride potentiation in GABA-A ρ1 receptor was concentration-dependent with the apparent enhancement occurring at concentrations greater than 10 μM. At the modest amiloride concentrations, we observed a change in the GABA-mediated current profiles, similar to heteromeric GABA-A receptors (Figure 1). At higher amiloride concentrations (>30 μM), the typical wild type recording profile returned. Amiloride's actions on the hGABA-A ρ1 receptor were similar to actions of allosteric modulators on heteromeric GABA-A receptors (Saunders and Ho, 1990; Rho *et al*., 1996). The change in receptor desensitization kinetics is similar to the plant alkaloid picrotoxin effects on deactivation kinetics of the GABA-A ρ receptor (Goutman and Calvo, 2004). Both of these allosteric modulators act at the GABA-A TM2 15' position. Furthermore, if amiloride's activity on the human GABA-A ρ1 receptor was similar to

pentobarbital, the diuretic should be able to directly gate the channel (Rho *et al*., 1996). Here, amiloride activated the channel modestly in the wild type hGABA-A ρ1 receptor (Supplemental Figure 1). However, barbiturates do not influence wild type GABA-A ρ receptors, requiring a single point mutation to confer the sensitivity (Amin, 1999). These ligands require a substitution of the GABA-A ρ1 TM2 15' position to an asparagine, the same amino acid at the GABA-A β2 and β3 subunit 15' sites.

Based on our modeling and electrophysiological data, we considered that an allosteric modulating amiloride site of interaction may be located away from the GABA agonist site. One such site was the intersubunit site, located between neighboring subunits' transmembrane domains where ethanol binds (Sauguet *et al*., 2013) and where propofol inhibits GLIC mediated current (Ghosh *et al*., 2013). The side chains of the 15' and 19' residues protrude into the intersubunit cavity. The resulting model containing a single amiloride molecule docked near this intersubunit site in the GLIC crystal structure provided further support for our focus on this site. However convincing the molecular docking model and electrophysiology are, there remains a possibility that mutations within the intersubunit site may be involved in amiloride or GMQ allosterism and not direct binding. Additional work is necessary to determine if the intersubunit site is the site of action for amiloride and GMQ in the GABA-A ρ1 receptor.

When the 15' residue was mutated from an isoleucine to a cysteine in the prokaryotic pLGIC homologue, propofol modulation switched from inhibition to potentiation (Ghosh *et al*.,

2013). Charge and/or size of the functional group at the TM2 domain 15' residue influenced propofol modification of the GLIC channel (Ghosh *et al*., 2013). In another example, the TM2 domain bends when glutamate chloride channel's bound by ivermectin and stabilization of the second and third transmembrane domains occurs (Althoff *et al*., 2014). Thus, to elucidate the site of interaction of amiloride, we mutated two residues that protrude into the intersubunit site (TM2 15' and 19' residues). Previous studies analyzed the influence of alcohol on the GABA-A ρ1 receptor, and that the TM2 15' (I15') was integral for inhibition (Mihic *et al*., 1997). Furthermore, the TM2 15' position has been implicated in the action of etomidate through the GABA-A β subunit (Belelli *et al*., 1997) and anesthetics through the GABA-A α receptor subunit (Mascia *et al*., 2000). Consistent with these changes in amiloride allosteric modulation following the I15'N mutation, we propose that the intersubunit site is critical for amiloride potentiation in the human GABA-A ρ1 receptor.

The hGABA-A ρ1 TM2 6' position was implicated in picrotoxin inhibition of several Cys-loop receptors, including the GABA-A ρ1 receptor (Pribilla *et al*., 1992; Gurley *et al*., 1995; Zhang *et al*., 1995). This residue has also been implicated in neurosteroid inhibition of the GABA-A ρ1 receptor, but with the appearance of a tail current, similar to picrotoxin inhibition and channel blocking (Li *et al*., 2007). In the hGABA-A ρ1 T6'F and N19'D mutant receptors, amiloride antagonized the GABA-mediated current similar to what was observed in heteromeric GABA-A receptors (Fisher, 2002). The threonine at the TM2 6' position is conserved among the inhibitory Cys-loop receptors (Figure 2). Because we observed amiloride inhibition in these mutant receptors, perhaps the TM2 6' position is not involved with direct amiloride binding

within hGABA-A ρ1 receptor. Instead, the hGABA-A ρ1 T6'F mutation alters gating of the channel and converts the amiloride potentiation to inhibition, similar to the N19'D mutation. The hGABA-A ρ1 I15'N mutant receptor ameliorated amiloride potentiation without amiloride antagonism (Figure 3). This change in amiloride activity in response to a mutation at the hGABA-A ρ1 TM2 domain 15' position was similar to changes in both barbiturate and neurosteroid activity following mutagenesis of the channel's TM2 15' residue. When mutated to a serine, this residue introduces barbiturate sensitivity to the otherwise barbiturate insensitive hGABA-A ρ1 receptor (Belelli *et al*., 1999).

The allosteric intersubunit can accommodate a large range of compounds. Resolved Cys-loop receptor structures have molecules bound within the intersubunit site that range in size from ethanol (84 Daltons) (Sauguet *et al*., 2013) to ivermectin (875 Daltons) (Hibbs and Gouaux, 2011). Amiloride is approximately 266 Daltons in size could fit into this intersubunit cavity. Furthermore, a simple reduction in 15' site side chain volume (I to N, from 101 to 63.7 $Å^3$) may not account for the loss of allosterism. However, this mutation increased amino acid side chain polarity. The asparagine side chain may mimic the presence of the amiloride or GMQ guanidinium group resulting in loss of activity (amiloride) or decreased potency (GMQ) due to steric hindrance between protein and ligand. Furthermore, this is reflected in the increase in GABA potency in the presence of amiloride (Figure 2, Table 1). The GABA-A ρ1 I15'N mutant receptor GABA EC_{50} approaches the same value as the WT receptor GABA EC_{50} in the presence of 300 μM amiloride (Figure 3B, Table 2). One possible explanation for this activity is that the I15'N mutation mimics, when activated, the receptor in the presence of amiloride (Supplemental

Figure 2), thus preventing amiloride potentiation. This would suggest that the 15' residue of the hGABA-A ρ1 receptor is integral in the potentiation activity of amiloride on this receptor. The isoleucine (whose amino acid R-group faces into the intersubunit site) creates an environment conducive for amiloride interaction. This same residue fails to accommodate a barbiturate or anesthetic, such as etomidate, to interact with this GABA-A ρ1 intersubunit site (Stewart *et al*., 2014).

Furthermore, GABA-A αβγ receptors are antagonized by GMQ in a competitive manner (Xiao *et al*., 2013). Here, GMQ inhibited the hGABA-A ρ1 receptor (Figure 4) and the intersubunit mutations reduced GMQ potency (a 47-fold reduction) (Figure 4E). The I15'N mutation reduced picrotoxin potency, but to a lesser degree (13-fold reduction). Removing this hydrophobic side chain and replacing it with the polar side chain of asparagine reduces GMQ potency. We considered that GMQ may inhibit via the picrotoxin site found on the cytoplasmic side of the GABA-A ρ1 channel pore based upon the rebound current observed during GMQ antagonism. The observed GMQ effects in our study, such as the slight rebound current at the end of GABA and GMQ co-application, is similar to the picrotoxin recordings of the GABA perch ρ1A receptor (Qian *et al*., 2005). The GABA-A ρ1 T6'F mutation resulted in a significant decrease in the IC_{50} and was similar to that observed in the GABA-A αβγ receptors (Figure 4) (Xiao *et al*., 2013). The GABA-A ρ1 T6'F mutation eliminated picrotoxin activity but enhanced GMQ potency (a 33-fold increase). Based on this, the phenylalanine at the TM2 6' position may have improved GMQ interaction at this site. However, additional studies are necessary to confirm that this TM2 6' position is a GMQ binding site.

Our results describe the allosteric modulation of the human GABA-A ρ1 receptor by the guanidine compounds amiloride and GMQ. A single point mutation, the hGABA-A ρ1 I15'N, eliminated amiloride positive allosteric modulation, and provides compelling evidence to suggest that the TM2 15' position (within the intersubunit site) contributes to the amiloride allosteric modulation site or to a guanidine compound binding site. Furthermore, the GABA-A ρ1 I15'N reduced GMQ potency. Both amiloride and GMQ increased GABA potency in the wild type hGABA-A ρ1 receptor, which suggests that these guanidine compounds are allosteric modulators. These results provide support the continued exploration of the molecular determinants of amiloride and GMQ allosterism in the GABA-A ρ1 receptor.

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Author contributions

Participated in research design: Heather D. Snell and Eric B. Gonzales

Conducted experiments: Heather D. Snell

Performed data analysis: Heather D. Snell and Eric B. Gonzales

Wrote or contributed to the writing of the manuscript: Heather D. Snell and Eric B. Gonzales

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Person to contact for reprint requests:

Eric B. Gonzales, Ph.D.

Assistant Professor

Department of Pharmacology & Neuroscience

UNIVERSITY OF NORTH TEXAS Health Science Center

3500 Camp Bowie Blvd.

Fort Worth, TX 76107

Phone: 817-735-2755

Fax: 817-735-0408

Email:eric.b.gonzales@unthsc.edu

FIGURES LEGENDS AND CORRESPONDING FIGURES

Figure 1. Amiloride potentiates the GABA-A ρ1 receptor GABA-induced current. (A) Representative traces of wild type hGABA-A ρ1 receptor currents generated by co-application of GABA EC_{50} (10 μM) with increasing concentrations of amiloride. (B) Potentiating amiloride concentration-response profile is shown. Data represents the mean ± SEM of 10 μM GABA and co-application of increasing amiloride 1, 3, 10, 30, 100, 300, and 1,000 μM) of $n \geq 4$ individual cells. The amiloride EC_{50} was 77.0 ± 6.3 μM with a Hill coefficient of 1.6 ± 0.2. (C) GABA concentration-response profiles in the absence and presence of amiloride are shown. A leftward shift of the GABA EC_{50} is depicted from the GABA EC_{50} (9.4 ± 0.1 μM GABA, control), 30 μM amiloride (7.8 ± 0.5 μM GABA), 100 μM (6.4 ± 0.6 μM GABA), 300 μM (5.9 ± 0.9 μM GABA) (Table 1). Each recording was normalized to the cell's maximal response (1 mM GABA).

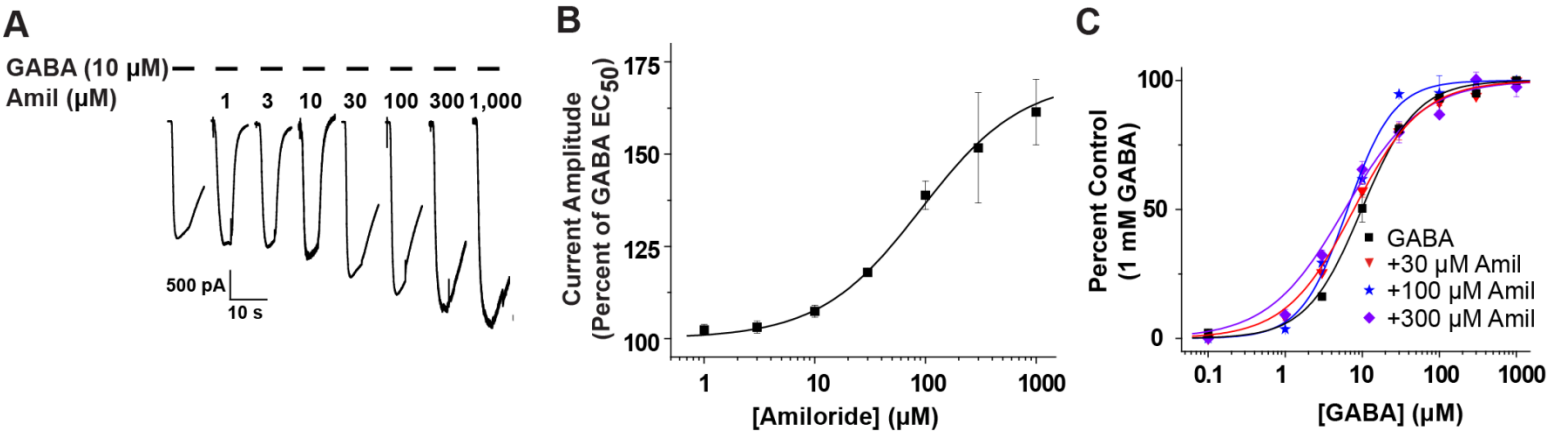

Figure 1. Amiloride potentiates the GABA-A ρ1 receptor GABA-induced current.

Figure 2. Sequence alignment of the second transmembrane domain among select Cys-loop receptor subunits. The residues are labeled according to Miller notation (Miller, 1989). Chemical structure of guanidine compounds amiloride and GMQ. Model of amiloride interacting with the intersubunit site of ethanol sensitive GLIC (Sauguet *et al*., 2013). Compound docked utilizing PatchDock online server.

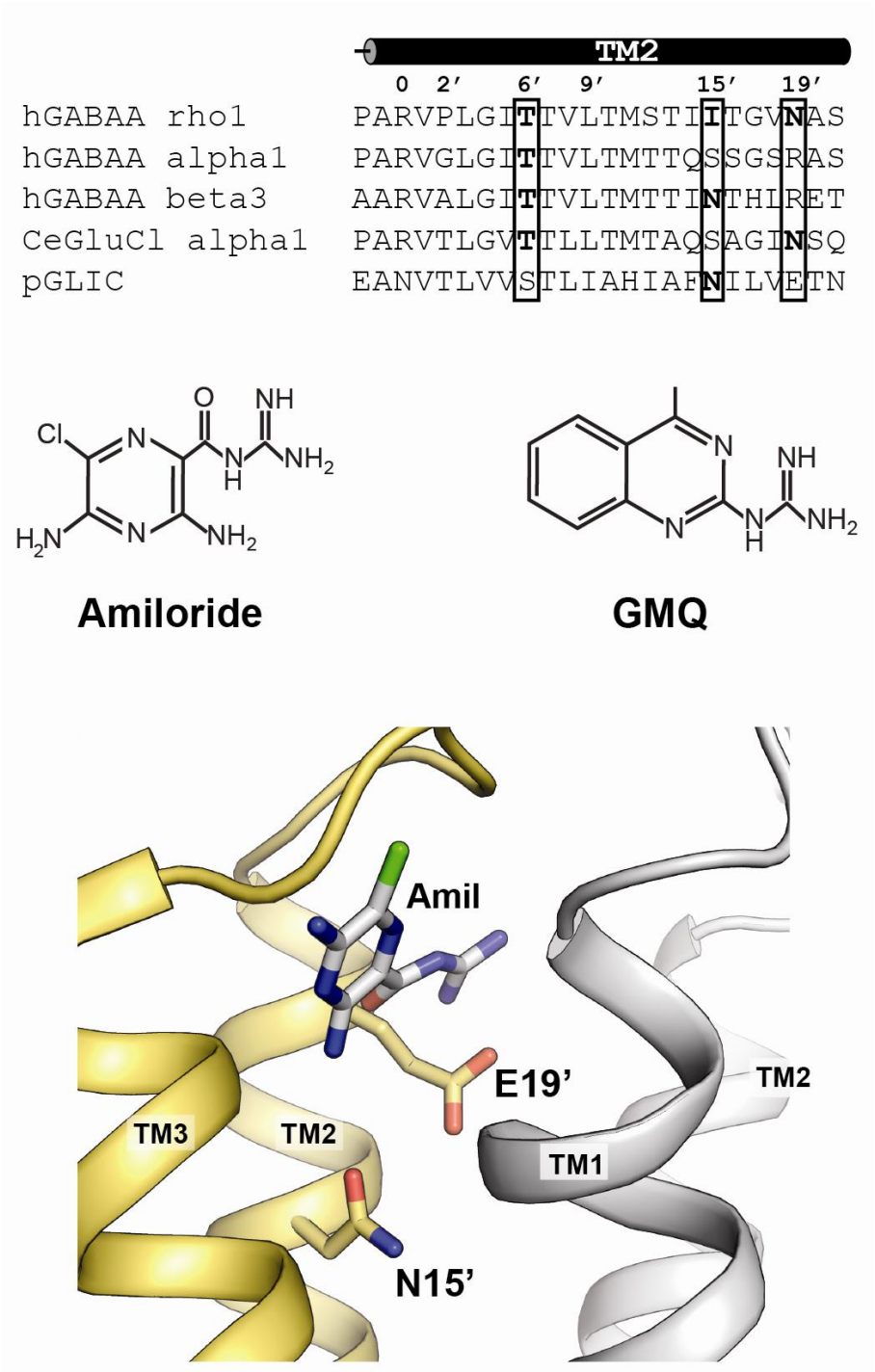

Figure 2. Sequence alignment of the second transmembrane domain among select Cys-loop receptor subunits.

Figure 3. Human GABA-A ρ1 receptor containing the TM2 6', 15', and 19' mutations display altered response profiles to amiloride. Comparison of amiloride response profile of wild type hGABA-A ρ1, and hGABA A ρ1 T6'F, I15'N, or N19'D mutant receptors. All constructs were transiently expressed in HEK293T cells. (A) Representative traces of hGABA-A ρ1 T6'F mutant receptor currents elicited upon co-application of 8 μM GABA (EC_{50}) with increasing concentrations of amiloride. (B) Representative traces of hGABA-A ρ1 I15'N mutant receptor currents elicited upon co-application of GABA EC_{50} (3 μM) with increasing concentrations of amiloride. All activation currents generated by applying GABA and amiloride test solutions for 5 second (s). (C) Representative traces of hGABA-A ρ1 N19'D mutant receptor currents elicited upon co-application of GABA EC_{50} (8 μM) with increasing concentrations of amiloride. (D) Comparison of normalized concentration-response profiles of amiloride inhibition in wild type hGABA-A ρ1, and hGABA-A ρ1 I15'N, hGABA-A T6'F, and hGABA-A N19'D mutant receptors compared to control the respective control concentration of GABA. The determined amiloide EC_{50} was 44.57 ± 14.24 μM for the wild type hGABA-A ρ1 receptor. IC_{50} values of 277.3 ± 88.6 μM with a Hill coefficient of 1.2 ± 0.3, and 66.4 ± 9.4 μM with a Hill coefficient of 0.4 ± 0.02 were obtained for hGABA-A ρ1 T6'F and hGABA-A ρ1 N19'D mutant receptor respectively. The hGABA-A ρ1 I15'N mutant receptor displayed an absence of amiloride modulation, and thus no EC_{50} or IC_{50} was obtained. Data is presented as the mean ± SEM, with a sample size of $n \geq 5$ cells.

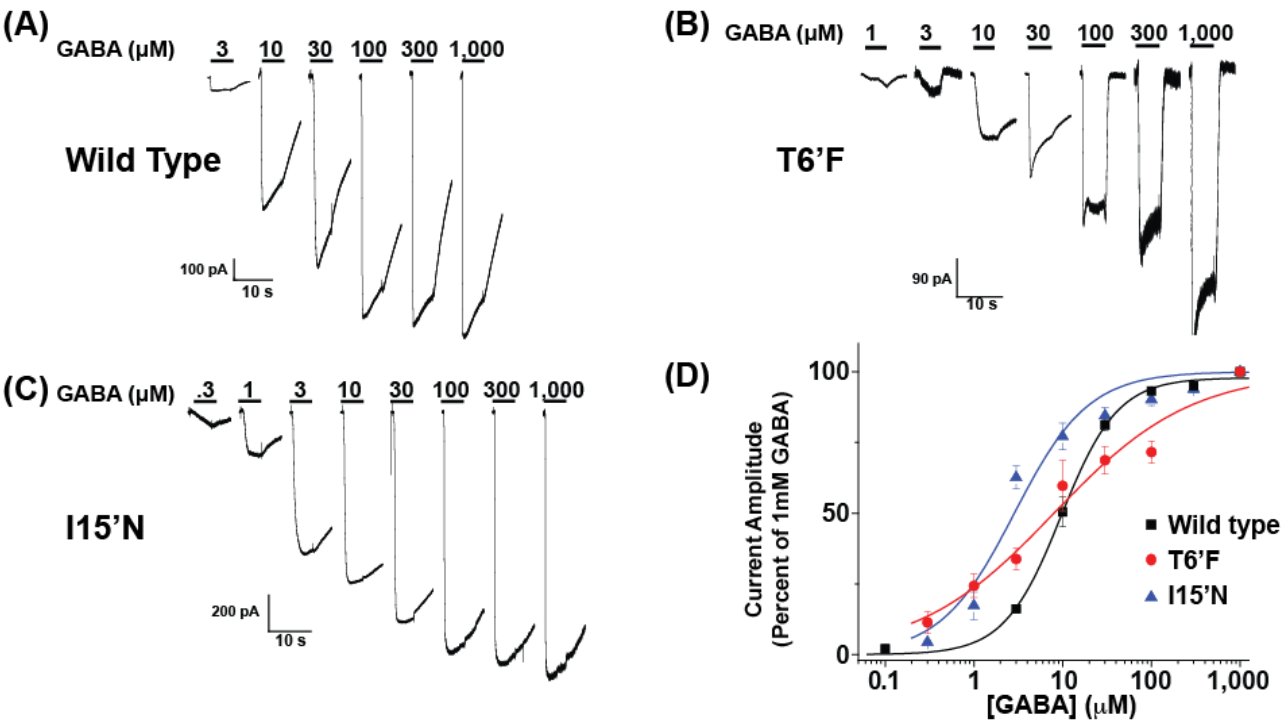

Figure 3. Human GABA-A ρ1 receptor containing the TM2 6', 15', and 19' mutations display altered response profiles to amiloride.

Figure 4. Human GABA-A ρ1 receptor containing the 15' and 6' mutations display altered response profiles to GMQ. (A) Representative traces of hGABA-A ρ1 receptor currents elicited upon co-application of GABA EC_{50} with increasing concentrations of GMQ. (B) Representative traces of hGABA-A ρ1 T6'F mutant receptor currents elicited upon co-application of GABA EC_{50} with increasing concentrations of GMQ. (C) Representative traces hGABA-A ρ1 I15'N mutant receptor currents elicited upon co-application of GABA EC_{50} with increasing concentrations of GMQ. All activation currents generated by applying GABA and GMQ test solutions for 5 second (s). (D) Representative traces of hGABA-A ρ1 N19'D mutant receptor currents elicited upon co-application of GABA EC_{50} with increasing concentrations of GMQ. (E) Comparison of normalized concentration-response profiles of GMQ inhibition in wild type hGABA-A ρ1, and hGABA-A ρ1 I15'N, and hGABA-A T6'F mutant receptors compared to the respective control concentration of GABA. The determined GMQ IC_{50} was 13.2 ± 0.6 μM with a Hill coefficient of 1.4 ± 0.1 for the wild type hGABA-A ρ1 receptor, 0.4 ± 0.1 μM with a Hill coefficient of .8 ± 0.2 for the hGABA-A ρ1 T6'F mutant receptor, and 630.5 ± 58.4 μM with a Hill coefficient of 1.5 ± 0.2 for the hGABA-A ρ1 I15'N mutant receptor. We were unable to fit the data for the N19'D mutation, and thus not IC_{50} was obtained. Data is presented as the mean ± SEM, with a sample size of $n \geq 5$ cells.

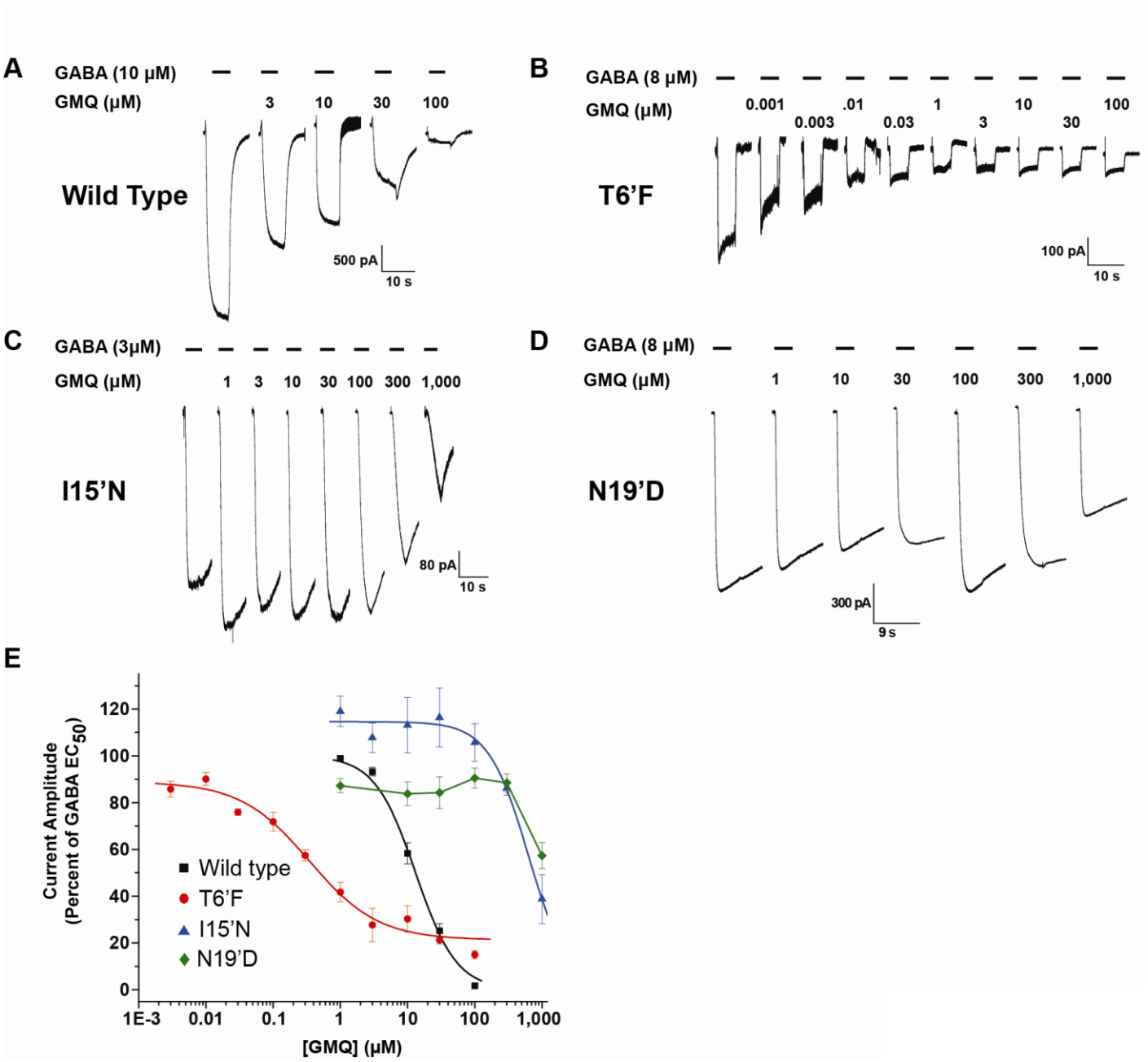

Figure 4. Human GABA-A ρ1 receptor containing the 15' and 6' mutations display altered response profiles to GMQ.

Figure 5. Comparison of picrotoxin inhibitory response profiles of wild type hGABA-A ρ1, hGABA-A ρ1 I15'N mutant receptor, and hGABA-A ρ1 T6'F mutant receptor. All constructs were transiently expressed in HEK293T cells. (A) Representative traces of wild type hGABA-A ρ1 receptor. (B) Representative traces of picrotoxin inhibition of the hGABA-A ρ1 T6'F mutant receptor. (C) Representative traces of picrotoxin inhibition of the hGABA-A ρ1 I15'N mutant receptor. All activation currents generated by 5 second (s) exposures to increasing concentrations of picrotoxin co-applied with the receptor's respective GABA EC_{50} concentrations. (D) Comparison of normalized concentration-response profiles of picrotoxin inhibition in wild type hGABA-A ρ1, and hGABA-A ρ1 I15'N and, hGABA-A ρ1 T6'F mutant receptors. Due to the difference in EC_{50} among each construct, each response was compared to the respective control concentration of GABA. The determined picrotoxin IC_{50} was 4.8 ± 0.2 μM with a Hill coefficient of 1.3 ± 0.1 for wild type hGABA-A ρ1 receptor, and 63.4 ± 12.0 with a Hill coefficient of 0.6 ± 0.1 for the hGABA-A ρ1 I15'N mutant receptor. The hGABA-A ρ1 T6'F mutant receptor displayed an insensitivity towards picrotoxin, and thus no IC_{50} was obtained. Data is presented as the mean ± SEM, with a sample size of $n \geq 5$ cells.

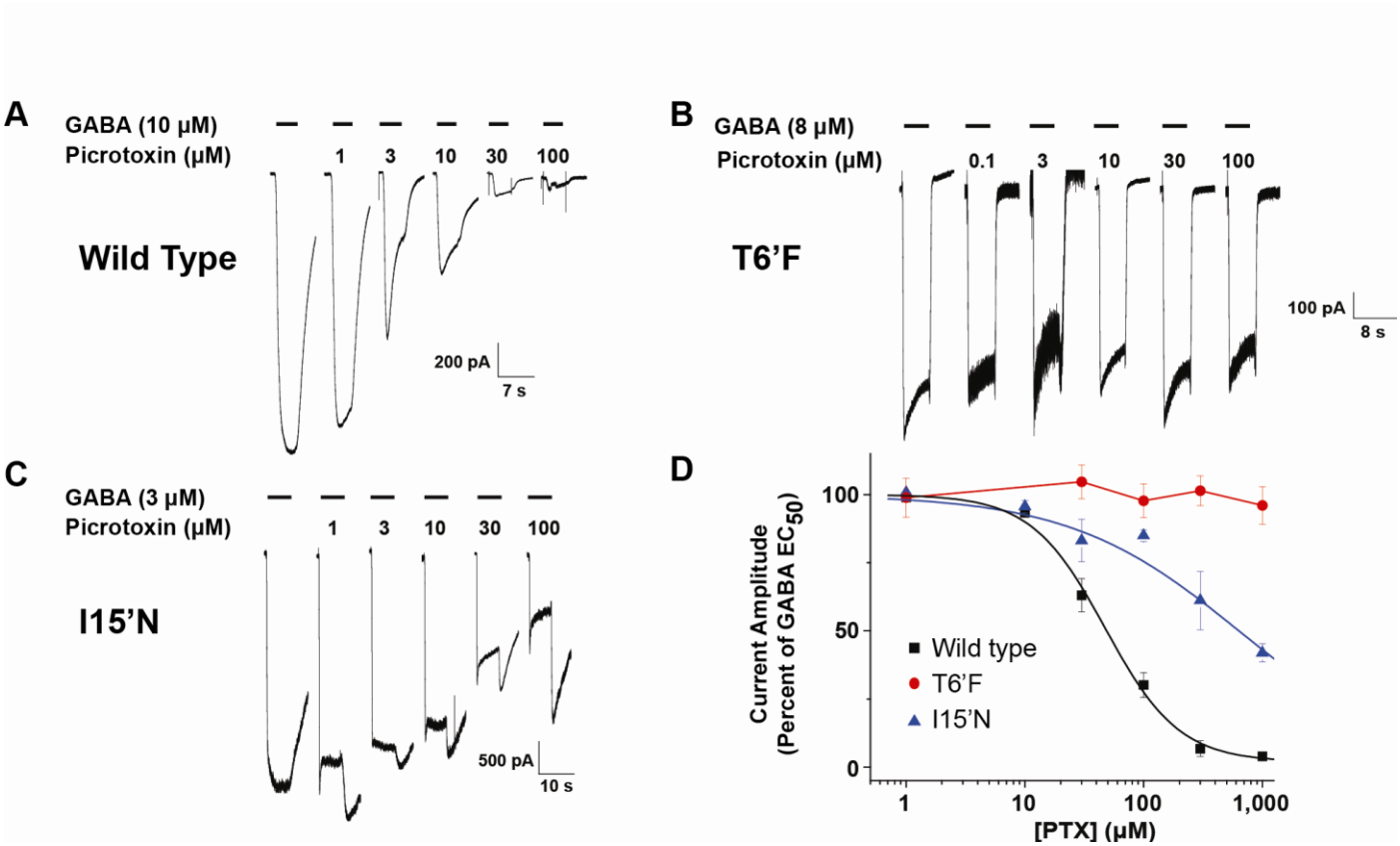

Figure 5. Comparison of picrotoxin inhibitory response profiles of wild type hGABA-A ρ1, hGABA-A ρ1 I15'N mutant receptor, and hGABA-A ρ1 T6'F mutant receptor.

Table 1. Amiloride increases the affinity of the wild type human GABA-A ρ1 receptor for GABA. Summary of EC_{50} and Hill co-efficient values for GABA, and co-application of increasing concentrations of GABA and amiloride (30, 100, and 300 μM on the wild type human GABA-A ρ1 receptor.

Compound	EC_{50}	n_H
	μM	
GABA	9.4 ± 0.1	1.1 ± 0.2
+ 30 μM amiloride	7.8 ± 0.5*	1.0 ± 0.1
+ 100 μM amiloride	6.4 ± 0.6**	1.4 ± 0.2
+ 300 μM amiloride	5.9 ± 0.9**	0.9 ± 0.1
+ 10 □M GMQ	7.2 ± 0.6**	1.9 ± 0.3
+ 100 □M GMQ	4.8 ± 0.9**	2.6 ± 0.7

n ≥ 4 cells

*, p<0.05

**, p<0.01

nH, Hill coefficient

Table 1. Amiloride increases the affinity of the wild type human GABA-A ρ1 receptor for GABA.

Table 2. GABA and allosteric modulator sensitivity in wild type and mutant GABA-A ρ1 receptors. Summary of EC_{50}/IC_{50} and Hill co-efficient values for GABA, and co-application amiloride, GMQ, and picrotoxin on the wild type hGABAA-ρ1, hGABA-A ρ1 T6'F, hGABA-A ρ1 I15'N, and hGABA-A ρ1 N19'D mutant receptors.

Receptor	EC_{50}	n_H	Amil EC_{50}/IC_{50}	Amil n_H	GMQ IC_{50}	GMQ n_H	PTX IC_{50}	PTX n_H
	μM		μM		μM		μM	
WT ρ1	9.4 ± 0.1	1.1 ± 0.2	(EC_{50}) 77.0 ± 6.3	1.6 ± 0.2	13.2 ± 0.6	1.4 ± 0.1	4.8 ± 0.2	1.3 ± 0.1
ρ1(T6'F)	8.0 ± 1.7	0.6 ± 0.1	(IC_{50}) 277.3 ± 88.6	1.2 ± 0.3	0.4 ± 0.1***	0.8 ± 0.2	n.d.	n.d.
ρ1(I15'N)	2.9 ± 0.6***	1.0 ± 0.2	n.d.	n.d.	630.5 ± 58.4***	1.5 ± 0.2	63.4 ± 12.0**	0.6 ± 0.1
ρ1(N19'D)	7.4 ± 1.5	1.0 ± 0.2	(IC_{50}) 66.4 ± 9.4	0.4 ± 0.02	n.d.	n.d.	n.d	n.d

n ≥ 4 cells

*, p<0.05

**, p<0.01

***p<0.001

nH, Hill coefficient

n.d., not determined

Table 2. GABA and allosteric modulator sensitivity in wild type and mutant GABA-A ρ1 receptors

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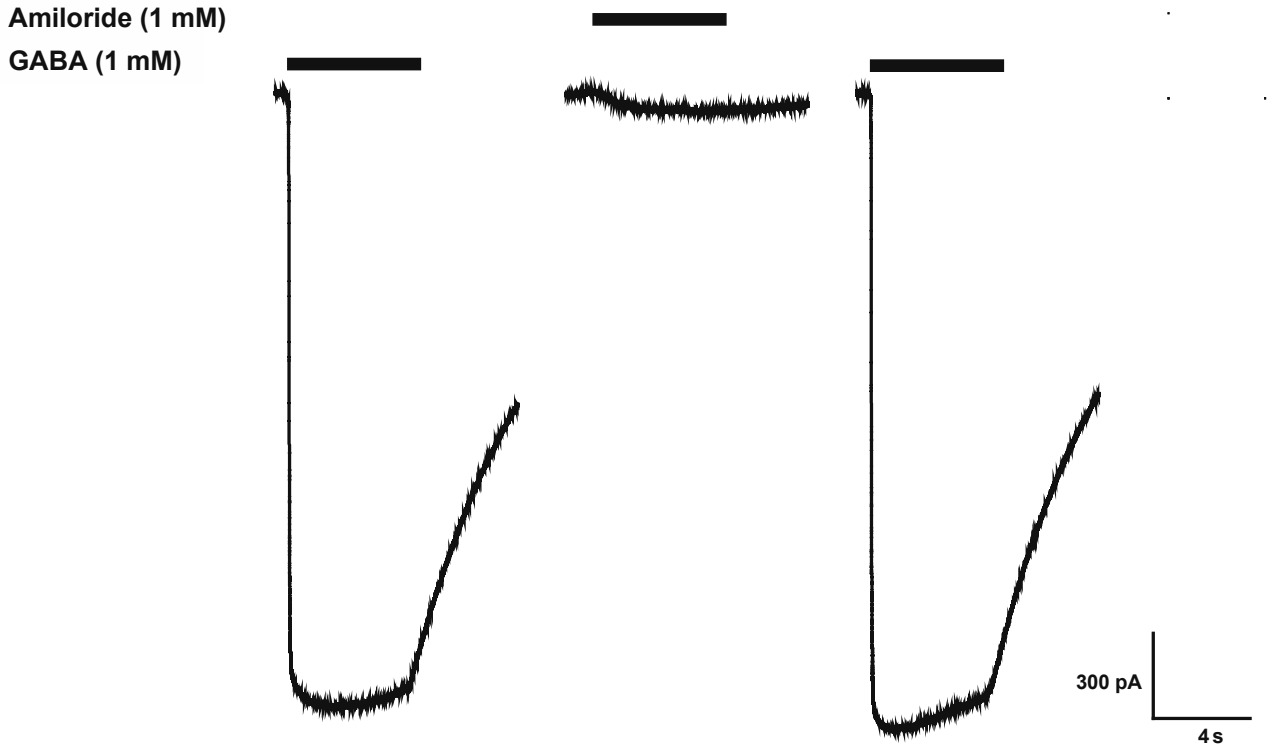
Amiloride and GMQ allosteric modulation of the GABA-A ρ1 receptor: influences of the intersubunit site.

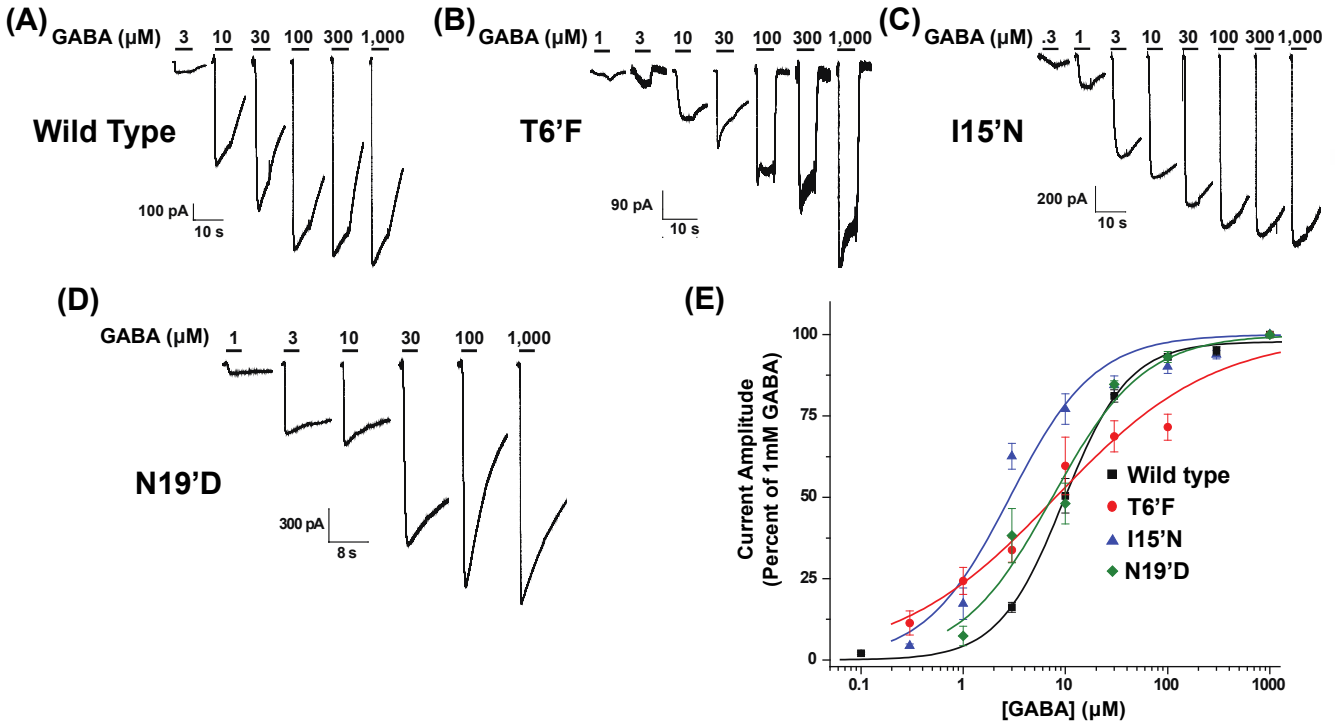
Heather D. Snell and Eric B. Gonzales

Supplemental Figure 1. Amiloride exhibits slight agonist activity in human GABA-A ρ1 receptors. Whole cell patch clamp electrophysiology recordings are shown. Patch-clamp recording traces of application of GABA (1 mM) used for control followed by application of amiloride (1mM). Current of the GABA-A ρ1 receptor was observed above an established cutoff of 20 pA ($n \geq 4$).

Supplemental Figure 1. Amiloride exhibits slight agonist activity in human GABA-A ρ1 receptors.

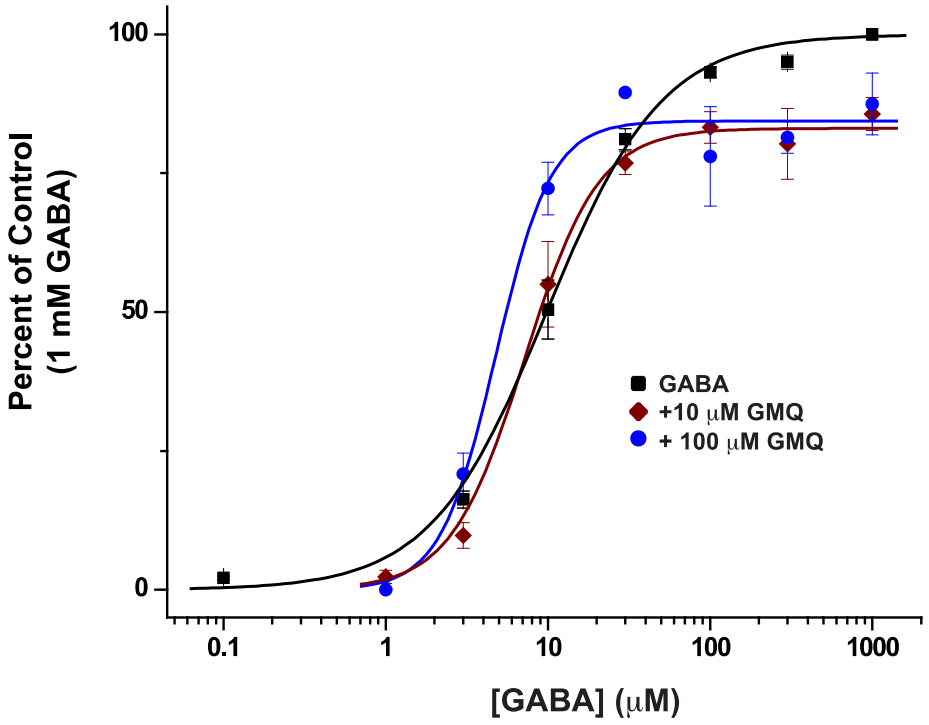


Supplemental Figure 2. Concentration-response profile comparison of wild type hGABA-A ρ1, T6'F, I15'N, and N19'D mutant receptors, transiently expressed in HEK293T cells. (A) Representative traces of wild type hGABA-A ρ1, (B) hGABA-A ρ1 T6'F, (C) hGABA-A ρ 1 I15'N, and (D) hGABA-A ρ1 N19'D mutant receptors are shown. All activation currents generated by 5 second (s) exposures to increasing concentrations of GABA. (E) Summary of concentration-response profiles of GABA-mediated current in wild type hGABA-A ρ1, T6'F, I15'N, and N19'D mutant receptors compared to the maximal response (1,000 μM GABA). The determined GABA EC_{50}s for WT, T6'F, I15'N, and N19'D are 9.4 ± 0.1 μM, 8.0 ± 1.7 μM, 2.9 ± 0.6 μM, and 7.4 ± 1.5 μM, respectively. The respective Hill coefficients are 1.1 ± 0.2, 0.6 ± 0.1, 1.0 ± 0.2, and 1.0 ± 0.2 respectively. Data is presented as the mean ± SEM, with a sample size of $n \geq 4$ cells.



Supplemental Figure 2. Concentration-response profile comparison of wild type hGABA-A ρ1, T6'F, I15'N, and N19'D mutant receptors, transiently expressed in HEK293T cells.

Supplemental Figure 3. Concentration-response profiles of increasing GABA concentrations in the presence of 10 μM and 100 μM GMQ. Each co-application was normalized to a maximal response of GABA (1,000 μM) in the absence of GMQ. The GABA + 10 μM GMQ and GABA + 100 μM GMQ EC_{50} values were 7.2 ± 0.6 μM and 4.7 ± 0.9 μM, respectively with Hill coefficients of 1.9 ± 0.3 and 2.6 ± 0.7, respectively. Both GABA and GMQ concentration-response profiles have a reduced maximal efficacy as compared to the control GABA profile. Data are represented as the mean ± SEM of a sample size of $n \geq 4$ cells for each concentration evaluated.



Supplemental Figure 3. Concentration-response profiles of increasing GABA concentrations in the presence of 10 µM and 100 µM GMQ.

Amiloride and GMQ both displayed different activities on the human GABA-A ρ1 receptor than reported in the heteromeric GABA-A αβγ receptor. Amiloride potentiated, and GMQ competitively inhibited the GABA-induced current in the GABA-A ρ1 receptor. The 15' residue was also as an important residue in the allosteric modulatory activity of amiloride. Having investigated and characterized the integral sites on the receptor important on this allosteric mechanism, we next sought to investigate the compound. It still remained unclear what part of the compound was important in this allosteric mechanism. It was hypothesized the guanidine group, shared by all guanidine compounds, is integral in potentiation of the GABA-A ρ1 receptor by amiloride. To analyze this, we utilized amiloride derivatives which contained the addition of substituents at different sites on the amiloride backbone in the proceeding experiments.

III. MOLECULAR DETERMINANTS OF POSITIVE ALLOSTERIC MODULATION BY AMILORIDE DERIVATIVES IN THE GABA-A ρ1 RECEPTOR

Authors

Heather D. Snell, Eric B. Gonzales*

Affiliations

Center for Neuroscience Discovery, Institute on Healthy Aging, UNT Health Science Center, Fort Worth, TX USA 76107

Running Title: HMA positively modulates the GABA-A ρ1 receptor

*Corresponding Author:

Eric B. Gonzales, Ph.D.

Assistant Professor

Center for Neuroscience Discovery

Institute on Healthy Aging

UNT Health Science Center

3500 Camp Bowie Blvd.

Fort Worth, TX 76107

Email: eric.b.gonzales@unthsc.edu

Phone: 817-735-2755

Fax: 817-735-0408

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Nonstandard abbreviations:

Amil: amiloride

ASIC: acid-sensing ion channel

DMSO: dimethylsulfoxide

GABA: γ-aminobutyric acid

HMA: 5-(N,N-Hexamethylene) amiloride

PAM: positive allosteric modulation

TM: transmembrane domain

Keywords: GABA-A receptor, amiloride, guanidine compound, allostery, ρ1, Cys-loop, electrophysiology, amiloride derivatives, allosteric modulation

ABSTRACT

Guanidine compounds have been identified as ion channel modulators. In the case of Cys-loop receptors, amiloride antagonizes the heteromeric GABA-A, glycine, and nicotinic acetylcholine members of this family. Recently, the guanidine compound amiloride was shown to have positive allosteric modulatory effects on the GABA-A ρ1 receptor. Mutagenesis revealed that the GABA-A ρ1 second transmembrane domain 15' mutation (I15'N) abolished the stimulatory activity of amiloride. What remains unclear is which portion of the amiloride molecule, the guanidine group or the pyrazine ring, interacts with the GABA-A ρ1 receptor. To address this question, we utilized three amiloride derivatives, benzamil, phenamil, and 5-(N,N-Hexamethylene) amiloride (HMA) to assess the interaction of these compounds with the GABA-A ρ1 receptor. Whole cell patch-clamp electrophysiology of the wild type GABA-A ρ1 receptor resulted in benzamil and phenamil eliciting no change in GABA induced current, while HMA has decreased potency compared to amiloride and exhibits similar potentiation of current. Furthermore, HMA directly activates hGABA-A ρ1 receptor with higher potency and efficacy than amiloride. Our findings suggest that the guanidine group on amiloride is integral in the potentiation of the GABA-A ρ1 receptor and substitution of the pyrazine ring leads to more potent and efficacious direct activation of the receptor. Focus should be made to preserve the guanidine group in generating additional GABA-A ρ1 positive allosteric modulators.

INTRODUCTION

The interaction of different GABA-A receptor ligands is dependent on the presence of specific subunits. For example, benzodiazepine sensitivity is dependent on the presence of the α1-3 or α5 subunits (Milic *et al*., 2012). GABA-A receptors with the α4 or α6 subunits are insensitive to classical 1,4-benzodiazepines, but are sensitive to neurosterioids and ethanol (Derry *et al*., 2004). The α6 subunit was demonstrated to be integral in the effects of the barbiturate pentobarbital, conveying both a higher affinity and efficacy for the direct activation of the GABA-A receptor (Thompson *et al*., 1996). This complex pharmacology remains consistent with non-classical GABA-A ligands, including guanidine compounds. The guanidinoacetic acids are agonists in the presence of heteromeric GABA-A receptors (Neu *et al*., 2002), whereas the same compounds exhibit antagonistic effects on the GABA-A ρ1 receptors (Chebib *et al*., 2009). Similarly, amiloride, a guanidine compound, was found to be a competitive antagonist for the GABA-A αβγ receptors, and this antagonism was dependent on the presence of specific α subunits, with α6 conveying a 10-fold increase in potency of amiloride for the GABA-A receptor (Fisher, 2002).

Amiloride's diuretic effect is contributed to the direct block of epithelial sodium channels where sodium reabsorption is inhibited (Loffing and Kaissling, 2003). Additionally, amiloride inhibits acid–sensing ion channels (ASIC), which are expressed in retina (Chu and Xion, 2012). In 2013, amiloride was proposed as a pharmacological intervention to relieve retinal degeneration in ischemic disorders such as diabetic retinopathy through the antagonism of retinal ASIC receptors. They concluded that amiloride (at 100 μM) reduced retinal degeneration. Inexplicably, amiloride at higher concentrations led to a worsening of retinal degeneration (Miyake at al., 2013).

Recently, we have shown that amiloride has a different effect on the GABA-A ρ1 receptor than that reported on the heteromeric GABA-A receptors (Snell and Gonzales, 2015). Unlike heteromeric GABA-A αβγ receptors, amiloride potentiated the GABA-mediated current in the human GABA-A ρ1 receptor, most highly expressed in the retina and implicated in the degeneration of retinal ischemic disorders. This potentiation of current was highest at concentrations above 100 μM and potentiation was abolished with the introduction of the GABA-A ρ1 (I15'N) mutation. Thus, we proposed categorizing amiloride as a positive allosteric modulator of the GABA-A ρ1 receptor.

While this mutation abolished the potentiation effect, it is still unclear if the guanidine or pyrazine ring is important for this effect of amiloride with the human GABA-A ρ1 receptor. Here, we investigated the importance of the guanidine group in amiloride's potentiation of the human GABA-A ρ1 receptor utilizing whole cell patch-clamp electrophysiology of three amiloride derivatives; phenamil and benzamil, which mask the guanidine group with the addition of a phenyl and a benzene ring, respectively; and 5-(N,N-Hexamethylene)amiloride (HMA), which adds a azepane ring structure to the pyrazine side of the molecule which leaves the guanidine group accessible. When co-applied with GABA to the human GABA-A ρ1 receptor, HMA displayed potentiation similar to amiloride, while the receptor was insensitive to phenamil and benzamil, which suggests that the guanidine group is integral for the positive allosteric mechanism of amiloride and its derivatives. Finally, when applied alone, HMA directly activated the human GABA-A ρ1 receptor with an increased efficacy as compared to amiloride.

METHODS

Cell Culture and Expression of human GABA-A ρ1 receptor

We transfected wild type and mutant hGABA-A ρ1 (I15'N) receptor cDNA into human embryonic kidney 293T cells, as described previously (Snell and Gonzales, 2015). The HEK293T cell line was maintained in T25 flasks at 37° C in a 5% CO_2 water-jacketed incubator. After reaching 80% confluency, HEK293T cells were plated on glass coverslips 2-4 hours before transfection. Cells were co-transfected with pNEGFP-EU (2 μg), human GABA-A ρ1 cDNA (2 μg) or human GABA-A ρ1 I15'N cDNA (2μg) as described previously (Snell and Gonzales, 2015). Whole cell patch-clamp electrophysiology was performed 18-24 hours following transfection.

Electrophysiology:

Whole cell patch-clamp electrophysiology was performed on an inverted fluorescence microscope with an Axopatch 200B amplifier, as previously described (Snell and Gonzales, 2015). Cells were patched wit borosilicate glass patch electrodes (resistance of 8-12 MΩ) (Chebib *et al*., 2009). Amiloride hydrochloride hydrate, phenamil methanesulfonate salt, benzamil hydrochloride hydrate, 5-(N,N-Hexamethylene)amiloride (HMA), and γ-aminobutyric acid (GABA) were obtained from Sigma Aldrich (St. Louis, MO). Stock solutions were made and stored at -20°C with test solutions being made the day of the experiment. Test solutions were pH were confirmed and corrected after diluting the test compounds. For the high concentrations of amiloride (1 mM), stock solutions were made using dimethylsufoxide (DMSO). An array of perpendicular capillary tubes was used to expose patched cells to the described test solutions at a

holding potential of -70 mV. Solution flow was controlled electronically using computer driven PTFE valves using a ValveLink8.2 controller (AutoMate Scientific).

Cells were bathed continuously in external solution. Upon successful establishment of the whole-cell patch clamp configuration, test solution was applied for 5 seconds, followed by a 90 second wash period for complete recovery. The approximate GABA EC_{50} for the wild type hGABA-A ρ1 and I15'N mutant receptors are 10 and 3 μM, as determined previously (Snell and Gonzales, 2015). The recording was aborted if re-establishment of the control peak current amplitude (within 10%) failed. Concentration response profiles were fit to a dose-response function using OriginLab 8.1.

Data Analysis:

Maximum peak current amplitude in each whole cell patch-clamp electrophysiological experiment were measured and normalized to the maximum peak current amplitude elicited by the receptor's respective control. Data is presented as the mean ± standard error of the mean (SEM) of individual cells. Statistical significance was determined using unpaired Student's *t*-test.

RESULTS

Our previous studies determined amiloride was a positive allosteric modulator for the human GABA-A ρ1 receptor, but it remained unclear whether the guanidine or the pyrazine group on the compound was important for this effect. To investigate the importance of the guanidine group, we utilized whole cell patch-clamp electrophysiology to study the human GABA-A ρ1 receptor in the presence of amiloride derivatives; 5-(N,N-Hexamethylene) amiloride (HMA), phenamil, and benzamil. We first chose an amiloride derivative with an exposed guanidine group. We co-applied GABA (10 μM) with increasing concentrations of HMA ranging from 1 μM to 1000 μM. Like amiloride, HMA potentiated the GABA induced current upon co-application with GABA, with a HMA EC_{50} of 373.8 ± 177.7 μM. There was a significant decrease in potency as compared to the EC_{50} of amiloride of 77.0 ± 6.3 μM (Snell and Gonzales, 2015). Potentiation of current was not significant at lower concentrations (0.3 to 1 μM), but at higher concentrations there was a significant increase in current as compared to the control (Figure 2). Furthermore, there was an increase in the standard error at higher concentrations of HMA, which is similar to the amiloride experiments and data collected with barbiturates, classical allosteric modulators of the heteromeric GABA-A αβγ receptors (Snell and Gonzales, 2015).

While potentiation of the hGABA-A ρ1 receptor was conserved for HMA, we next sought to characterize the activity of amiloride derivatives with inaccessible guanidine groups on the GABA-induced current. We co-applied GABA EC_{50} with increasing concentrations of either phenamil or benzamil (Figure 3, Figure 4). With a GABA EC_{50} and increasing concentrations of phenamil, there was a significant decrease in current at 100 μM phenamil (Figure 3). Upon co-application of increasing concentrations of benzamil and GABA EC_{50}, there was no significant

increase in GABA induced current as compared to the control at any concentrations of compound, thus the hGABA-A ρ1 receptor was insensitive to benzamil (Figure 4).

Previously, we reported the mutation of the second transmembrane domain 15' residue of the hGABA-A ρ1 receptor displayed insensitivity to amiloride (Snell and Gonzales, 2015). To further assess the importance of the guanidine group, we sought to test these derivatives on the hGABA-A ρ1 I15'N mutant receptor (Figure 5, Figure 6). Upon co-application of increasing concentrations of HMA with GABA EC_{50}, there was a significant increase in current at 100, 300, and 1,000 μM (Figure 5A, 5B). Unlike in the wild type hGABA-A ρ1 receptor, there was a decrease in efficacy and peak current at 100 μM HMA (Figure 5B). Thus, the mutation of this residue decreased the PAM activity of HMA on the hGABA-A ρ1 receptor, but unlike amiloride, the receptor was sensitive to HMA. Upon co-application of GABA EC_{50} and benzamil, the hGABA-A ρ1 I15'N receptor was insensitive to all concentrations except 100 μM, which displayed a significant increase in peak current amplitude compared to the control (Figure 6A, 6B). Co-application of phenamil resulted in a significant increase in GABA-induced current at 10 and 100 μM (Figure 6C, 6D).

Amiloride has been shown to directly activate the hGABA-A ρ1 receptor (Snell and Gonzales, 2015). However, it remains unclear if these compounds exhibit direct activity on this receptor. Upon application of maximum concentration of phenamil and benzamil (1,000 μM) there was no current induced (Supplemental figure 1). Application of HMA (1000 μM) induced current similar to ~80% of GABA EC_{50} peak current amplitude (Figure 7). The direct activation of the channel was concentration-dependent effect.

DISCUSSION AND CONCLUSIONS

Previously we elucidated the intrinsic activity of amiloride, the prototypical ASIC blocker, on the human GABA-A ρ1 receptor (Snell, Gonzales, 2015). In our hands, amiloride potentiated the GABA induced current, and this potentiation was removed when the 15' residue of the second transmembrane domain was mutated from an isoleucine to an asparagine, the amino acid residue present in the β subunit of the GABA-A receptor. Here we continued our investigation into the chemical determinants of guanidine compound interaction with the hGABA-A ρ1 receptor. Amiloride derivatives phenamil and benzamil, which had inaccessible guanidine groups failed to elicit a concentration dependent change in the GABA induced current. HMA, which contains an accessible guanidine group, elicited potentiation similar to amiloride but with less potency. The 15' residue mutation eliminated concentration dependent effects for all amiloride derivatives. Finally, we observed robust direct activation of the hGABA-A ρ1 receptor by HMA.

The amiloride derivatives 5-(N,N-Hexamethylene) amiloride (HMA), benzamil, and phenamil have been synthesized utilizing amiloride as a backbone, but pharmacologically have different receptor interactions. These interactions include the Na^+/ H^+ exchanger (Masereel B *et al*., 2003) ENaCs, and ASICs. In the case of HMA, the structure contains a hexamethylene group substituted for two amino hydrogens, forming an azapane ring (Giansanti V *et al*., 2012). Benzamil and phenamil have either a hydrophobic benzene ring or a hydrophobic phenol group substituted for a guanidine hydrogen, respectively (Grinstein *et al*., 1988). These compounds provide an opportunity to assess the importance of the accessibility of the guanidine group for the modulation of the hGABA-A ρ1 receptor. We hypothesized that the presence of the

hydrophobic ring structures in benzamil and phenamil would prevent the guanidine group from interacting with the hGABA-A ρ1 receptor, and thus prevent potentiation, while HMA would preserve potentiation due to an exposed guanidine group.

The blocking mechanism of amiloride and its derivatives is dependent on the protonation of the guanidine group, evident due to the fact that guanidine itself can block Na^+ channels (Benos, 1982). At physiological pH, amiloride is protonated and thus positively charged (Warncke and Lindemann, 1985). Benzamil and phenamil are less protonated at physiological pH due to the substitution of hydrophobic groups for and the hydrogen on the guanidine group. The loss of the positive charged guanidine group may be contributing to the lack of modulation of the hGABA-A ρ1 receptor. HMA displayed an activity similar to the PAM activity of amiloride on the hGABA-A ρ1 receptor (Figure 2). While the potentiation was still present, there was a significant decrease in the potency as compared to amiloride. This increase could be due to the presence of the azapane ring, causing HMA to be larger than amiloride. This increase in size could prevent the compound from entering the inter subunit site of the hGABA-A ρ1 receptor as easily as amiloride.

Many studies have assessed the influence of structure of a compound on its activity in the presence of the GABA-A receptors. Alkyl-substituted butyrolactones have differential pharmacology on the GABA-A receptors depending on the position of their substitution. Lactones with α-position substitutions are positive modulators, while β-substituted lactones are frequently inhibitors (Williams *et al*., 1997). For example, positive and negative enantiomers of α-benzyl-α-methyl-γ-butyrolactone (α-BnMeGBL) modulate the GABA-A α1β2γ2 receptor differently; with the positive enantiomer potentiating the receptor with a 2-fold greater maximum

current than the negative enantiomer (Gonzales *et al*., 2004). Similar to butrylactone activity in the GABA-A α1β2γ2 receptor, neurosteroids differentially modulate the GABA-A ρ1 receptor based on the substitution of the neurosteroid structure (Hosie *et al*., 2006, Morris *et al*., 1999). Similarly, the substitution of the ring structure in HMA could influence amioride derivative activity. Benzamil, however, did not elicit any significant increase in GABA-induced current as compared to the control (Figure 4). This insensitivity could be due to the inaccessibility of the guanidine group to the receptor, thus inhibiting interaction and abolishing the potentiation seen with both amiloride and HMA. Additionally, phenamil inhibited the GABA induced current at 100 μM, but not in a concentration dependent manner (Figure 2, Figure 3). This inhibition could be due to another site, a low affinity site that is responsible for this concentration-independent effect.

Amiloride failed to potentiate GABA-mediated current in the hGABA-A ρ1 (I15'N) mutant receptor (Snell and Gonzales, 2015). This site is also implicated in both barbiturate and neurosteroid activity in GABA-A αβγ receptors (Saunders and Ho, 1990). We sought to investigate if this site was important for HMA potentiation. Upon co-application of GABA with increasing concentrations of HMA, the hGABA-A ρ1 I15'N mutant receptor was insensitive to most concentrations of HMA except for 100, 300, and 1,000 μM, where we observed a significant increase in GABA induced current (Figure 5A, Figure 5B). While there was potentiation of current, the observed current was less than that observed for HMA potentiation of the wild type hGABA-A ρ1 receptor (Figure 2). This reduction in potentiation, and shift towards insensitivity suggests that this residue is also important in PAM activity of HMA. Phenamil was also inactive in the hGABA-A ρ1 I15'N mutant receptor similar to the wild type receptor (Figure

6A, Figure 6B). Benzamil, however displayed a significant increase in GABA-induced current at 100 µM in the hGABA-A ρ1 I15'N mutant receptor (Figure 6C, Figure 6D). This suggests that the may be a low affinity site that accommodates phenamil and benzamil interaction with the hGABA-A ρ1 receptor.

In addition to the PAM effect of HMA, we observed robust direct activation of the hGABA-A ρ1 receptor in the presence of this amiloride derivative (figure 7). Upon application of HMA alone, there was direct activation of the receptor, most significantly at the highest concentration examined (1,000 µM) (Figure 7). Previously, we reported that amiloride directly activated the hGABA-A ρ1 receptor (Snell and Gonzales, 2015). In comparison, HMA was more potent and efficacious as a direct activator of the hGABA-A ρ1 receptor. This increase in efficacy could be due to the addition of the hexamethylene for the two hydrogens on the amiloride backbone (Figure 1, Figure 8). One possible explanation could be that the site of action for amiloride is larger than an amiloride molecule and that HMA's large chemical substitution on the pyrazine ring results in direct activation with higher potency and efficacy. Thus, we concluded, the guanidine availability is integral for PAM activity, while chemical substitution on the pyrazine ring influences direct activation of the hGABA-A ρ1 receptor (Figure 8).

Here, we reported the importance of the guanidine group for positive allosteric modulation of the hGABA-A ρ1 receptor by amiloride derivatives. Furthermore, we observed the direct activation of the hGABA-A ρ1 receptor by HMA. Based on our data, an accessible guanidine group is critical for amiloride and amiloride derivative interaction with the human GABA-A ρ1 receptor. The hydrophobic substitution on the amiloride pyrazine ring may be responsible for direct and positive allosteric modulation of these receptors. This opens up a new

line of inquiry for the development and identification of hGABA-A ρ1 receptor allosteric modulators.

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AUTHOR CONTRIBUTIONS

Development of idea and experimental design: HDS and EBG

Data collection: HDS

Data analysis: HDS and EBG

Manuscript development and editing: HDS and EBG

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FOOTNOTES

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2. Society for Neuroscience (SFN) national conference, Chicago, IL, October 16-21, 2015.

Person to contact for reprint requests:

Eric B. Gonzales, Ph.D.

Assistant Professor

Center for Neuroscience Discovery

Institute on Healthy Aging

UNT Health Science Center

3500 Camp Bowie Blvd.

Fort Worth, TX 76107

Email: eric.b.gonzales@unthsc.edu

Phone: 817-735-2755

Fax: 817-735-0408

FIGURE LEGENDS AND CORRESPONDING FIGURES

Figure 1. Chemical structure of guanidine compounds 5-(N,N-Hexamethylene) amiloride (HMA), phenamil, and benzamil. 5-(N,N-Hexamethylene)amiloride (HMA), has an added azepane ring structure on the pyrazine side of the molecule. Phenamil and benzamil, contains a n added phenyl and a benzene ring, respectively.

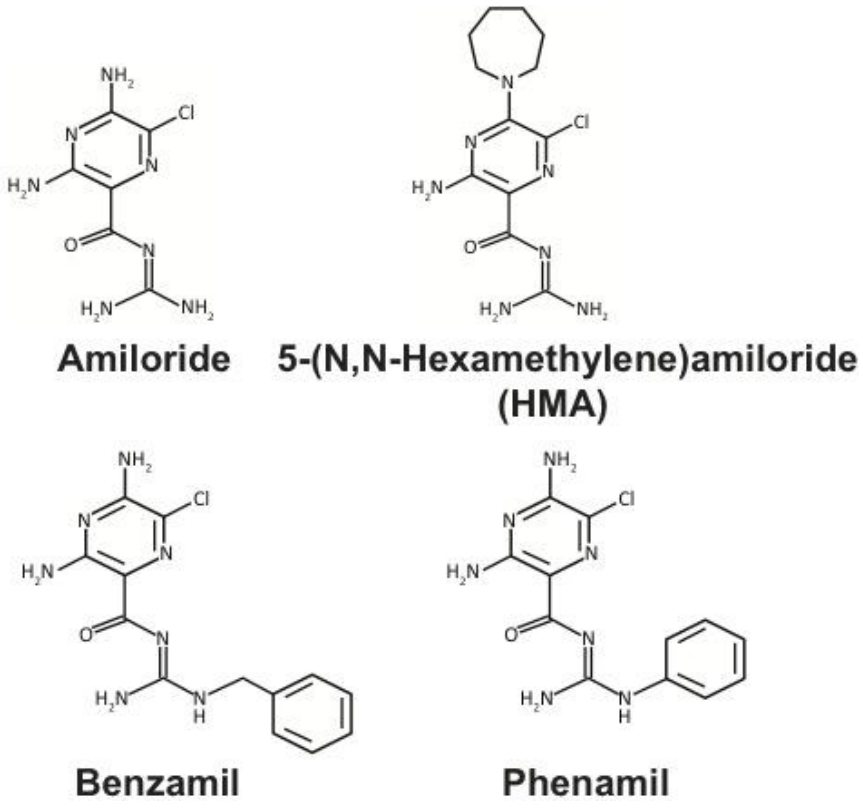


Figure 1. Chemical structure of guanidine compounds

Figure 2. 5-(N,N-Hexamethylene) amiloride (HMA) potentiates the hGABA-A ρ1 receptor. (A) Resulting representative current generated by the co-application of EC_{50} GABA (10μM) with increasing concentrations of HMA in the presence of wild type hGABA-A ρ1 receptor. (B) Potentiation of the hGABA-A ρ1 receptor in the presence of increasing HMA. Data presented as the mean ± SEM at the GABA EC_{50} concentration co-applied with increasing concentrations of HMA. Data representative of $n \geq 4$ individual cells. HMA EC_{50} was 373.8 μM ± 177.7 with a Hill coefficient of 2.9 ± 4.0. Significance is indicated by: *, p 0.05; #, p 0.005; ‡, p 0.0005

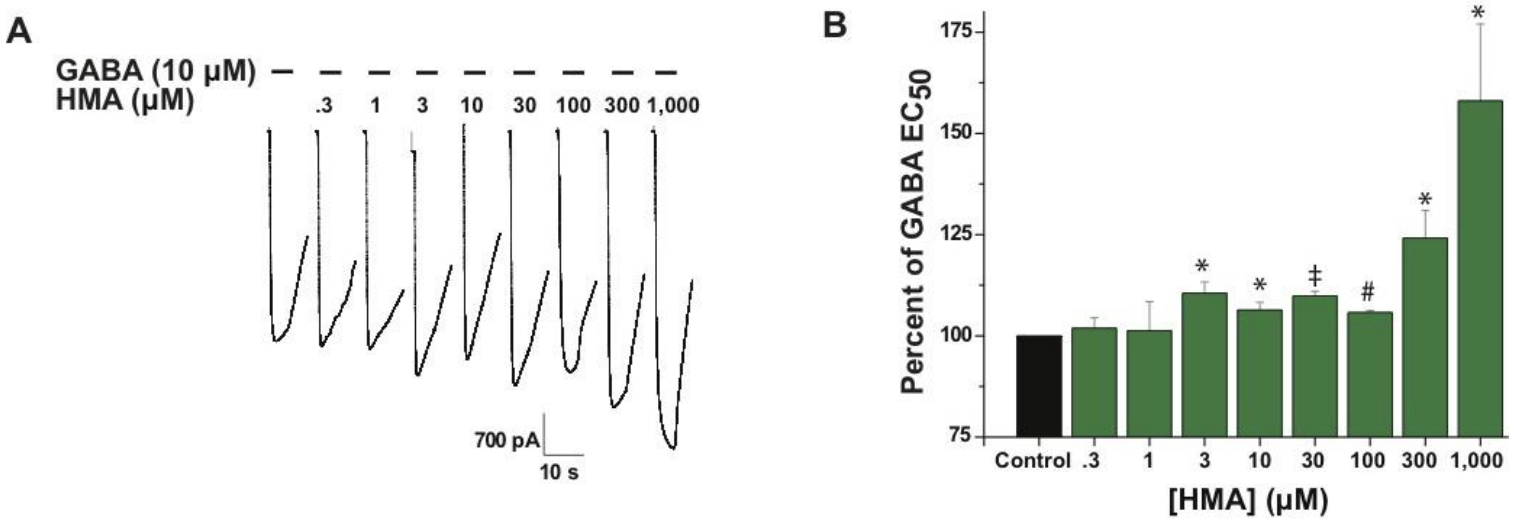


Figure 2. 5-(N,N-Hexamethylene) amiloride (HMA) potentiates the hGABA-A ρ1 receptor.

Figure 3. Phenamil inhibits the hGABA-A ρ1 receptor at a single concentration. (A) Resulting representative traces generated by the co-application of GABA EC_{50} (10μM) with increasing concentrations of phenamil in the presence of the wild type hGABA-A ρ1 receptor. (B) Phenamil displayed a slight decrease in peak current amplitude upon co-application with GABA EC_{50}. While there was a decrease in current, the only concentration that was significant was 100 μM. Data are the mean ± SEM of n ≥4 cells. Significance is indicated by *, p 0.05

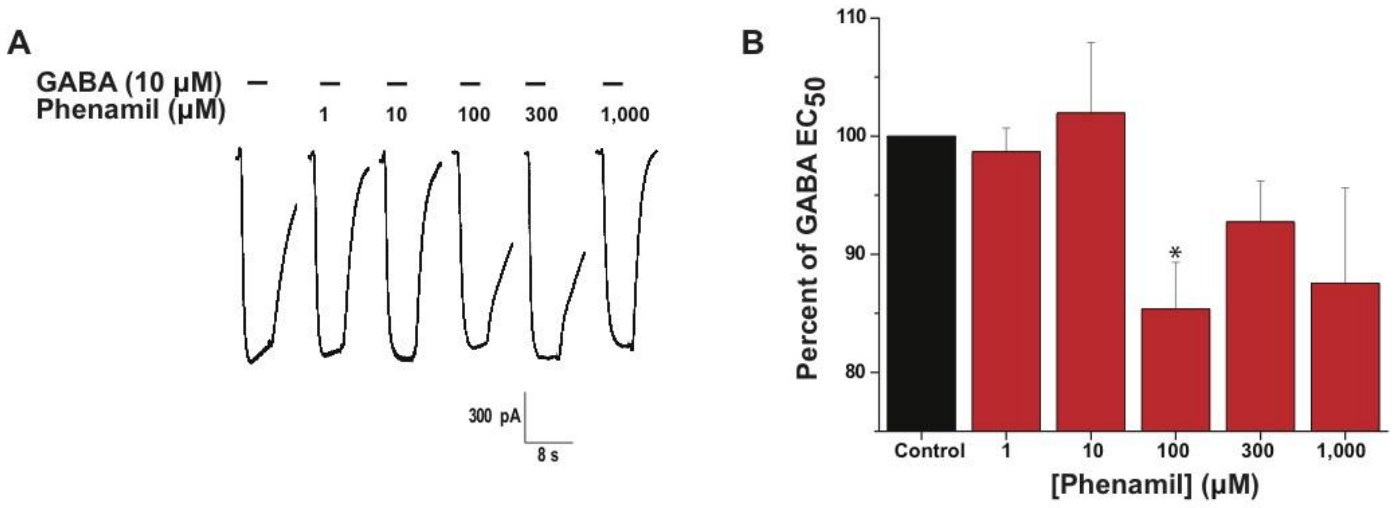


Figure 3. Phenamil inhibits the hGABA-A ρ1 receptor at a single concentration.

Figure 4. The hGABA-A ρ1 receptor is insensitive to the amiloride derivative benzamil. (A) Resulting representative responses generated by the co-application of GABA EC_{50} (10 μM) with increasing concentrations of benzamil in the presence of the wild type hGABA-A ρ1 receptor. (B) Benzamil failed to elicit potentiation in current of the hGABA-A ρ1 receptor, thus no EC_{50} was obtained for this compound. Data are the mean ± SEM of n ≥4 cells.

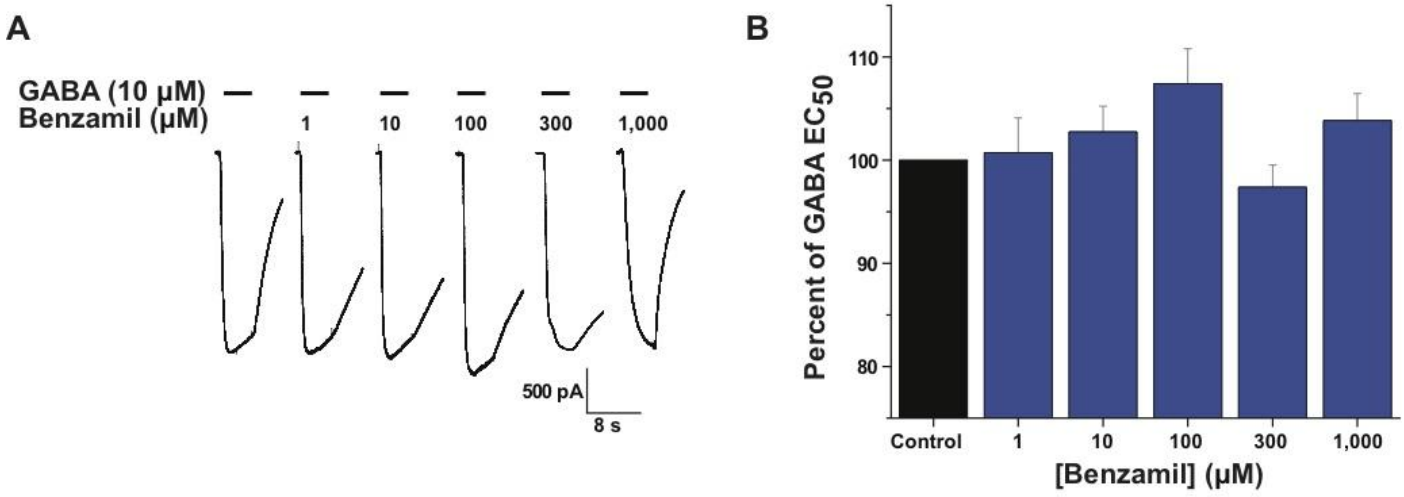


Figure 4. The hGABA-A ρ1 receptor is insensitive to the amiloride derivative benzamil.

Figure 5. HMA potentiates the hGABA-A ρ1 I15'N mutant receptor with a decreased potency and efficacy as compared to the wild type hGABA-A ρ1 receptor. (A) Representative responses to increasing concentrations of HMA co-applied with GABA EC_{50} (3 μM) on the hGABA-A ρ1 (I15'N) mutant receptor. (B) Comparison of normalized concentration responses of HMA on the hGABA-A ρ1 (I15'N) mutant receptor. Significant current was elicited at 100, 300 and 1,000 μM HMA. Data are the mean ± SEM of n ≥4 cells. Significance is indicated by: ‡, p 0.0005.

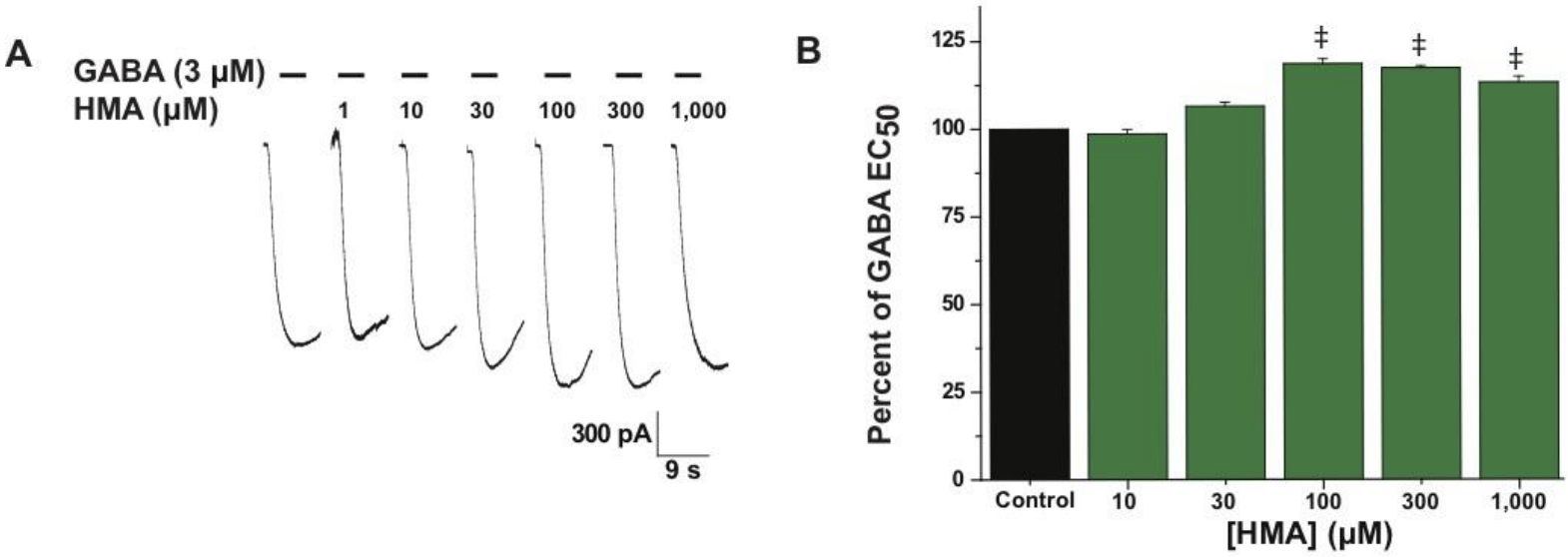


Figure 5. HMA potentiates the hGABA-A ρ1 I15'N mutant receptor with a decreased potency and efficacy as compared to the wild type hGABA-A ρ1 receptor.

Figure 6. Phenamil and benzamil fail to elicit a concentration-dependent increase in GABA-induced current on the hGABA-A ρ1 (I15'N) mutant receptor. (A) Representative current traces of increasing concentrations of phenamil co-applied with GABA EC_{50} (3 μM) on the hGABA-A ρ1 (I15'N) mutant receptor. (B) Comparison of normalized concentration-response of co-application of EC_{50} GABA and phenamil in the presence of the hGABA-A ρ1 I15'N mutant receptor. Significant current was elicited at 100, 300 and 1,000 μM HMA. (C) Representative current traces of increasing concentrations of phenamil co-applied with GABA EC_{50} (3 μM) in the presence of the hGABA-A ρ1 I15'N mutant receptor. (D) Comparison of normalized concentration-response profile of co-application of GABA EC_{50} and increasing concentrations of benzamil. Only 10 and 100 μM benzamil were significant. Data are the mean ± SEM of n ≥4 cells. Significance is indicated by: * p 0.05, # p 0.005

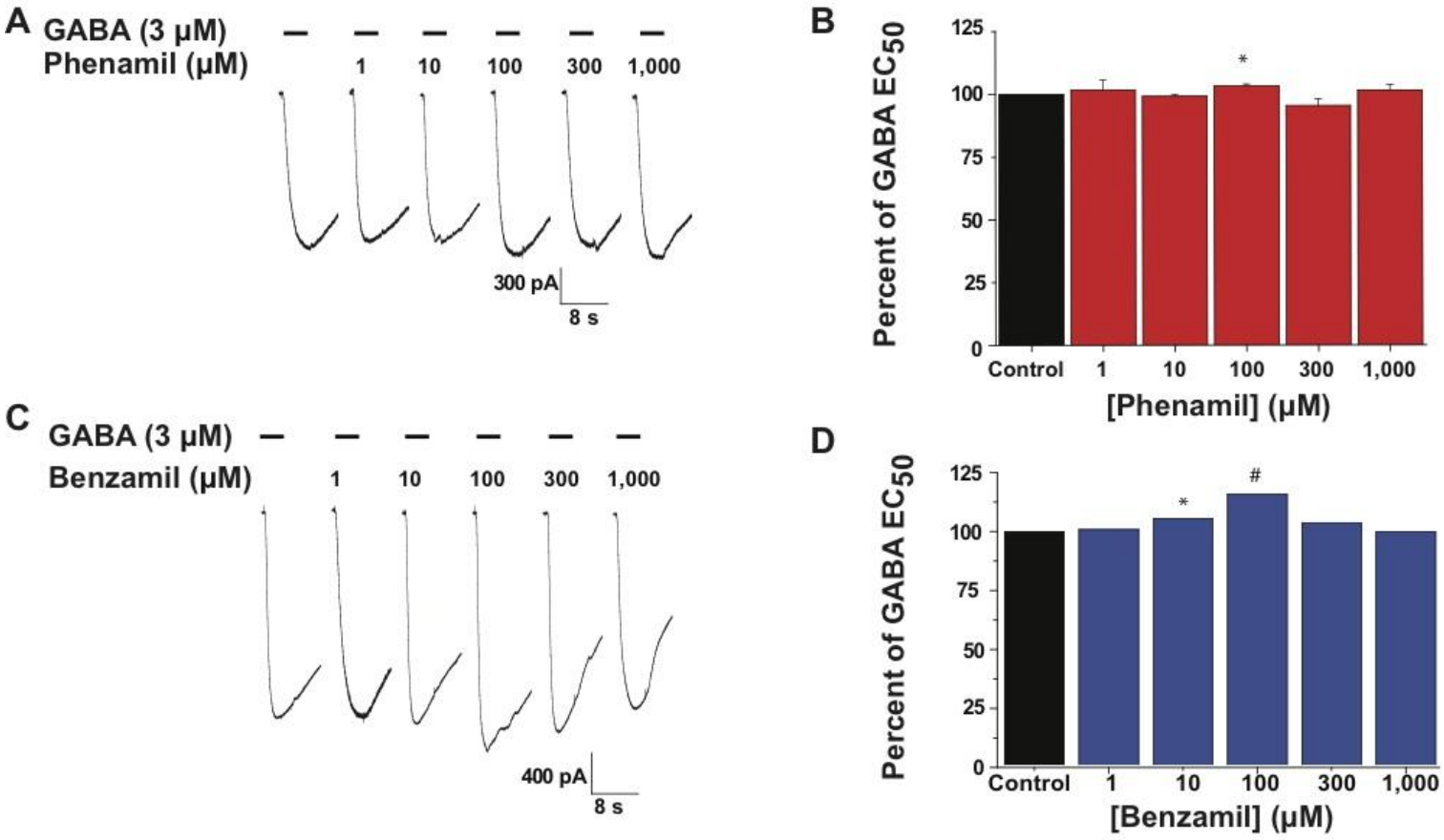


Figure 6. Phenamil and benzamil fail to elicit a concentration-dependent increase in GABA-induced current on the hGABA-A ρ1 (I15'N) mutant receptor.

Figure 7. HMA elicits direct activation of the hGABA-A ρ1 receptor. (A) Representative current of application of HMA in the presence of the hGABA-A ρ1 receptor. Application of HMA elicited activation of the hGABA-A ρ1 receptor at 10, 100, and 1,000 μM. Traces are from different cells. n≥ 3 (B) Normalized responses of HMA direct activation of the hGABA-A ρ1 receptor. Application of 1,000 μM HMA elicited ~80 % of the control (EC_{50} GABA). Data are the mean ± SEM of n ≥4 cells.

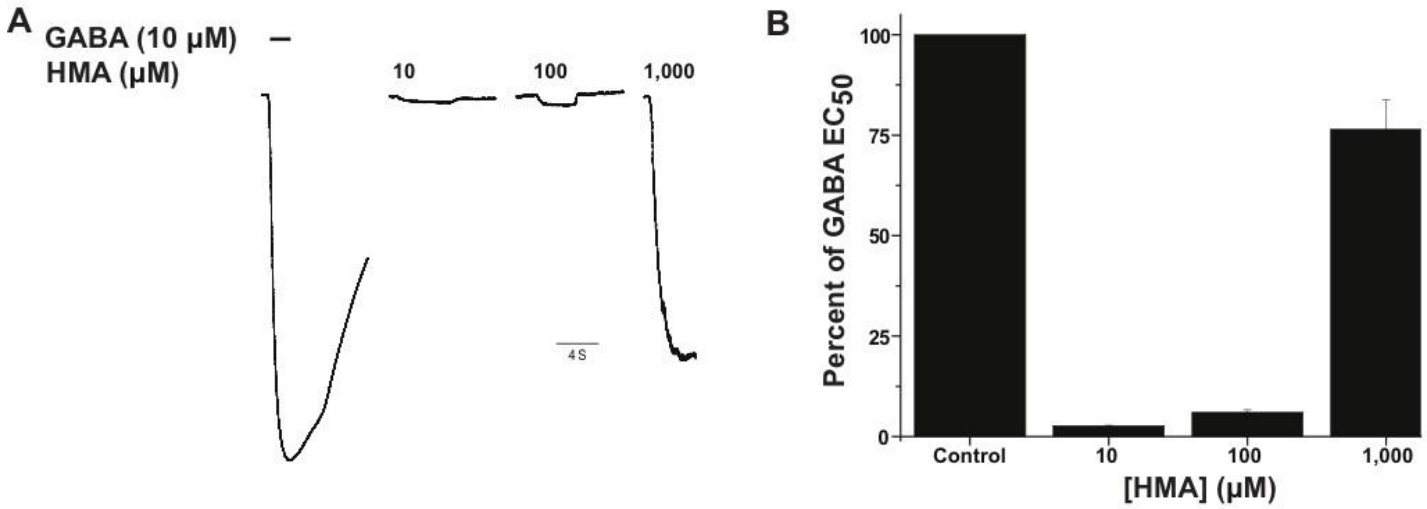


Figure 7. HMA elicits direct activation of the hGABA-A ρ1 receptor

Figure 8. Proposed effects of amiloride derivation on the human GABA-A ρ1 receptor. When the guanidine group is accessible and the pyraszine ring remains un-changed, as in the case of amiloride, there is PAM activity and weak direct activity on the human GABA-A ρ1 receptor. HMA's structure is produced from derivation of the pyraziine ring, but the guanidine group remains accessible, resulting in conservation of PAM activity, and more robust direct activation of the receptor. If there is substitution on the guanidine group, however, the compound elicits no PAM activity or direct activation of the receptor, as in the case of benzamil and phenamil

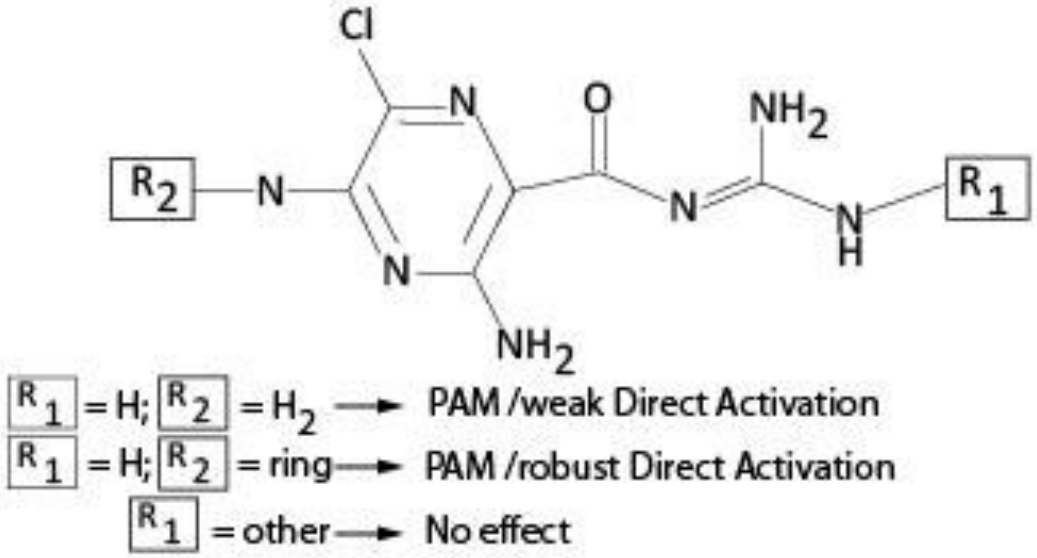
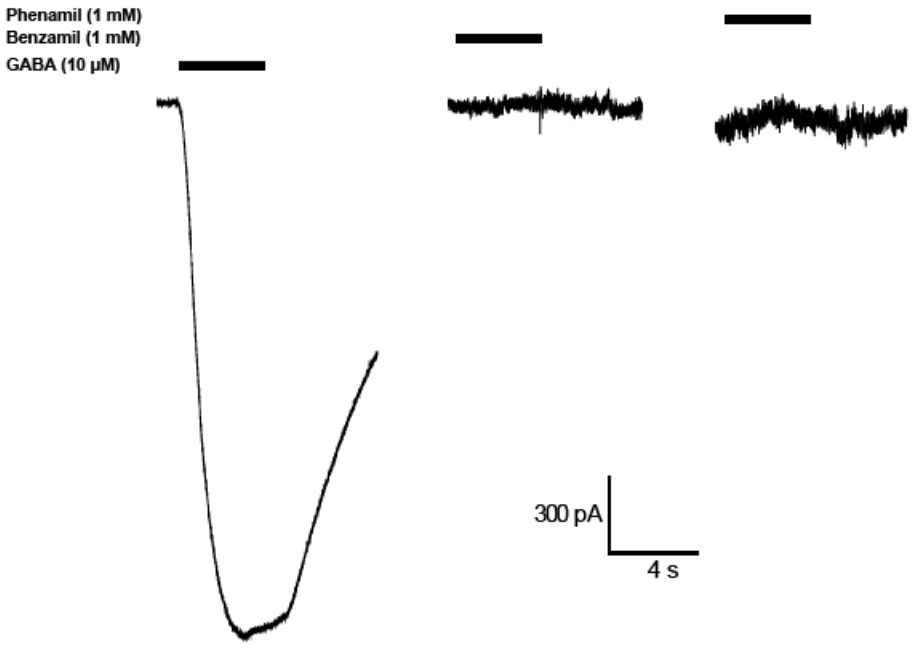


Figure 8. Proposed effects of amiloride derivation on the human GABA-A ρ1 receptor.

Supplemental Figure 1. Phenamil and Benzamil fail to elicit current from the hGABA-A ρ1 receptor when applied alone. EC_{50} GABA was applied alone in the presence of the human GABA-A ρ1 receptor and elicited am induced current. Maximum concentration (1 mM) benzamil and phenamil were then applied to the receptor alone. Neither of the compounds elicited activation of the receptor in the absence of GABA.



Supplemental Figure 1. Phenamil and Benzamil fail to elicit current from the hGABA-A ρ1 receptor when applied alone.

II. GENERAL DISCUSSION

These studies show evidence of positive and negative allosteric modulators for the GABA-A ρ1 receptor. Additionally, these studies elucidated the importance of the 15' residue in the second transmembrane domain of the receptor in the modulatory mechanism of amiloride, suggesting a possible previously unknown site on the receptor, which is not present in the heteromeric GABA-A αβγ receptors. Lastly, we characterized the molecular aspects of the group on the compound important in eliciting amiloride's modulatory activity on the GABA-A ρ1 receptor. The guanidine group was proven to be integral in conveying the modulatory activity, while the pyrazine ring was shown to be important in eliciting direct activation of the receptor. The data presented in this dissertation contributes to the field's understanding of novel effects of amiloride and guanidine compounds, and a better understanding of the GABA-A ρ1 receptor's functional and structural characteristics. This research has further implications in fields such as diabetes and hypertension, because diabetics often take diuretics like amiloride to treat their hypertensive symptoms. Our data would suggest amiloride consumption could exacerbate symptoms of diabetic retinopathy, a disorder common among those with advanced stages of type 1 and type 2 diabetes.

The guanidine compounds interact with the 15' residue of the second transmembrane domain

The 15' residue of the second transmembrane domain of the GABA-A ρ1 receptor is integral in the allosteric modulatory activity of amiloride and HMA (Snell and Gonzales, 2015).

While the presented data suggests the 15' residue of the GABA-A ρ1 receptor plays a role in the gating or mechanism of the allosteric modulation, it could also be the site of interaction. This conclusion was unable to be confirmed through binding experiments, mainly due to the lack of high affinity competitive compounds for amiloride at that site. Even with the lack of binding experiments, the importance of the site was validated with mutagenesis.

This same residue has been implicated in barbiturate binding, specifically in the GABA-A β subunit. Mutation of this residue in the GABA-A β subunit from an asparagine (N) to an isoleucine (I), the residue present in the GABA-A ρ1 receptor, results in insensitivity to barbiturates (Stewart *et al*., 2014). It would be interesting to determine if this mutated residue in the GABA-A β subunit conveys sensitivity to amiloride potentiation. The insensitivity conveyed upon mutation of the 15' residue, however, suggests that this could be the site of interaction for amiloride and to a lesser extent HMA (Figure 1). We hypothesize that these compounds bind to the 15' site of the second transmembrane domain of the GABA-A ρ1 receptor and elicit potentiation of the GABA-induced current (Figure 1). Due to the inaccessible guanidine group, phanamil and benzamil are unable to interact with the 15' site, and thus do not potentiate the GABA-induced current. Further mutagenesis investigation of the GABA-A ρ1 receptor could lead to the discovery of other novel compound interaction and binding sites.

Amiloride administration could exacerbate diabetic retinopathy

Amiloride, is classically prescribed as adjunctive treatment of hypertension (Svendsen *et al.,* 1983). Due to its weak natriuretic, diuretic, and antihypertensive activity, amiloride is rarely

prescribed or taken alone for the treatment of hypertension (Bull and Laragh, 1968). Hypertension is a common comorbidity with diabetes, with prevalence often increasing as the diabetes progresses. During the years of 2009-2012, the National Diabetes Statistics Report for 2014 stated 71% of adults aged 18 years or older with diagnosed diabetes had blood pressure at hypertensive levels or used prescription compounds to lower their blood pressure. It is unknown whether hypertension is caused by the diabetes, or vice-versa. It is hypothesized that the diabetic nephropathy, or kidney disease, seen with high prevalence in Type 1 diabetics, may play a role in the development of hypertension (Oster *et al*., 1990). Less is known about the causation of hypertension and type 2 diabetes, but the correlation remains.

Diabetic retinopathy occurs with both type 1 and type 2 diabetes. The National Eye Institute estimates between 40 and 45 percent of Americans diagnosed with diabetes have diabetic retinopathy, making it the number one cause of blindness in America. There remains no cure or prevention other than controlling blood sugar, blood cholesterol, and blood pressure levels. This disorder is a microvascular disorder resulting in lesions including a thickened capillary basement membrane and defective blood-retinal barrier (Gillow *et al*., 1999). A defective blood-retinal barrier (BRB) leads to an increase in the permeability of this barrier, thus compounds that otherwise would not pass into the retina now enter the tissue, leading to deleterious effects (Kaur *et al*., 2008).

Thus, compounds commonly taken by patients with diabetic retinopathy, such as diuretics, could penetrate the retina, and lead to worsening of the symptoms of diabetic retinopathy. In 2002, a study investigating diabetes and atherosclerosis noted that of the patients participating in the study, those taking diuretics had a significantly increased prevalence of retinopathy in the

later stages of the disorder (Klein *et al*., 2002). They, however, did not state which diuretics these patients were taking, nor for what duration. While the exact number is unknown, many patients with diabetes could be taking amiloride as a treatment for their hypertensive symptoms.

Taken with the data presented in this dissertation, if allowed access to the retina through the compromised retinal-blood barrier, amiloride would both potentiate the GABA-induced current and directly activate the GABA-A ρ1 receptor. Amiloride was proposed as a pharmacological intervention to slow the progression of retinal degeneration in ischemic disorders such as diabetic retinopathy through the antagonism of retinal acid sensing ion channels (ASICs). They concluded that 100 μM amiloride reduced retinal degeneration. Inexplicably, amiloride at higher concentrations led to a worsening of retinal degeneration (Miyake at al., 2013). The GABA-A ρ1 receptor, as previously stated in the introduction, has been implicated in diabetic retinopathy. Potentiation of this receptor would lead to an increase in retinal vascular leakage, and worsening of the retinal disorder. Here, correlation may not mean causation. Certain factors, such as; taking amiloride for hypertensive symptoms, and having diabetic retinopathy a result of either type 1 or type 2 diabetes, could result in the worsening of diabetic retinopathy.

Thus, closer care needs to be taken when prescribing medication for patients with hypertension and diabetes, and amiloride could be contraindicated with diabetes, or at least diabetic retinopathy symptoms. Additionally, compounds such as metformin, which is prescribed for the treatment of Type 2 diabetes and contains an exposed guanidine group, could contribute to the potentiation of the GABA-A ρ1 receptor both in the retina and in the brain. This data could also lead to a closer examination and study of other compounds that contain a guanidine group.

The results in this dissertation suggest that any compound containing an exposed guanidine group could act as an allosteric modulator for the GABA-A ρ1 receptor. This data leads the way to the investigation of other guanidine containing compounds, with the potential to be more efficacious or potent than amiloride and HMA at the GABA-A ρ1 receptor. Based on our data and observations, the medications provided to diabetics should be carefully evaluated due to the possible role in diabetic retinopathy. Furthermore, data in this dissertation could aid in the formulation of compounds that could be utilized to alleviate pathologies, while eliminating possible side effects in the retina and other locations in the brain where the GABA-A ρ1 receptor is expressed. These compounds would need to contain a masked, or inaccessible guanidine group, while retaining affinity for the target receptor.

FIGURE LEGEND AND CORRESPONDING FIGURE

Figure 1. Schematic depicting the interaction of amiloride and its derivatives with the human GABA-A ρ1 receptor. Utilizing the accessible guanidine group, both amiloride and HMA are able to interact with the human GABA-A ρ1 receptor. Benzamil and phenamil, both containing inaccessible guanidine groups, are unable to interact with the receptor at the intersubunit site, thus potentiation of current is lost.

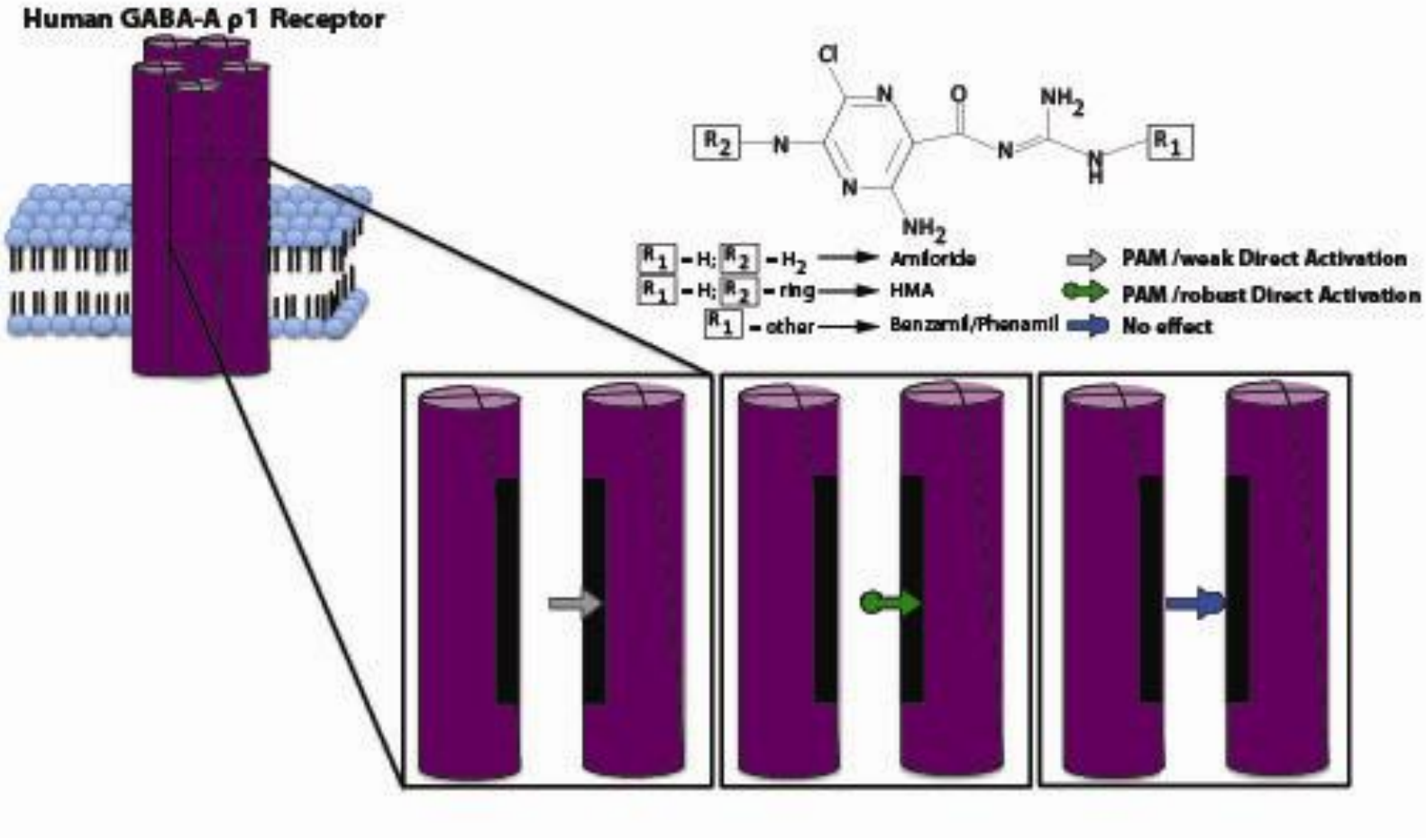


Figure 1. Schematic depicting the interaction of amiloride and its derivatives with the human GABA-A ρ1 receptor

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Stewart DS, Pierce DW, Hotta M, Stern AT and Forman SA (2014) Mutations at Beta N265 in gamma-Aminobutyric Acid Type A Receptors Alter Both Binding Affinity and Efficacy of Potent Anesthetics. *PloS one* **9**:e111470.

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III. CONCLUSIONS AND FUTURE DIRECTIONS

While much is known about the GABA-A heteromeric receptors containing the GABA-A αβγ subunits, the GABA-A ρ family of receptors remains a neglected field of pharmacology and structural science. The expression of this GABA-A subunit family goes beyond the retina and has been discovered more widely expressed than previously thought throughout the human body. Like-wise, its function has become more involved in processes such as learning and memory, fear, and anxiety, and thus a better understanding of pharmacological compounds that interact with this receptor could aid in treating pathologies in these areas. Our studies have identified a novel group of compounds that was thought not to interact with the GABA-A ρ receptor family. These compounds are classified as antagonists for heteromeric GABA-A receptors, thus these studies contribute to the differential pharmacology seen between the GABA-A subunits. Our studies identify and validate amiloride and similar guanidine compounds such as HMA as positive allosteric modulators for the GABA-A ρ1. This discovery could lead to the future development of subtype selective allosteric modulators for the GABA-A ρ1 receptor.

These studies may contribute to a further understanding of the GABA-A ρ receptor family function and structure. Amiloride and HMA are shown to act as positive allosteric modulators, while GMQ acts as a negative allosteric modulator of the GABA-A ρ1 receptor. While we utilized modeling and site-directed mutagenesis to investigate certain sites on the second transmembrane domain, we chose select mutations for the 15' site. Further studies utilizing the mutation of this site to other residues with a range of biochemical properties, such as; volume, hydrophilicity, hydropathy, and polarity, would help investigate the site's

importance in the allosteric modulatory mechanism (Table 1). Additionally, binding studies would aid in discovering the exact site of interaction, which still remains unknown. There is also a secondary site at which benzamil, phenamil and GMQ elicit their activity. Further investigation into important residues in the second transmembrane domain would assist in revealing the possible allosteric site on the GABA-A ρ1 receptor.

While these compounds have been characterized for the GABA-A ρ1 receptor, their activity on the ρ2 and ρ3 receptor remains unknown. The investigation of these compounds on the other ρ subunits, or heteromers composed of different combinations of ρ1, ρ2 and ρ3 receptor subunits, would further determine if the activity of these guanidine compounds is specific for the ρ1 receptor, or shared among other ρ subunits. These findings and future experiments have implications in the GABA-A receptor field, as it will contribute to the development of novel therapeutics that influences the actions of the GABA-A ρ1 receptor.

Table 1. Physiochemical properties of proposed amino acid mutations for the 15' residue

Amino Acid	Letter Abbr.	Side Chain Volume (Å^3)	Hydrophilicity (kcal/mol)	Polarity	Hydropathy Index
Alanine	A	26.3	-0.5	0	1.8
Arginine	R	129.0	3.0	52.0	-4.5
Asparagine	N	63.7	0.2	3.38	-3.5
Aspartate	D	53.3	2.5	49.7	-3.5
Cysteine	C	39.7	-1.0	1.480	2.5
Glutamine	Q	85.6	0.2	49.9	-3.5
Glycine	G	0	0	0	-0.4
Histidine	H	95.5	-0.5	51.6	-3.2
Isoleucine	I	101.1	-1.8	0.130	4.5
Phenylalanine	F	129.7	-2.5	0.350	2.8
Serine	S	30.4	0.3	1.67	-0.8
Threonine	T	56.2	-0.4	1.66	0.7
Tryptophan	W	167.9	-3.4	2.10	-0.9
Tyrosine	Y	133.3	-2.3	1.61	-1.3
Valine	V	75.3	-1.5	0.13	4.2

APPENDIX A: CIRRICULUM VITAE

Education

- Louisiana State University: Bachelor of Science, Bachelor of Art
    - Graduation date: May 2010
    - Major: Biochemistry and English Literature
    - Minor: Chemistry and Latin
- University of North Texas Health Science Center: Doctor of Philosophy
    - Anticipated Graduation date: October 2015
    - PhD Candidate
    - Department of Pharmacology and Neuroscience

Honors/Scholarships/Awards

- Future Leaders of America: Medicine, summer 2005
- Tiger scholarship, Fall 2005
- Dean's List of Academic Excellence, Fall 2009
- Minority Opportunities in Research and Education (MORE) fellowship, 2010-2012
- Institute for Aging and Alzheimer's Disease Research (IAADR) Associate Fellowship, 2011-2012
- Society for Advancement of Chicanos and Native Americans in Science (SACNAS) Travel Scholarship Award to National Conference, Fall 2011
- Summer Program in Neuroscience, Ethics, & Survival (SPINES) Fellow at the Marine Biology Laboratory (MBL) in Woods Hole Massachusetts, Summer 2012
- Post Course Research Fellow at Marine Biological Laboratory (MBL) in Woods Hole Massachusetts, Summer 2012
- Online Coursera course: Drugs and the Brain (taught by Henry Lester), 2012-2013
- UNTHSC Graduate Student Association Scholarship, 2013-2014
- Student Leadership Professional Development Fund (SL/PDF) Travel Award, Spring 2014
- Institute of Aging and Alzheimer's Disease Research (IAADR) Associate Fellowship, 2013-2014
- FASEB MARC Program Poster/Oral Presentation Travel Award- Experimental Biology National Conference, Spring 2014
- Neurobiology of Aging Training Grant Fellowship, 2014-2015
- UNTHSC Moorman Family Scholarship, 2014-2015
- Neuroscience Scholar Program Associate Fellow (Society for Neuroscience), 2014-2015
- Society for the Advancement of Chicanos and Native American in Science (SACNAS) Travel Scholarship Award to National Conference, Fall 2014
- Student Leadership Professional Development Fund (SL/PDF) Travel Award, Fall 2014

- UNTHSC Student Bridge Grant, 2015-2017
- Neurobiology Course scholarship at Marine Biological Laboratory (MBL) in Woods Hole Massachusetts, Summer 2015
- 2015 SPINES symposium travel award, Chicago Illinois, Fall 2015

Research Experience

- Medical Entomology lab, 2007-2009
    - Faculty Mentor(s): Dr. Lane Foil, Department of Entomology
    - Louisiana State University
    - Research Project: "*The control of leishmaniasis disease."*
- Biochemistry lab, 2007-2008
    - Faculty Mentor(s): Dr. E Hawkins, Department of Biochemistry
    - Louisiana State University
    - Research Project: "*Isolation and cloning of the adh gene."*
- Biochemistry lab, 2008-2010
    - Faculty Mentor(s): Dr. Roger Lane, Department of Biochemistry
    - Louisiana State University
    - Research Project: "*CD69 effect of neoangiogenesis of tumor cells.*"
- Department of Molecular Biology & Immunology, August 2010- November 2010
    - Faculty Mentor: Dr. Abha Sharma, Department of Molecular Biology & Immunology
    - University of North Texas Health Science Center
    - Rotation Research Project: *"Treatment of non-small lung carcinomas with chemotherapeutic agents"*
- Department of Pharmacology and Neuroscience, December 2010-present
    - Faculty Mentor: Dr. Eric B Gonzales, Department of Pharmacology and Neuroscience
    - University of North Texas Health Science Center
    - Research Project: "*Crystallization and Characterization of a Gamma-Aminobutyric acid A Receptor*"
- Marine Biological Laboratory, July- August 2012
    - Faculty Mentor: Dr. Steven Zottoli
    - Research Project: *"Touch sensitivity of supramedullary neurons in the cunner, Tautoglobrus adspersus"*

Presentations

- 2011-UNTHSC Research Appreciation Day (RAD) abstract (poster): "*Pre Crystallization Screening of a Gamma-Aminobutyric Acid A Receptor"*
- 2011-Society for the Advancement of Chicanos and Native Americans in Science National Conference (SACNAS) National Conference abstract (poster): "*Pre Crystallization Screening of a Gamma-Aminobutyric Acid A Receptor"*

- 2012-UNTHSC Research Appreciation Day (RAD) abstract (poster): "*Pre Crystallization Screening of a $GABA_A$-ρ (GABAC) receptor*"
- 2012-MBL Summer Program in Neuroscience and Survival (SPINES) Symposium abstract (oral presentation): *"Pre Crystallization Screening of a GABAA-ρ receptor"*
- 2012-Society for Neuroscience Diversity Forum abstract (poster): "*Pre Crystallization Screening of a $GABA_A$-ρ (GABAC) receptor"*
- 2013-UNTHSC Research Appreciation Day (RAD) abstract (poster): "*PROGRESS TOWARDS CRYSTALLIZING A GABAA RECEPTOR"*
- 2013-Nation Directors of Graduate Studies meeting (Poster): "*PROGRESS TOWARDS CRYSTALLIZING A GABAA RECEPTOR"*
- 2013-Society for Neuroscience Diversity Forum abstract (Poster): "*Crystallization of a homomeric GABAA receptor***"**
- 2013-Society for Neuroscience abstract (Poster): "*Crystallization of a homomeric GABAA receptor***"**
- 2014-18th Annual UT Austin Neuroscience Symposium abstract (Poster): "*Crystallization of a homomeric GABAA receptor***"**
- 2014-Research Appreciation Day abstract (Poster): "*NOVEL GABAA-RHO1 INTERACTIONS WITH ACID SENSING ION CHANNEL LIGANDS"*
- 2014-Experimental Biology National Conference abstract (Poster): "*Novel GABAA-rho1 interactions with acid sensing ion channel ligands*"
- 2014-Society for the Advancement of Chicanos and Native Americans in Science National Conference abstract (Oral): "*Allosteric Modulation of the Human GABA-A rho1 Receptor By ASIC Non-proton Ligands"*
- 2014-Society for Neuroscience Diversity Forum abstract (Poster): *"Characterizing the amiloride potentiation site in GABAA rho1 receptors"*
- 2014-Society for Neuroscience abstract (Poster): *"Characterizing the amiloride potentiation site in GABAA rho1 receptors"*
- 2015-Research Appriciation Day abstract (Poster): "*Characterizing the amiloride potentiation site in GABAA rho1 receptors"*
- 2015-15th Annual Neurobiology of Aging Trainee Symposium (Oral): "*Characterizing The Guanadine Compound Interaction Site In GABAA Rho1 Receptors"*
- 2015- Society for Neuroscience abstract (Poster): "Characterization of guanidine compound interaction with the GABA-A □1 receptor"
- 2015- SPINES symposium abstract (Oral): "Characterization of guanidine compound interaction with the GABA-A □1 receptor"

Publications

Snell HD and Gonzales EB (2015) Amiloride and GMQ allosteric modulation of the GABA-A r1 receptor: influences of the intersubunit site. *J Pharmacol Exp Ther*. June; **353**(3): 551-559. PMID: 25829529

Organizations

- National African American Scholars, 2005-present
- Student Affiliates of American Chemical Society, 2008-present
- Residential Life Judicial Board, 2008-2009
- Louisiana State Club Volleyball, 2006-2010
- Black Graduate Student Association, 2010-present
- UNTHSC Graduate School Association, 2010-present
- UNTHSC tour guide, 2011-present
- The American Society for Pharmacology and Experimental Therapeutics, 2011-present
- Society for Advancement of Chicanos and Native Americans in Science, 2011-present
- UNTHSC Graduate School Association Officer (Secretary), 2012
- UNTHSC Graduate School Association Officer (Vice-President), 2012-2013
- UNTHSC Graduate School Association Officer (President) 2013-2014
- UNTHSC Graduate Student Association Committee Head (Volunteer and Outreach) 2014-2015
- UNTHSC Graduate Student Association Student Leadership Professional Development Fund Representative 2014-2015
- Marine Biology Laboratory (MBL) Corporation, 2012-present
- Society for Neuroscience, 2012-present
- American Association for the Advancement of Science, 2013-present
- UNTHSC K-12 Outreach Student Ambassador, 2014-2015
- Sigma Xi Research Society, 2015-present