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Development of Cellular High Throughput Assays to Determine the Electrophysiological Profile of GABA(A) Receptor Modulators for Neurology and Immunology

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DEVELOPMENT OF CELLULAR HIGH THROUGHPUT ASSAYS TO DETERMINE THE ELECTROPHYSIOLOGICAL PROFILE OF $GABA_A$ RECEPTOR MODULATORS FOR NEUROLOGY AND IMMUNOLOGY

by

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ABSTRACT

DEVELOPMENT OF CELLULAR HIGH THROUGHPUT ASSAYS TO DETERMINE THE ELECTROPHYSIOLOGICAL PROFILE OF $GABA_A$ RECEPTOR MODULATORS FOR NEUROLOGY AND IMMUNOLOGY

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Gamma (γ) -aminobutyric acid (GABA) is the major inhibitory neurotransmitter found in the mammalian central nervous system. Its effect stems from its ability to cause the opening of ion channels which causes an influx of negatively charged chloride ions or an efflux of positively charged potassium ions. This hyperpolarization of the neuron lowers the threshold for neuronal firing. This has an overall inhibitory effect on neurotransmission, decreasing the excitability of the neuron and diminishing the likelihood of a successful action potential occurring. There are two classes of GABA receptor: ligand-gated $GABA_A$ receptor ($GABA_AR$) and metabotropic $GABA_B$ receptor ($GABA_BR$). The $GABA_AR$ is a pentameric receptor containing two binding sites for GABA and once bound, the channel opens to allow the influx of chloride ions. However, $GABA_AR$ not only contains GABA binding sites but also binding sites that modulate the actions of GABA. This includes the benzodiazepine-binding site which occurs at the α and γ2 interface.

$GABA_ARs$ draws a great deal of attention as pharmaceutical targets for treating anxiety, insomnia, epilepsy, schizophrenia, and cognitive deficiencies, among others. Benzodiazepines (BZD) are regularly used as sedatives and anxiolytics. Although there are many alternatives to treating anxiety, none have matched either the efficacy nor the rapid onset of BZDs. However,

these drugs have come to be associated with undesirable symptoms, most notably development of tolerance, addiction, as well as withdrawal symptoms.

Over the past decade, there has been an emerging understanding of the specific subunit composition which mediates the diverse spectrum of BZD pharmacological effects which has generated great interest in developing α-subtype selective drugs. There are at least nineteen different individual $GABA_AR$ subunits that assemble the 5-subunit structure into different combinations to form the native receptor (α1-6, β1-3, γ1-3, δ, and minor subunits). Of these potential combinations, the receptors containing two of the α1-6, two of any β subunits, and one of the γ2 subunit are the most prevalent in the brain. Receptors containing α1/2/3/5 are known as BZD sensitive receptors while α4/6 are BZD insensitive. Studies have shown that the subtype containing the α1 is responsible for sedation, anti-convulsant effects, ataxia, amnesia, and addiction while subtypes responsible for anxiolysis are primarily α2, α3, and perhaps α5 based on one report.

Electrophysiological techniques are critical in determining the enhancement of chloride conductance and calculating potency and efficacy of the drugs but data collection is limited by slow throughput. Herein the development of higher throughput cellular assays to determine BZD subtype selectivity is described. First, an assay was created and optimized using transiently transfected cells on automated patch clamp. However, this assay suffered from variable reproducibility. Next, receptor subtypes were recombinantly expressed in stable cell lines using a single plasmid and antibiotic. These cells can be reliably used to determine subtype specificity of compounds. The overall potency and efficacy of the drugs were also tested on commercially available human neuronal induced pluripotent stem cells (IPSC) which would more accurately

reflect the mixture of receptor subtypes natively expressed on human neurons. Next a fluorescence assay, which utilizes an enhanced yellow fluorescent protein that quenches in the presence of selective anions, was optimized and tested in order to determine if the assay was suitable to perform structure activity relationship studies. Finally, the $GABA_AR$ was found to be present in leukocytes so multiple cell sources were tested to determine their subunit composition and electrophysiological behavior.

To

my parents,

my husband,

and anyone who reads this, thank you

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC-automated patch clamp

ASD-autism spectrum disorder

ATP-adenosine triphosphate

BBB-blood brain barrier

BRET-bioluminescence resonance energy transfer

BZD-benzodiazepine

BZR-benzodiazepine receptor

Carb-carbenicillin

CDC-Center of Disease Control

cDNA-complementary DNA

CHO-Chinese hamster ovary

CL-cell line

Clswell-volume-relgulated Cl- channels

CNS-central nervous system

COX-2-cyclooxygenase-2

CRAC-calcium release-activated calcium

CRH-corticotropin-releasing hormone

dNTP-deoxynucleoside triphosphates

eBFP-enhanced blue fluorescent protein

EC-effective concentration

eCFP-enhanced cyan fluorescent protein

ECS-extracellular solution

eGFP-enhanced green fluorescent protein

ER-endoplasmic reticulum

eYFP-enhanced yellow fluorescent protein

FACS-fluorescence-activated cell sorting

FBS-fetal bovine serum

FST-forced swim test

GABA-gamma (γ) -aminobutyric acid

$GABA_AR$-$GABA_A$ receptor

GABA-T-GABA transaminase

GAD-glutamate decarboxylase

GAT-GABA transporter

HDM-house dust mite

HEK-human embryonic kidney

hiPSCs-human induced pluripotent stem cells

HPAA-hypothalamic-pituitary adrenal axis

hPBMCs-human peripheral blood mononuclear cells

ICS-intracellular solution

ICSS-intracranial self-stimulation

Ig-immune globulin

IHF-integration host factor

IL-interleukin

IMAC-inner membrane anion channel

Int-integrase

IPSC-induced pluripotent stem cells

LNCaP- lymph node prostate adenocarcinoma

LPS-lipopolysaccharide

Luc-luciferase

MBR-mitochondrial benzodiazepine receptor

MDD-major depression disorder

MIN-mouse insulinoma

MS-multiple sclerosis

MTX-methotrexate

NK-natural killer

NOD-non-obese prediabetic type 1 diabetes

NREM-non-rapid eye movement

OVA-ovalbumin

PBR-peripheral benzodiazepine receptor

PCP-phencyclidine

PCR-polymerase chain reaction

PD-Parkinson's disease

PHA-phytohemagglutinin

pLIGICs-pentameric ligand-gated ion channels

PLO-poly-L-ornithine

PMA-phorbol 12-myristate 13-acetate

qRT-PCR-quantitative reverse transcription polymerase chain reaction

REM-rapid eye movement

RT-PCR-reverse transcription polymerase chain reaction

S-sensitized

SAR-structure activity relationship

S/C-sensitized and challenged

SDCN-sacral dorsal commissural nucleus

SNP-

SSADH-succinate-semialdehyde dehydrogenase

SSRI-selective serotonin reuptake inhibitor

SV-simian vacuolating

TCR-T-cell receptor

TM-transmembrane

TNF-tumor necrosis factor

TRP-transient receptor potential

TSPO-translocator protein

TST-tail suspension test

VDAC-voltage-dependent anion channel

VGAT-vesicular GABA transporter

VIAAT-vesicular inhibitory amino acid transporter

VTA-vental tegmental area

Xis-excisionase

YFP-yellow fluorescent protein

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Thank you to Dr. Alexander Arnold, whom I owe the most thanks to in terms of my graduate career and research opportunities. Whose endless quests for funding has allowed me to pursue a carefree life of indulgent research expenditures and week-long conferences. As an undergraduate, he allowed me into his lab and inspired me to continue my education into graduate school. It was truly a privilege to have worked under his advisement.

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It is herein that I reveal who first inspired my interest in Chemistry was Dr. Dennis Bennett, who I had the pleasure of having as my first chemistry professor in a CHM 102 course. His fast-paced and fun approach to chemistry introduced a wonderful and fantastical world to me. Though I was an anonymous undergraduate among hundreds of others, the impact his lectures had on me influenced my life.

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CHAPTER 1: INTRODUCTION

1.1 History of $GABA_A$ Receptor

γ-Aminobutyric acid (GABA) is an amino acid neurotransmitter that was first discovered in the late 1800's and was only known to exist in plants and microbes where it serves a metabolic role in the Krebs cycle [3]. In 1950, Eugene Roberts isolated a significant amount of an unidentified ninhydrin-reactive material in extracts of fresh brains of mouse, rat, rabbit, guinea-pig, human, and frog. Only traces of this substance were found in other tissues, urine, and blood. The material was then isolated via paper chromatograms and based on the co-migration on paper chromatography in three different solvent systems, the substance was revealed to be GABA. Subsequent isotope derivative methods verified the findings [4]. Ernst Florey, in 1953, independently observed that an unknown compound from horse brain inhibited the crayfish stretch receptor as well as inhibited the patellar reflex in cats [5]. Florey purified the molecule from 100 pounds of cow brain with the help of Alva Bazemore of Merck Inc. and identified the active compound as Factor I (where 'I' represented inhibitory action). Thus, Ernst Florey proposed that GABA was an inhibitory neurotransmitter in the brain. However, some of the findings were in question. Firstly, GABAs large abundance was 1000-fold higher than known monoamine neurotransmitters. Secondly, its simple structure and its appearance in the Krebs cycle seemed to suggest that it was a metabolite rather than signaling molecule [6]. In 1956, topically applied solutions of GABA exerted inhibitory effects on electrical activity in the brain [7]. The research into GABA flourished during this period and 3 years later led to the first truly interdisciplinary neuroscience conference ever held where most of the key players in this field gathered to discuss their findings [8]. However, its role in the mammalian CNS was largely in question until the late 1960s when GABA was found to be the major inhibitory neurotransmitter in the central nervous system (CNS). Research

demonstrated proof of postsynaptic action, presence in inhibitory nerves, release from terminals of nerves and the rapid inactivating mechanism at synapses which were carefully reviewed and extensively documented at the time [8,9].

GABA is synthesized by the decarboxylation of L-glutamic acid in the vertebrate CNS, a reaction catalyzed by glutamate decarboxylase (GAD) which localizes primarily in inhibitory neurons [10]. However there is gathering evidence of the presence of both GAD and GABA in non-neuronal tissues and the peripheral nervous system which will be discussed further in Chapter 6: IMMUNOLOGICAL ROLE OF GABAA RECEPTOR ON T-LYMPHOCYTES. The reversible transamination of GABA with α-ketoglutarate is catalyzed by GABA-transaminase (GABA-T)

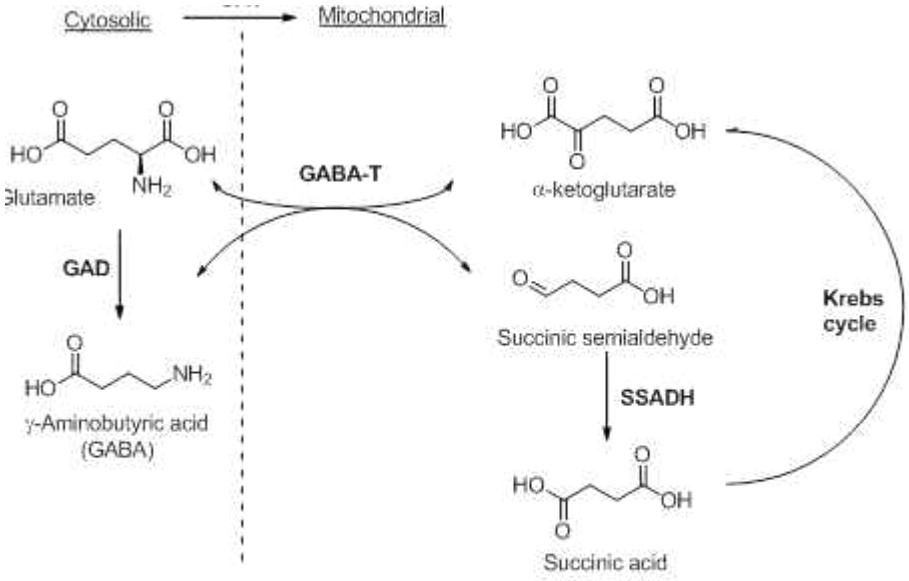


Figure 1. Schematic of GABA shunt metabolic pathway.

and results in the products of succinic semialdehyde which is then converted to succinic acid by succinate-semialdehyde dehydrogenase (SSADH). Steady state concentrations of GABA in the brain is usually monitored by GAD and not by GABA-T. In many inhibitory nerves, both GAD and GABA-T are found throughout the neuron. While GAD is highly concentrated in the

presynaptic terminals, GABA-T is contained in mitochondria of all neuronal regions [11]. GABA transporter (GAT) then removes GABA from the synaptic cleft, terminating inhibitory synaptic transmission and regulates spill-over to neighboring synapses [12]. The entire process is known as the GABA shunt, shown in Figure 1, and is detailed further in a review [11].

In the 70s and 80s, research was subsequently turned to defining the nature of the receptor that GABA acts on. Early electrophysiological studies on the GABA receptor showed inhibitory hyperpolarizing responses [13] resulting from an increased membrane chloride conductance [14] that were blocked competitively by the alkaloid bicuculline [15]. Not long after, another study attempting to identify receptors on peripheral nerve terminals found that GABA reduced release of noradrenalin in the rat heart, this effect was not blocked by bicuculline. This action could be reproduced with application of baclofen, which in contrast, was unable to produce responses in neurons of the CNS. This new receptor was named $GABA_B$ receptor to differentiate it from the more familiar type which was termed $GABA_A$ receptor ($GABA_AR$) [16,17]. During this time, another bicuculline-insensitive receptor was found and termed $GABA_C$, however it was later determined by the IUPHAR Nomenclature Committee to be a subgroup of the $GABA_A$ receptor class [18,19]. When bovine brain receptor was purified in the early 1980s [20], an analysis of the genes of the $GABA_AR$ and the glycine receptor the year before revealed the existence of what would later be called the Cys loop ligand-gated ion channel superfamily of neurotransmitter receptors, also known as the pentameric ligand-gated ion channels (pLIGICs) [21]. With a known method of receptor purification, the same lab headed by Eric A. Barnard of the Imperial College of Science and Technology in the United Kingdom isolated two distinct $GABA_AR$ proteins [22] which were later named the α and the β subunits [21]. Shortly after, the presence of additional subunits was suggested [23,24] and molecular studies on the amino acid sequence similarities revealed that the

receptor subunits could be divided into eight classes which could be further divided into subdivisions. It would appear that the final estimate is that *Homo sapiens* contain six α-, three β-, three γ-, three ρ-, with one of each of the δ, ε, π, and θ subunits. Furthermore, splice variants for five subunits has been described with the best studied being γ2L (L indicated "long") which includes an insert of eight amino acids between the transmembrane domains TM3 and TM4 [25]. Additional isoforms can occur in other species such as the fourth β and a fourth γ subunit identified in chickens, however these have yet to be described in mammals [26,27]. Each subunit is comprised of a large (200 amino acids) extracellular N-terminal domain which putatively includes the ligand-binding site, four hydrophobic (20 amino acids each) presumed to be membrane-spanning domains labeled TM1-TM4, and a small extracellular C-terminus illustrated in Figure 2. The membrane spanning helices are connected by short peptide sequences with the second helix expected to form the inner wall of the chloride ion channel. TM2 is hydrophobic in the upper two thirds of the channel with a funnel large enough to accommodate water molecules. Further into the channel, there is a narrow hydrophilic region filled with the hydroxyl groups from threonine and serine residues. These groups act as a selectivity filter where water molecules are sloughed from the chloride ions hydration shell and replaced by side chains that line the channel walls. The N-terminal domain contains a highly conserved loop consisting of 13 amino acids due to a disulfide bridge between cysteine residues (C-x-[LIVMFQ]-x-[LIVMF]-x(2)-[FY]-P-x-D-x(3)-C) and are

therefore known as cys-loop receptors. Some subunits have a larger intracellular loop occurs between TM3 and TM4, contributing to phosphorylation sites as is the case of γ2L. Regardless of

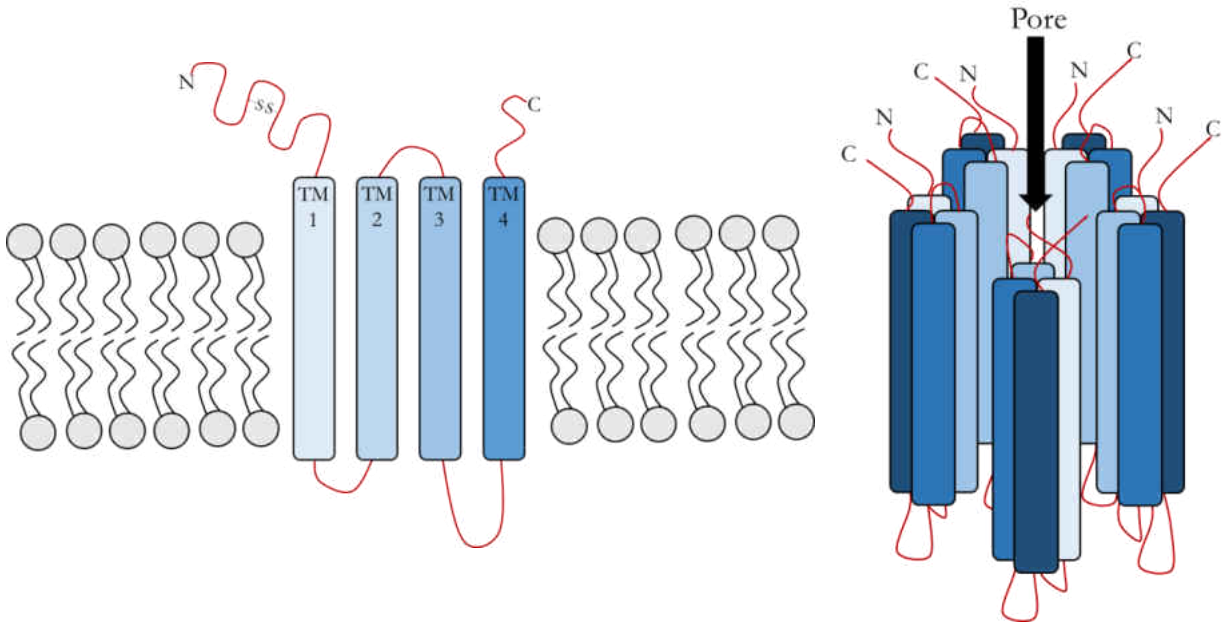


Figure 2. Schematic structure of the $GABA_AR$. LEFT: Monomeric subunit of the $GABA_AR$ imbedded in a lipid bilayer. The four transmembrane helices (TM1-TM4) are depicted as rods. RIGHT: Five individual subunits assemble to create the pentameric receptor and the central chloride anion conduction pore. TM2 is arranged to form the inner wall of the channel with TM1, TM3, and TM4 form the outer wall.

these differences, the subunits range from 50 to 60kDa and sequence homology within a subunit class is around 70% but between classes this number can drop to around 30% [11].

Studies on the stoichiometry of the receptor at first yielded the assumed 2α, 1β, 2γ theory [28], however this was replaced with the now accepted theory for the 2α, 2β, 1γ schema [29-31]. Taking into account the number of subunits, the stoichiometry, splice variants, heterogeneity, and relative position of the subunits; the number of possible receptor isoforms might exceed hundreds of thousands of subtypes mediating different biological responses [32]. However, it would appear that incompatibilities as well as developmental and functional transcriptional control and limitations by the temporal and spatial pattern of subunit expression and assembly dramatically reduces the

probability of isoform expression. In fact, some studies suggest that there are less than 20 widely occurring combinations with the major contribution being α1β2/3γ2, α3β3γ2, and α2β3γ2 [33].

Functional $GABA_A$ receptors, upon activation, selectively conducts chloride ions across neuronal cell membranes. This results in a hyperpolarization of the neuron which has an inhibitory effect on neurotransmission by diminishing the chance of a successful action potential occurring and thereby decreasing the excitability of the neuron. Similarly, $GABA_BR$ also causes hyperpolarization however these involve efflux of K^+ rather than influx of Cl^-. The assembled

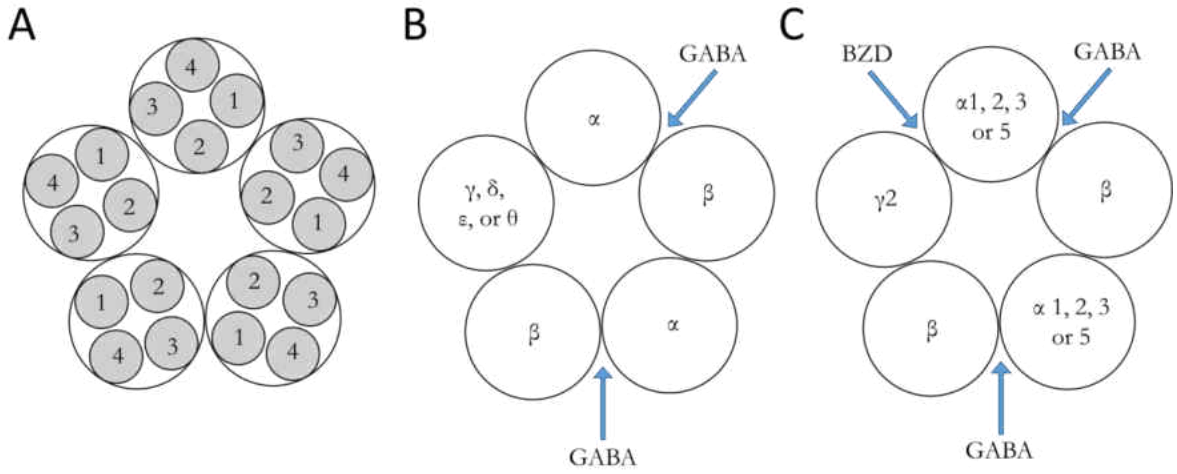


Figure 3. Schematic representation of the $GABA_AR$ subunit arrangement in the synapse. (A) The directionality of the transmembrane helices (I-IV) with TM2 lining the pore; (B) The most common arrangement of subunits is two α, two β, and one γ. The γ subunit can be replaced by either the δ, ε, or θ. The GABA binding site is located between the α and β subunits so each receptor contains two of these sites; (C) When the subunit is γ2 and the α is either the α1, α2, 3α, or α5, a benzodiazepine site is formed at the interface of these subunits.

$GABA_A$ receptor not only contains GABA binding sites, which are believed to be at the interface between the α and β subunits, but also binding sites that modulate the actions of GABA. This includes the benzodiazepine-binding site which occurs at the interface of the α and γ2 subunit depicted in Figure 3.

Benzodiazepines (BZDs) are not the only group of compounds that bind and modulate the $GABA_AR$. Other compounds that interact with the $GABA_AR$ include: neuroactive steroids,

barbiturates, ethanol, gaseous and intravenous anesthetics, picrotoxin, and zinc, among others [34]. It has been estimated that there are at least 12 distinct sites on the $GABA_AR$: GABA binding site, picrotoxin sites, sedative-hypnotic barbiturate site, neuroactive steroid sites, benzodiazepine sites, ethanol sites, inhaled anesthetic sites, furosemide site, loreclezole site, Zn^{2+} site, other divalent cation sites, La^{3+} site and a recently discovered site between the α and β interface. However, this estimation is now considered conservative due to overlap and interaction between the distinct sites [35]. Most recently, a novel benzodiazepine site has been found between the α-α interface [36]. Agents that act to increase GABA-mediated synaptic inhibition either by direct activation of the receptor or enhancement of the action of GABA are known as positive modulators with positive efficacy. These can bind allosterically at sites remote from the GABA binding site. Molecules that reduce the action of GABA on the receptor are known as negative allosteric modulators, known interchangeably as inverse agonists with negative efficacy. Finally, if a compound blocks the actions of both positive and negative allosteric modulators, they are referred to as neutralizing allosteric modulators or antagonists with no efficacy. There exists a diverse range of agents that act on the $GABA_AR$ and oftentimes the terminology is misused.

An extensive review has been performed to determine the immunocytochemical distribution of the most common subunits in the adult rat brain [37]. The summary of which is depicted in Figure 4 [38]. Presently it is estimated that 12-24 isoforms represent the most abundant $GABA_AR$ and according to mRNA localization and immunohistochemical staining. The α1 subunit is the most abundant subunit in the CNS [37,39-41] with only a few regions lacking it [42]. Colocalization of α1 with β2 has been noted using mRNA localization and double immunofluorescence detection [39,43]. In addition, the presence of γ2 in nearly all brain regions has often been described as colocalizing with α1β2 [39,40,44]. Therefore, the most abundant receptor type

has been concluded to be α1β2γ2. In fact, α1, β1, β2, β3, and γ2 are found throughout the brain

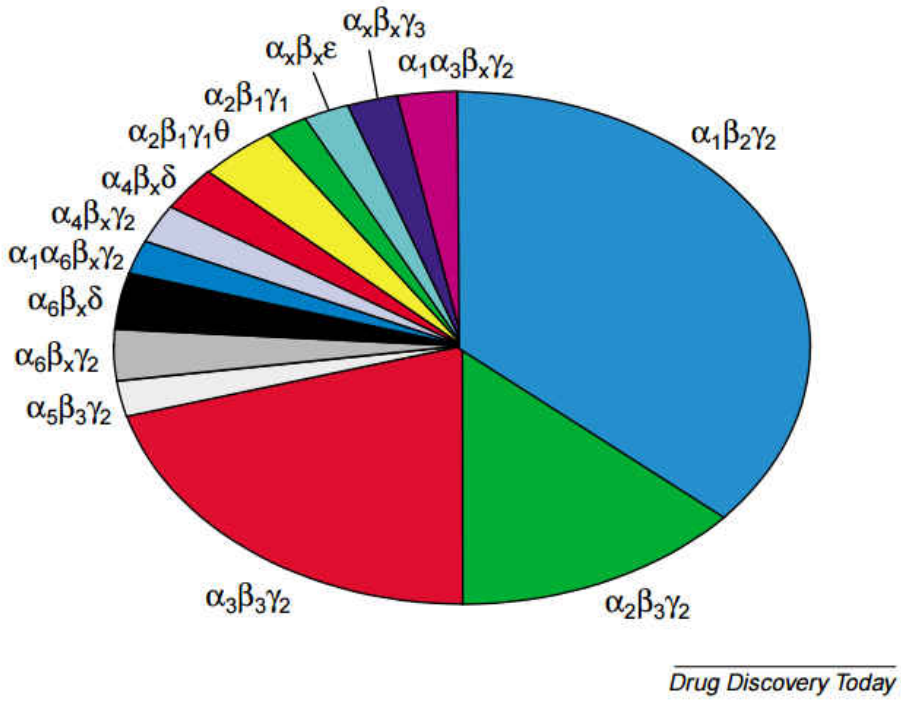


Figure 4. Pie chart by Paul J. Whiting, representing the approximate abundance of $GABA_AR$ subtypes in the rat brain. Subscript x indicates that the subdivision of the subunit is not known. It should be noted that these receptors are the most abundant ones and other subunit combinations do exist. Reprinted with permission from Elsevier.

but all the others: α2, α3, α4, α5, α6, γ1, γ3, and δ are confined to specific locations in the brain. Relative abundances of subtypes found in rat brain is illustrated in Figure 4. Notably, 25% of the α1 available subunits co-assemble with different α subunits and a mixed BZD effect results. It should be noted that abundance does not necessarily correspond with physiological importance and until the whole circuitry of brain regions can be fully comprehended, even minor amounts of subunits cannot be ignored. Rather, a specialized function is suggested by their specific

localization in the CNS. The importance of which may be evident by the results of knock-out and knock-in mutations in animal models that will be discussed in 1.3 History of Subtype Selectivity.

Table 1. Regional Distribution of GABAAR Subunits in the Adult Rat Brain. Table recreated from data. Where xxx indicates extremely high expression, ss is high, x is low, and o is very low.

Region	α1	α2	α3	α4	α5	α6	β1	β2	β3	γ1	γ2	γ3	δ	ε	θ
Olfactory bulb															
Glomercular layer	xx	x	xx	o	x	-	-	xx	xx	-	xx	o	x	-	
Ext. plexiform layer	xxx	x	xx	o	x	-	xx	xxx	xxx	-	xxx	o	o	-	
Granular layer	xx	xx	o	x	xx	-	-	x	xx	-	xx	-	o	-	
Mitral cell layer	xx	-	o	-	xx	-	x	xx	-	-	xx	o	x	-	
Olfactory tubercle	x	x	-	xx	x	-	x	x	xx	-	x	x	x	-	
Cerebral cortex															
All layers	xx	x	x	xx	x	-	xx	xx	xx	-	xx	o	x		
Outer layers	xx	x	x	x	x	-	xx	xx	xx	-	xx	o	x	-	
Inner layers	xx	x	xx	x	x	-	xx	xx	xx	-	xx	o	x	-	
Hippocampus															
Molecular layer	x	xx	-	xx	x	-	xx	x	xx	-	xx	-	x	-	
Hilar neurons	xx	-	x	-	-	-	o	xx	-	-	xx	-	x	-	
Strat. Oriens/radiatum	xx	xx	-	x	xx	-	xx	x	xx	-	xx	o	-	-	
Septum															
Medial	xx	x	x	-	o	-	o	xx	x	-	xx	o	-	x	x
Lateral	xx	xx	x	x	o	-	xx	x	x	x	xx	o	-		
Basal ganglia															
Striatum/n. accumbens	x	xxx	x	xx	xx	-	x	x	xxx	x	x	o	x	x	x
Globus pallidus	xx	o	o	x	o	-	o	xx	o	xx	xx	o	o		
Subst. nigra	x	x	x	o	x	-	x	x	-	x	x	x	o	xx	x
Thalamus															
Reticular nucleus	x	-	xx	x	x	-	xx	-	xx	-	xx	o	o		
Ventr. Lat. Geniculate	xxx	x	x	x	o	-	x	xx	x	-	x	o	o	xx	x
Dors. Lat. Geniculate	xxx	-	-	xxx	o	-	x	xxx	x	-	x	x	xxx		
Medial and central	x	xx	x	o	o	-	xx	xx	xx	xx	x	x	x	xx	x
Hypothalamus															
Ventromedial	x	xx	x	o	xx	-	xx	x	xx	x	xx	x	x	xx	xx
Supraopticus	xxx	xxx	x	x	o	-	xxx	xx	x	-	x	x	x		
Paraventricular	xx	xxx	-	-	x	-	xx	x	xx	-	x	x	x	xx	x
Arcuate	x	x	x	x	x	-	x	x	x	-	-	x	x	xx	x
Med. Preoptic area	xx	xx	x	-	x	-	x	x	x	-	xx	x	x	xx	x
Amygdala															
Lateral	xx	xx	xx	x	o	-	xx	xx	xx	-	xx	x	o	x	x
Basolateral	xx	xx	xx	x	o	-	xx	xx	xx	-	xx	x	o		
Medial and central	x	xx	x	o	o	-	xx	xx	xx	xx	xx	x	x	x	x
Cerebellum															
Granule cell layer	xxx	x	o	o	x	xxx	x	xxx	xxx	x	xx	-	xxx		
Molecular layer	xx	xx	-	-	xx	-	x	x	-	-	x	o	-		
Midbrain/Pons															
Ventral teg.area	xx	x	o	-	o	-	xx	x	xx	x	xx	xx	o	-	-
Raphe nuclei	xx	xx	x	-	o	-	xx	xx	x	-	xx	xx	xx	x	x
Inferior colliculus	xx	-	-	-	o	-	x	xx	o	-	o	o	o		
Olive superior	o	-	x	-	o	-	xx	-	x	-	x	o	x		
Medulla															
Trig. sensory complex	xx	-	xx	o	o	-	x	x	x	x	xx	x	x		
Dors. cochlear nucleus	xx	x	xx	o	x	xx	o	x	o	o	x	x	xx		
Solitary tract nucleus	xx	o	xx	-	xx	-	x	o	x	o	xx	x	xx		

$GABA_AR$ with different subunit composition have different physiological and pharmacological properties and are often differentially expressed in certain subcellular regions. For example, $GABA_A$ receptors composed of the α1, α2, α3, or α5 combined with β and γ subunits are benzodiazepine-sensitive and, with the notable exception of α5 receptors, are primarily located postsynaptically and mediate phasic inhibition [45-47]. The α4 and α6 subunits primarily assemble with the β and δ subunits to form a specialized extrasynaptic receptors which mediate tonic inhibition and are insensitive to benzodiazepine modulation [47]. Synaptic and extrasynaptic receptors also differ in their desensitization kinetics with synaptic receptors having rapid desensitization and extrasynaptic displaying slow kinetics [48]. An excellent summary of the regional distribution of GABA published by W. Sieghart and G. Sperk can be seen in

Table 1 [49].

It should be kept in mind, however, that the extent of expression and distribution of the $GABA_AR$ in rodents does not necessarily correspond to that of the human brain. For example, rodent hippocampus is nearly devoid of α3 while it is strongly expressed in areas of the human hippocampus. There are also differences in the expression of α1 which has no staining in human CA3 pyramidal cells while rodents have moderate immunoreactivity in the analogous CA3 dendritic fields. Further, α1 and α2 are abundant in hilar mossy cells in humans but absent in rodents [50]. Finally, basal dendrites of dentate granule cell show strong staining in the human brain while rats lack basal dendrites in the granule cells [51,52]. The interspecies differences can profoundly influence the interpretation of findings when data gathered from animal models is translated to treatment of human disease. A more complete immunohistochemical study of human brain tissue would be greatly beneficial for the field of neuroscience, particularly in studying the brains of

those who suffer from neurological disorders. This would also allow further insight into how these diseases develop and progress. Particularly in assessing population imbalance of neurotransmitters and receptors (so called “Chemical Imbalance theory”) and/or the hereditary genetics, environmental stressors, or psychological trauma which may influence or trigger these diseases.

1.2 History of Benzodiazepines

Table 2. Compounds referenced in Chapter 1: Introduction

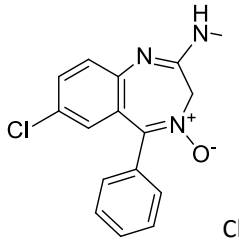
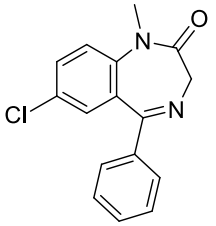
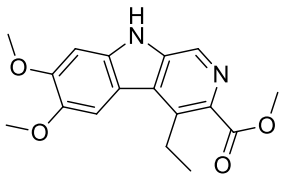
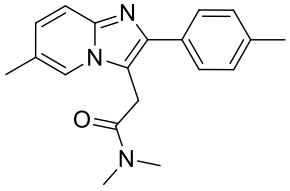
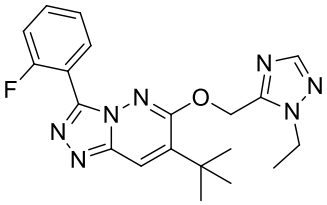
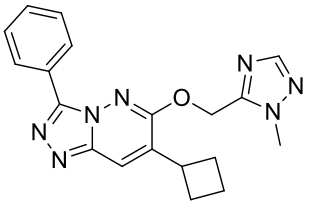
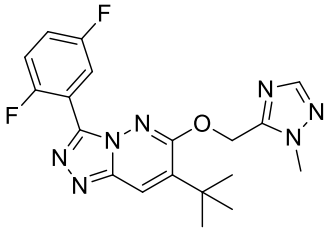
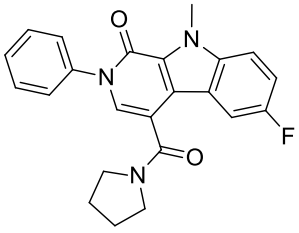
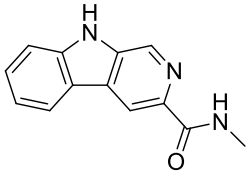
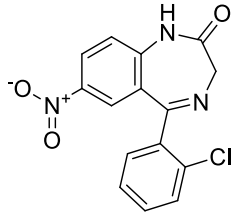
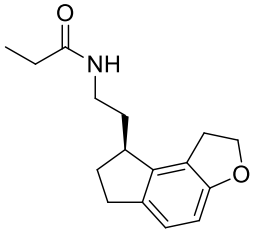
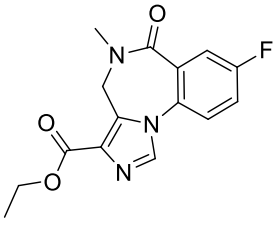
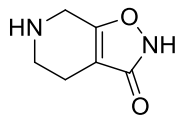
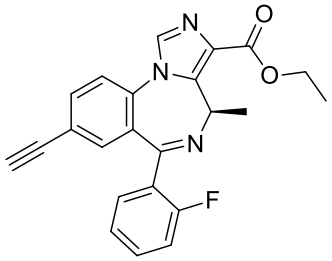
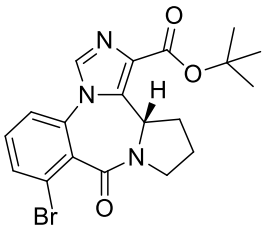
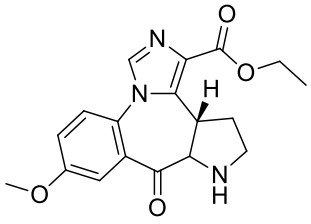
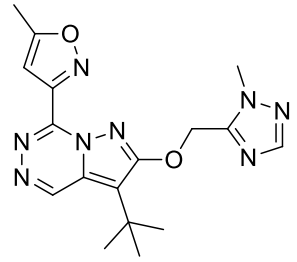
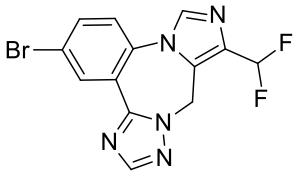
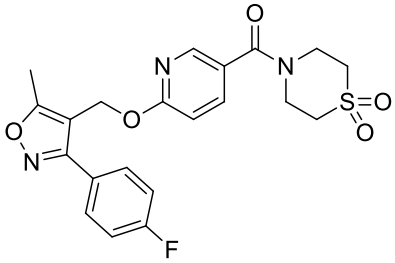
 Chlordiazepoxide	 Diazepam
 DMCM	 Zolpidem
 TPA023	 TPA123
 L-838,417	 SL651498
 FG-7142	 Clonazepam
 Ramelteon	 Flumazenil

Table 3. Compounds referenced in Chapter 1: Introduction, cont'd

 Gaboxadol	 SH-053-2'F-R-CH_3
 Bretazenil	 L-655,708
 MRK-016	 RO4938581
 RG1662	

In 1955, Leo Sternbach, a chemist working at Hoffman-La Roche research facility at Nutley, New Jersey was studying heptoxdiazines in the hopes of discovering compounds with psychopharmacological activity. Eventually he realized that the drugs that he'd thought were heptoxdiazines were in reality benzoxadiazepines and synthesized about 40 benzoxadiazepines. He noted that "basic groups frequently impart biological activity" and performed a reaction of 6-chloro-2-chloromethyl-4-phenylquinazoline-3-oxide with methylamine which yielded the

unexpected rearrangement product 7-chloro-2-N-methylamino-5-phenyl-3H-1,4,-benzodiazepin-4-oxide. Thus Ro 5-0690 had been stabilized with a methylamine, instead of a secondary or tertiary amine, and was then shelved. Years later, it was rediscovered after a laboratory cleanup and was "nicely crystalline" and thus submitted for pharmacological evaluation with a battery of animal tests. Surprisingly, the compound exhibited strong sedative, anticonvulsant, and muscle relaxant effects. Ro 5-0690 was the first anxiolytic 1,4-benzodiazepine with the generic name of chlordiazepoxide (Table 2) and the brand name of Librium [53]. Further improvements on the molecular structure and the ironic removal of the basic nitrogen moiety brought about diazepam (Table 2) which was found to be 3- to 10-fold more potent and marketed under the name Valium in 1963. Given that these benzodiazepines were less toxic and less likely to cause dependence than other drugs on the market, competitors were quick to begin studying the drugs and popularity skyrocketed. From the late 1960s through the 1970s, sales of diazepam topped those of all other drugs in the United States. However, over-prescribing exposed the side effects and a negative public perception developed [54]. Benzodiazepines were quickly associated with abuse and dependence[55]. Soon enough, reports in the media warned of their illicit and non-medical use among the youth and counter-culture; even extending to a Rolling Stones' song as "Mother's Little Helper", referring to their widespread use among middle-class housewives [56]. Reinforcing effects of BZDs are well studied in a variety of experimental conditions employing i.v. self-administration protocols in which subjects are trained to push a lever in order to receive the drug via a chronic venous catheter [57-61]. Benzodiazepines were the center of the largest class-action lawsuit against drug manufacturers in the United Kingdom wherein 14,000 patients and 1,800 independent law firms alleged drug manufacturers were aware of the dependence potential but knowingly withheld this information from doctors. The court case never reached a verdict after allegations that

witnesses had a direct conflict of interest in their testimonies. However, the result was that individual benzodiazepines and soon the entire class of compounds saw the advent of guidelines and legislation giving guidance on their use [62].

However, newly developed BZDs remain widely prescribed and are considered generally safe and an effective treatment for many anxiety disorders since abuse and dependence do not seem to occur in the majority of patients. Studies have also shown that tolerance to their sedative side-effects, but not their anxiolytic action, usually occurs after 2 weeks of continuous use [63]. In addition, BZDs are remarkably safe in overdose due to their allosteric effect [64]. Full antagonists or agonists are more likely to cause severe CNS effects due to obstruction of the necessary neurotransmission and desensitization of receptors. There are currently more than two dozen BZDs in clinical use in the United States. All BZDs enter the cerebral tissue rapidly with durations of action ranging from 2 hours (diazepam) to 72 hours (lorazepam). Although there are many alternatives to treating anxiety, none have matched either the efficacy or the rapid onset of BZDs [65].

Diazepam, also referred to as 1,4 benzodiazepine, referring to the position of the nitrogen in the diazepine core is the prototypic scaffold that other analogues have followed. The structure-activity relationship for benzodiazepine anxiolytics has been described and the minimum needed for binding includes a heteroaromatic ring, which participates in π-π stacking with aromatic amino

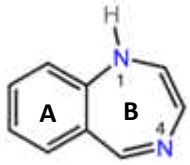


Figure 5. The 1,4-benzodiazepine ring system.

acid residues in the receptor, and a proton-accepting group that interacts with a histidine residue, preferably in a coplanar spatial orientation with the aromatic ring A. These components are often referred to as 'ring A' and 'ring B' respectively and shown in Figure 5 [65].

Molecular modeling and structure-activity relationship (SAR) studies based on rigid ligands in Milwaukee has resulted in the creation of a benzodiazepine pharmacophore. Pioneered by research members in the group of James M. Cook, the unified Milwaukee-based pharmacophore/receptor model was created on the in vitro binding affinities of over 150 ligands which bind to the benzodiazepine site on the $GABA_AR$: benzodiazepines [66,67], β-carbolines [68-70],

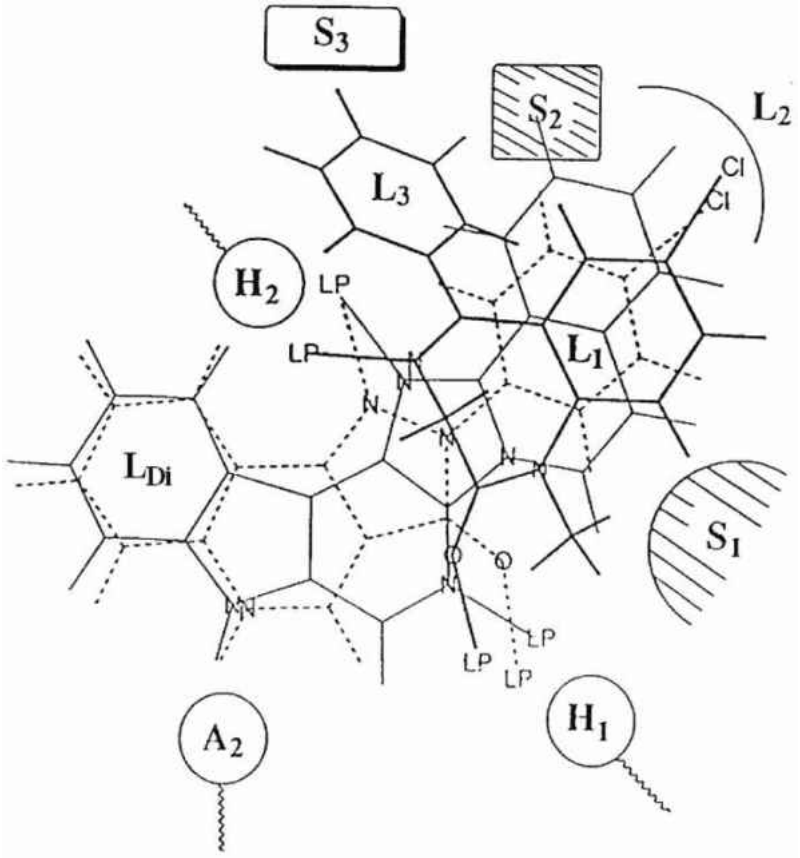


Figure 6. The unified Milwaukee-based pharmacophore/receptor model. The pyrazolo[3,4-c]quinolin-3-one CGS-9896 (dotted line), a diazadiindole (thin line), and diazepam (thick line) aligned within the unified pharmacophore/receptor model for the Bz BS. H_1 and H_2 represent hydrogen bond donor sites within the Bz BS while A_2 represents a hydrogen bond acceptor site necessary for potent inverse agonist activity in vivo. L_1, L_2, L_3 and L_{Di} are four lipophilic regions and S_1, S_2, and S_3 are regions of negative steric repulsion. LP = lone pair of electrons on the ligands (modified from the figure in Clayton, *et al.*). [1,2]

triazolopyrimidines [71], pyridodiindoles [72,73], imidazo-pyridines [74], and pyrazoloquinolines [75]. Analysis of the gathered data show that the binding site of benzodiazepine sensitive receptors are very similarly and a two-dimensional representation has been produced, Figure 6, with compounds from three separate classes (quinolinone, diazediindole, and benzodiazepine) depicted within.

The majority of pharmacological effects of BZDs can be attributed to binding at the benzodiazepine site located on the $GABA_AR$. These sites are often referred to as the benzodiazepine receptor (BZR). However, it should be noted that this terminology also encompasses the peripheral benzodiazepine receptor (PBR), also known as Bz3, ω3, mitochondrial benzodiazepine receptor (MBR), inner membrane anion channel (IMAC) and most recently referred to as translocator protein (TSPO). TSPO will be discussed in further detail within Chapter 6.

New research has shown that BZD compounds can bind to a novel site between the α and β that prevents further modulation by drugs [76]. Covalent binding at this site renders it unresponsive at high concentrations, resulting in a bell-shaped curve; this feature has been suggested to contribute to the high degree of safety of the drugs.

The most common side-effect of BZDs is sedation, evidenced by tiredness, drowsiness, light-headedness, difficulty concentrating, thinking or staying awake, apathy, confusion, muscle weakness, ataxia, dysarthria, blurring of vision, diplopia, vertigo and anterograde amnesia. These are generally dose-dependent and show great variability between individuals. In addition, pharmacokinetic changes and a reduction of baseline cognitive and motor function can result in worsened sedative side-effects among the elderly [77] which can increase the risk factor for falls and fractures. Other possible side-effects include weight gain, skin rash, nausea, headache, and sexual

dysfunction. Importantly, as will be discussed in further detail in Chapter 6- 6.1 Introduction, BZDs can also lead to reduction in the upper airway muscle tone, respiratory depression, and increase risk of secondary infection [78]. Paradoxically, increased feelings of anxiety, anger, or hostility have been described with benzodiazepine use, however they appear to be very rare [79].

The abuse and dependence liability of BZD consumption has been a matter of much concern and debate. In nonsubstance abusing populations, abuse of BZDs is quite rare and the majority of patients do not take more than the prescribed dosage [80,81]. Those with a history of drug abuse are much more likely to abuse BZD [81]. Discontinuation of BZD therapy can cause discomfort in individuals in the form of three types of discontinuation syndromes: recurrence, rebound, and withdrawal [63,82]. Of the three, only withdrawal clearly manifests as physical dependence. Recurrence is the reappearance of the same symptoms for which the drug was prescribed while rebound is characterized by the return of symptoms to a higher level of intensity. Rebound occurs in 15-30% of BZD patients, ranging from mild to moderate severity with a generally short duration time [82]. In contrast, BZD withdrawal syndrome is generally characterized by autonomic responses: sweating, tachycardia, mild systolic hypertension, tremulousness, dizziness, tinnitus, excessive sensitivity to light, sound or touch, altered taste, nausea, abdominal discomfort, depressed or dysphoric mood, fatigue, restlessness, and agitation. Infrequently there can also be confusion, psychotic symptoms, and seizures. The syndrome can last from 3-6 weeks followed by recurrence of the original disorder. Gradual tapering upon discontinuation can reduce the occurrence of these syndromes [63,82].

1.3 History of Subtype Selectivity

The exact symptoms generated from the consumption of BZDs appears to depend on what regions of the brain are involved and the $GABA_AR$ therein expressed. In addition, it would seem that some CNS diseases are caused by either an underexpression or overexpression of the receptors on the nerve cells, causing them to be 'out of balance'. Restoration of this 'chemical imbalance' is a major aim of therapies targeting GABAergic inhibition. To achieve this safely and rapidly is the goal of current development of new BZDs. Since the BZR on the $GABA_AR$ is located at the interface of the α and β subunit and there are 6α and 3γ subtypes, at least 18 different BZD binding sites exist. However exchange of γ2 with either of the other γ subunits can alter the BZD affinity and potency dramatically [83].

The most important evidence for subtype-dependent $GABA_AR$ pharmacological effects is the advent of mice which had been genetically altered for the study of the roles of individual subunits. Advances in gene targeting such as constitutive or conditional knockout and knock-in techniques have improved the ability to study the possible neurobiological traits of anxiety in mice [84]. Constitutive knockout, wherein a mouse lacks a gene of interest during all stages of development, has greatly increased our understanding of the roles of $GABA_AR$ subtypes. A landmark study performed by the lab of Hanns Mohler, generated mice lacking an individual subunit of the $GABA_AR$, the γ2, which rendered the mice benzodiazepine-insensitive [85]. Homozygous mice with the knockout of both γ2 genes were not viable so heterozygotes with half the usual complement of the γ2 subunit were studied. These mice were not only less sensitive to benzodiazepines but exhibited symptoms of hypervigilance and anxiety in an elevated plus maze and light/dark choice tests. These mice became a genetically defined model of trait anxiety which reproduces the molecular, pharmacological, and behavioral features of human anxiety disorders

[86]. Following Mohler's success, knockout mice lacking other $GABA_AR$ subunits were generated. Though knockout mice were the first model studied, they were soon followed by knockin mice, and finally triple-point-mutated knockin mice. Whereas knockout mice are generated by removing the gene in question but knockin mice are mutated at a specific amino acid to render the subtype BZD insensitive. The modification of a single amino acid is a very slight change in comparison to the deletion of a complete gene. Knockin mice have less genetic abnormalities during development, reduced lethality, and less unknown compensatory changes. Classical benzodiazepines bind to the α1βγ2, α2βγ2, α3βγ2, or α5βγ2 and show very little to no affinity for the α4βγ2 and α6βγ2 subtypes and reduced affinity towards receptors containing the γ1 and γ3. This selectivity is attributed to a single amino acid which is histidine in the α1, α2, α3, and α5 but an arginine in the α4 and α6 subunits [87]. Point-mutated mice that change the histidine to the arginine are utilized as knockin mice to ascertain the role of individual subunits to the variety of effects from BZD application. Studies utilizing single point-mutated mice look at which side effects are lacking when a single subtype is mutated (loss-of-function) while studies with triple point-mutated mice search for which side effects remain when all but one subtype is mutated (restriction-of-function).

Heterozygous knockout of the α1 subunit was successful despite its ubiquitous nature. The resulting mice exhibited a 30% loss in body weight as well as a tremor when handled [88]. Surprisingly the mice were more sensitive to motor-impairment and sedative effects of diazepam [89]. Knockin H101R of the α1 subunit created mice that lacked drug-induced sedation and the diazepam-induced amnesia, suggesting that the α1 is responsible for anterograde amnesia action and sedative action of diazepam. Interestingly, the characteristic effect of BZD hypnotics on sleep electroencephalogram (EEG) remained in α1 knockin mice, indicating that the sedative action of

BZDs is not related to the changes in sleep EEG, in which α1 does not seem to be involved [90]. In addition, diazepam only partially protected against pentylenetetrazole-induced myoclonic seizures, suggesting α1 involvement in the anticonvulsant activity of diazepam [91]. DMCM (methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate, Table 2) failed to elicit convulsions which may indicate involvement of the α1 in the anti-seizure activity [92]. Triple point-mutated mice exhibited impairment of motor coordination when diazepam was applied, while previous studies with single point-mutated mice failed to support this involvement [91,93,94]. There has been controversy as to the involvement of the α1's role in the addictive properties of BZDs. Unlike sedation, muscle relaxation, and memory impairment; abuse and dependence does not serve any conceivable clinical purpose and are always considered undesirable [95,96]. It is estimated that 0.1-0.2% of the adult population of the United States (300,000-600,000) abuse or are dependent upon benzodiazepines [97]. In mice, the α1 was found to be necessary to the oral midazolam self-administration with a two-bottle choice drinking sucrose paradigm [98]. In addition, zolpidem (Table 2), an α1-preferring compound, exhibited abuse by polydrug users [99] as well as self-administration in primates [100,101]. The electrophysiological evidence also shows addiction-like disinhibition, evoking synaptic plasticity in α1 containing $GABA_AR$ in excitatory glutamatergic afferents on dopaminergic neurons in the ventral tegmental area (VTA), thus increasing the firing of dopaminergic neurons by decreasing the activity of GABAergic interneurons [98]. It should be noted that all addictive drugs increase dopamine concentrations in the mesolimbic dopamine system [98,102]. This strongly implicates the α1 as the mediator for addiction.

However, in direct contrast, further work done has shown that simply abolishing specificity towards α1 does not eliminate reward behavior. In one study, TPA023 (Table 2), a weak partial agonist at α2/3 but no efficacy at α1/5, with TPA123 (Table 2), a weak partial agonist of α1/2/3/5,

were applied to baboons in a self-administration study. TPA123 had moderate self-injection rates and exhibited BZD-like withdrawal syndrome [103]. L-838,417 (Table 2), a compound with that is an agonist at α2/3 and an antagonist for the α1, however, did maintain self-administration in a similar primate model [61]. Suggesting that abolishing the efficacy at the α1 does not eliminate abuse liability unless coupled with the reduction of α2/3 specificity. Zolpidem and diazepam examined in knockin mice using the intracranial self-stimulation (ICSS) paradigm agree with the findings that α2/3 are vital to the creation of abuse liability [104]. The inherent difference between the two bottle choice paradigm and the ICSS paradigm is the 'like' and 'want' aspects of them; in which the latter involves active seeking of the reward instead of preference [105]. The ICSS paradigm can be viewed as the animals' willingness to work to obtain stimulation and is significant for individuals already in a high-reward state or when there is a motivational component involved [103]. Furthermore, another experiment with zolpidem, an α1-preferring BZD agonist was given to knockin mutants for α1, α2, or α3 with no reward enhancing effects and showed that α1 knockin reduces the reward behavior but α2 knockin is capable of abolishing it [104]. Further studies that state that physical dependence, measured with rapid precipitated withdrawal assay by inverse agonist-induced seizures, reported that chronic treatment with zolpidem (selective 1), L-838,417 (selective 2, 3, 5), TPA023 (selective 2, 3, 5), and SL651498 (selective 2, 3, Table 2) did not respond to administration of seizure inducing partial inverse agonist FG-7142 (Table 2). Considered together, this suggests that physical dependence does not occur with subtype-selective compounds. In addition, non-selective partial agonists also suppressed FG-7142 induced seizures, implying that high efficacy might also be a requirement for physical dependence [106,107]. Taking all of this information into consideration indicates a group involvement of all three subtypes in creating a reward-enhancing genotype. It has been suggested that α1 may be required for long-lasting

neuronal changes of addiction, as indicated by the electrophysiological studies, while the α2 and α3 are responsible for the immediate reinforcing effect [104]. It should be noted that although BZDs do produce self-administration behavior above levels seen in comparison with a vehicle, they appear to be relatively weak reinforcers in general compared to other drugs of abuse like barbiturates [108,109].

Knockin at α2 H101R attenuates the anxiolytic action of diazepam in the light-dark choice test and the elevated plus maze test but exhibit normal sedative and anticonvulsant response. In addition, the muscle relaxant effect of diazepam had disappeared [94]. These initial results seem to indicate that the α2 is the sole subtype responsible for diazepam's anxiolytic effect, however TP003, an agonist with relative selectivity towards the α3, displays anxiolytic activity [110]. In addition, L-838417 still had anxiolytic activity in α2 knock-in mice. Indicating possible involvement of the α3 in the anxiolytic effect of BZDs. However it should be noted that $GABA_AR$ bearing the α2 subunit require 20-25% receptor occupancy for anxiolysis [94] while α3-containing receptors require 75% of the receptors be occupied to elicit anxiolytic response [110]. Consequently, α3 is considered a backup target for anxiety.

The genetic deletion of α2 leads to behavioral despair as seen in the results of an FST and TST [111]. In addition, single nucleotide polymorphisms (SNPs) genetic association studies have implicated the α2 in alcohol dependence [112,113] and illicit drug dependence [114,115]. In direct contrast, a recent triple point-mutated mouse study found that tolerance can be avoided when only α2 $GABA_AR$ are targeted. All other subtypes (α1/3/5) exhibit fast tolerance development with rapid diminishing of BZD effects during prolonged treatment of eight days [93].

Knockout mice lacking α3 displayed an increase of spontaneous locomotor activity in the open field with loss of prepulse inhibition of the acoustic startle response [116]. When α3 knockin H126R mice were treated with diazepam there was reduction in muscle relaxant activity and similar deficits in sensorimotor gating from pre-pulse inhibition of acoustic startle [117]. This suggests that an α3 selective positive modulator may be able to treat sensorimotor gating deficiency, which occurs in such psychiatric conditions like schizophrenia. Previously it had been suggested that the anxiolytic activity of diazepam was partly due to its action on α3 rich reticular activating systems. However, single point knockin mice had anxiolytic activity in the light-dark choice test and elevated plus-maze test, comparable to the wild-type mice [94]. In addition, the same study revealed that there was no change in sedative and anticonvulsant activity of diazepam. However, muscle relaxant activity was slightly reduced, suggesting involvement of the subtype [117]. It is interesting to note that even though there is exclusive expression of α3 in the reticular nucleus of the thalamus, involved in regulating thalamocortical oscillations which generate sleep spindles, it seems this subtype is not involved in the sleep inducing effects of diazepam. However, a study into generalized absence epilepsy with knockin mice showed that anti-absence drug clonazepam (Table 2) showed no effect on mice with a mutated α3, suggesting that the subunit is involved in this particular form of epilepsy. Study of triple knockin mice suggested that, like α1, the α3 is involved in the impairment of motor coordination by diazepam. In addition, double point-mutated mouse studies exhibited that both of these subunits appear to be required for tolerance [93].

α5 subunits are primarily localized in the hippocampus and make up less than 5% of the total population of $GABA_AR$ in the brain. They are primarily responsible for generating tonic conductance and play a key role in cognition. Knockout of the α5 in mice causes better performance in the Morris water maze model of spatial learning [118]. The knockout does not seem

to alter normal motor performance or coordination but show deficits in sensorimotor gating from pre-pulse inhibition of acoustic startle [118]. Knockin at H105R reduced the muscle relaxant action of diazepam and recapitulated the learning and memory results from the knockout [119]. Compounds selective towards the α5 have been capable of enhancing spatial learning [120].

α6 knockout mice caused an inhibition of δ subunit expression and mice were significantly more impaired by diazepam in rotorod tests [121]. Selective knockout of the α6 leads to a post-translational loss of the δ since they are strongly coexpressed [122]. Allelic variants in the α6 have been connected to abdominal obesity and cortisol secretion but the significance is still unknown [123].

Knockout of the β3 resulted in a cleft palate but 90% of the mice died within 24 hours of birth. The survivors exhibit an epileptic phenotype [124,125], hyperactivity, poor learning and memory, poor motor coordination, repetitive behavior consisting of running continuously in tight circles, and seizures [125]. Loss of the β3 led to dramatic reduction in the levels of expressed $GABA_AR$ In addition, when mice with the knockout were treated with oleamide, an endogenous sleep promoting fatty acid, it was found to be inactive; indicating an involvement of the β3 in sleep [126].

The very lethal effect of knocking out the γ2 results in a defect in postsynaptic clustering of the $GABA_A$ receptor as well as decreased single channel conductance [127]. Morbidity was expected as γ2 is in 90% of all $GABA_A$ receptors [85]. Heterozygous knockout results in mice with reduced synaptic clustering. They also displayed heightened response in trace fear conditioning and anxiety-related behavior which was relieved by diazepam [86].

The removal of the δ subunit results in mice that have spontaneous seizures and greater sensitivity to pharmacologically induced seizures [128]. Interestingly, neurosteroid-sensitive extrasynaptic δ subunit is downregulated during pregnancy due to the elevated amounts of neurosteroids in the system. Post-partum drop in neurosteroids with the inadequate levels of δ $GABA_AR$ levels leads to decreased inhibitory function. Consequently, there is an increase in neuronal excitability until the levels of δ are reestablished. In animal studies, when there is a delay in δ $GABA_AR$ recovery, severe despair behavior is observed in mice which leads to mothers cannibalizing their offspring [129]. Similar changes have been reported during puberty which could explain why this developmental stage is often associated with an increase in susceptibility to stress-related disorders [130]. In addition, rats that are stress-induced through social isolation have an upregulation of extrasynaptic δ subunits [131]. The δ subunit also seems to be involved in schizophrenia and may offer a target to treat insomnia as discussed further in 1.3.1.6 Role of Genetics and Selective Targeting.

1.3.1 $GABA_AR$ AND DISEASE

Quantitatively, GABA is the most important inhibitory neurotransmitter in the CNS. Excitation and inhibition is a precarious balancing act whereupon overstimulation of the GABAergic system can cause sedation, depression, amnesia and ataxia while attenuation can result arousal, anxiety, convulsions, and insomnia. It has been suggested that GABA systems are involved in the pathogenesis of anxiety, depression, insomnia, cognitive dysfunction, Down syndrome, autism, schizophrenia, and epilepsy, among others [132]. The existence of the benzodiazepine site is a mystery in itself with many evolutionary biologists theorizing why they occur. One theory proposes the existence of an endogenous agonist that is produced by the brain to reduce anxiety. Others theorize that, in contrast, the receptors mediate the activity of endogenous

inverse agonists (negative allosteric modulators) which can promote brain arousal. Another theory is that there are no endogenous ligands and the site is merely be a 'fine-tuning' feature that results from the particular protein conformation [133]. Whatever the reason for its existence, the GABAergic system is one of the most important neurotransmitter networks in the mammalian CNS.

1.3.1.1 Anxiety

According to the National Institute of Mental Health, anxiety disorders are among the most common mental illnesses with nearly 40 million adults in the United States age 18 and older, or 18%, being affected [134]. In a 1999 study commissioned by the Anxiety and Depression Association of America, anxiety disorders cost the U.S. more than $42 billion a year, virtually one-third of the country's $148 billion total mental health bill [135]. A study six years later claimed that the total annual cost may be upwards to $60 billion [136]. From an evolutionary perspective, anxiety is a useful and innately driven form of distress that arises in response to actual or threatened danger. Not long ago, it was believed that anger and harm are assessed by three levels of the triune forebrain: the rational (neomammalian), emotional (paleomammalian), and instinctive (reptilian). Anxiety is a de-escalating strategies mediated by these more primitive paleomammalian and reptilian forebrains [137]. Since then, the triune brain model, popularized by Carl Sagan's "The Dragons of Eden", has fallen out of favor among neuroscientists in the post-2000 era but has yet to be replaced with another working theory.

Anxiety can be grouped into six different models with many patients presenting multiple forms of the illness: social anxiety disorder (prevalence 13%), phobias in general (11%), post-traumatic stress disorder (8%), generalized anxiety disorder (5%), panic disorder (4%), and obsessive-compulsive disorder (2%). Generalized anxiety disorder is defined by excessive and

uncontrollable worry for at least 6 months, accompanied by three of the six associated symptoms of restlessness, fatigability, concentration difficulties, irritability, muscle tension, or sleep disturbance [138]. When compared to other anxiety and mood disorders, generalized anxiety disorder may commonly manifest at early ages, with many patients reporting an onset in adulthood as a result of psychosocial and emotional stress. Anxiety disorders develop from a complex set of risk factors, including but not limited to: genetics, brain chemistry, personality, and life events. The latter two being somewhat conjectural in nature and difficult to quantify. However, there is a gathering evidence that genetic variance causes altered expression and function of proteins that regulate the network of brain neurotransmitter systems (i.e. receptors, ion channels, transporters and enzymes) which can be associated with complex behavioral traits [139-141].

Selective serotonin reuptake inhibitors (SSRIs), which are a mainstay for anxiety treatment, are too slow acting for acute situations and often require several weeks to work. So a fast-acting BZD with little to no side effects would present a major improvement to therapeutic treatment.

1.3.1.2 Insomnia

Sleep is a complicated process involving many autonomic, physiological, and biochemical changes that transition the brain from a state of wakefulness, slow wave non-rapid eye movement (NREM) sleep and paradoxic rapid eye movement (REM) sleep. In a statement released in 2005, the NIH has concluded that insomnia occurs, at least occasionally, for around 30% of adults and is a chronic problem among 10-15% of adults with 40% of insomniacs also having a co-existing psychiatric disorder [142]. The condition affects daytime functioning, quality of life, and mental and physical health [143]. The first historically relevant use of sedative-hypnotics in the United States began in the Industrial Revolution when laudanum, which was opium mixed with alcohol, was

packaged and marketed as sleep aids. In the early 20th century, barbiturates became the leading prescribed hypnotic until the 1960s when benzodiazepine receptor agonist hypnotics replaced them [144].

BZDs are considered much safer than barbiturates and the FDA-approved several BZR agonists for the treatment of insomnia. However, nonbenzodiazepine agents are toted as even safer and the safest being a recent targeting of the melatonin receptor by agonist ramelteon (Table 2) which has no abuse liability or interference in cognitive function. There are benzodiazepines that are specifically promoted as sleep inducers but depending on the dosage, any approved BZD may be employed. Studies suggest that specific stimulation of the a1 would primarily result in sedative-hypnotic effects [145]. BZDs increase total sleep, reduce nocturnal wakefulness, and decrease the time required to fall asleep. The effectiveness of BZDs are known to decrease after long-time use due to tolerance so the majority of BZDs are usually recommended for short term use [146]. Rebound insomnia may occur following abrupt discontinuation but tapering the dose should offset the severity of the effect [147].

1.3.1.3 Epilepsy

The word "epilepsy" is derived from the Greek word *epilambanein*, meaning to seize, and was coined by Hippocrates in his famous 400BC essay *On the Sacred Disease* in which he described the disorder as a disease of the brain and promoted treatment through diet. Convulsive disorders have been described as a loss of the normal inhibitory control mechanisms and chemical super-sensitivity that increases excitability of the neurons [148]. Brain-wave studies measured by EEG show that abnormal excessive or synchronous neuronal activity in the brain during seizure [149]. Epilepsy is defined as the occurrence of two or more seizures not provoked by any identifiable

cause. According to a study performed by the World Health Organization in 2012, it is estimated that 1% of the population currently has epilepsy with the majority, nearly 80%, living in developing countries. There is an average of 180,000 new cases of epilepsy each year; 30% of cases are in children. There are different forms of epilepsy but they all originate in the brain due to irregularities in neuronal activity caused by disturbance of physicochemical function and electrical activity. There is very little understood about the causes of 'true' idiopathic epilepsy, which is distinct from symptomatic 'acquired' epilepsy, which results from brain injury, tumor, infection, or chronic alcohol use. In true epilepsy, around 70% of all epilepsy cases, the cause for the disease is unknown. In general, BZDs are not usually the first choice for long-term treatment of epilepsy due to tolerance development but in emergency management the short-term use during periods of increased, repeated, or prolonged seizure is very effective. Thus BZDs are still considered first-line agents for emergency management of acute seizures and status epilepticus [150].

1.3.1.4 Schizophrenia

In the mid-19th century, psychiatrists began taking note of a disorder progressing to chronic deterioration among the youth with unknown causes. In France it was called démence précoce, while in Scotland it was known as "adolescent insanity". In Germany, Emil Kraepelin gathered all information surrounding this varied disease with the underlining pattern of severe cognitive and behavioral decline; he named the illness "dementia praecox". Initial definitions of the illness have changed considerably. Currently it is known that schizophrenia is a heritable psychiatric disorder that can impair cognition, perception, and motivation. The disease manifests late in adolescence or early in adulthood. The route that leads to the diseased state is unknown however there have been shared pathological features such as the disproportionate loss of grey matter [151] and the drop in dendritic spine density which help transmit electrical signals [152].

According to a study by the National Institute of Mental Health, approximately three million Americans or 1.1% of the US adult population have been diagnosed with schizophrenia with delusional thinking and hallucinations. Unfortunately although there is treatment available for the psychotic symptoms, there is not yet a method to treat or prevent the cognitive impairments or the deficits of normal emotional response that personifies the most constant features of this disorder.

1.3.1.5 Cognitive Deficiencies

Cognition is a highly complex construct of mental activities that includes problem-solving, learning and memory, reasoning and judgment, understanding, knowing, creativity, intuition, self-awareness, and mental time-travel. Cognition and mood are closely linked however the underlying mechanisms appear to differ. Drugs that alleviate mood related symptoms such as depression and anxiety do not necessarily improve cognition, sometimes even worsening the deficit [153,154]. Among many psychiatric and neurological disorders, cognitive impairment is associated with bipolarism, depression, generalized anxiety disorder, panic disorder, PTSD, OCD, ADHD, ASD, Alzheimer's disease, Parkinson's disease, Down Syndrome, autism, and schizophrenia [132].

According to the Center of Disease Control (CDC), age-related cognitive impairment affects 16 million people in the United States and the impact as the Baby Boomer generation passes age 65 is expected to increase that number drastically. The estimated 4.7 million Americans with Alzheimer's disease may rise to 13.2 million by 2050 [155]. Alzheimer's disease and related dementias are estimated to be the third most expensive disease to treat in the United States. In 2010, the Medicaid nursing facility expenditure per state for individuals with Alzheimer's disease

is estimated to be $647 million [156], which does not reflect home and community-based care or prescription drug costs.

1.3.1.6 Role of Genetics and Selective Targeting

The role that genetics plays is well studied but complex. There is a large number of hereditable mutations in genes that regulate ion channel function called ion channelopathies [157].

Anxiety appears to have a strong genetic component. Selective breeding of mice over generations can produce high- and low-anxiety lines, suggesting a strong genetic component to this behavior [158]. In addition, individuals with generalized anxiety disorder appear to have high comorbidity rates with other psychiatric disorders including panic disorder, major depression, dysthymia, social phobia, and specific phobia [159-163]. Analysis of multivariate genetic comorbidity statistics shows that generalized anxiety disorder and major depression have common genetic origins [164] and vulnerability to the two overlaps to a substantial extent genetically [165]. When imaged by positron emission tomography, patients with panic anxiety exhibited reduction in $GABA_AR$ expression, a deficit that is most likely involved in the disease [166]. When patients with panic disorder are intravenously given flumazenil (Table 2), a BZR antagonist, it provokes panic in most patients however control subjects without panic disorder exhibited no such response [167]. The increased sensitivity may suggest an abnormality in $GABA_AR$ distribution or in the receptors themselves. This effect appears to be specific in patients with severe episodic anxiety since patients with generalized anxiety, post-traumatic stress disorder, and depression do not panic when given flumazenil. Interestingly, there is also a significant hereditary factor in panic disorder which may suggest that $GABA_A$ receptor irregularity is passed on due to transmission of a mutated receptor gene.

$GABA_AR$s play a fundamental role in sleep and BZD treatment for sleep disorders persist today, despite tolerance. Mutation in the β3 has been observed in a patient with chronic insomnia. Characterization of the mutant receptor showed a slower rate of desensitization than with the normal $GABA_AR$ [126]. BZDs approved to treat insomnia are estazolam (ProSom), flurazepam (Dalmane), quazepam (Doral), temazepam (Restoril), and trizolam (Halcion); which exhibit selectivity for the α1 [168]. Gaboxadol, a δ selective compound (Table 3), was able to promote slow wave sleep and increase total sleep time while simultaneously shortening sleep latency, and decrease wakefulness. However, due to side-effects such as hallucinations and disorientation, gaboxadol failed phase III clinical trials [169]. The popularity and persistence of BZDs in sleep disorder therapy speaks for the usefulness of the $GABA_AR$ as a target.

Epileptic heritable mutations of the $GABA_AR$ are known to occur in diseases such as Angelman syndrome. As a result of deletions in the β3 subunit, patients with Angelman syndrome develop severe mental retardation, delayed motor development, and epilepsy [170]. The β3 knockout mice also have a phenotype similar to those with this neurodevelopmental disorder [124,171]. The gene encoding the α1 (*GABRG1*) has been implicated in juvenile myoclonic epilepsy and the mutated γ2 (*GABRG2*) has also been discovered in two families with generalized epilepsy syndrome characterized with febrile seizures, and childhood absence epilepsy [172,173].

Research has shown biological markers associated with schizophrenia include neurocognitive dysfunction, brain dysmorphology, and neurochemical abnormalities. Studies in genetic association have targeted multiple candidate loci and genes but failed to demonstrate that any specific gene abnormality, or a combination of genes, is sufficient to cause schizophrenia. Very recent findings have revealed that the C4 genes is associated with schizophrenia, the C4

protein is localized in neuronal synapses, dendrites, axons, and cell bodies and is responsible for synaptic pruning. Patients with an overexpression of synaptic pruning protein have a higher risk of developing schizophrenia [174]. In schizophrenia there is a decline in the production of cortical GABA leading to a downregulation in cognitive function. As expected, there is a compensatory upregulation of $GABA_AR$ however not enough to regain normal function [175]. α3 knockout and partial knockout of α5 both show a deficit in sensorimotor gating which may point to potential involvement of these subunits in the pathophysiology of schizophrenia [176,177]. Post-mortem brains of individuals suffering from schizophrenia have a reduced expression of the δ subunit. In addition, the α5 has been identified as a susceptibility locus for schizophrenia [178]. Partial positive modulator with α5 selectivity SH-053-2'F-R-CH3 (Table 3) in the rat model of schizophrenia has demonstrated therapeutic potential by reducing the number of spontaneously active dopaminergic neurons in the VTA to those of control animals [179]. Non-selective positive modulator bretazenil (Table 3) was shown to be effective treatment in 40% of patients exhibiting acute episodes of schizophrenia in a range of symptom severity levels with the most severe side-effects being sedation [180]. Findings point to compatibility with a BZD that acts on α2, α3 and α5 which would have antipsychotic and cognitive enhancing qualities.

Irregularity in the GABAergic system has also been correlated to the pathophysiology of autism spectrum disorders. Knock-in mice with an H105R in the α5 subunit in the hippocampus show improved spatial performance in the Morris water maze and enhancement of trace fear memory [181]. Partial α5 knock-in mice exhibit improved trace fear conditioning, appetitive/food-related conditioning, and novel object recognition [116,119,182]. Development of agonists for the α5 has had very little success but might be an option for compounds that promote memory suppression that may provide useful for phobias and PTSD. The use of BZDs as analgesia has seen beneficial

towards the anterograde amnesia which can be a useful outcome when used in invasive surgery or other traumatic events. L-655,708 (Table 3), a novel partial inverse agonist with heightened affinity for the α5, succeeded in enhancing the performance in the Morris maze [183]. MRK-016 (Table 3), an inverse agonist selective for the α5, rescued acquisition and memory consolidation [184] and also exhibited antidepressant qualities [185]. However, it was poorly tolerated in the elderly and was not investigated further [186]. A highly selective α5 imidazotriazolobenzodiazepine called RO4938581 (Table 3) was able to assuage phencyclidine (PCP) induced cognitive inhibition and enhance performance of an object retrieval task. These selective inverse agonists would also find use in promoting recovery after stroke. The increase in tonic inhibition is observed after stroke, mediated by extrasynaptic $GABA_AR$ containing the α5 or δ subunit. Lowering the number of the these subunits genetically improved recovery after stroke and administering BZD inverse agonist specific for α5 produced early and sustained recovery of motor function. However, the timing of drug delivery is very important as a $GABA_AR$ agonist given during the stroke can decrease stroke size [187]. Tests in mice have proven that early administration can exacerbate stroke damage but when treatment is delayed by 3 days, functional recovery is promoted without affecting stroke size [188]. This evidence suggests that a selective α5 inverse agonist may be useful as the first clinical treatment for promoting recovery after stroke or other devastating brain injuries. Selective inverse agonist also have therapeutic potential in patients with Down syndrome. Down syndrome, though clearly caused by trisomy 21 linked to Olg1 and Olg2 genes, is connected to increased presynaptic GABA release which leads to excessive inhibition and obstruction of synaptic plasticity [189]. α5 selective partial inverse agonist RO4938581 was capable of improve deficits in synaptic plasticity and neurogenesis without affecting motor coordination or sensorimotor abilities. Interestingly, the compound exhibited anxiolytic properties and suppressed hyperactivity [190]. A related compound,

RG1662 (Table 3), by Hoffmann-La Roche has entered into clinical trials for the treatment of cognitive disabilities associated with Down syndrome. Cntnap2 knockdown, a mouse model for syndromic autism spectrum disorder (ASD) there is reduction in GABAergic interneurons and abnormal network activity [191]. Increasing evidence has indicated that dysfunction of GABAergic neurotransmission is related to ASD [192]. In Scn1a heterozygous knockout mice, which is a model of a syndromic form of ASD (Dravet's syndrome), mice display hyperactivity, social interaction deficits, impairment of context-dependent spatial memory, and decreased GABAergic neurotransmission [177]. Application of clonazepam in these knockout model rescues abnormal social behavior and deficits in fear memory [193]. $GABA_AR$ subtypes involved are unknown but postmortem study has observed reduced expression of α4, α5, and β1 [194]. The absence of the α5 subunit in ASD patient brains has also been verified through PET scans [195].

In addition to the five diseases that have been linked and treated with $GABA_AR$ drugs, there are other possible applications. The hypothesis that targeting the GABAergic system to treat depression is a new prospect that has not yet been sufficiently verified. Global brain gene expression analysis links the GABAergic dysfunction to suicide and major depression disorder (MDD). Studies have shown that patients suffering from MDD have reduced CNS GABA concentrations [196,197]. In addition, there is differential expression of GABRA5, GABRB1, GABRD, GABRG1, GABRG2 [198]. The γ2 subunit has shown possible involvement as the heterozygous knockout increased behavioral despair in immobility in the forced swim test (FST) and the tail suspension test (TST) [199].

In conclusion, therapeutics aimed at $GABA_AR$ are various and wide-ranging and drugs which target specific subtypes can elicit specific behavioral effects. Due to the receptors

pervasiveness as the major inhibitory neurotransmitter, it is involved in many vital processes. Thus, dysfunction or altered expression can profoundly affect CNS function, translating to behavioral changes in an individual. Although BZDs have fallen out of favor due to their side effect profile, there remains an amazing potential in reducing these off-target effects by increasing subtype selectivity.

CHAPTER 2: ELECTROPHYSIOLOGICAL CHARACTERIZATION OF TRANSIENTLY TRANSFECTED HEK293T CELLS

2.1 Introduction

Ion channels are proteins in the lipid bilayer that selectively allow ions entrance or exit across cell membranes. They have been ubiquitously found in every cell membrane studied: plant cells, bacteria, unicellular and multicellular organisms and even in the membranes of many intracellular organelles. The lipid bilayer is a very hydrophobic and impermeable to charged molecules, despite the size. In solution, inorganic ions like Na^+ and Cl^- form a hydration shell and will not pass across the membrane without an ion channel or ion pump. The activity of these membrane proteins are so keenly controlled that the difference in the charge balance from the intracellular to the extracellular domain can generate a voltage difference called the membrane potential (V_m). Typically, the V_m is around -70mV to -40mV; this is known as the resting membrane potential. The flow of cations in or anions out makes the inside of the cell become more positively charged and is known as depolarization while the flow of cations out or anions in makes the inside more negatively charged and is called hyperpolarization. This change can make the V_m fluctuate between -90mV to +60mV. The plasma membrane acts as a resistor and a capacitor. Its resistor characteristics depends on the number of open ion channels; if many are open, the membrane has a lowered resistance but if they are closed, the membrane has a very high resistance. A capacitor is formed from the separation of charge. The intracellular side is more negative than the extracellular side so both sides have an electromagnetic gradient across the membrane. Anions in the cytosol are pulled towards the membrane and cations accumulate near the membrane on the outside. This scenario posits an energy state for the gathered ions and the amount of charge can be described as $Q = E_m C$, where Q is the charge stored, E_m is the potential difference across the

membrane and C is the membrane capacitance in farad. These differences between the two sides arises from the concentration differences and electrical due to the ionic charge differences. This is called an electrochemical gradient. The capacitance is proportional to the surface area of the membrane and inversely proportional to the thickness and can be described with the equation $C = \frac{A\varepsilon_r}{d}$ where A is the area, ε_r is the dielectric constant and d is the membrane thickness. So capacitance can indicate the membrane surface area under investigation. Since the membrane is approximately 25Å, it is able to act as a very thin electrical capacitor. The resistance can be estimated applied to Ohm's law (E=I*R), where E is the membrane potential, I is the membrane current, and R is the membrane resistance. The electrochemical equilibrium of a neuron can be estimated using the Goldman equation: $E_m = \frac{RT}{F} \ln \frac{P_K[K]_{out} + P_{Na}[Na]_{out} + P_{Cl}[Cl]_{in}}{P_K[K]_{in} + P_{Na}[Na]_{in} + P_{Cl}[Cl]_{out}}$, where E_m is the membrane potential in volts, P is the permeability of the membrane to each ion in meters per second, and $[ion]_{out}$ and $[ion]_{in}$ are the concentrations in the outer and inner membrane. The Goldman equation is just an extended version of the Nernst equation and removing the permeability will collapse it back to its simpler form: $E_m = \frac{RT}{zF} \ln \frac{[A^-]_o}{[A^-]_i}$, where z is the number of moles of electrons transferred between membranes, $[A^-]_o$ is the concentration of ion outside the membrane, $[A^-]_I$ is the concentration of ion inside the membrane.

The space between the pre- and post-synaptic chemical synapses is known as the synaptic cleft. In the pre-synaptic terminal there are small membrane-bound organelles called synaptic vesicles which are filled with one or a mixture of neurotransmitters. These neurotransmitters bind and potentiate a postsynaptic electrical response. Though neurons generate electrical signals to transmit information, they are not intrinsically good conductors of electricity. So neurons evolved to produce action potentials which acts as a booster system to propagate a signal through the

nervous systems. A signal is sent by achieving enough stimulus that the resting potential increases and the voltage reaches a threshold to trigger an action potential. Excitatory signals (postsynaptic potentials) increase the likelihood of the action potential firing while inhibitory signals (inhibitory postsynaptic potentials) lower the resting potential and reduce the likelihood of an action potential. $GABA_AR$ thus have an inhibitory effect as the influx of chloride anions lowers the resting potential from the threshold for neuronal firing.

It was not until 1952 that a technique was invented to study ion channel function. Sir Andrew Huxley and Sir Alan L Hodgkin studied the workings of the squid giant axon [200]. For their work, they used Kenneth Cole's technique called a voltage clamp and were able to measure changes in the conductance of ions of the membrane of the squid axon in response to modification of the membrane voltage. In order to measure the membrane potential, two electrodes were placed across the membrane. The first electrode is placed outside the cell and the second must be inside. However, the cell membrane's central function is the hold the contents of the cell so as not to leak, thus it was a central concern as to how to access the intracellular space. It was discovered that squid giant axons could be cut at the end and dried (air gap). By doing this, a wire electrode could be inserted into the cytoplasm through the cut end without leakage between the two spaces. Huxley and Hodgkin's experiments and calculations proved the ionic theory of nerve impulses and led to a set of ground-breaking differential equations which are still essential for describing neuronal circuits [201]. Huxley and Hodgkin were awarded with a Nobel Prize in Physiology and Medicine in 1991 and has defined our understanding of how the central nervous system and the brain works. After this landmark study, Erwin Neher and Bert Sakmann created a voltage-clamp recording method in 1976 and called it patch-clamp. Neher discovered that by applying negative pressure inside the pipette, a high seal resistance could be attained [202]. After formation of the giga-ohm seal,

the membrane patch was broken by applying high negative pressure and the cytoplasmic side is connected to the pipette to allow for readings. This technique enables the study of the real-time current fluctuations through single ion channels [203]. The patch-clamp technique uses a thin glass pipette and fuses it to the cell membrane through gentle suction. This technique was further improved by scientists in 1981 when a team was able to resolve changes as small as 0.5pA.

There are many different recording modes in patch clamp. Whole-cell mode indicates that when a cell is patched, the cell-attached part is ruptured to gain access to the intracellular domain. Important features of this mode includes several factors. First, the pipette tip must be wide enough to allow washout of the cytoplasm by the intracellular solution filling the pipette. The washout can make readings more uniform by controlling the intracellular contents; but relevant cytosolic factors that may be vital to function could be lost. Secondly, leak resistance R_{leak} represents seal quality between the glass of the pipette and the membrane. This value should be higher than the current input resistance of the probe to ensure that no significant amounts of current will leak away. In manual patch-clamp, the R_{leak} should be higher than 10GΩ. A high seal resistance is important since it allows the resolution of minute current changes from background noise. Third, to perform manual patch-clamp, an elaborate rig is required to procure good results. A stable platform is the foundation to successful current readings, quick movements and vibrations can break seals so isolation of the rig in the building or the lab can enhance data collection. A microscope in the rig will be helpful in the visualization of the preparation but some skilled electrophysiologists have been known to work without one. Stable manipulators are needed to position the micropipette and introduce or position the tip onto the cell membrane in a controlled environment. Finally, the electronics to perform the recording and analysis are necessary [204].

Next, the solutions used should be carefully considered. The range of intracellular and extracellular ion concentrations appears to be relatively constant in cell types, organs and across species [204]. There are large differences between sodium, potassium, and calcium concentrations seen in Table 5. This range roughly translates to those typically used, Table 4, when performing

Table 5. The intracellular and extracellular concentrations found in animal fluids.

Ion	Intracellular range (mM)	Extracellular range (mM)
Na^+	5-20	130-160
K^+	130-160	4-8
Ca^{2+}	50-1000 nM	1.2-4
Mg^{2+}	10-20	1-5
Cl^-	1-60	100-140
HCO_3^-	1-3	20-30

Table 4. Typical concentrations used in patch clamping of a mammalian cell. pH is set in the ECS using NaOH and the ICS by using KOH.

Chemical	ICS concentration (mM)	Extracellular range (mM)
Na^+	5	126
K^+	147	6
Ca^{2+}	0	1.2
Mg^{2+}	1.2	2.5
Cl^-	150	125
GTP	0.1	0
ATP	5	0
HEPES	20	10
Glucose	11	11
Sucrose	0	67

whole-cell patch clamp. Besides the ionic concentrations, the pH and the osmolarity play a big part in obtaining good seals and thus good current readings. Physiological pH is about 7.4 in the extracellular solution (ECS) while the intracellular solution (ICS) is slightly more acidic at 7.2-7.3. Osmolarity can affect the volume of the cells and the osmotic force on the membrane. The cell membrane will be effected if the difference is large but increasing the ICS osmolarity can swell and smooth the wrinkled membranes of cells to improve successful seal formation of the patch. Normally sucrose or glucose are used for this purpose since they don't interfere with channel function. However, the addition of calcium and glucose can facilitate the growth of microorganisms in the stock solutions. In addition, if perishable ATP is used in the intracellular solution then solutions must be kept frozen. These factors should all be carefully considered when choosing the buffer stocks and storage conditions.

2.2 Instrumentation

Electrophysiological techniques are critical in determining the enhancement of chloride conductance and calculating potency and efficacy of the drugs but data collection is limited by slow throughput. Several types of automated patch clamp (APC) systems have been developed to allow for high-throughput data collection. The IonFlux System uses plates with microfluidic channels underneath the wells to enable high-throughput electrophysiology measurements. Plates are divided into experimental patterns in which specific wells are designated for cells, compounds, and recording buffer solution. The 96 well plate contains 8 experimental patterns with 12 wells: one cell suspension well (inlet), one well for waste (outlet), two cell recording wells (traps) and eight compound addition wells. Once the reagents and cells are loaded and placed in the instrument, a pneumatic interface seals against the top to regulate the air pressure and vacuum into the wells. The interface contains an array of electrodes that lower into the recording wells, ensuring

enough volume in these wells to reach the electrode is important. The plate is primed with fluid by applying positive pressure to all the channels. Once priming is complete, positive pressure is used to introduce the cells into the main flow channel. Trapping begins by applying negative pressure to the wells of the trapping zones where cells are attached and sealed similarly to manual patch pipettes. Whole cell voltage clamp is achieved once the cells are attached. Each of the trapping zones captures 20 cells which are used to make one patch clamp recording. There are two trapping zones per experimental pattern so there are 16 parallel recordings total in a 96 well plate. Once trapped, the IonFlux software can be programmed to apply voltage sweeps across the electrodes and measure the ionic current through the cell membranes. Open channel resistance should drop to approximately 1MΩ or less. After recordings are established, up to 8 different compounds or concentrations can be applied in rapid succession. Fast compound addition, washout, and exchange with simultaneous and continuous recording makes this system an accelerated system for drug discovery and development. The system itself reduces lab space required for manual forms of patch-clamp to a small benchtop footprint. The disadvantage is that the speed of data collection is countered by the expense of the microfluidic plates which are hundreds of dollars per plate. In contrast, manual patch clamp is time-consuming but very cost effective once the rig is set up. Giga ohm seal formation is very difficult to achieve in high throughput systems, and accordingly the IonFlux 20 cell trap has resistance seals consistently in the 7MΩ range which compares to a 140MΩ seal for a single cell. However, Molecular Devices has recently released a single cell trapping plate that is capable of establishing a gigaseal to produce high quality recordings with slightly lower success rates. Coming from a technical aspect, manual patch clamp requires a very fastidious and experienced hand while IonFlux's plate reader setup can be learned comparatively quickly and eliminates most human-related errors. In a plate reader

format, cells are brought to the patching site rather than having the pipette get brought to the cell. Therefore, there is no optical access to the cells in the microfluidic plate and no ability to choose the best cell to patch. However, there are many additional advantages such as the ability for precise temperature control and uniform control of compound applications. In addition, the summation of the population of captured cells greatly improves success rates and uniformity of recordings [205]. It should be noted that the IonFlux utilizes Ag/AgCl electrodes that only performs well in solutions containing chloride ions. It is vital to replenish the chloride ions on the electrode, if the AgCl is exhausted by the current flow, bare silver could come in contact with the solution and silver ions can leak and poison proteins.

The IonFlux experiment can be divided into four different phases which can be programmed in the software with different well pressures, voltage applied to the cells, and signals recorded. The phases are: priming the microfluidic channels, capturing the cells, breaking the cell and gaining whole-cell recording, and the data acquisition phase. During the priming, compound and trapping pressure values should be kept from 10-15psi and the main channel pressure value at 1 psi for a 2-4 minute duration. This flushes out any storage solution left in the channels and any bubbles that may be in the microfluidic channels. These are high pressure settings since there's not yet cells patched. The compound/trapping pressures and main channel pressure should not exceed a factor of 20:1 or the compound will flow upstream into the cell suspension inlet well and contaminate the readings. Trapping phases introduce the cells into the main channel and suction is applied to pull the cells to the pipette series. Because the velocity of the main channel is quite high, trapping protocol necessitates a dwell, or a pause, to stop the flow in the main channel and allow the cells to be drawn to the trap openings. This docking period can take multiple cycles to ensure that all the traps are filled with cells. Main channel pressure should be kept at 0.1-0.2psi while the

compound pressures are inactive, the main channel is pulsed for 0.5sec with 3-5sec dwells. This should be cycled 10-15 times. The trapping vacuum should be 5inHg, too low and the traps may not fully fill, too high and the cells may be damaged. The breaking period is characterized by a burst of suction to break the cell and gain whole-cell patch. Though many cells break spontaneously during the trapping phase, it is necessary to ensure that all cells are in the same patch-clamp configuration. The main channel pressure should continue to hold at 0.1psi with a trap pulse value of 8-10 in Hg and duration of 5 sec. The dwell value should be 5inHg to hold the cells in place and the dwell time should be 5 secs. This series should be repeated 3-5 cycles to ensure that all the cells are opened. The most important and tunable phase of the experiment is the data acquisition phase. To minimize diffusive mixing that will dilute the compounds, a ratio of 200:1 between compound pressure and main channel pressure should be maintained. This will deliver 90% of compound and 10% flow of cell suspension. It is incredibly important that the cell suspension main channel flow be kept active at all times. If the main channel is off then compound will start to flow upstream and contaminate the cell suspension which is the wash buffer that rinses the patched cells. The main channel pressure should be kept at 0.1-0.3 psi, adjusting this can speed the compound delivery though it should not be raised above 0.3 psi because the seal can degrade. Compound pressure pulse value is 10-15 psi, higher pressures can lead to faster compound delivery. To reiterate, the ratio of the compound pressure to main channel flow must be kept between 60:1 and 200:1, breaching this range can lead to diluted compound delivery or compound contaminating the cell suspension well. Compound delivery duration can be modified to as fast as 100 msecs depending on the ion channel under study and the experiment performed. The trap dwell should continue to hold at 3-5 in Hg to keep the cells sealed in place.

2.3 Assay Format

The original format of the assay for testing compounds recommended by the IonFlux was to split the compound ensemble into 2 halves. The first half would contain GABA EC_3 and compound while the second half would only contain the compound. The wells would then open to preincubate the cells with just compound, followed by a burst from wells containing both GABA and compound. This method was very reliable and took into account slow kinetics of the compounds which may require longer exposure times to bind to the receptor. However, data collection was limited to 3 data points which does not result in a dose-response curve, the scheme is depicted in Figure 7.

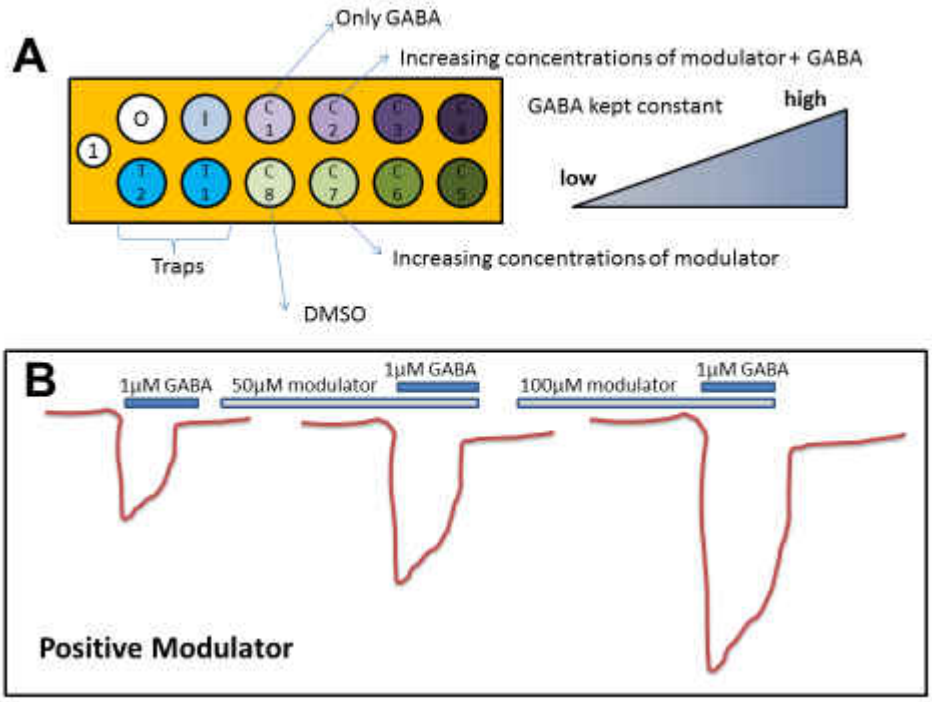


Figure 7. Plate layout and Depiction of Data Acquisition Phase of the Original Method Used for Drug Testing. A) illustrates a single ensemble of the IonFlux 16 plate, O indicates the outlet, I is the inlet, C denotes compound wells, and T are the trap wells. GABA is kept constant while testing the compound but the modulator increases in concentration across the plate from left to right. B) illustrates the current readings during such an experiment. The bars above the current sweep indicate the duration that the cells are exposed to GABA and the drug.

2.4 Assay Optimization

2.4.1 INTRODUCTION

To study the $GABA_AR$, transient transfection was used to generate the receptors in human embryonic kidney cells (HEK293T). Transfection allows the negatively charged phosphate backbones of DNA to enter cells with a negatively charged membrane. This can be done by coating the DNA with cationic lipid based reagents which can fuse with the lipid bilayer [206]. Transient transfection coupled with automated patch clamp is capable of providing maximum flexibility with high throughput capabilities. Initial experiments were performed with positive modulator HZ-166, Figure 9, and competitive antagonist bicuculline, Figure 8.

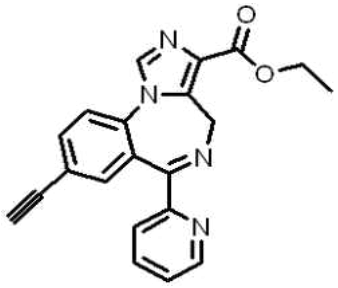


Figure 9. Structure of the HZ-166 imidazobenzodiazepine synthesized by the Cook Lab at UWM.

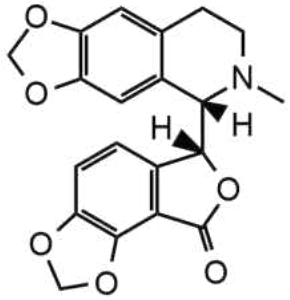


Figure 8. Structure of $GABA_AR$ antagonist Bicuculline.

2.4.2 EXPERIMENTAL

Plasmid Propagation Reagents

Plasmids containing the *Mus musculus* genes for α1-6, β3, and γ2 $GABA_AR$ subunits were generously received from the Werner Sieghart Lab in Austria. These plasmids arrived on paper disks eluted with RNase Free Water (Fisher, BP24701). 1 µL of the elution was added to a tube of NEB 5-alpha competent E. coli cells (New England BioLabs, C2987H) and flicked to mix. The

mixture was placed on ice for 2 minutes, undisturbed and immediately heat shocked at exactly 42°C for 30secs. The tube was moved to ice for 2min, after which 950 µL of SOC was added to the mixture. 50 µL and 100 µL were spread onto a 100 µg/mL carbenicillin (GoldBio, C10325) plate and grown overnight at 37°C. A colony was chosen and used to inoculate LB broth (Fisher, BP9733-500) containing carbenicillin. The resulting culture is centrifuged at 6,000 x g for 15 min at 4°C to pellet the bacteria and the supernatant removed. The plasmid DNA is then extracted using a gravity-flow anion-exchange HiSpeed Plasmid Maxi Kit (Qiagen, 12663). The pellet was resuspended in buffer and an alkaline lysis is performed before the lysate is cleared by filtration. The lysate is then added to a primed HiSpeed tip to bind DNA, wash, and finally elute. Isopropanol was added to the elution to precipitate the DNA and collected using the QIAprecipitator. The final elution from the QIAprecipitator yields ultrapure plasmid DNA. The DNA concentration was determined by UV at 260 nm using the Tecan Infinite M1000 plate reader. Protein impurities were assessed at 280 nm.

Cell Culture Reagents and Instrumentation

A commercially available human embryonic kidney (HEK 293T) cell containing the simian vacuolating (SV) virus 40 T-antigen origin of replication [207] was used in all the stable cell lines. HEK 293T cells were purchased (ATCC) and cultured in 75 cm^2 flasks (CellStar) coated in matrigel (BD Bioscience, #354234), a gelatinous protein secreted by mouse sarcoma that facilitates cell adhesion to the flask. Cells are grown in DMEM/High Glucose (Hyclone, SH3024301) media to which non-essential amino acids (Hyclone, SH30238.01), 10 mM HEPES (Hyclone, SH302237.01), 5 x 10^6 units of penicillin and streptomycin (Hyclone, SV30010), and 10% of heat-inactivated premium US-sourced fetal bovine serum (FBS) (Biowest, SO1520HI)

were added. Cells were rinsed with phosphate buffered saline (Hyclone SH30256.01) without calcium or magnesium. Cells are harvested using 0.05% Trypsin (Hyclone, SH3023601) or Detachin (Genlantis T100100) which both disrupts the cell monolayer and proteolytically cleaves the bonds between the cells and flask. The latter is more gentle and used for patch-clamp study. The media utilized in transient transfections contains the same components only the FBS was heat-inactivated and dialyzed FBS (Atlanta Bio, S12650H), then cells were rinsed and shaken in Serum Free Media (Hyclone, SH30521.01).

Cell transfection was conducted by lipid-based methods using Lipofectamine with PLUS reagent (Life Technologies, #15338020). Hygromycin was used for clone selection (Invitrogen 10687-010). Cells were counted on a hemocytometer, 20 µL of cell suspension are aliquoted onto the slide and 3 counting areas whose volume is 100 nL are averaged and multiplied by $1x10^4$ to give a concentration of cells in cells/mL.

Electrophysiological Reagents and Instrumentation

The following concentrations were optimized for study of $GABA_AR$s. The extracellular and intracellular solutions recommended by the manufacturers of the IonFlux. The extracellular solution contains: 238 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM Glucose, and 10 mM HEPES at pH 7.4. The intracellular solution contains: 60 mM KCl, 15 mM NaCl, 70 mM KF, 5 mM HEPES, and 5 mM EGTA at pH 7.25. These buffer components and concentrations were later altered slightly during the troubleshooting phase of creating the recombinant cell lines see Chapter 3: GENERATION OF GABAA STABLE RECOMBINANT CELL LINES.

Buffer components contained: NaCl (Fisher, BP358-1), KCl (Fisher, BP366-1), $MgCl_2$ (Sigma, M8266), $CaCl_2$ (Acros Org, 123350025), Glucose (Sigma, G0350500), KF (Sigma,

229814), HEPES (Fisher, BP410-500), CsCl (Sigma, 203025), EGTA (Tocris, 28-071-G), and Mg^{2+}ATP (Sigma, A9187). Intracellular solutions pH was adjusted with KOH and extracellular with NaOH.

2.4.3 RESULTS AND DISCUSSION

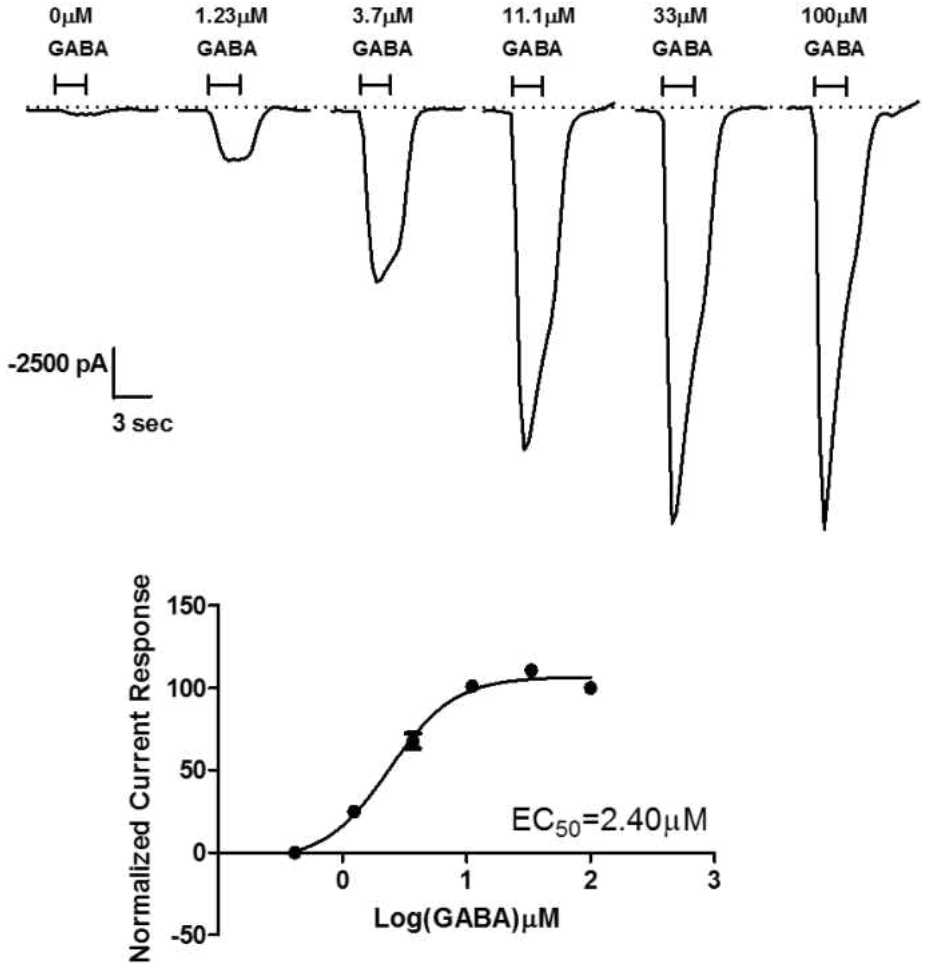


Figure 10. Stable Recombinant Millipore cells exposed to varying GABA concentrations, N=16.

Initial tests were run using α1β3γ2 stably-expressing cells provided by Fluxion (Millipore, CYL3053). These cells were tested using the agonist GABA (Figure 10) and competitive antagonist Bicuculline (Figure 11).

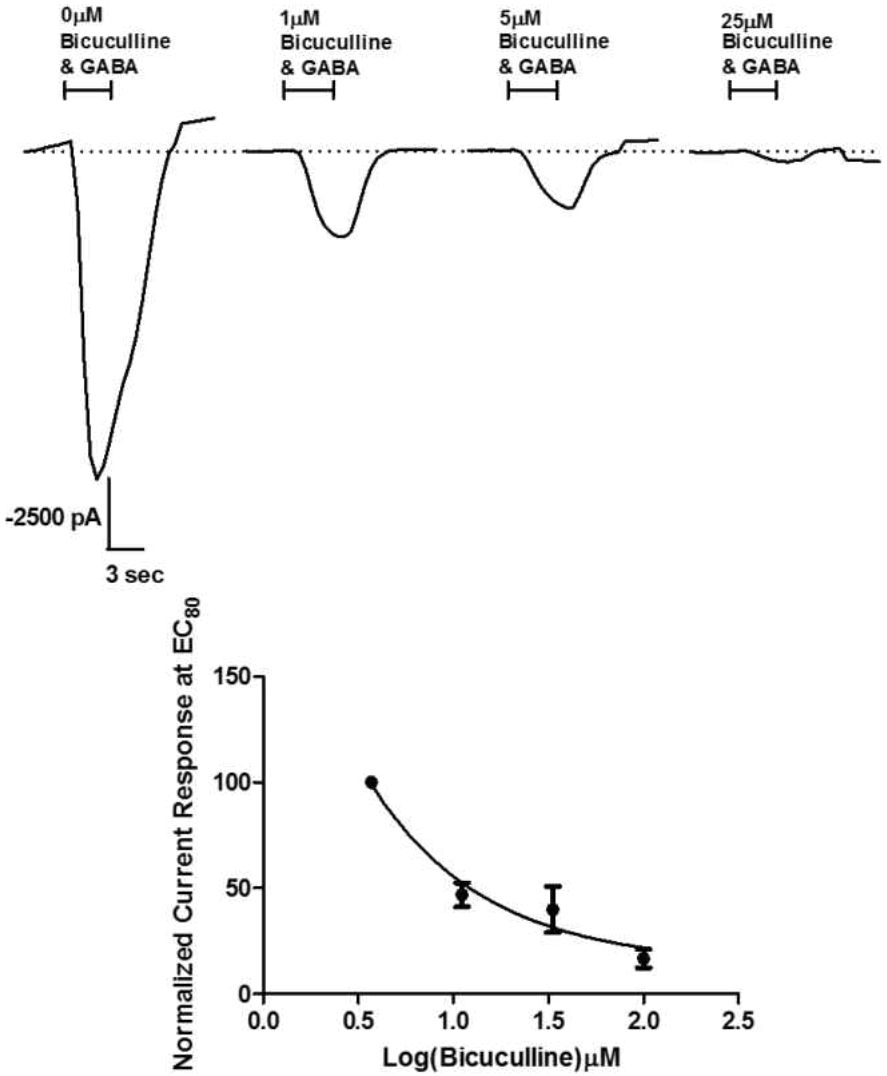


Figure 11. Stable Recombinant Millipore cells exposed to 10uM GABA EC_{80} and increasing concentrations of negative modulator Bicuculline. N=4

Initial tests with transiently transfected cells yielded very poor results when compared to the Millipore cells: with transfected cells having current readings ranging from -500pA to -5,000pA (Figure 12) compared to Millipore's recombinant cell response which ranged from to -4,000pA to 20,000pA (Figure 13).

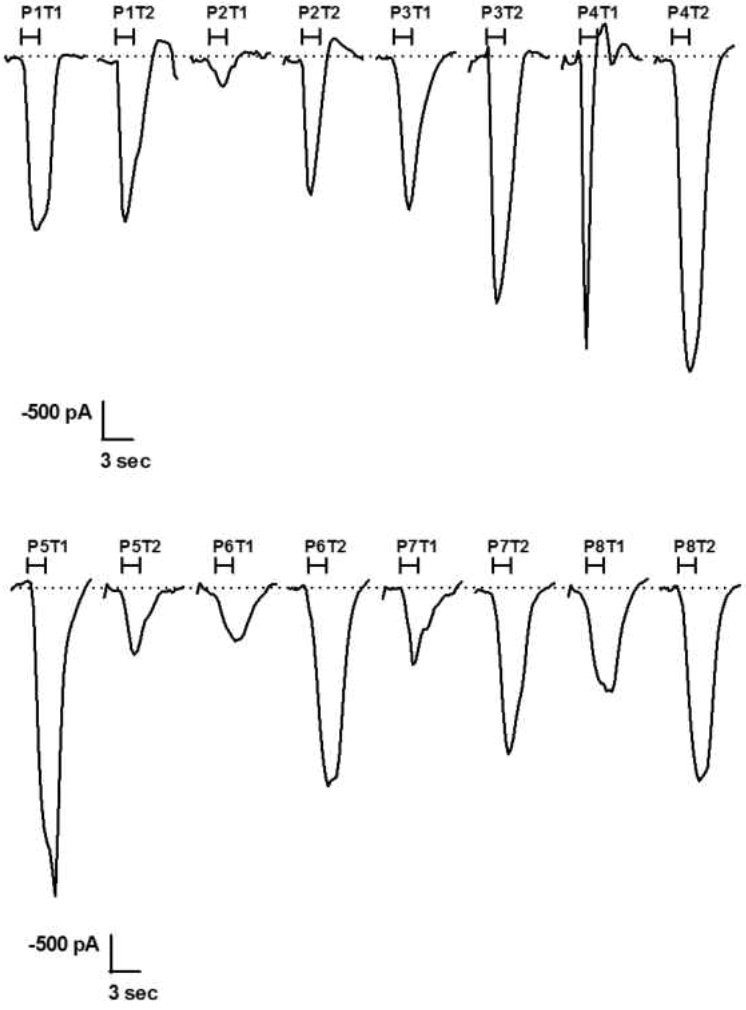


Figure 12. The maximum inhibitory current achieved in transiently transfected cells expressing the α1β3γ2 $GABA_AR$ exposed to GABA. Pattern (P) and trap (T) of each of the sweeps is seen above.

A series of extensive experiments optimizing the lipofection transfection method and cellular preparation is summarized in Table 6. All transfections were performed between 40-80% confluency as too few cells cause the culture to grow poorly with no cell-to-cell contact and too many cells result in contact inhibition and make the cells resistant to uptake of foreign DNA, resulting in lower transfection efficiencies [208]. In general, actively dividing cells take up introduced DNA better than quiescent cells. In addition, 1 volume of DNA was considered to be 5µg. A range of DNA concentration is usually suitable for transfection but anywhere below or above the range

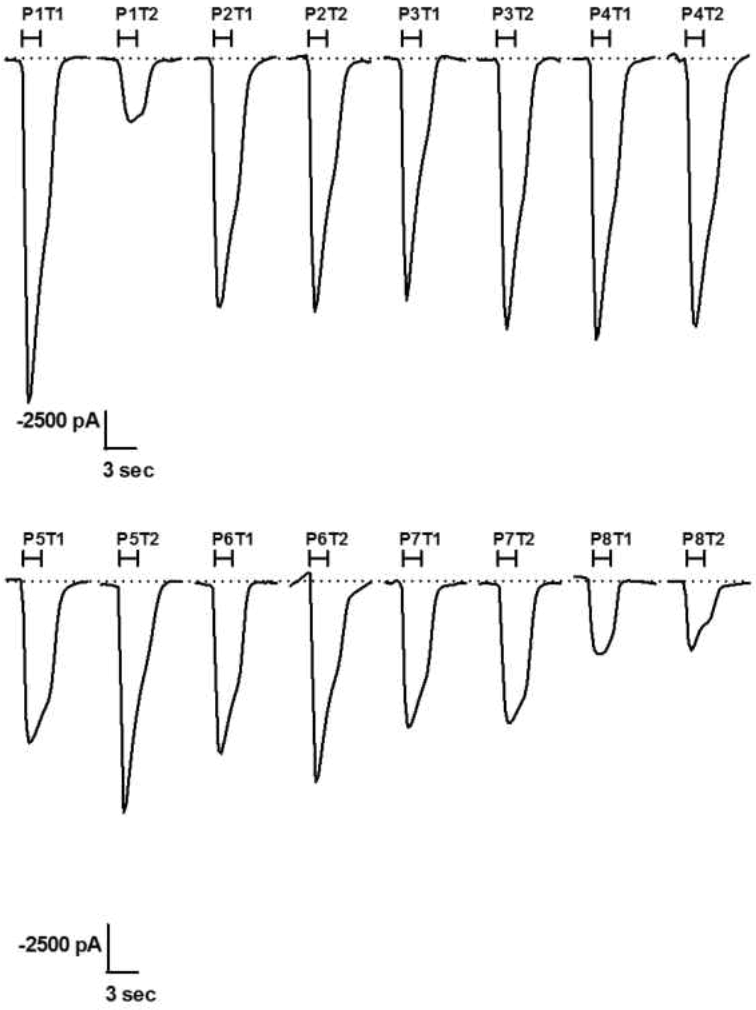


Figure 13. The maximum inhibitory current achieved in the stable recombinant Millipore cells expressing the α1β3γ2 $GABA_AR$ exposed to GABA. Pattern (P) and trap (T) of each of the sweeps is seen above.

and transfection efficiencies will decrease. Too little DNA can result in very little gene production, too much DNA can be toxic to cells. The α1, β3, and γ2 plasmids were used for the transient transfection so as to be able to compare results with the stably-expressing α1β3γ2 Millipore cells. Initial conditions yielded very poor current response when cells were patch-clamped and exposed to increasing concentrations of GABA. In order to assess the optimal expression levels and functionality, the number of responding traps and the intensity of current response were studied. It was found that growth in media containing dialyzed FBS (Atlanta Bio, S12650H) followed by

rinsing and shaking cells in Serum Free Media (Hyclone, SH30521.01) greatly improves the seal and reduces background noise (conditions 7-9); possibly by removing excess proteins and lipids

Table 6. Optimization of transient transfection and cellular preparation

Condition #	α:β:γ ratio	Transfection (hrs)	Lipofectamine (μL)	Recovery (hrs)	Wash in SFM (min)	Media	Responding traps	Ave I_{min} (pA)
0	1:1:1	4	92.1	N/A	N/A	HI-FBS	1/8	-2000
1	4:4:2	24	92.1	N/A	N/A	HI-FBS	2/8	-3000
2	6:6:3	24	92.1	N/A	N/A	HI-FBS	1/8	-1000
3	6:6:3	48	92.1	N/A	N/A	HI-FBS	2/8	-2000
4	6:6:3	24	46	N/A	N/A	HI-FBS	0/8	-1000
5	8:8:4	24	92.1	N/A	N/A	HI-FBS	0/8	-1000
6	2:2:1	4	92.1	N/A	N/A	HI-FBS	4/8	-4000
7	2:2:1	4	92.1	30	N/A	HI-FBS	4/8	-4000
8	2:2:1	4	92.1	30	30	HI-FBS	7/8	-3000
9	2:2:1	4	92.1	30	30	DI-HI-FBS	8/8	-2000
10	1:1:1	4	92.1	N/A	30	DI-HI-FBS	8/8	-2000
11	1:1:1	24	92.1	N/A	30	DI-HI-FBS	8/8	-8000

from the cell surface. The duration of transfection and the ratio of the plasmids matters less than

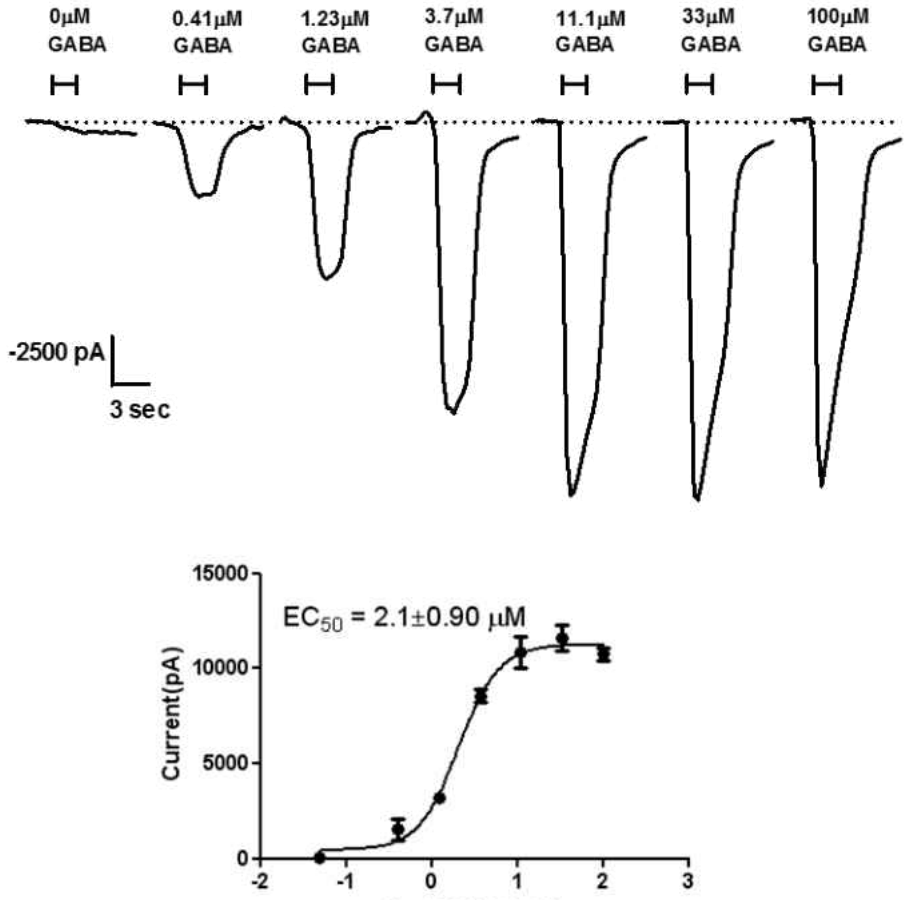


Figure 14. Optimized transiently transfected α1β3γ2 cells exposed to GABA

the time needed for the cells to begin expressing large quantities of the $GABA_AR$ (condition 9-10) making the recovery time in fresh media unnecessary and proving cells can instead be left with the lipofection reagent still in solution. Transfections using 5000ng of each plasmid with a 1:1:1 ratio of α:β:γ yielded synonymous results; despite logic dictating that the optimal ratio should be 2:2:1 since receptors assemble with 2α:2β:1γ; the amounts used can be reduced to 1:1:1 without significant effects. The final result seen in condition 11 where plasmids were transfected with a 1:1:1 ratio for 24hrs in media containing dialyzed heat-inactivated FBS and a 30min wash in SFM led to maximal success in trapping and current response. The dose-response curve from this transfection condition (Figure 14) yielded data similar to those of the stably-expressing Millipore cells.

The same optimized transfection protocol was utilized for the 6 different subtypes with measurable current response in all of them. Figure 15-Figure 21 shows the current sweeps of the entire run and the corresponding dose-response curve from each of the six subtypes. The α2β3γ2

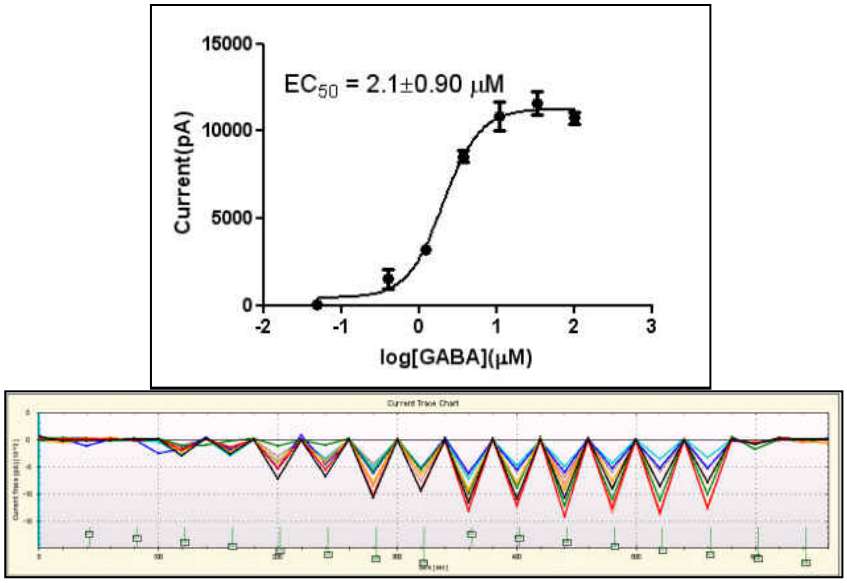


Figure 15. Dose-response curve and current sweeps of α1β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA.

(Figure 16) and α3β3γ2 (Figure 17) subtypes exhibited robust GABA responses comparable to the

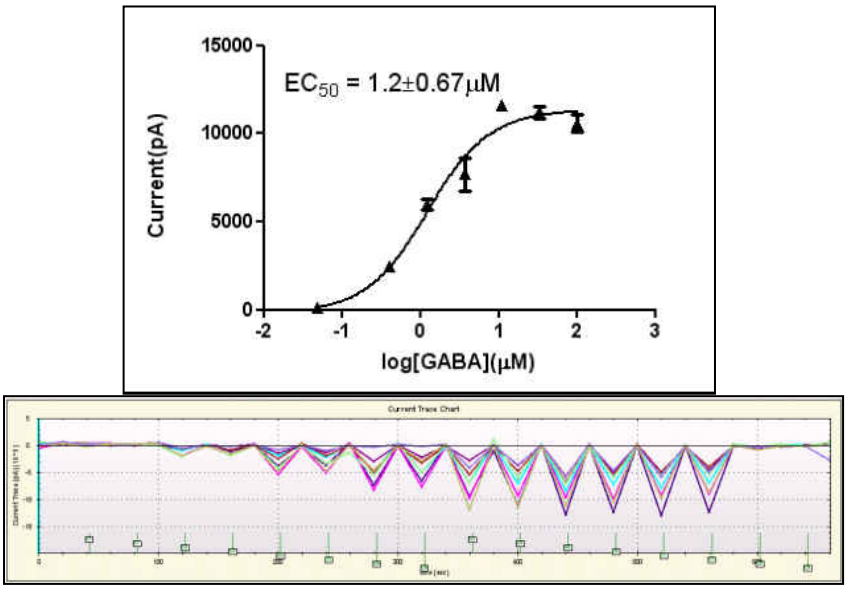


Figure 16. Dose-response curve and current sweeps of α2β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA.

α1β3γ2 (Figure 15) with current reaching a maximum of -10,000pA. The α4β3γ2 (Figure 18)

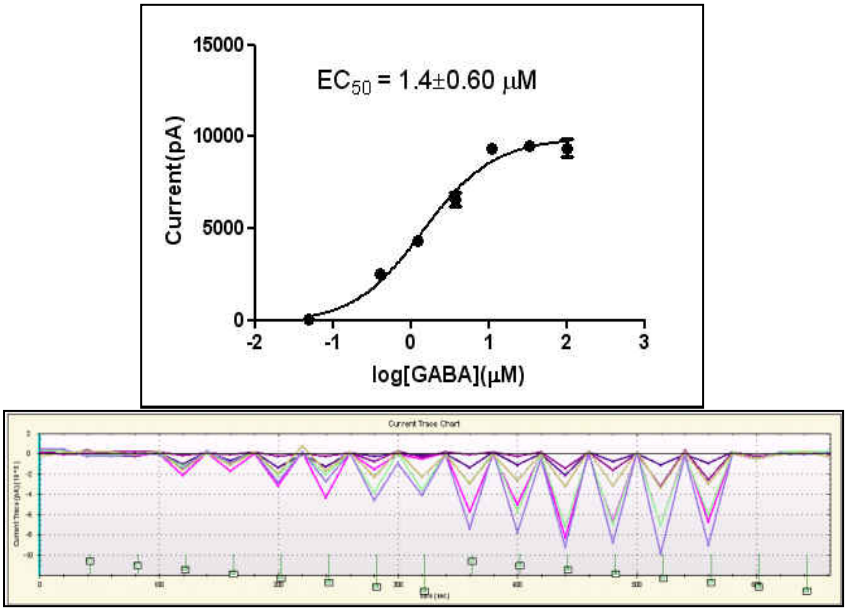


Figure 17. Dose-response curve and current sweeps of α3β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA.

transfected cells performed the worst and exhibited a high level of background synonomous with

its low maximum current responses at -1,000pA. The high background and low current response of the α4β3γ2 $GABA_A$ receptors may be indicative of failure of the receptor to assemble correctly

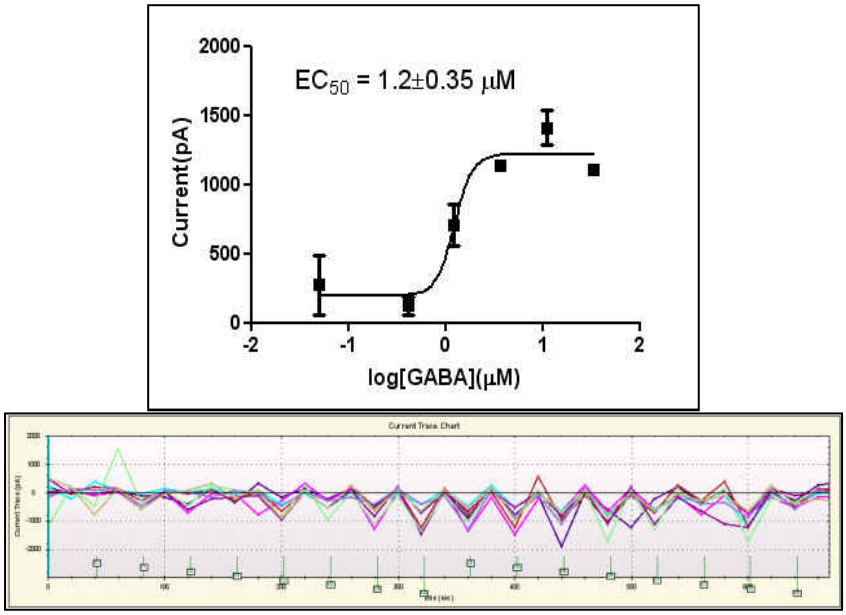


Figure 18. Dose-response curve and current sweeps of α4β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA.

in the membrane, compromised expression of the α4 protein, or a characterisitic of the receptor

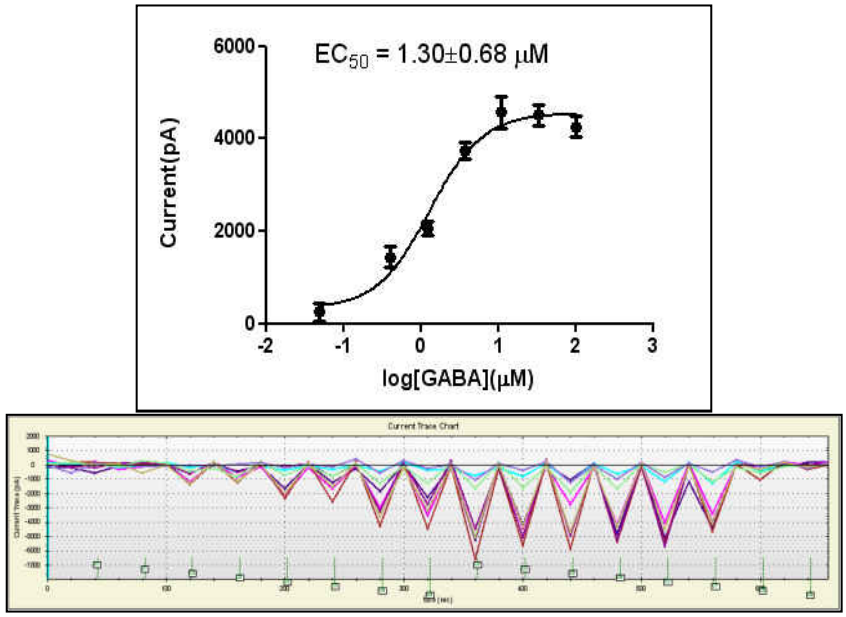


Figure 19. Dose-response curve and current sweeps of α5β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA.

subtype itself. The α5β3γ2 subtype (Figure 19) exhibited robust response but at slightly lowered maximum readings at -4,000pA. Transfection of the α6β3γ2 (Figure 20) had a robust response to GABA but was oversaturate at the lowest amount of GABA which was not observed with any of

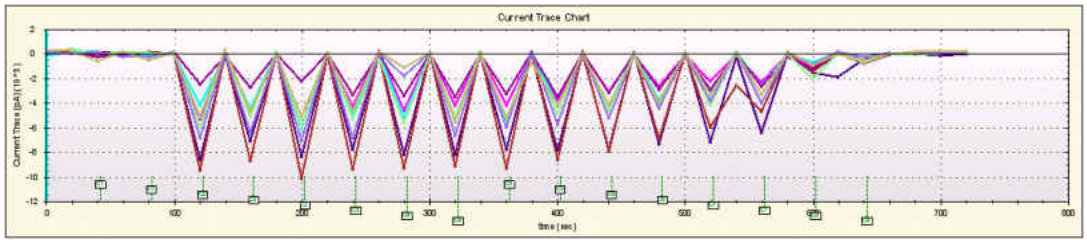
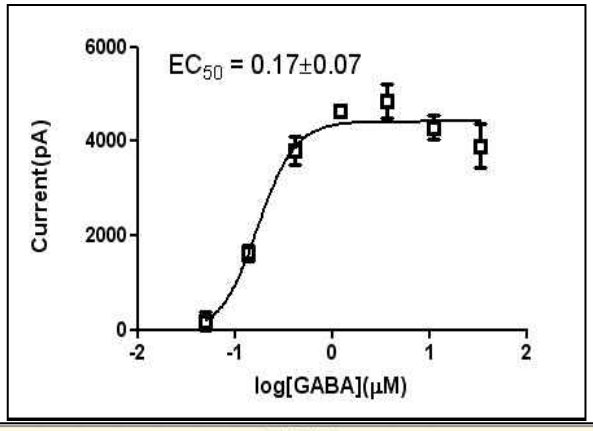


Figure 20. Current sweeps showing oversaturation at the lowest concentration of GABA (0.4μM) in HEK293T cells transfected with the α6β3γ2.

the other subunits. This is in line with what is observed in other electrophysiological studies where α6 is consistently reported as the most GABA sensitive [83]. When the range of the GABA application was lowered from 100 μM to 33 μM (Figure 21), an intermediate response was observed. In retrospect, an even lower concentrations of GABA should have been used to produce a more sigmoidal shape. The lowest concentration is already part of the linear portion of the curve



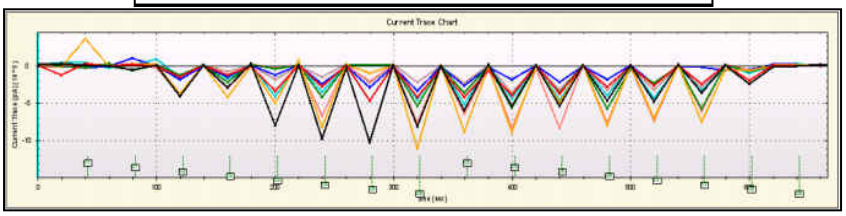


Figure 21. Dose-response curve and current sweeps of α6β3γ2 transiently transfected HEK293T cells exposed to successively larger concentrations of agonist GABA. After reduction of the $GABA_{MAX}$ from 100μM to 33μM.

and the maximum response level (EC_{100}) is already achieved at concentrations as low as 1.23 µM so lowering the maximum concentration of GABA to 5-10 µM may be acceptable for additional experiments.

After GABA dose response curves were studied, testing proceeded to other types of compounds: positive allosteric modulator BZD HZ-166 (Figure 22) and competitive antagonist

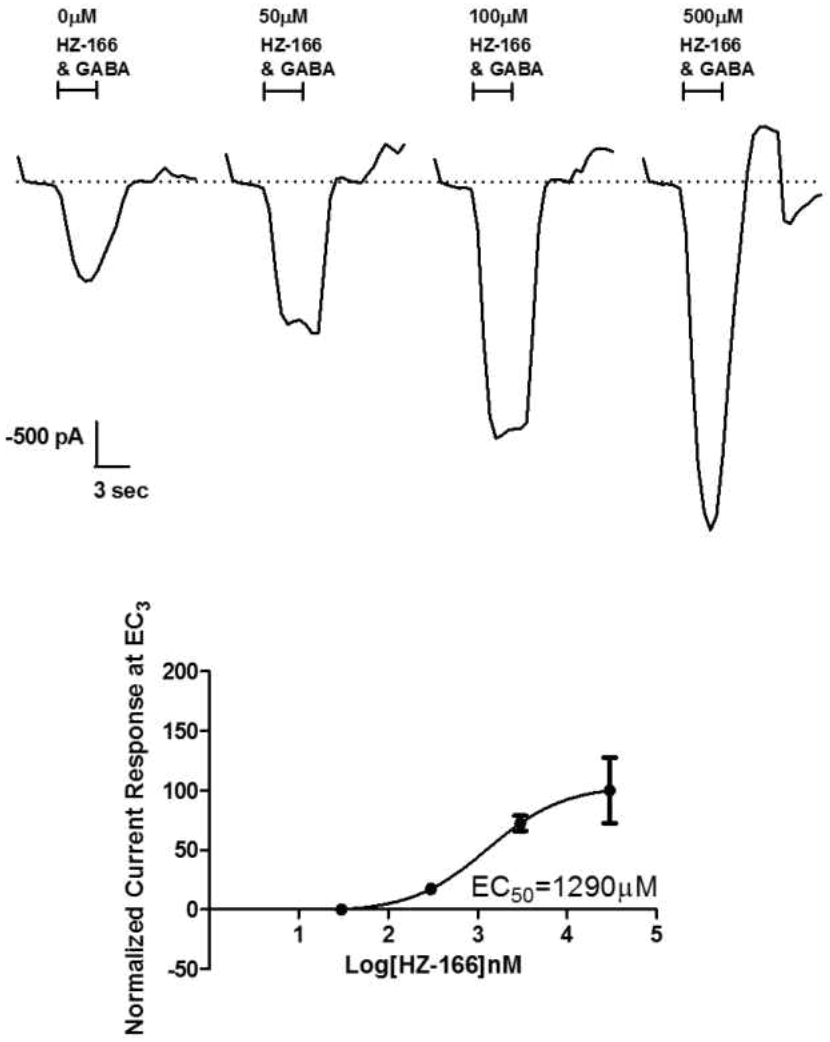


Figure 22. Transiently transfected α1β3γ2 cells exposed to 2µM of GABA and increasing concentrations of positive modulator HZ-166.

bicucculine (Figure 23).

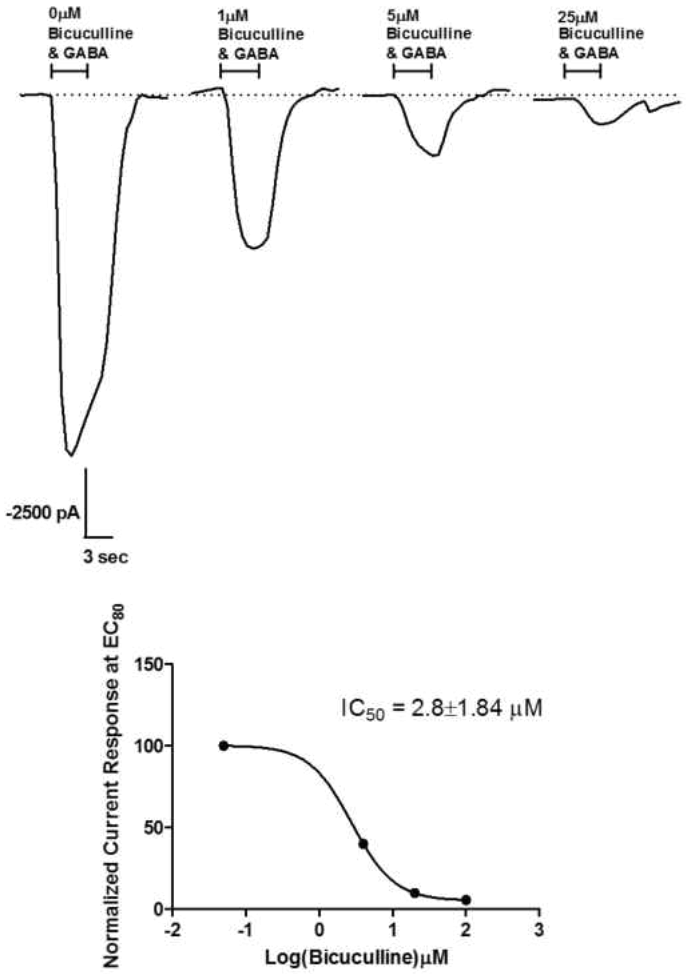


Figure 23. Bicuculline dose response curve in transiently transfected α1β3γ2 cells with 10μM GABA.

The throughput of the assay itself when testing modulators is a major obstacle to overcome. Taking into consideration that the $GABA_AR$ is a fast action ionotropic chloride ion channel and the rapid rate of onset known to benzodiazepine is a result of high affinity binding with rapid kinetics [209], we surmised that it may be possible to utilize all compound application wells to increase data output. With this modified method, a constant amount of GABA and an increasing concentration of positive modulator is placed in 7 of the compound wells, saving a single channel solely for GABA application to set a baseline. The ensemble layout is depicted in Figure 24. This would double the number of data points aquired from each trap. However, proof

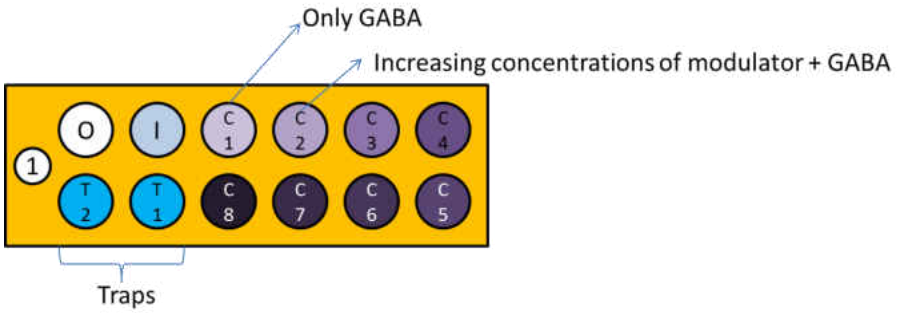


Figure 24. Ensemble setup for utilization of all compound wells

of concept was important since it was possible that the acquired EC_{50} would be shifted right if the rate of binding of the BZD is delayed due to simultaneous exposure to both an agonist and positive modulator. Thus the same experiment was performed but with lowered GABA concentrations to

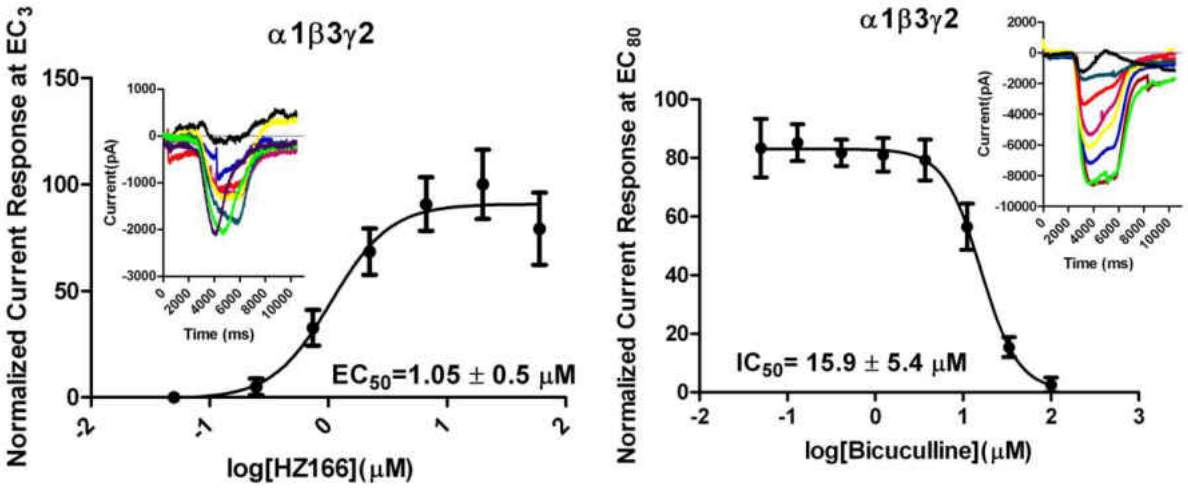


Figure 25. Dose response curves from improved assay forma. EC_3 concentrations taken as 0.1μM and EC_{80} as 3μM.

the calculated concentration for agonist EC_3 and EC_{80}. Previously published studies perfomed by a collaborator have exhibited very similar results with EC_{50} values around 1μM for HZ-166 seen in Figure 26 [210]. Study of the other $GABA_AR$ subtypes using transient transfection yeilded a similar

range of EC_{50} concentrations between 100 nM-1.0 μM This cooresponds with the theory that the potency of positive modulators has a strong coorrelation between the slow infusion of drugs in manual patch clamp of frog oocytes and our format using ultra fast application of compounds on HEK293T cells using the microfluidic technology of automated patch clamp. Analysis of the

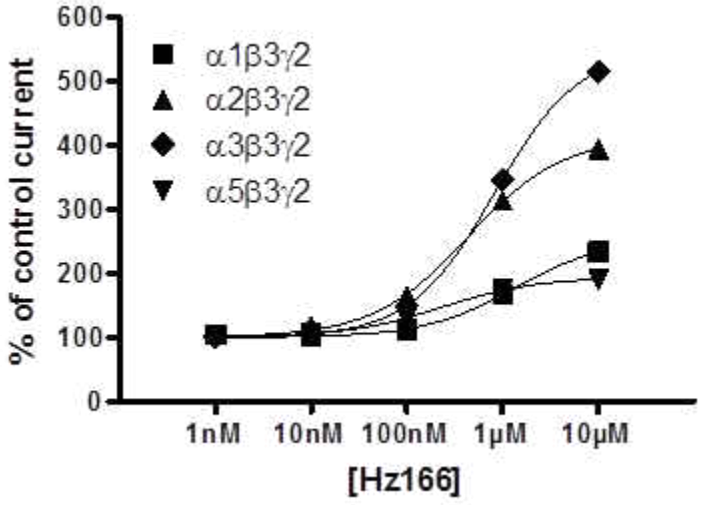


Figure 26. Results of HZ-166 performed in frog oocytes injected with $GABA_AR$ subunit plasmid DNA via manual patch clamp. Figure modified from Rivas 2009, referenced in text.

efficacy, seen in Figure 28, presented heightened potentiation towards the receptor subtypes containing the α2/3 and lowered effects towards the α1/5. Unusually high results of traditionally benzodiazepine-insensitive subtypes α4/6 were observed. This may be due to the recorded sensitivity of βγ2 constructs which have been known to assemble with expression systems of α4

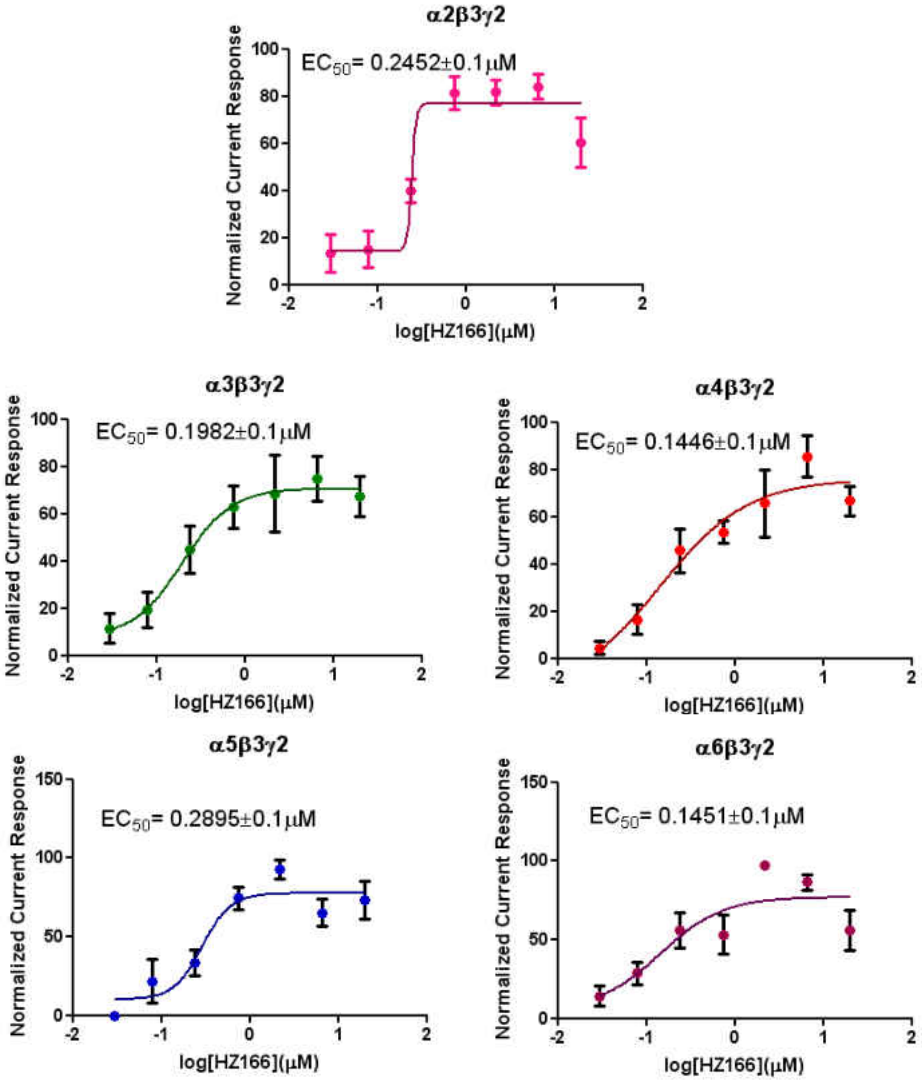


Figure 27. GABA dose response curves of transiently transfect cells containing differing alpha subunits, N=8

and are stongly potentiated with diazepam with high potency [211]. The efficacy and potency observed for α1/2/3/5 appear to be in satisfactory agreement to proceed with further analysis using the fast application strategy for other positive modulators.

With the issue concerning low data output having been resolved, another shortcoming was the method of data analysis. Initial analysis strategies for modulators called for normalization of

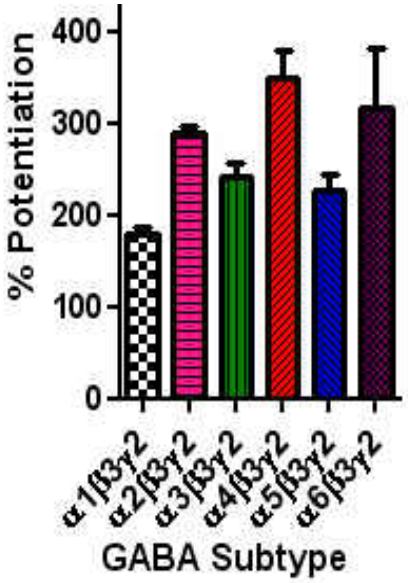


Figure 28. Graph of the efficacy of HZ-166 in different α subtypes transiently transfected into HEK293T cells

the data and automated export by the IonFlux software. Normalization naturally ignores the efficacy (potentiation) in favor of determining compound potency (EC_{50}). Creating a drug with maximum efficacy not potency appears to be the most important quality to determine subtype selectivity [212]. Examination of the manner in which data is exported from the IonFlux also revealed some problems. Figure 29 is a screenshot which illustrates the limitations of using the software's automated data export program when quirks and shifts in response are present. The blue bar indicates the area in which the lowest reading is exported for analysis. Notice that not all traps respond simultaneously and the I_{min} of the sweep occurs at different times for many of the readings. Automated export of the data in the blue bar ignores this behavior.

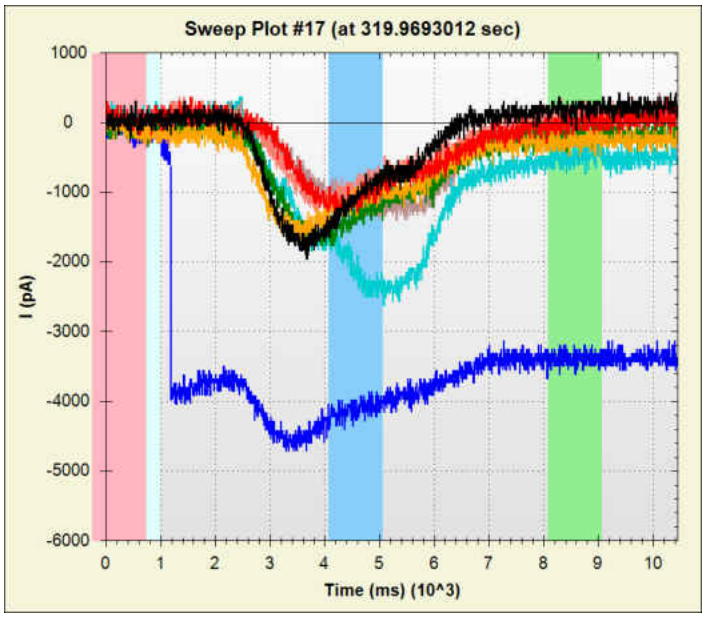


Figure 29. Real time view of the sweeps viewable from the IonFlux software.

Manual export using the raw sweep data is time consuming but necessary in order to gain more genuine response curves. Since each one of the raw sweep files contains 10,260 data points per sweep, processing speeds of the computer used for analysis is limited; particularly if using Microsoft Excel. Thus sweeps must undergo initial smoothing whereupon 342 data points are averaged into 1. This yield 30 data points per sweep. These sweeps are loaded into a customized

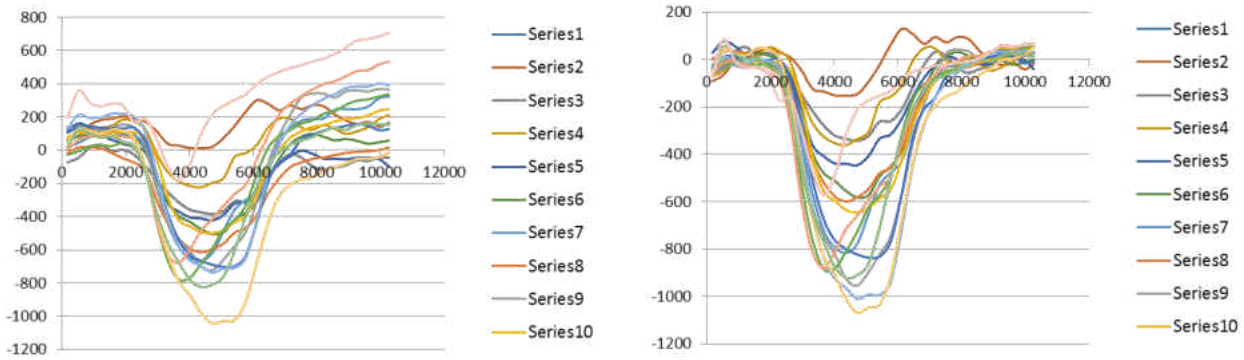


Figure 30. Smoothed sweeps before and after baseline correction

32-sheet Excel sheet where Event of the sweep is reorganized into data collected per trap. For

baseline correction, the first 6 points and the last 6 points of the sweep are averaged and a slope and intercept are calculated. From this data, a line is constructed and subtracted from the smoothed sweep. The results from this step can be seen in Figure 30. The minimum currents of each entire sweep is then isolated and divided by the minimum current achieved with GABA alone and multiplied by 100. This established % potentiation. When a normalized GABA dose response is needed, the sheets are altered in such that the minimum current of each sweep is subtracted from the minimum current elicited with just GABA and then divided by the difference between the lowest current achieved in the trap and the lowest current from GABA application alone. This process is illustrated in Figure 31.

A well-timed opportunity involving work with a pharmaceutical company allowed for the

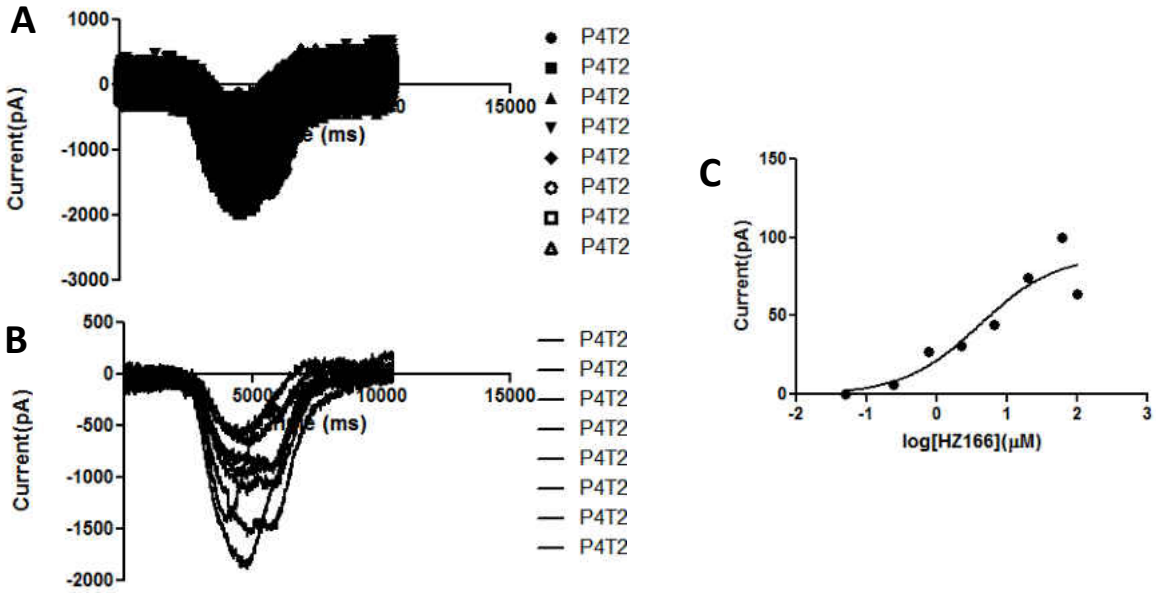


Figure 31. Improved method of data analysis. A) is the original output of data sweeps assembled by the IonFlux software. B) Smoothing of the data points reveals the individual sweeps. After the I_{min} is isolated, a dose response curve C) can be created.

testing of this new method. The pharmaceutical company Concert Pharmaceuticals provided a known subtype selective, non-benzodiazepine that has been reported to exhibit partial agonism at

α2 and α3 and antagonism at α1 called L-838417. However, this compound, synthesized by Merck, was not advanced into clinical development due to poor preclinical pharmacokinetic profile. They had created an analog of L-838417 by incorporating deuterium atoms in place of hydrogen at key positions (Figure 32) in hopes of improving metabolic stability but retaining pharmacological

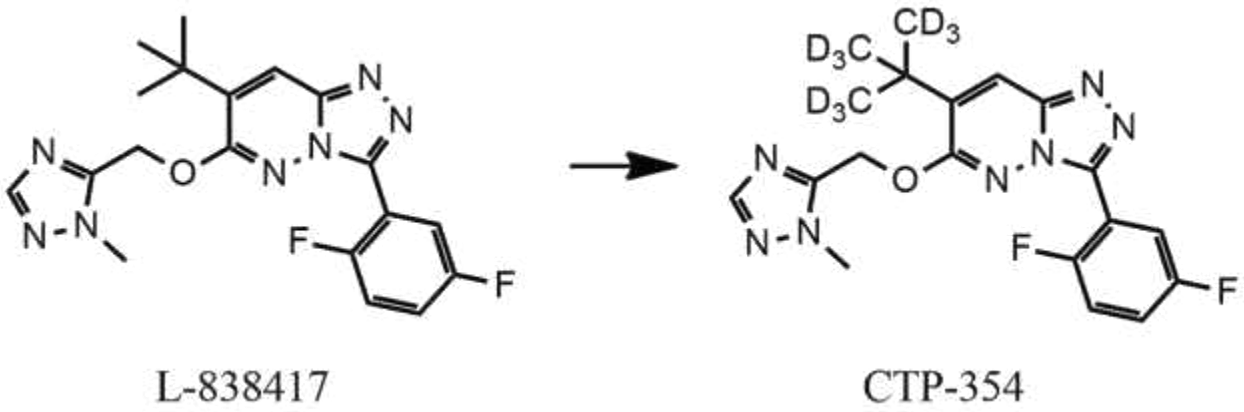


Figure 32. CTP-354 containing 9 deuterium atoms.

selectivity. Initial tests, seen in Figure 33, with the two compounds on the biologically relevant subtypes of α1/2/3/5 did not reveal any significant differences between the deuterated and non-deuterated compounds. In addition, neither did they exhibit subtype selectivity with antagonism towards the α1 receptor subtype. Previous studies performed using human $GABA_AR$ transiently expressed in *Xenopus laevis* oocytes revealed that efficacy towards the α1/2/3 were +0%, +11%, and +21% respectively when tested with EC_{20} GABA [213]. Since initial tests were performed at EC_3, this parameter was replaced with GABA EC_{20} concentrations to allow for better comparison. This change brought a much higher variance of results between the two compounds, as seen in Figure 34. For example, observation of drug response at α1 containing receptors reveals two very different profiles. The L-838417 would appear to be unchanged from the previous results in EC_3 GABA concentrations; with an efficacy in the 200s range. However the deuterated compound

CTP-354 exhibited bell shaped curve with a heightened response followed by a steady trend

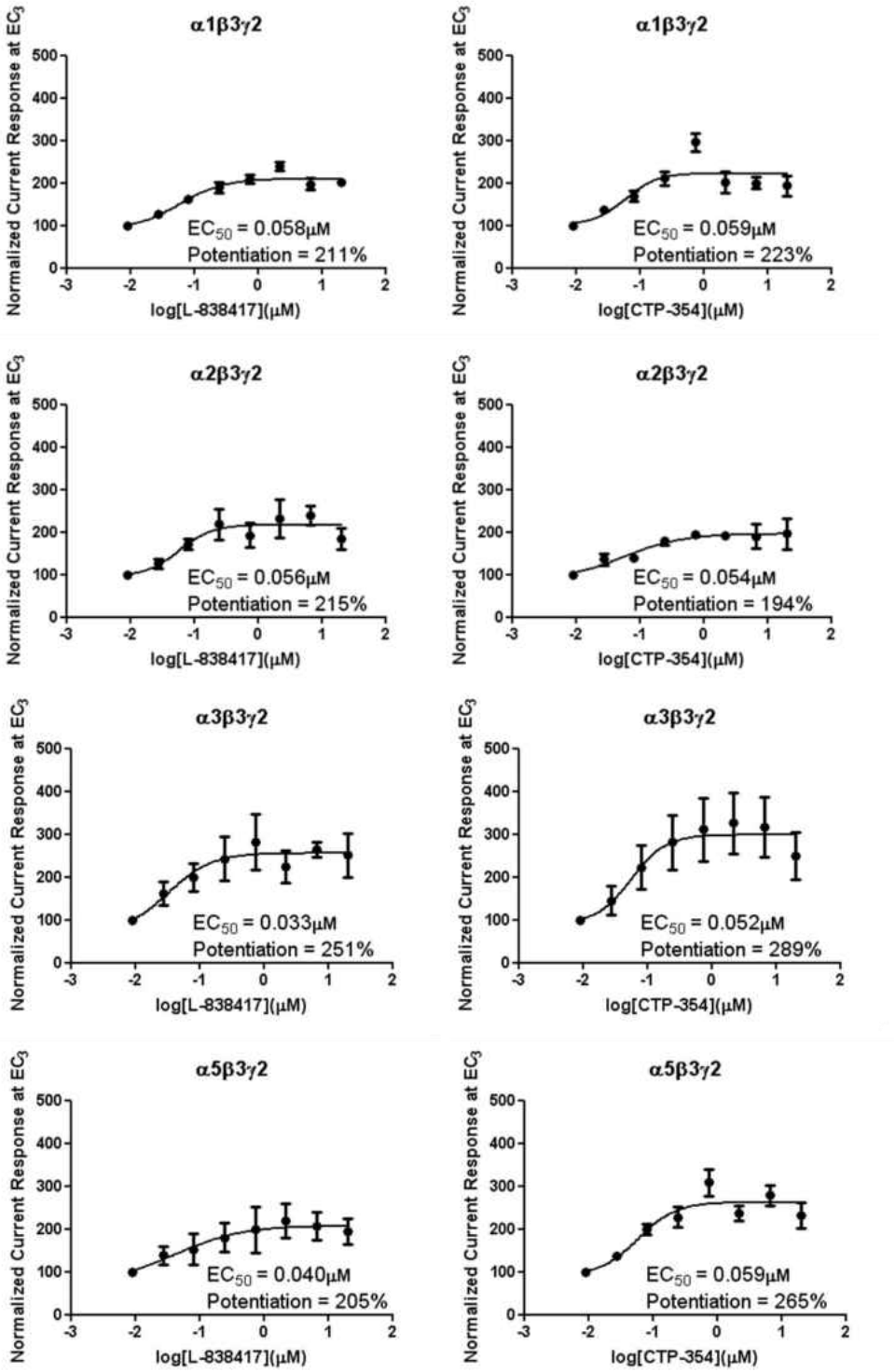


Figure 33. Dose response curves of non-deuterated L-838417 vs deuterated CTP-354 in transiently transfected HEK293T cells with GABA EC_3 of 0.1μM, N=4

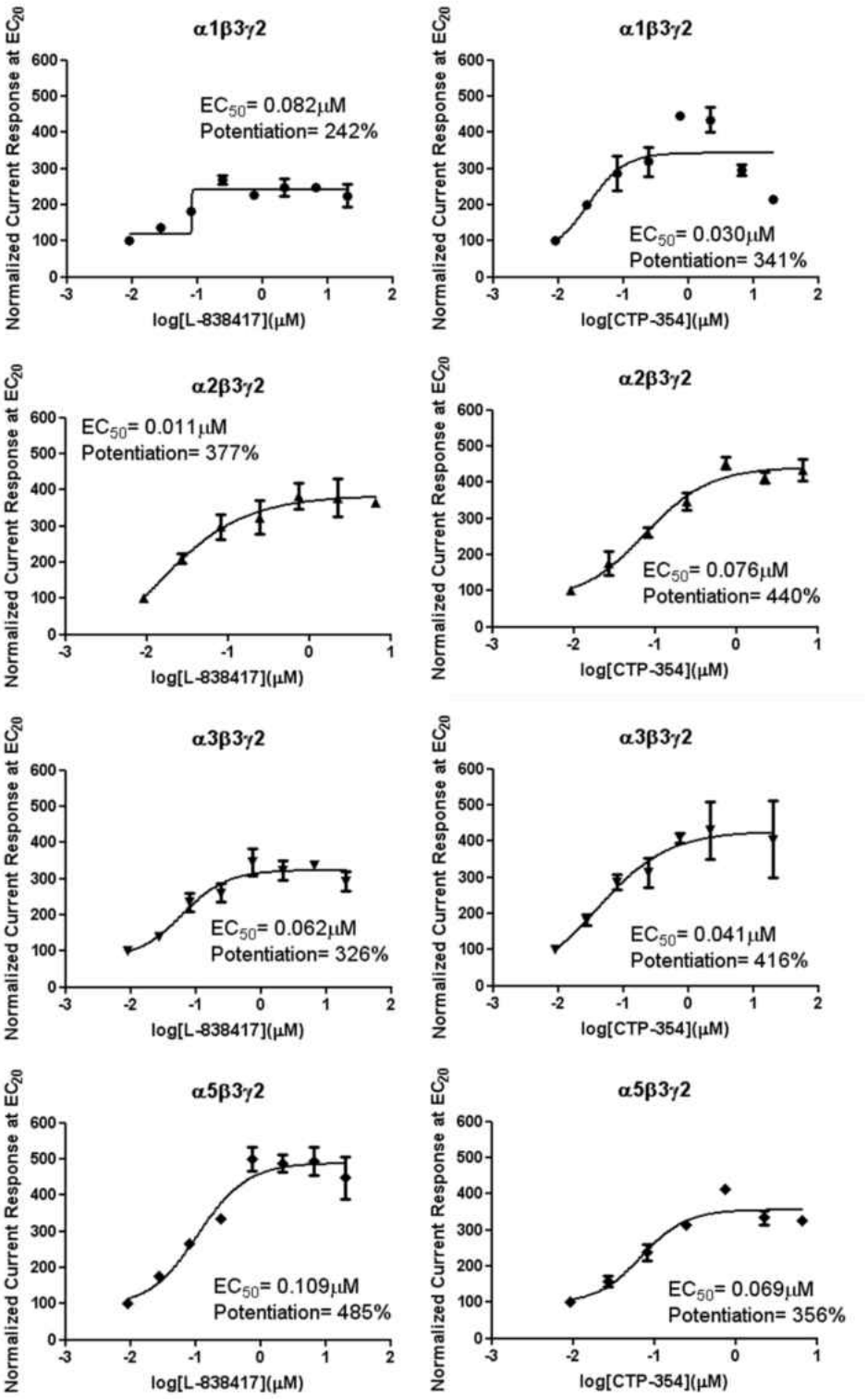


Figure 34. Dose response curves of non-deuterated L-838417 vs deuterated CTP-354 in transiently transfected HEK293T cells with GABA EC_3 of 0.1μM, N=4

downwards. This may be indicative of desensitization, high concentrations of the compound

directly blocking the chloride channels, or compound non-specifically interrupting membrane function. Comparison with manual patch clamp experiments, results provided by Concert Pharmaceuticals, offered a very different dose response profile, seen in Figure 35 [214]. The α2/3/5

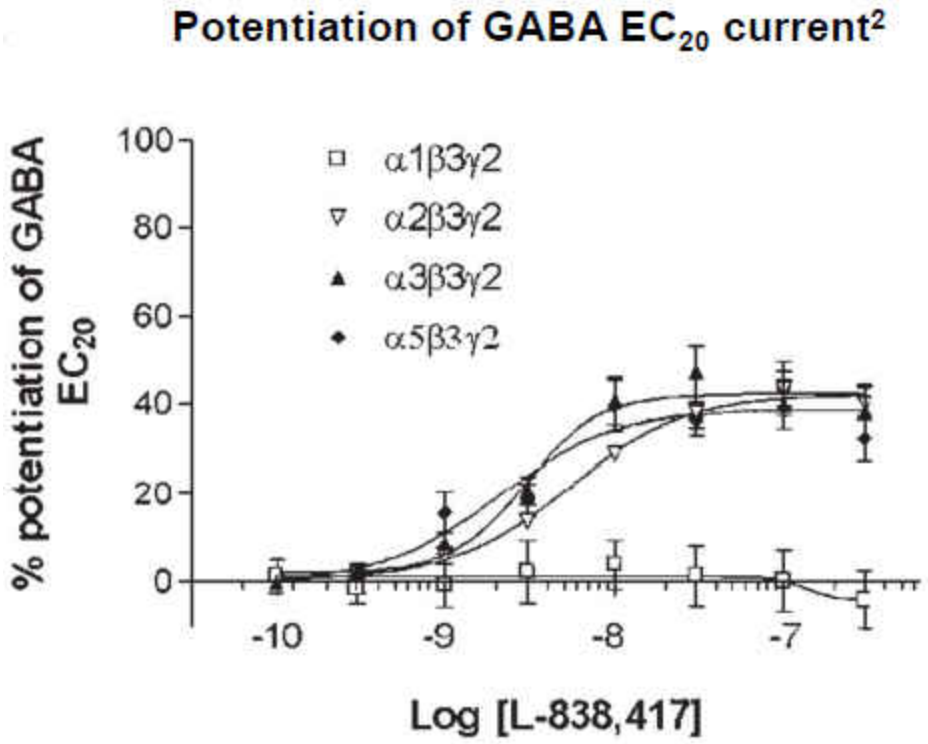


Figure 35. Dose response curve for L-838417 using oocytes transfected with human $GABA_AR$. Figure used with permission from Nature Neuroscience, reference cited in text.

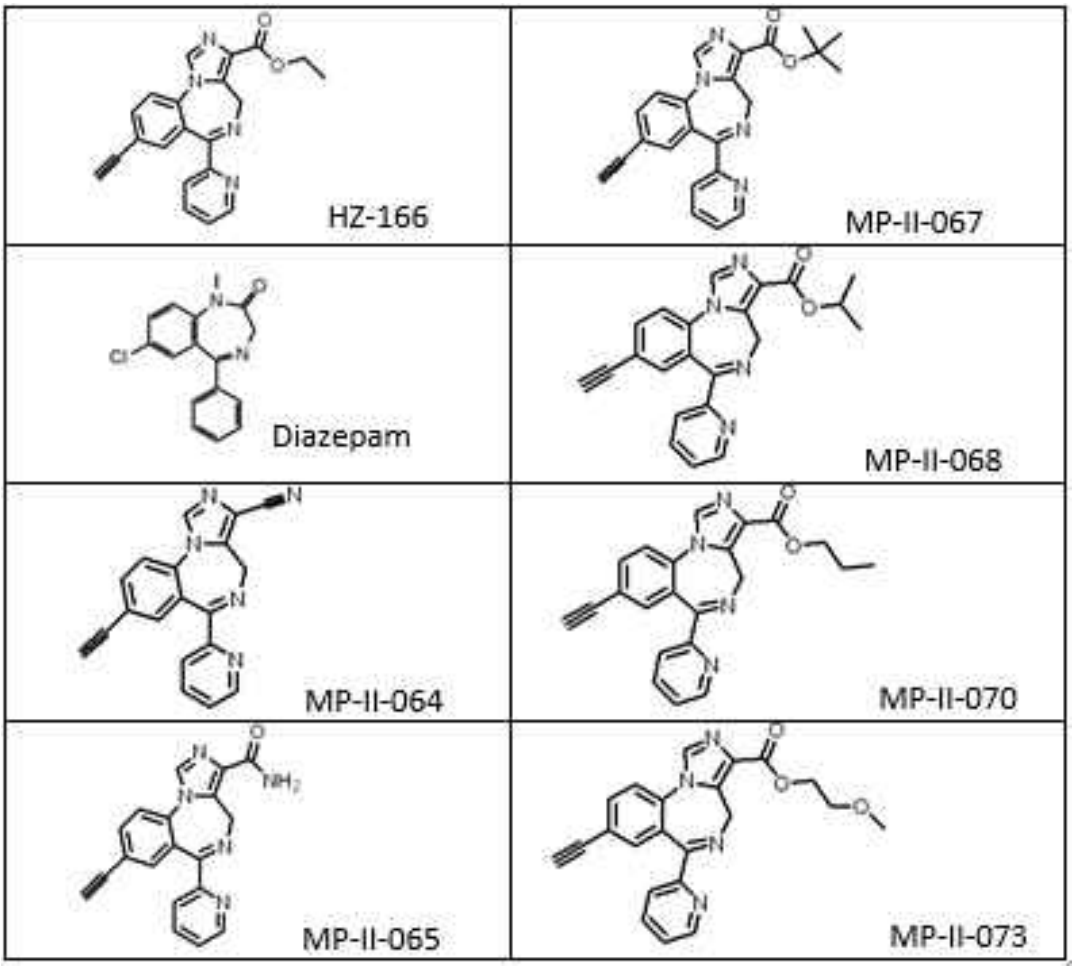
has equal efficacy while the α1 subtype remained at the baseline with mild antagonism at the highest concentration. Given these results, the company withdrew interest in further testing.

Despite this unusual result, the high-throughput screening campaign of compounds synthesized by Dr. Cook was launched. The compounds under interest were anticipated to be a series of α3 selective drugs. In order to asses this, preliminary testing was performed on one of the compounds which had exhibited on transiently transfected cell lines containing differing α

subtypes. In addition, our method of using transiently transfected cells makes it necessary to test compounds in parallel with a control to assess the relative difference.

It was critical to determine what concentration to use of the GABA when determining the effect of a BZD since the BZD has no intrinsic affect without the agonist present. Experiments were, at first, going to be performed at EC_{30}, however the results would most likely not be comparable to experiments performed by our collaborators who tested at EC_3 concentrations. It should be pointed out that transient transfection might generate a second low affinity BZD site that

Table 7. Structures of the drugs utilized in the high throughput screening campaign. HZ-166 and diazepam were used as controls while compounds beginning with the initials MP were test compounds.



reacts toward μM concentrations of BZD in combination with low EC_{3-8} concentrations of GABA [215]. So in order to reduce the effect of the second binding site but keep results comparable, GABA EC_{10} concentration was applied in unison with the modulators.

Along with two control compounds, HZ-166 and diazepam, 6 experimental compounds were tested in parallel to assess their relative efficacy. Their structures can be seen in Table 7. The MP-II-0XY compounds were similar to HZ-166 scaffold but differ in the ethyl ester functional group on the imidazole. MP-II-064, which contains a nitrile group, was tested by the Sieghart Lab in Austria and exhibited lowered efficacy towards the α1. Thus this compound was tested first on

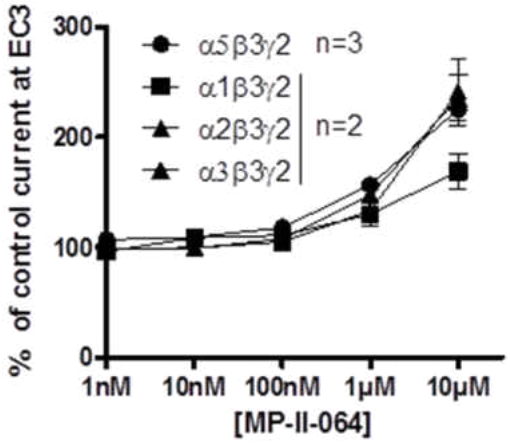


Figure 36. Manual patch clamp experiments on frog oocytes performed by the Sieghart Lab. The HZ-166 was tested in the presence of GABA EC_{20} while the MP-II-064 was tested with GABA EC_3.

the α1/2/3/5 to compare the results. MP-II-064 showed lowered efficacy and potency for all subtypes when tested by our collaborator, seen in Figure 36. When the same experiment was tested using our experimental methods, the transient cells had a much higher current response in general with potentiation values in the thousands range, seen in Figure 37. Overall, the compound had

lower efficacy than HZ-166 on all but the α3, which was slightly higher though had a significantly

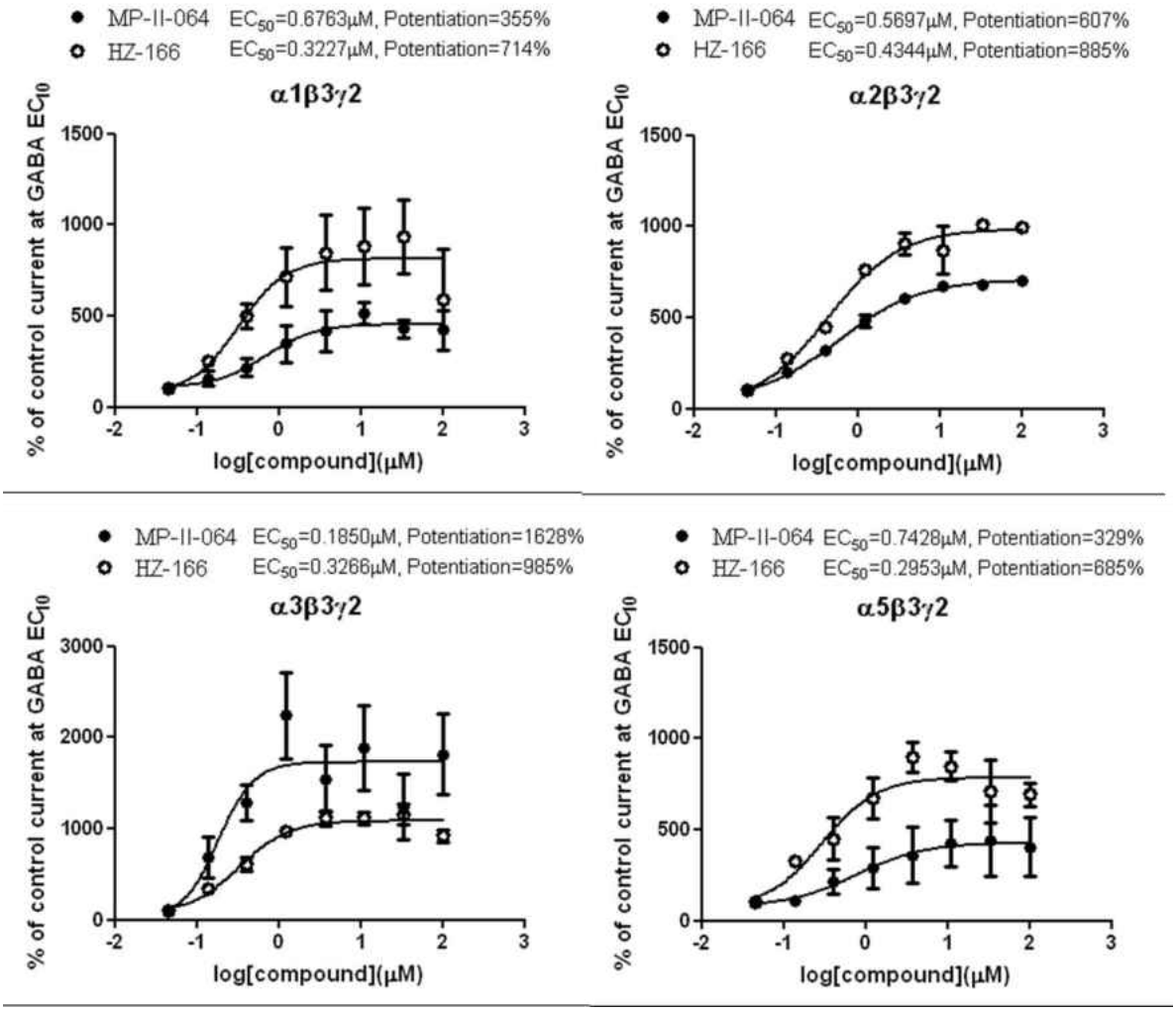


Figure 37. Dose response curve comparing HZ-166 and MP-II-064 using transiently transfected cells.

higher standard deviation than the other experiments. It was also noted that accompanied GABA agonist concentrations differed greatly. While the Sieghart Lab performed their tests with HZ-166 and MP-II-064 with GABA EC_3, ours were performed in mid-range at EC_{10}. In addition, we had tested our compounds at a maximum of 100μM instead of 10μM and since the MP-II-064 curves in Figure 36 had not reached saturation, higher concentrations may have revealed differences in the efficacy.

Similar experiments were performed with other experimental compounds, seen from Figure 38 to Figure 42, though these lack manual patch clamp data for comparison.

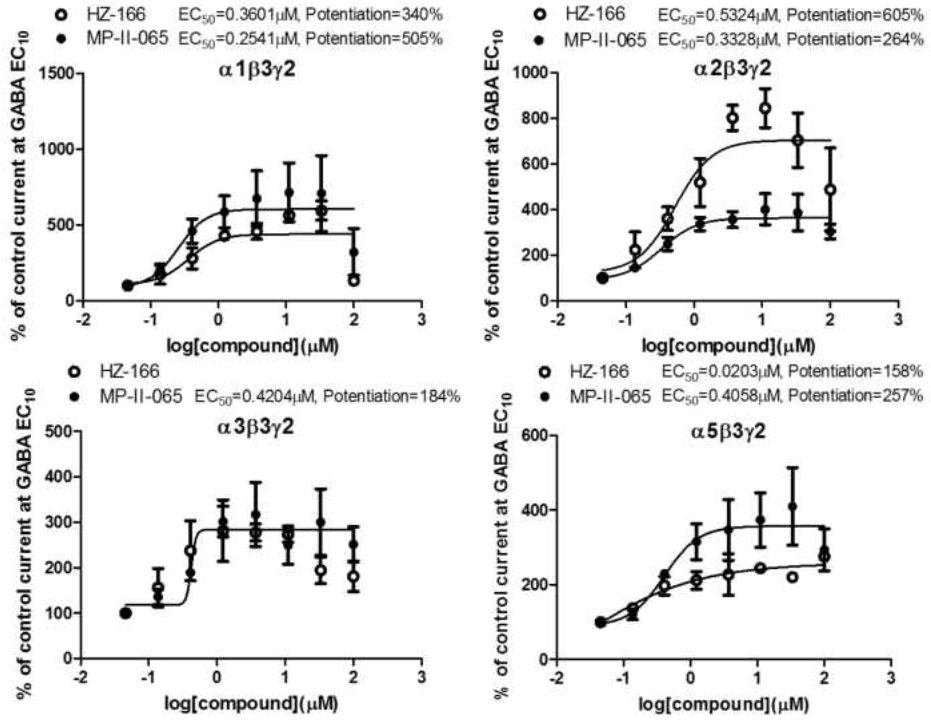


Figure 38. Dose response curve comparing HZ-166 and MP-II-065 using transiently transfected cells.

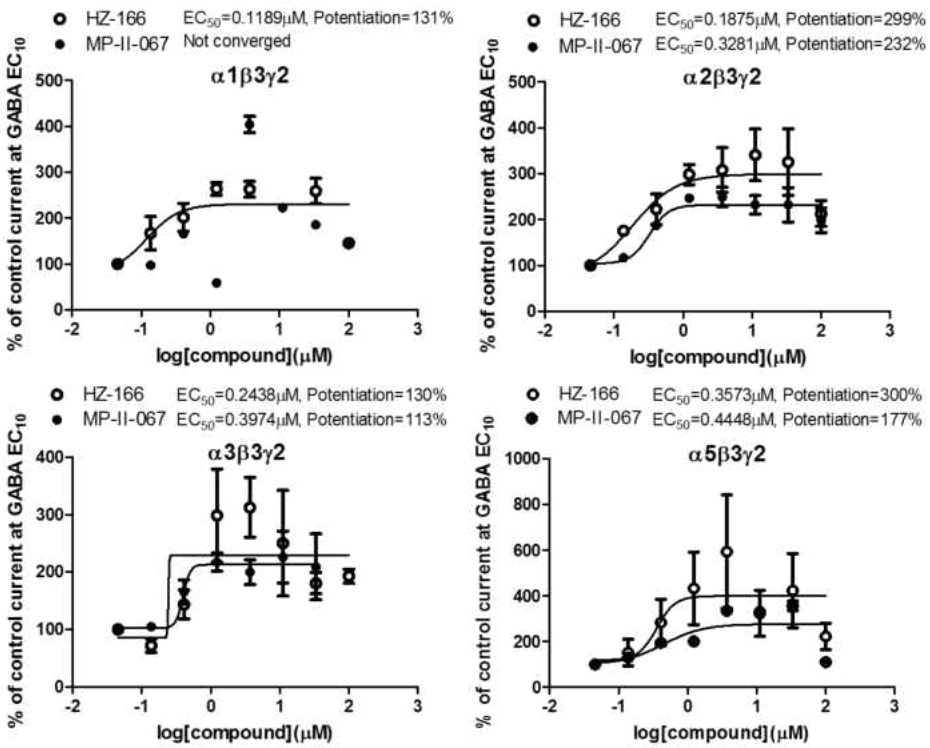


Figure 40. Dose response curve comparing HZ-166 and MP-II-067 using transiently transfected cells.

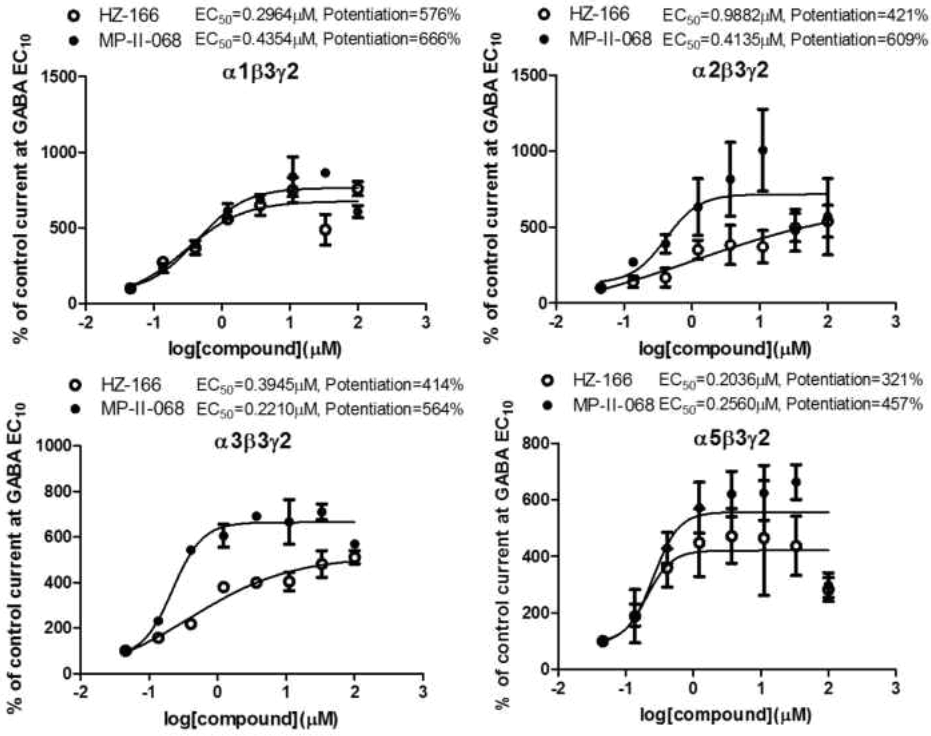


Figure 39. Dose response curve comparing HZ-166 and MP-II-068 using transiently transfected cells.

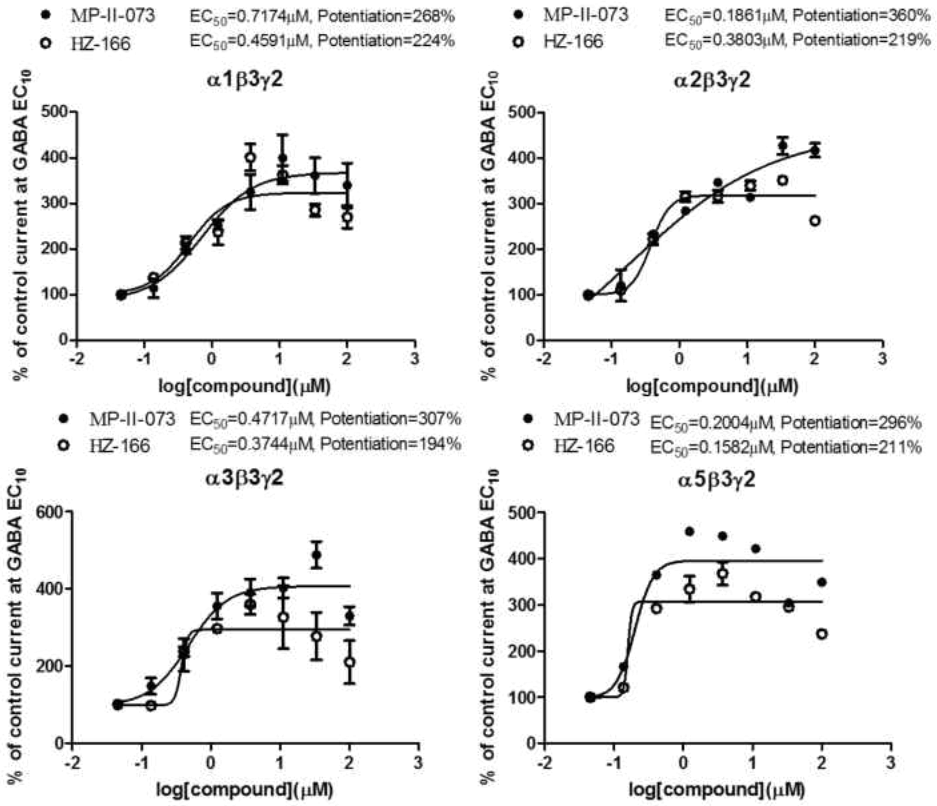


Figure 42. Dose response curve comparing HZ-166 and MP-II-073 using transiently transfected cells.

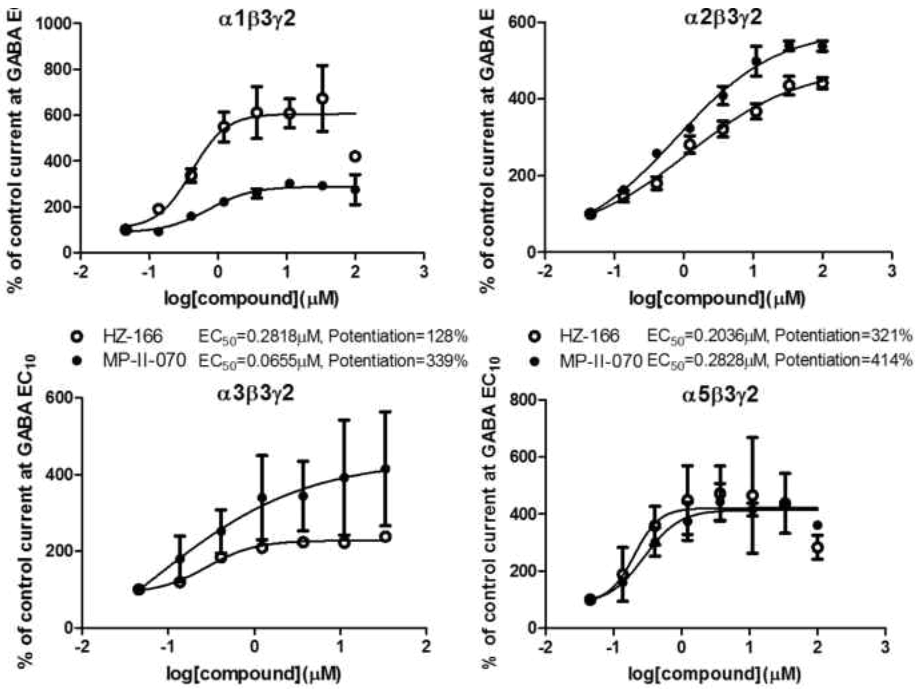


Figure 41. Dose response curve comparing HZ-166 and MP-II-070 using transiently transfected cells.

This series of compounds were tested in 7 independent experiments with transiently transfected HEK293T cells using the α1, β3, and γ2 plasmids using the IonFlux. The summarized

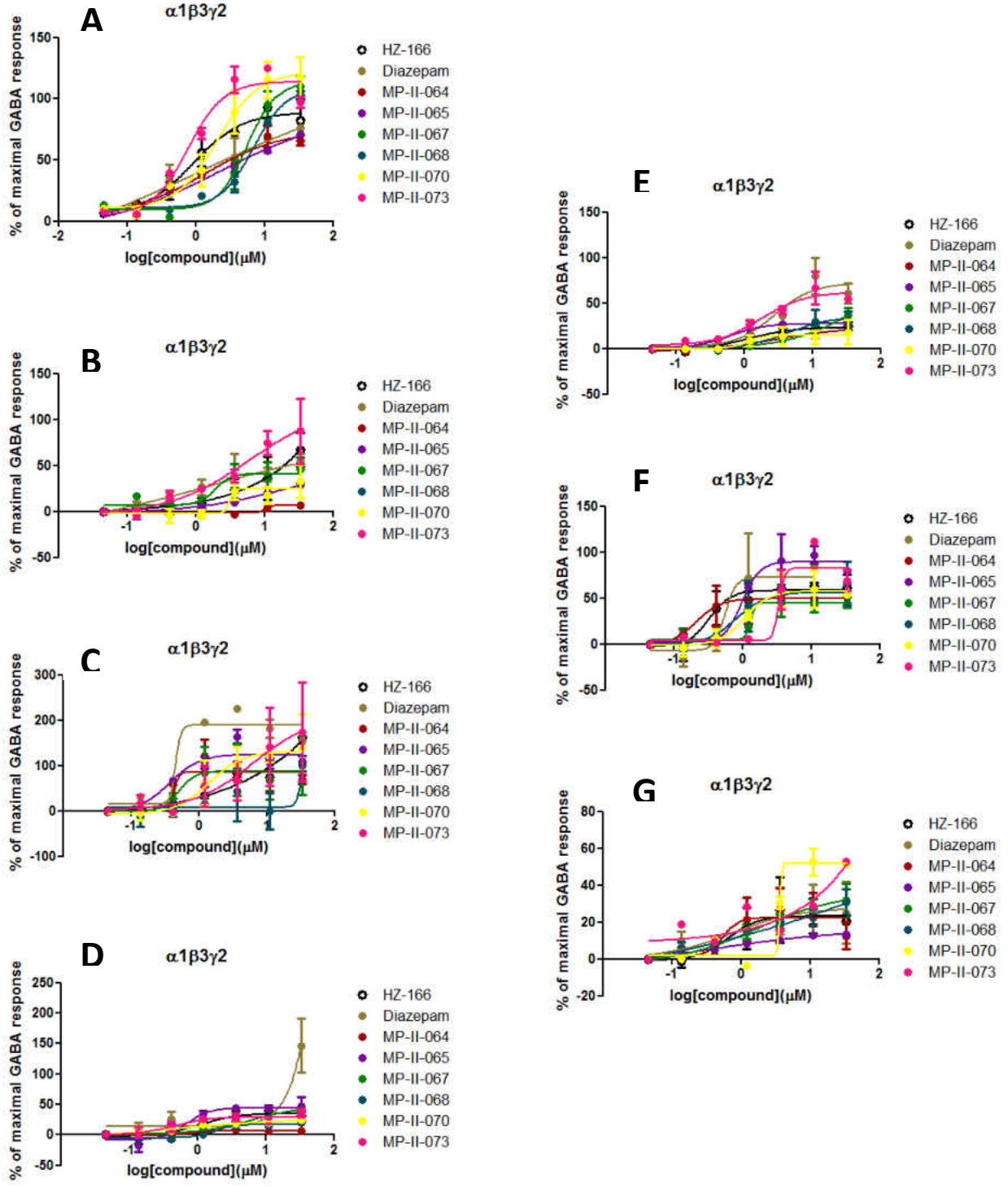


Figure 43. Dose response curves from seven independent assays for the high-throughput screening campaign of the Cook Lab compounds using α1β3γ2transiently transfected cells.

results, show in Figure 43, have been normalized to the highest achieved current result per experiment and then baseline corrected. The curves show a high variation in results from an experiment to experiment basis. The relative efficacy values from each experiment, seen in Table 8, illustrate the variation between experiments. The top table, highlighted by column to show the range by experiment, shows how particular experiments such as the first and third, which are dark green, will exhibit a higher general efficacy for all compounds. The bottom table, highlighted by row to show the range by compound, shows an overall pattern occurs where a particular compound

Table 8. Relative efficacies of the compounds tested by experiment. The above table has highlighted color scale by experiment (column) while the lower table is highlighted by compound (row) with green showing a higher numerical value and yellow indicating lower values.

Experiment	HZ-166	Diazepam	MP-II-064	MP-II-065	MP-II-067	MP-II-068	MP-II-070	MP-II-073
1	88.67	120.3	73.59	89.5	113.7	109.1	122.1	113.9
2	N/A	77.64	6.366	54.49	40.96	N/A	24.85	117
3	N/A	189	85.41	124.3	87.53	N/A	131.1	210.2
4	35.12	N/A	7.218	44.09	47.05	16.88	26.82	29.47
5	23.37	71.76	22.43	27.36	92.75	34.61	14.7	61.92
6	58.36	72.52	49.31	89.49	45.07	56.07	57.9	83.12
7	23.23	27.68	22.32	16.83	35.78	N/A	52.04	1519000
Experiment	HZ-166	Diazepam	MP-II-064	MP-II-065	MP-II-067	MP-II-068	MP-II-070	MP-II-073
1	88.67	120.3	73.59	89.5	113.7	109.1	122.1	113.9
2	N/A	77.64	6.366	54.49	40.96	N/A	24.85	117
3	N/A	189	85.41	124.3	87.53	N/A	131.1	210.2
4	35.12	N/A	7.218	44.09	47.05	16.88	26.82	29.47
5	23.37	71.76	22.43	27.36	92.75	34.61	14.7	61.92
6	58.36	72.52	49.31	89.49	45.07	56.07	57.9	83.12
7	23.23	27.68	22.32	16.83	35.78	N/A	52.04	1519000

will test repeatedly high among the group (MP-II-073 dark green) while another will consistently produce low results (MP-II-064 light yellow). Though this information may be useful for strongly specific compounds, the ones that test within mid-range are indeterminable.

2.4.4 CONCLUSIONS

From these findings, it can only be concluded that the method of testing transiently transfected cells on automated patch clamp is not a feasible approach to generating reproducible numerical values for determining compound efficacy. In addition, assaying many different batches of transfected cells for data with low reproducibility had very low efficiency to the high cost of plates and reagents.

The formation of heteromeric, dimeric, or homomeric receptors different from the typical 2α:2β:1γ can dramatically vary in their level of expression depending on the recombinant expression system. And despite optimism that the deviation would be stifled by averaging of the cellular population when cells are patched in series, the reproducibility remained lower than expected. Even assuming that the receptor successfully forms a pentameric formation with the α, β, and γ, there is a possibility that the order of assembly is altered. Figure 44 illustrates the possible pentameric assemblies when all subunits are involved [31].

Since the expression of the genes varies from cell to cell and the transfection is not successful in every cell, the variability of expression and the statistical nature of cell capturing in the instrument plates creates an environment in which one has a chance of capturing both high expressing cells, low expressing cells, untransfected cells, or debris. In addition, it is difficult to assess whether the high expressing cells have functionally assembled the biologically relevant pentameric receptor. The ability of recombinant systems to assemble both βγ and αβ receptors has been elucidated and even homomeric receptors containing only β3 subunits have been found to be robustly expressed [216,217]. Despite the statement that γ2 is necessary for diazepam sensitivity of the $GABA_AR$, there have been studies whereupon the combination of αβ elicited a significant

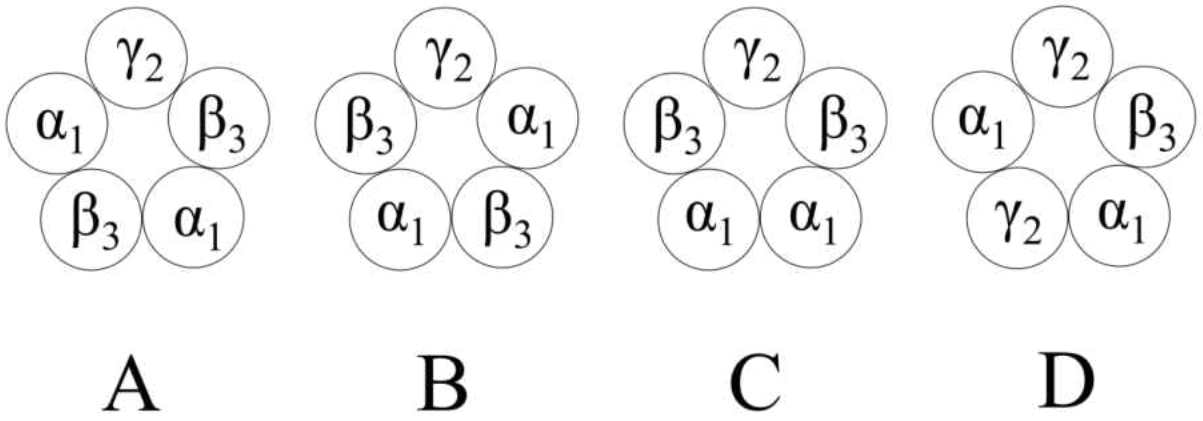


Figure 44. The possible assembly of recombinant α1β3γ2 formed in HEK 293T cells transfected. The most likely arrangement formed is (A) and (B) configurations. Conformation (C) has a low probability of formation and would be unresponsive to BZDs. (D) would be improbable but if formation occurs then multiple BZD sites would be present.

potentiation from diazepam within the μM range [215]. Additionally βγ assembly has been shown to occur in oocytes and diazepam potentiated these receptors with high potency at an EC_{50} of 50nM and efficacy of +76% [211].

In any case, despite efforts to optimize cell preparation, viability, transfection, patch-recordings, data-throughput, and data analysis; the low reproducibility of results remains a major point of contention. To overcome these shortcomings, a proposed solution takes us to Chapter 3: GENERATION OF GABAA STABLE RECOMBINANT CELL LINES.

CHAPTER 3: GENERATION OF GABA$_A$ STABLE RECOMBINANT CELL LINES

3.1 α1β3γ2 GABA$_A$R Recombinant Cell Line

3.1.1 INTRODUCTION

Transiently transfected cells express the gene of interest but do not integrate it into their genome so the new gene will not be replicated. During transient expression, target gene expression is temporary (24-72 hours for RNA, 48-96 hours for DNA) and the foreign gene is lost through cell division or other factors. In contrast, stable transfection introduces genetic materials with a

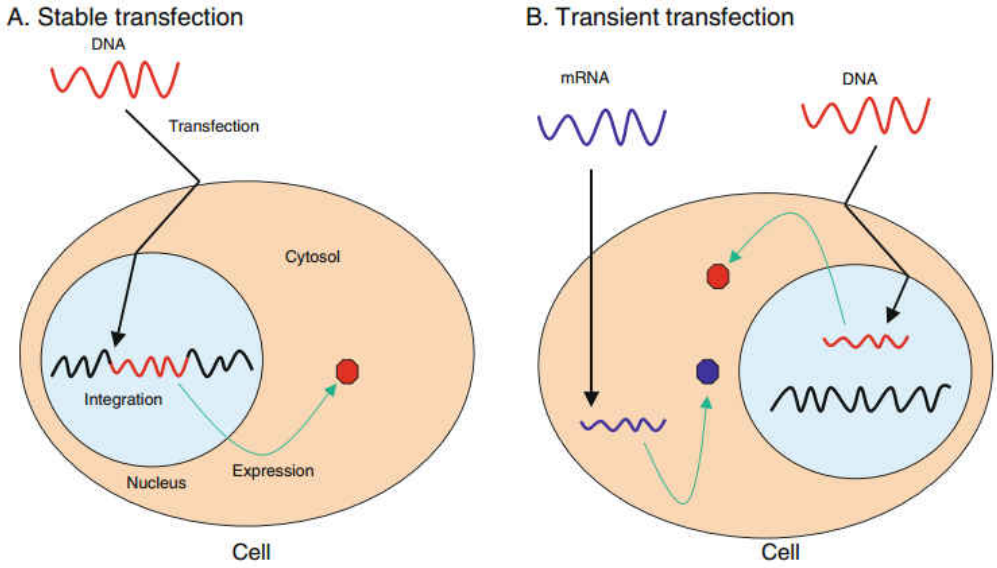


Figure 45. Diagrams of the two different types of transfections. A) Stable transfection: whereupon the foreign DNA (red wave) is delivered to the nucleus and is integrated into the host genome (black wave) and expressed sustainably. B) Transient transfection: following delivery of the DNA or mRNA into the nucleus, proteins (colored circles) are translated for a temporary period. Open Access source cited in text.

marker gene for selection and sustain expression even after the host cells replicate as illustrated in Figure 45 [218]. The integration is usually non-specific and only occurs in a small number of cells. The descendants of DNA-altered cells will carry and express the gene resulting in a stably-transfected cell line. Stable, long-term expression of a gene is achieved when a plasmid is introduced to the cell, travels to the nucleus, and finally integrates into the chromosomal DNA.

Since this chromosomal integration is a rare event, successful stably-transfected cells have to be selected and cultured with a selection agent. Only cells with the plasmid integrated will survive in the presence of the selection reagent. In the case of antibiotic selection, only the cell which have integrated the antibiotic resistant gene, contained on the plasmid with the gene of interest, will survive.

$GABA_AR$ contains multiple protein subunits to form the pentameric receptor and thus transfection necessitates multiple genes of interest. Cotransfection with multiple plasmids can leads to heterogeneous cell populations with very variable expression levels of each protein. In addition, the use of multiple antibiotics to select for the expression of multiple plasmids can negatively affect the recovery and proliferation of some cell lines. Common chemical transfection methods such as lipofection can be used for plasmid delivery into the cell. Choosing a clone with appropriate expression levels for all genes can be time-consuming. For the isolation process, single cells are isolated by plating into multi-well plates to obtain 100% clonal purity. Stably-transfected cells, in contrast to transient expression, express a gene of interest long-term at defined and reproducible levels. For the selection process, the antibiotic concentration is crucial since cells differ in their susceptibility to antibiotics and the activity of the antibiotic can vary considerably from batch to batch. So generating an antibiotic kill-curve is necessary if a new stock is used. After transfection, the cells grow and develop the proteins for antibiotic resistance during the initial selection. This step can take up to four weeks to extensively eliminate any contamination with non-resistant cells. After 1-2 weeks, the antibiotic concentration can be lowered. The optimal confluency for the time of transfection is normally 60-80%. Variation in cell densities can result in lower transfection efficiencies [208].

Using a stably-transfected cells line has several benefits. Firstly, discontinuing use of lipofection reagent is fiscally beneficial and would lead to a better intact cell membrane which is crucial for electrophysiological measurements. As a result, this would lead to a better seal with high resistance and less noise. Furthermore, since all cells are a genetically homogenous and clonal population, they maintain the same levels of $GABA_AR$ protein expression and thus offer high reproducibility of results.

The pJTI destination vector used in this study contains a φC31Integrase to mediate stable integration of the gene into the genome of the cell line. φC31 integrase, unlike recombinases such as Cre and Flp, does not have a corresponding excisionase enzyme, making the integration virtually irreversible [219]. Furthermore, the integration is site-specific for attB and attP phage attachment sites on extrachromosomal vectors and in human cells the integration will be distributed among a set of pseudo attP sites 90% of the time which is dramatically specific over random integration. These degenerate pseudo sites have partial sequence identity to attP and is still recognizable by the enzyme and may randomly present in large genomes like those of mammals [220]. The CMV promotor was added to generate the highest gene expressions possible, this has been reported as the best promotor for recombinant protein production in HEK293T cells with high efficiency long term transgene expression [221].

3.1.2 MOLECULAR CLONING

3.1.2.1 Introduction

Gateway technology utilized in the molecular cloning of the multi-gene system is based on the bacteriophage lambda site-specific recombination system which integrates lambda DNA into the E. coli chromosome. This process involves DNA att site recombination sequences with proteins

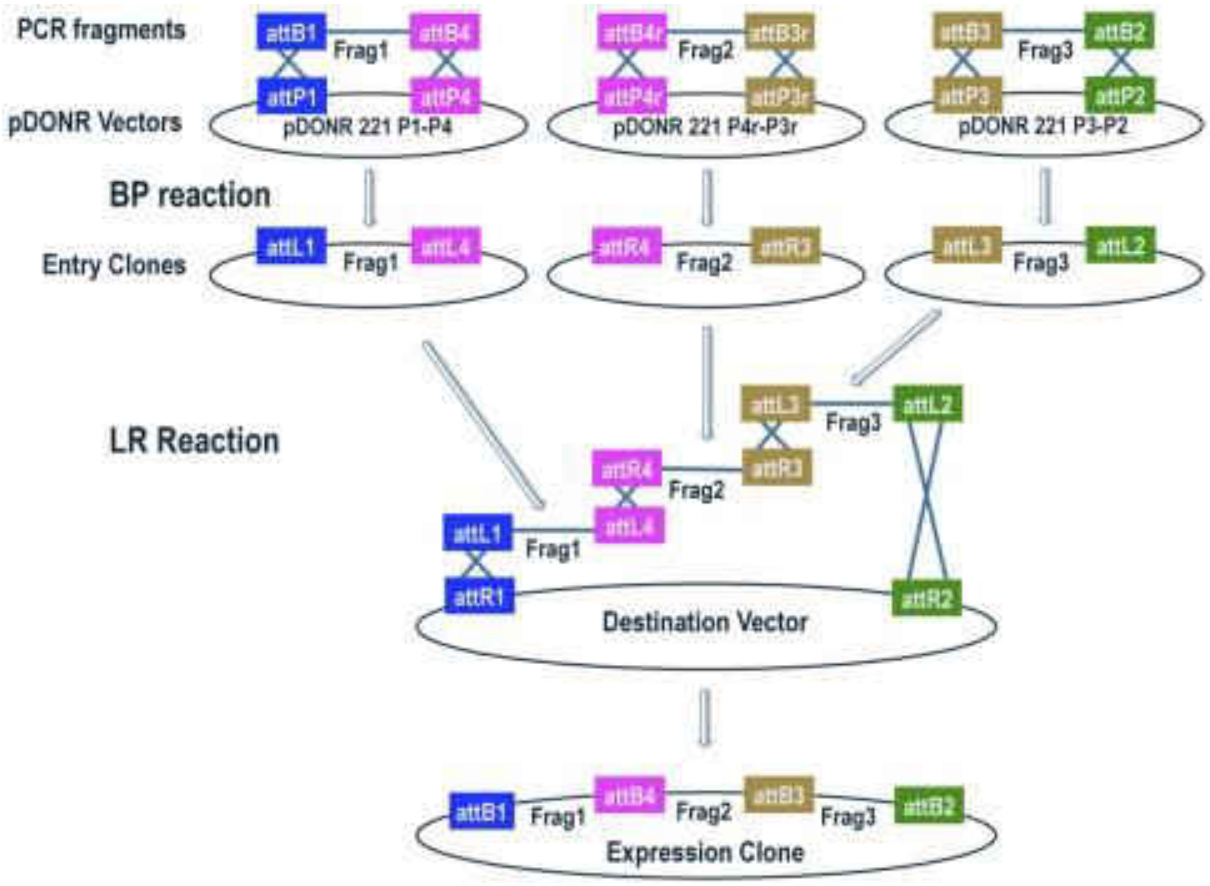


Figure 46. Multisite gateway 3-fragment recombination system assembling the target expression construct. Open Access cited in text.

that mediate the selective recombination reaction. At the att sites, attB on the E. coli chromosome and attP on the lambda chromosome. These sites serve as binding sites for recombination proteins [222]. After recombination occurs between the attB and attP sites, attL and attR sites are formed; facilitated by the bacteriophage λ integrase (Int) and E. coli integration host factor (IHF) proteins, a mixture called the lysogenic BP reaction. The recombination of the attL with the attR creates an attB expression clone catalyzed by Int, IHF, and Excisionase (Xis) proteins, a mixture called the LR Clonase enzyme mix. The recombination reactions are very specific for the sites. Invitrogen's Multisite Gateway technology manipulates this strategy to generate donor vectors. The three fragment system employs the following PCR fragments to donor vectors: aatB1 and attB4 react

with attP1 and attP4, attB4r and attB3r with attP4r and attP3r, attB3 and attB2 with attP3 and attP2. These newly created sites are the attL1 and attL4, attR4 and attR3, and attL3 and attL2 respectively. These sites reassemble with the final destination vector which contains an attR1 and attR2. This final reaction regenerates the attB1, attB4, attB3, and attB2 sites. This process is illustrated in detail in Figure 46 from [223].

3.1.2.2 Experimental

Plasmid Propagation

Plasmids, BP clonase, and LR clonase were acquired from a kit for the Multisite Gateway Technologies (ThermoFisher, 12537100). The ccdB effects of the DONR and DEST (donor and destination) vectors meant that propagation of the plasmid has to be done in ccdB Survival 2 $T1^R$ E. coli stain (ThermoFisher, A10460). Then plated on LB (IBI, IB49120) containing 50 µg/mL kanamycin (GoldBio, K12025) and 20µg/mL chloramphenicol. The destination vector should be cultured on LB plates containing 100µg/mL carbenicillin (GoldBio, C10325) and 20µg/mL of chloramphenicol (GoldBio, C1055). The plasmid DNA is then extracted using a gravity-flow anion-exchange HiSpeed Plasmid Maxi Kit (Qiagen, 12663). The pellet was resuspended in buffer and an alkaline lysis is performed before the lysate is cleared by filtration. The lysate is then added to a primed HiSpeed tip to bind DNA, wash, and finally elute. Isopropanol was added to the elution to precipitate the DNA and collected using the QIAprecipitator. The final elution from the QIAprecipitator yields ultrapure plasmid DNA. The DNA concentration was determined by UV at 260nm using the Tecan Infinite M1000 plate reader. Protein impurities were assessed at 280nm.

Cloning protocols

One Shot Mach1 T1 Phage-Resistant chemically competent cells (ThermoFisher, C862003) were used to generate DONR plasmids. PCR fragments were generated with flanking attB sites seen in Table 9 and ordered from Integrated DNA Technologies (IDT). The template DNA was combined with 1 µL of 20µM of each of the primers, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM $MgSO_4$, 5 µL of 10X High Fidelity PCR Buffer, add 0.2 µL Platinum Taq DNA Polymerase High Fidelity (ThermoFisher, 11304-011) and the reaction mixture was diluted to 50 µL reaction. The thermal cycler was set for 30 PCR cycles with an initial denaturation step 94°C for 2 minutes, denaturing step at 94°C for 15secs, annealing step at 55°C, and an extension phase at 68°C for 2 minutes.

Table 9. Primers used to integrate recombination sites.

Primer	Primer sequence (5'-3')
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTNN—sequence-
attB2r	GGGGACAGCTTTCTTGTACAAAGTGGNN—sequence-
attB4	GGGGACAACTTTGTATAGAAAAGTTGNN—sequence-
attB1r	GGGGACTGCTTTTTTGTACAAACTTGN—sequence-
attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTN—sequence-
attB3	GGGGACAACTTTGTATAATAAAGTTGN—sequence-

Gene fragments containing the attB sites were purified using 150 µL of TE, pH 8.0 (Qiagen, 12662) to a 50 µL amplification reaction containing the product. 100 µL of 30% PEG 8000 with 30 mM $MgCl_2$ (Fisher, BP233-1/Fisher, AB-0359) were added and vortexed to mix and centrifuged immediately at 10,000 x g for 15 minutes at room temperature. The supernatant was removed and the clear pellet was dissolved in 50 µL of 1 x TE, pH 8.0. The recovered product was run on a 1% agarose gel with 0.5 g of agarose (MidSci, BE-A500) with 50 mL of 1xTBE buffer, microwaved in bursts of 30 secs until the agarose was dissolved. 5 µL of an ethidium bromide replacement, Bullseye DNA Safestain (MidSci, C138) was added to visualize the DNA under

ultraviolet light. The agarose was poured into a small gel tray with a well comb. The gel was allowed to solidify at room temperature for 20-30 min. The gel was run at 80-150 V for around 30 min until the dye line was 50-75% down the gel.

50 fmoles of the attB PCR product was added to a mixture with 150 ng of pDONR vector, in 8 µL of TE buffer. 2 µL of BP Clonase II enzyme mix (ThermoFisher, 11789100) was added and vortexed to mix and incubated at 25°C for 1 hour. Afterwards, 1 µL of Proteinase K (ThermoFisher, 25530049) solution is added to the reaction and incubated for 10 min at 37°C.

The BP reaction is transformed using One Shot Mach1 T1 Competent Cells (ThermoFisher, C862003). 2 µL of the BP recombination reaction was added to a vial of One Shot Mach1 T1 chemically competent E. coli and incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42°C without shaking and immediately transferred to ice for 2 min. SOC medium, 250 µL, was then added and incubated at room temperature. The tube was secured and shaken horizontally at 225 rpm at 37°C for 1 hr before being spread on pre-warmed Kanamycin (GoldBio, K-120) selective plates (20 µL and 100 µL) which were incubated overnight at 37°C. Colonies were isolated and grown in LB broth containing Kanamycin and purified using the IBI High Speed Plasmid Mini Kit (MidSci, IB47101).

The DONR entry clones were combined at 10fmoles each with the destination vector at 20 fmoles with 1X TE buffer, pH 8.0 added to 8 µL. The LR Clonase II Plus enzyme (ThermoFisher, 11791100) was thawed and vortexed before 2 µL were added to the reaction vessel and vortexed for 2 seconds twice. The reaction was then incubated at 25°C for 16 h. The reaction was then incubated with 1 µL of Proteinase K (ThermoFisher, 25530049) for 10 min at 37°C to discontinue the reaction. 2-3 µL of the reaction mix was used to transform into One Shot Mach1 T1 competent

cells (ThermoFisher, C862003) or MAX Stbl2 Competent Cells (ThermoFisher, 10268019). The cells were plated onto Carbenicillin (GoldBio, C10325) selective plates (20 µL and 100 µL) which were incubated overnight at 37°C. Colonies were isolated and grown in LB broth containing Kanamycin and purified using the IBI High Speed Plasmid Mini Kit (MidSci, IB47101).

3.1.2.3 Results and Discussion

pDONR plasmids were replenished using a Qiagen Maxi prep in OneShot ccdB survival 2T1 competent cells. PCR product was run on a 1% agarose gel. Initial results to generate Entry Clones from the DONR plasmids exhibited integration of the α1 and β3 but not the γ2 gene. When the quality of the PCR product was assessed during genetic sequencing at GeneWiz, the product was found to be of poor quality. Multiple colonies were purified but attempts yielded the same results. A repeat with the PCR reaction with subsequent PEG purification followed by BP clonase and transformation led to a successful recombination of γ2 into the DONR plasmid. Jump-In LR Clonase II Plus recombination reaction was performed using 10 fmoles of each Entry Clone plasmid and 20 fmoles of the destination vector, DEST. The reaction was transformed into Mach1 T1 competent cells and plated onto Carbenicillin (Carb) plates. Growth yielded >200 colonies, all of which failed to grow in LB broth with Carb. When fresh plates were poured, growth was around 20 colonies. Two colonies were sequenced but lacked the γ2 gene. Re-sequencing the γ2 Entry Clone led to a failed priming so the original source plasmid, pCI-$GABA_AR$-γ2, PCR was performed again with >200 colonies growing on a Kanamycin selective plate. 5 colonies were isolated with all exhibiting high background or poor quality DNA. Repeating the transformation, colony isolation, LB growth and purification yielded sufficient DNA quality and sequencing within the γ2 gene fragment showed a successful insertion. The LR recombination reaction was repeated and mixture was transformed and plated out onto Carb plates. Around 15 colonies grew

and were sequenced with no priming at the α1 or β2 genes. Repeating the reaction resulted in formation of >20 colonies but Mini-prep of five colonies yielded negligible amounts of DNA except for 1 which only contained the γ2 insert. 8 more colonies were selected, grown and purified via mini-prep. The fifth plasmid yielded all three inserts which were verified with genetic sequencing with GeneWiz. The sequence of the entire construct can be found in the supplementary material.

A maxi-prep was performed to amplify the plasmid with growth kept below 30°C. Since the plasmid is large, it is unstable and has a low copy number and needs to grow for longer periods of time. It may be prone to mutate in regular competent cells so Stbl2 cells can be used instead.

3.1.2.4 Conclusions

DONR plasmid construction presented little trouble as long as the PCR product was of sufficient quality. The efficiency of the recombination of the three DONR plasmids was lower than expected despite reports that the combined success frequency of producing the correct clone was reported to be 51.8% in other publications [223]. However it should be noted that such numbers represent single-fragment Gateway cloning and multi-fragment success was not reported separately.

3.1.3 TRANSFECTION AND CLONE ISOLATION

Stable cell lines are an essential tool in research for mass production of recombinant protein and antibodies as well as for assay development, functional studies, and for gene editing CRISPR experiments. Currently most biopharmaceutical companies employ the methotrexate (MTX) amplification technology or the Lonza glutamine synthetase (GS) system for selection, using Chinese hamster ovary (CHO) cells due to their robust growth in suspension cultures. MTX

inhibits the dihydrofolate reductase enzyme necessary for cellular metabolism while methionine sulphoximine in the GS system inhibits glutamine synthetase [224,225]. If transfection of multiple genes on different plasmids is involved, cell clones obtained will have multiple random genome integration with highly heterogeneous results. Choosing a cell clone expressing all genes of interest in edition to having high, stable expression can be costly and time-consuming, taking 6 to 12 months at a time. Thus, advancements in this area to increase speed and efficiency of generating and picking stable cell clones is at the forefront of concern for many companies [226].

The pJTI destination vector employs site-specific recombination to develop clones with more reproducible and predictable efficiency. The vector also contains a hygromycin phosphotransferase gene which confers hygromycin B resistance. Though there are automated methods of picking cell clones such as fluorescence-activated cell sorting (FACS)-based screening, ClonePix fluorescence halo system, or Cell Xpress laser fluorescence detection; the traditional method is the serial limiting dilution approach. This approach is simple and ensures monoclonal purity but is time and labor intensive. It involves sequential diluting in well-plates with single cells selected from the survivors of multiple rounds of selection and expansion. This traditional method was utilized for selection of the clones discussed further.

3.1.3.1 Introduction

Generating a stable cell line with the use of antibiotic selection necessitates the optimization of the antibiotic concentration used. The concentration is cell type dependent and antibiotic batch dependent. A kill curve is a dose-response experiment whereupon cells are exposed to increasing concentrations of antibiotic to determine the minimum concentration

required to kill all the cells over the course of one week. Two different assay formats were tested to determine the optimal antibiotic concentration.

Trypan Blue is a 960 Dalton molecule that enters cells with compromised membranes and binds to intracellular proteins. Trypan Blue has a very high affinity for serum proteins in media so the cells must be pelleted and resuspended in serum-free media or salt solution. Cells are physically counted instead of using an automatic instrument measurement so human error can be a problem. However, this assay is quick and commonly used as standard dye exclusion technique for viable cell counting.

CellTiter-Glo determines the number of viable cells though quantification of the ATP present in the well. ATP (adenosine triphosphate) indicates living, or metabolically active, cells. The mixture results in the cell lysis and the generation of a luminescent signal indicative of ATP presence. The reaction that converts luciferin into oxyluciferin, generates luminescence as a byproduct. For this reaction to occur, the presence of Mg^{2+}, molecular oxygen, and ATP are necessary. Promega's luciferase ($LucPpe2^{m}$) is a stable form of the luciferase found in the firefly Photuris pennsylvanica (LucPpe2) and is pH and detergent resistant.

10µg total DNA was used for the transfection. After transfection, 48-72 hours passed to allow for sufficient recovery and to avoid low cell viability. Cells were then transferred to 6-well plates containing fresh medium at a less than 25% confluency. After cells successfully adhered to the culture dish (3-5 h), antibiotic pressure was applied with Hygromycin B. Visual toxicity of the cells were noted daily and media was replaced every 2-3 days. The surviving polyclonal line was then processed further using the limiting dilution method to expand single cells into colonies in separate wells. Only those cells with single, well-defined colonies and identifiable foci were

manually picked and expanded for further analysis. Very low survival rates are a hallmark of this method as secreted factors from neighboring cells are absent [227].

3.1.3.2 Experimental

Cell Culture Reagents and Instrumentation

A commercially available, human embryonic kidney (Hek 293T) cell containing the simian vacuolating (SV) virus 40 T-antigen origin of replication [207] was used for all the stable transfections. Human embryonic kidney (HEK) 293T cells were purchased (ATCC) and cultured in 75cm^2 flasks (CellStar) coated in matrigel (BD Bioscience, #354234), a gelatinous protein secreted by mouse sarcoma that facilitates cell adhesion to the flask. Cells are grown in DMEM/High Glucose (Hyclone, SH3024301) media to which non-essential amino acids (Hyclone, SH30238.01), 10 mM HEPES (Hyclone, SH302237.01), 5x10^6 units of penicillin and streptomycin (Hyclone, SV30010), and 10% of heat-inactivated premium US-sourced fetal bovine serum (FBS) (Biowest, SO1520HI) were added. Cells were rinsed with PBS or phosphate buffered saline (Hyclone SH30256.01) without calcium or magnesium. Cells are harvested using 0.05% Trypsin (Hyclone, SH3023601) or Detachin (Genlantis T100100) which both disrupts the cell monolayer and proteolytically cleaves the bonds between the cells and flask; the latter is more gentle and better suited for patch-clamp studies due to the proprietary mixture of proteases and collagenases. The media utilized in transient transfections contains the same components only the FBS was heat-inactivated and dialyzed (Atlanta Bio, S12650H).

Transfection Reagents and Instrumentation

15 µg of the pJTI-constructs were used for transfection reactions. Cell transfection was conducted by lipid-based methods using Lipofectamine with PLUS reagent (Life Technologies,

#15338020). Hygromycin was used for clone selection (Invitrogen 10687-010). Cells were transformed at 60-70% confluency and the population was thinned for accurate clone isolation.

Clone Selection Reagents and Instrumentation

After lipofection for 24 h in the 75cm^2 flask, cells were redistributed onto a 60cm^2 petri dish (MidSci, TP93100) and allowed to adhere overnight. 300µg/mL of hygromycin was added to the dish after 24 h. Media was exchanged every 2-3 days over a period of 10-15 days until 90% of the cells were dead. Large, well defined and separated colonies were chosen and placed in a matrigel-treated Nunc 384 well-plate (ThermoSci, 12-566-1) containing 80 µL of media. Once the well had sufficient growth of 50-60% confluency, the cells were moved to a matrigel-treated Nunc 96 well-plate (ThermoSci, 12-566-71) containing 150 µL of media. The wells that met 50-60% confluency were again moved to a matrigelled 24 well-plate (Corning, 3738) with 500 µL of media and 300µg/mL Hygromycin. Cells were again allowed to come to confluency before being moved to a 6 well-plate (MidSci, TP92006) with 2 mL of media and continued exposure to 300 µg/mL hygromycin.

Cells were centrifuged at 1000 rpm for 2min before being resuspended in PBS (Hyclone SH30256.01). 200 µL of the suspension was added to 300 µL and 500 µL of 0.4% Trypan Blue solution (Sigma, T8154) and mixed thoroughly. The mixture was incubated for 10 min before being counted with a hemocytomer. Cells were counted by adding 20 µL of cell suspension onto the slide. Three counting areas whose volume is 100 nL are averaged and multiplied by 1x10^4 to give a concentration of cells in cells/mL.

CellTiter-Glo cell viability assay was performed in a 96 or 384-well plate. Cells in the 96-well plate (ThermoSci, 12-566-71) were cultured with 100 µL of media while the 384-well plate

(ThermoSci, 12-566-1) should contain 25 µL of media. Control wells without cells were prepared to obtain a value for background luminescence. 1 volume of CellTiter-Glo reagent is added to the wells and the plate is placed on an orbital shaker for 2 minutes to induce cell lysis. The plate is further incubated at room temperature for 10 min before reading the luminescence. The luminescence was recorded using the Tecan Infinite M1000 plate reader with an integration time of 1000 ms. Since the plates are clear on the bottom to provide viewing of the cells, an adhesive bottom seal was applied prior to reading to maximize the luminescence intensity of each well (Perkin Elmer, 6005199).

qRT-PCR Reagents and Instrumentation

Cells were lysed using the QIAshredder (Qiagen) and RNA was isolated using the RNAeasy kit (Qiagen). RNA concentration was determined by UV at 260 nm using the Tecan Infinite M1000 plate reader. The Quantifast SYBR green RT-PCR Kit (Qiagen) was used for the real time PCR following the manufacturer's instructions. Primers used are listed in Table 10.

Table 10. qRT-PCR primers used for recombinant stable cell lines containing the α1β3γ2

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
α1 (gabra1)	CTCCTACAGCAACCAGCTATACCC	GCGGTTTTGTCTCAGGCTTGAC
β3 (gabrb3)	CCTACTAGCACCGATGGATGTT	GATGCTTCTGTCTCCCATGTAC
γ2 (gabrg2)	CGCTCTACCCAGGCTTCACTAGC	TCGGGCCGAAGTTTGTTGTCGT
GAPDH (gapdh)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Primers were ordered from Integrated DNA Technologies as 25nmole DNA oligos with standard desalting. qRT-PCR experiments were carried out on the Mastercycler 4 (Eppendorf) in 96-well twin.tec PCR plates (Eppendorf) with 20 µL reaction volumes.

qRT-PCR Protocol

Cells were harvested once 80% confluent using 0.05% Trypsin or Detachin solution and pelleted by centrifuging at 1000 rpm for 2 minutes. Media was aspirated and the cell pellet was resuspended in RTL buffer. The mixture was pipetted into the QIAshredder spin column and spun for 2 minutes at max rpm. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The solution was then transferred to an RNAeasy spin column and spun down for 15 sec at 10,000 rpm. Then washed with buffers to purify the bound RNA with the flow through discarded. After the column is washed and dried, RNA is eluted by addition of 30-50 µL of RNase-free water. Total RNA concentration was determined by UV at 260 nm and protein contamination was assessed at 280 nm. The RNA was then diluted and used for qRT-PCR with the QuantiFast SYBR Green RT-PCR Kit (Qiagen). The cycling conditions used was 10 min at 50°C (reverse transcriptase), 5 minutes at 95°C (PCR initial activation step), 10 sec at 95°C (denaturation), and 30 sec at 55°C (annealing and extension) for a total of 50 cycles. Data was taken in triplicate and the relative mRNA expression levels were calculated using wells containing no template and normalizing to housekeeping gene GAPDH.

Automated Patch-Clamp Electrophysiology

The buffers were made from NaCl (Fisher, BP358-1), KCl (Fisher, BP366-1), $MgCl_2$ (Sigma, M8266), $CaCl_2$ (Acros Org, 123350025), Glucose (Sigma, G0350500), HEPES (Fisher, BP410-500), CsCl (Sigma, 203025), and EGTA (Tocris, 28-071-G). The extracellular and intracellular solutions recommended by the manufacturers of the IonFlux. The extracellular solution contains: 238 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM Glucose, and 10 mM HEPES at pH 7.4. The intracellular solution contains: 60 mM KCl, 15 mM NaCl, 70 mM

KF, 5 mM HEPES, and 5 mM EGTA at pH 7.25. These concentrations were later optimized twice, see 3.2.1.3.2 Experimental.

To record $GABA_A$ currents, cell arrays were voltage clamped at a hyperpolarizing holding potential of -80mV. Cells were centrifuged at 380 g for 2 min and gently resuspended in ECS. This was repeated two more times before the cells were dispensed into the plate.

3.1.3.3 Results and Discussion

The kill curves generated were assessed. The curve generated using Trypan Blue studied various concentrations on a single time axis, Figure 47, with HEK293T cells exposed to hygromycin B for seven days in culture. The Trypan Blue assay seen in Figure 47 determined that after a week of Hygromycin B exposure, nearly all cells were dead at a concentration of 400 µg/mL. The Trypan Blue assay relies on visual assessment and counting of cells by the experimenter, while this method

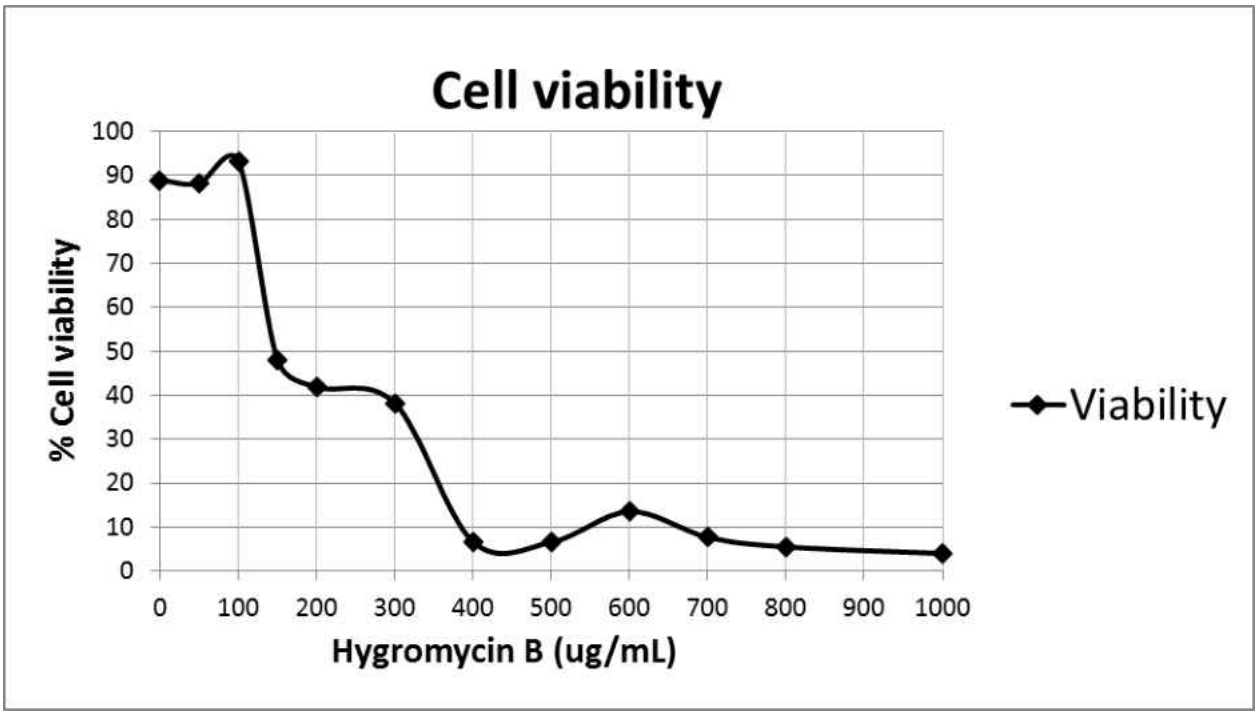


Figure 47. HEK293T cell viability after 7 days in culture with Hygromycin B. Assessment using Trypan Blue dye where the % viable cells = [1.00 - (number of blue cells/number of total cells)] x 100. Data was collected in duplicate and averaged.

is low-cost and simple, it is also time-consuming. When 'time' is added as an additional factor, it is faster and more convenient to use an instrument to record measurements such as absorbance, florescence, or luminescence. The Cell-Titer Glo assay is one such option; a luminescence based cell viability assay designed for multi-well plates. Using this method, various concentrations of antibiotic over a long period of time could be tested.

The result of the Cell-Titer Glo assay, seen in Figure 48, shows an incubation time at less than five days is insufficient to produce cell death as high as at 500 µg/mL. At ten days, the cell

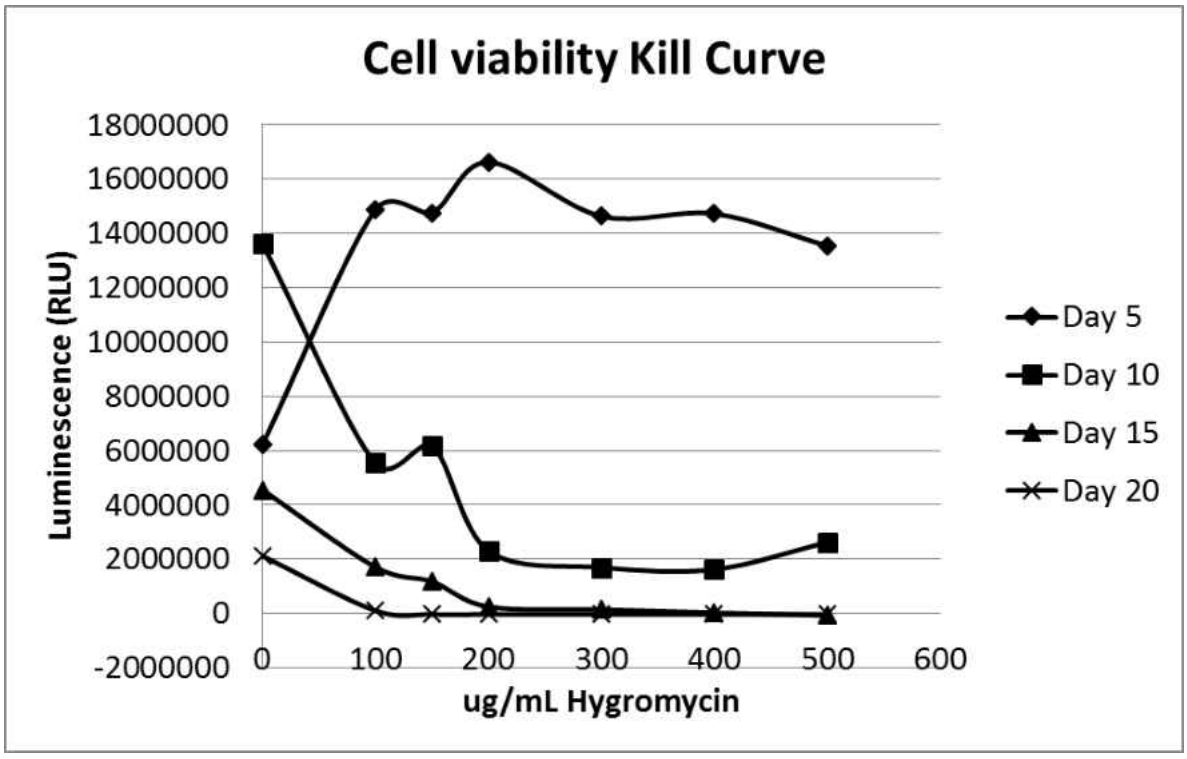


Figure 48. Cell viability over a period of 20 days. Assessment using CellTiter-Glo assay in a 96 well plate. Media and antibiotic was exchanged every 5 days. Data taken in doublet and averaged.

viability plummets at the lowest concentration (100 µg/mL) of antibiotic, however the curve does not flatten out until reaching 200 µg/mL. The curve at ten days was above the baseline- most likely caused by residual ATP after the single media exchange over five days, however it was expected

the luminescence of $2x10^6$ RLU corresponded to nearly complete cell death. After fifteen days, cell viability is low even without the presence of antibiotic due to the overgrown conditions in the well. Both the exposure time and the antibiotic concentration can affect the quality and purity of clones chosen; high selection stringency generates surviving clones with high transcript levels while insufficient incubation times can lead to the survival of cells which have not incorporated the gene of interest. Thus, in order to minimize overuse of antibiotic yet keep the high selection stringency, the selection window was chosen to be 10 days with 300 µg/mL of Hygromycin B. This concentration yielded almost no surviving cells within the shortest period of time.

In order to assess that the cloned plasmid would result in expression of the subunits, qRT-PCR was performed on cells which were transiently transfected with the plasmid. The results, seen

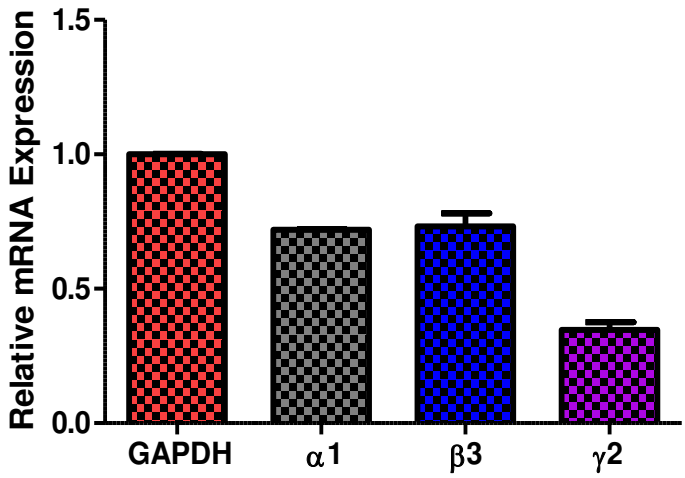


Figure 49. qRT-PCR of transiently transfected HEK293T cells containing the α1β3γ2 plasmid construct.

in Figure 49, shows the successful expression of the cassettes in the cell line via mRNA quantification.

The electrophysiological response of the transiently transfected cells were also recorded. The GABA dose response, seen in Figure 50, confirmed that the transfection with the plasmid resulted in functional $GABA_A$ receptors.

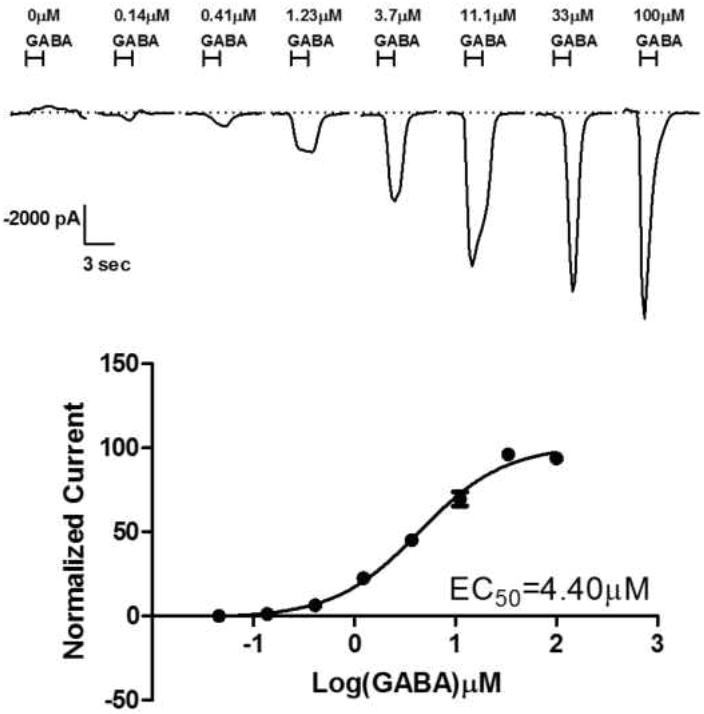


Figure 50. Transiently transfected HEK293T cells expressing the $GABA_AR$ α1β3γ2 construct exposed to increasing concentrations of GABA. N=4.

After transient transfection with the α1β3γ2 construct in a $75cm^2$ culture flask, cells were allowed to recover for 48 hours. After which, cells were separated into 6 well culture plates at a 20% confluency, allowed 2-3 hours to adhere to the matrigel-treated surface, and exposed to 300μg/mL of Hygromycin B. After ten days with frequent media exchange, well defined single-celled colonies were chosen and placed in a 384-well plate without the presence of the selective antibiotic. After reaching an 80% confluency in a 384 well plate, the cells were expanded further into a 96-well plate, a 24-well plate, and finally a 6 well plate before undergoing a second round of antibiotic pressure. Surviving cell cultures were selected and expanded further for analysis.

3.1.3.4 Conclusions

Kill curve generation was both fast and convenient using the Cell-Titer Glo assay but pricing can be a limiting factor. In comparison, the Trypan Blue was inexpensive but necessitated removal of the cells from the plate, staining, incubating, and manual counting. Cell-Titer Glo also eliminates most of the human error of manual counting of the cells and would only have possible inaccuracies of pipetting error.

Transient transfection with the α1β3γ2 construct resulted in high mRNA expression levels and large current changes (-8000pA) in response to the application of GABA during electrophysiological recordings. In the future, these large plasmid constructs can be reliably used if transient transfection of cells with the $GABA_AR$, in place of transfecting three individual plasmids. In addition, the maintenance and propagation of the plasmid only requires a single growth and purification rather than three separate; lowering the amount of consumables used.

Selection of the cells was time-consuming but performed with relative ease. Separating the cells to an initial starting point of 20% confluency allows for well separated colonies to flourish but also led to a very high amount of initial cell death, making single cellular recovery take even longer in a 384-well plate. Quite a few colonies, which presented an extremely healthy morphology during the first selection, died after being moved to the solitary confinement in the 384-well plate. Thus, two rounds of selection was performed, an initial selection after 10 days in culture with hygromycin and 20 days in culture with hygromycin.

3.1.4 CHARACTERIZATION OF CLONE

3.1.4.1 Introduction

Characterization of the cell clones was done electrophysiologically with the IonFlux system and also genomically using qRT-PCR. Polymerase chain reaction (PCR) is a method that utilizes a thermal cycling pattern that heats and cools the DNA in the presence of primers, deoxynucleoside triphosphates (dNTP) and the polymerase enzyme. There are three major steps to PCR: denaturation into single stranded DNA, cooling to allow annealing of the primers to the strands, and extension of the copy by DNA polymerase and dNTPs. Quantitative reverse transcription-PCR is different in a few ways. Firstly, it is real-time, meaning the process can be monitored with the fluorescent dye in parallel to the experiment (qPCR). Secondly, instead of

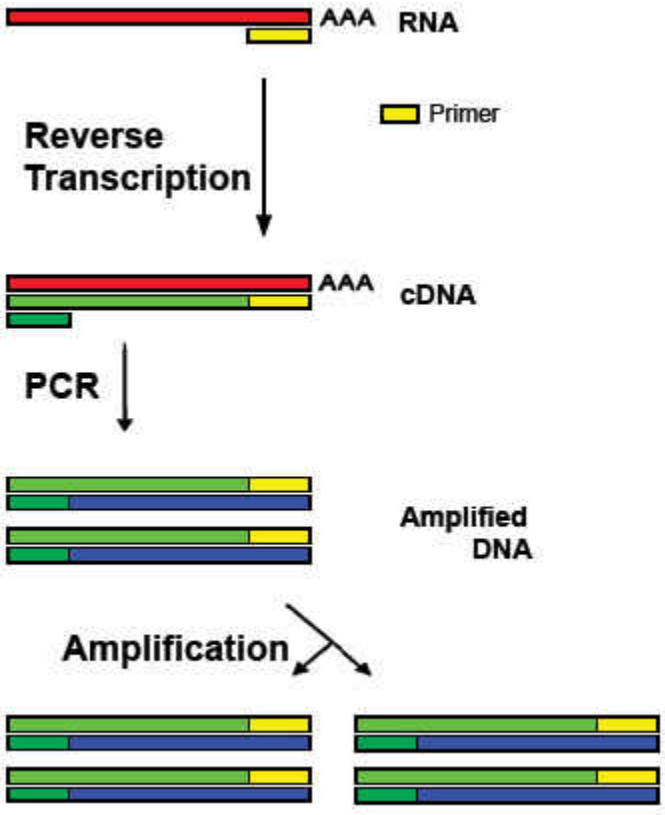


Figure 51. Reverse transcription polymerase chain reaction. Created by Jpark623 and used with permission. CC BY-SA.

DNA polymerase, reverse transcriptase is added in order to reverse transcribe the RNA of interest (RT-PCR) into its complementary DNA (cDNA) as seen in Figure 51. The combined technique is known as qRT-PCR. Qiagen uses the SYBR Green probe which binds to the minor groove of double stranded DNA and produces a fluorescent signal, illustrated in Figure 52. Reverse

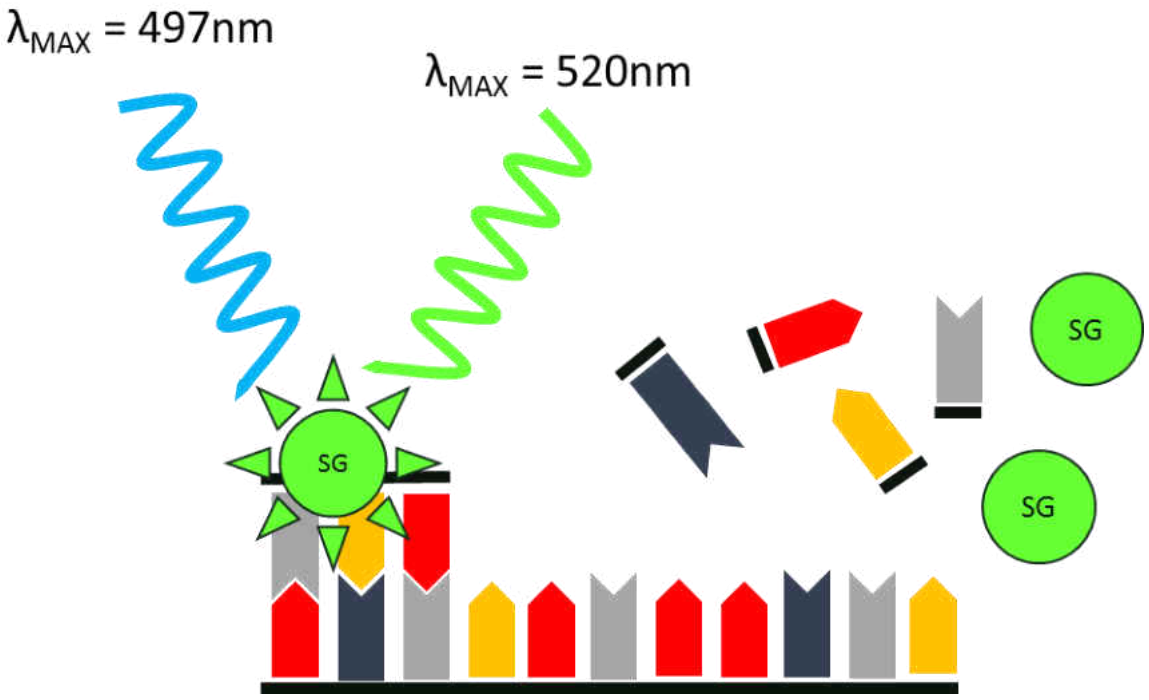


Figure 52. Biochemical illustration of the SYBR Green dye process. SYBR green binds to double-stranded DNA and the resulting DNA-dye complex absorbs blue light (λ_{max}=497nm) and emits green light (λ_{max}=520nm). During PCR, DNA polymerase amplifies the target sequence which creates new copies of double-stranded DNA so there is an increase in fluorescence intensity proportional to the amount of PCR product produced.

transcriptase of reverse transcribes mRNA to cDNA results in an increase of double stranded cDNA. The SYBR Green binds to the increasing product, leading to an increase in fluorescence after each cycle [228].

3.1.4.2 Experimental

qRT-PCR Reagents and Instrumentation

Refer to 3.1.3.2 Experimental

qRT-PCR Protocol

Refer to 3.1.3.2 Experimental

Automated Patch-Clamp Electrophysiology

Refer to 3.1.3.2 Experimental

3.1.4.3 Results and Discussion

Isolated clones were grown to 80% in a single well of a 6-well plates before being separated into three wells of the 6-well plate. One well was used in the qRT-PCR and the second well, if expressing sufficient quantities of the mRNA, would electrophysiologically examined with the IonFlux, the final well was saved to continue on the cell line for future study and for cryogenic preservation.

Among the hundreds of cells selected for the expansion, six cell lines quickly reached the appropriate confluency for examination. qRT-PCR was performed to ascertain that expression of the subunits. The data is seen in Figure 53. Interestingly, the β3 expression prevailed in all the cells

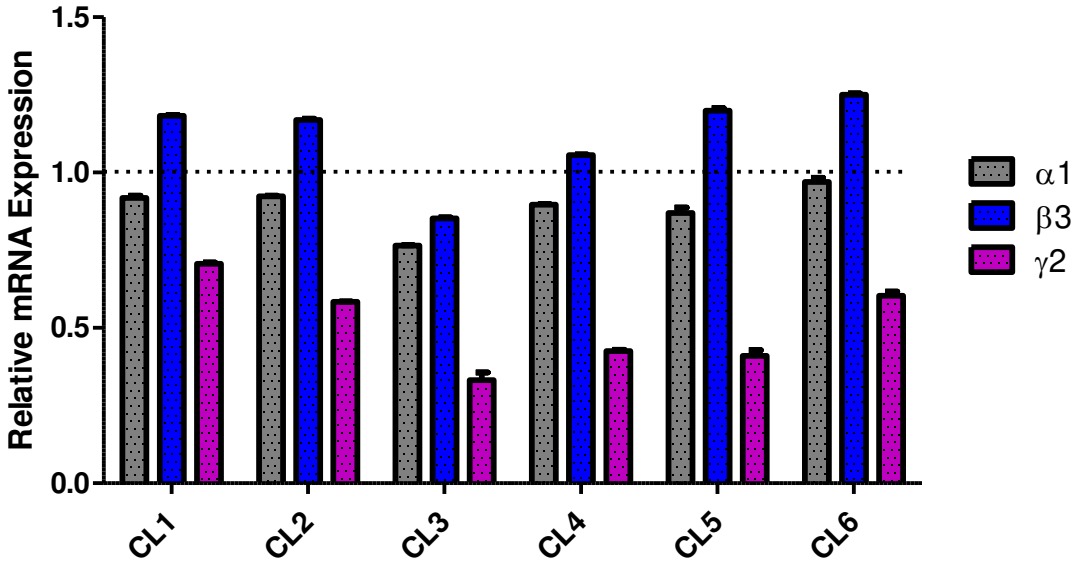


Figure 53. mRNA quantification of six recombinant cell lines (CL) containing the $GABA_AR$ α1β3γ2 construct. Results normalized to GAPDH (1.0 indicated by dashed line).

while the lowest expression was consistently the γ2 subunit. The cell line 1, 2, 4, and 5 were examined further on the IonFlux.

The normalized current response to GABA application, seen in Figure 54, exhibits nearly

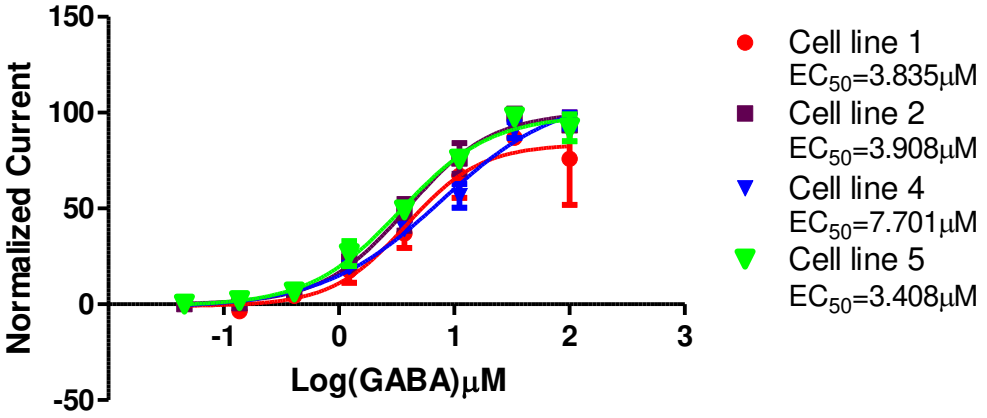


Figure 54. Isolated cell line (CL) clones expressing the $GABA_AR$ α1β3γ2 construct exposed to increasing concentrations of GABA. N=4

identical curve shapes with similar EC_{50} values. The current sweeps for these dose response curves can be seen in Figure 55. The largest response was from cell line 5 which, with four traps, averaged a maximum response of 14,250pA, cell line 2 responded with an average response of 9,875pA, cell line 4 had an average response of 8,375pA, and finally cell line 1 which responded with an average of 5,000pA. Incidentally, cell line 5 was the slowest growing clonal line while cell line 1 was the fastest. Thus these two cell lines were further characterized with testing of positive modulators diazepam and HZ-166.

The first trial with the BZD compounds elicited potentiation within the range of 200% for both compounds in both cell lines, Figure 56 with the sweeps shown in Figure 57. The responses

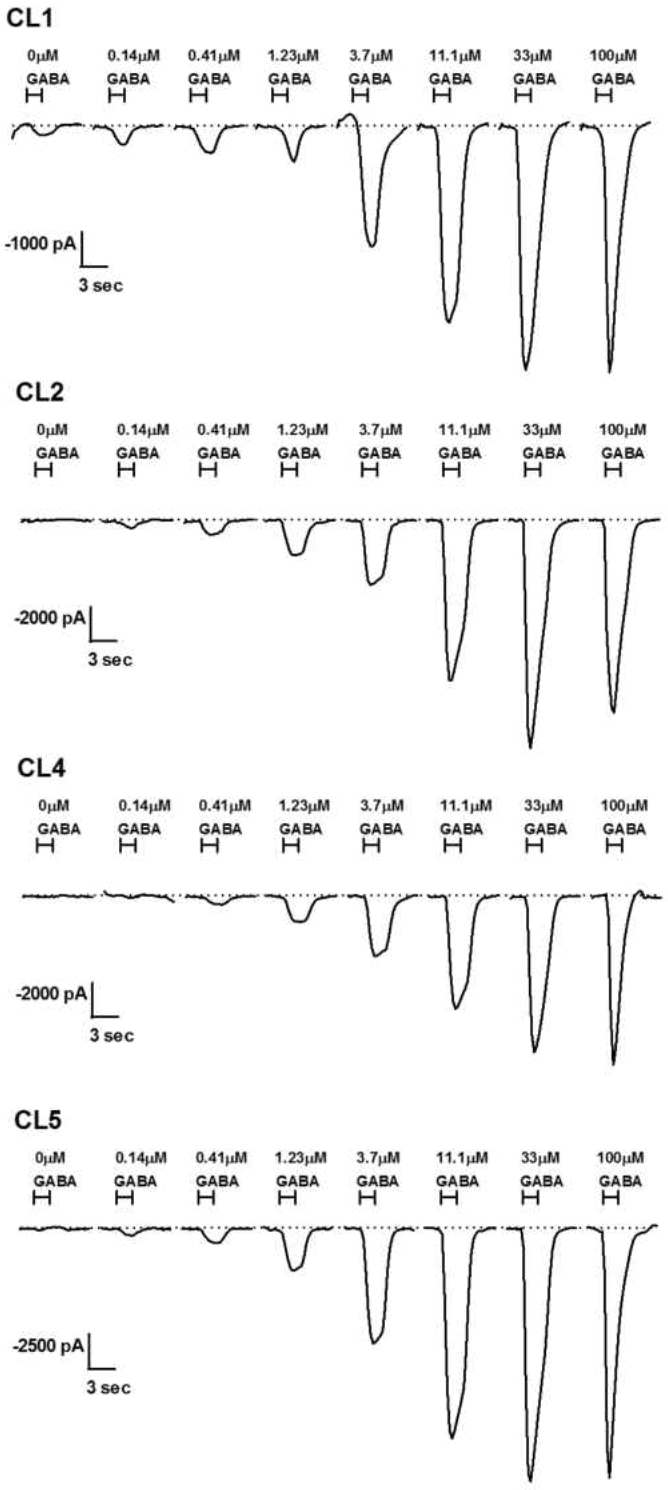


Figure 55. Recombinant stable cell lines (CL) expressing the $GABA_AR$ $\alpha1\beta3\gamma2$ exposed to increasing concentrations of GABA

from both cell lines corresponded well to each other but the response to diazepam was lower than

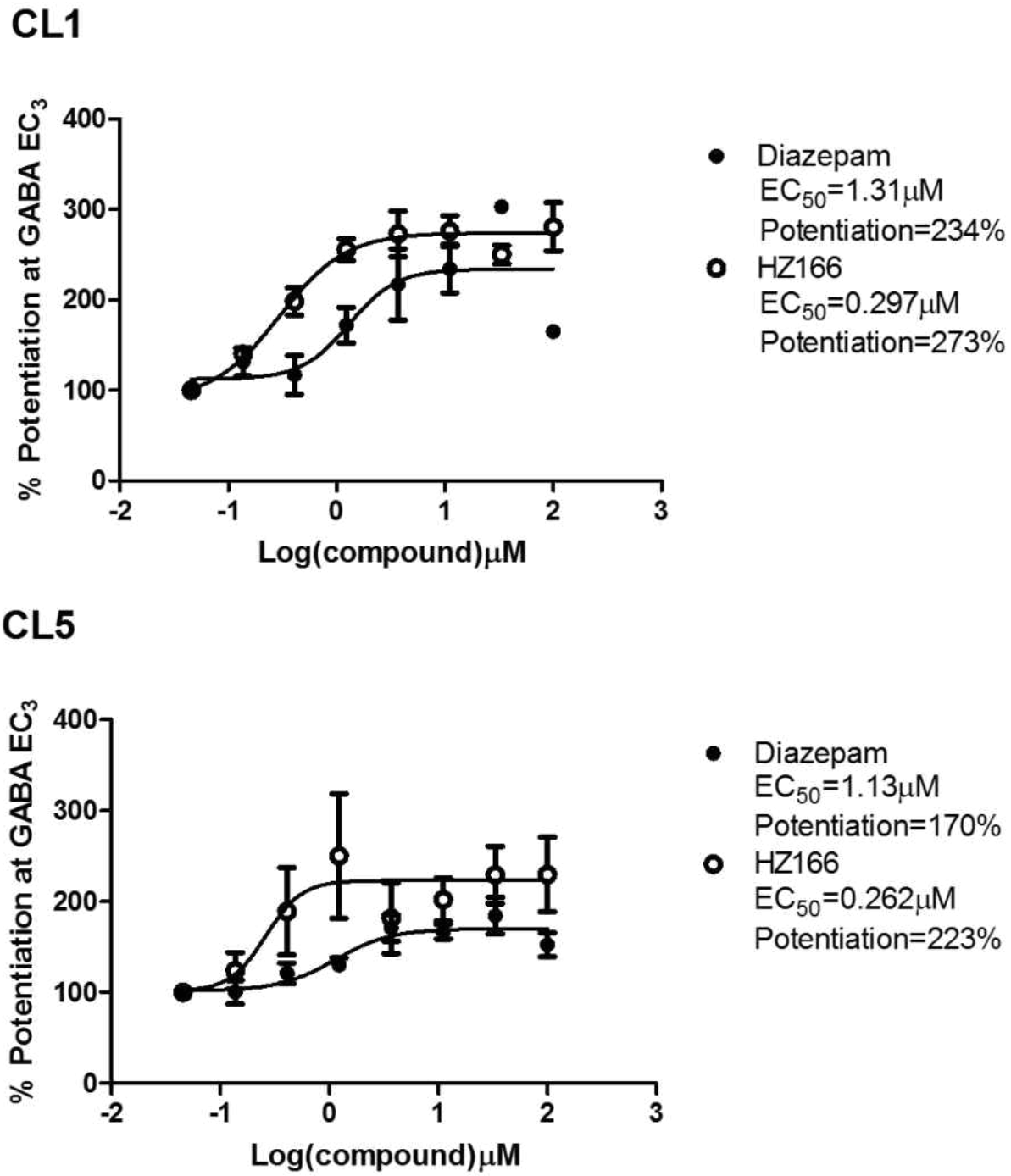


Figure 56. Stable cell lines 1 and 5 containing the $GABA_AR$ α1β3γ2 exposed to increasing concentrations of diazepam and HZ-166 in combination with a constant concentration of GABA EC_{20} or 0.1μM. Compounds were solubilized in 1% max DMSO. N=6

anticipated. Upon further inspection it was discovered that the compound solutions and patch clamp buffers had been created months prior to the experiment. Fresh extracellular, intracellular,

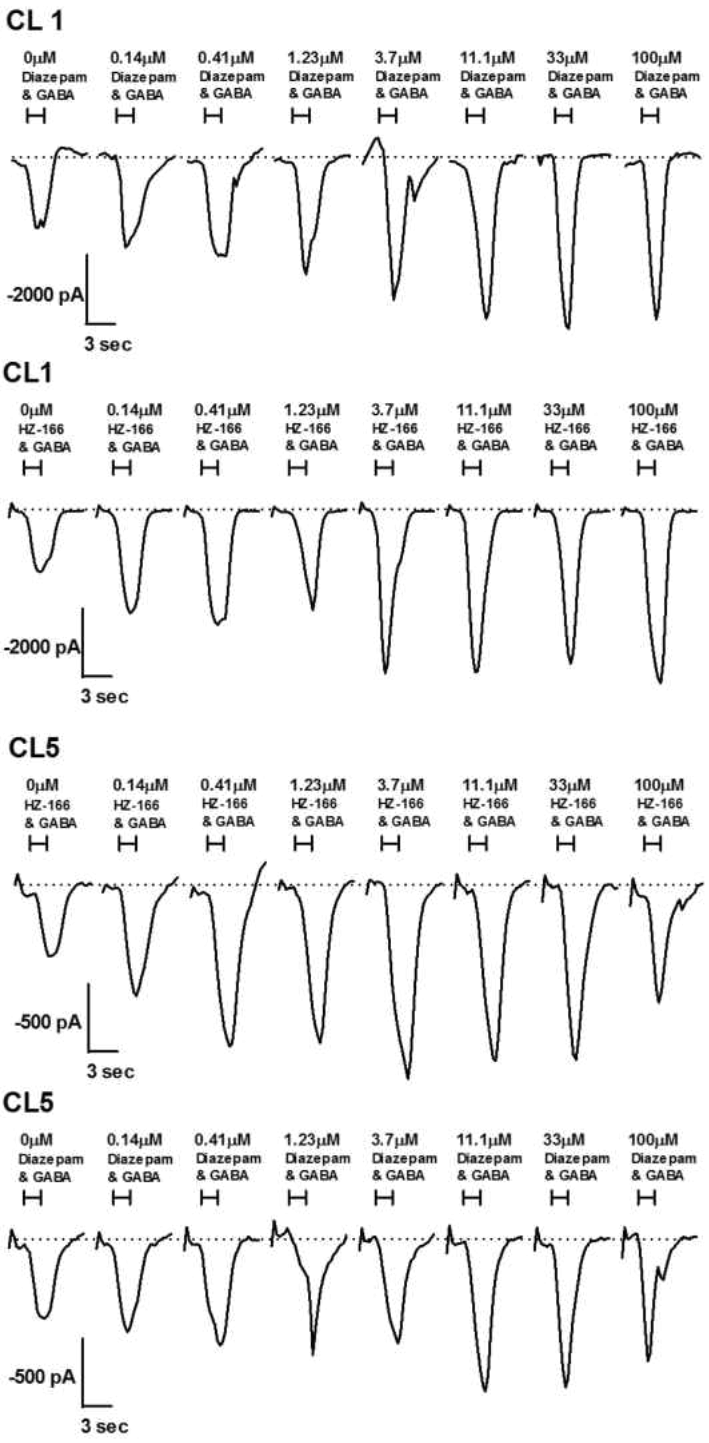


Figure 57. Current sweeps of recombinant stable cell lines (CL) expressing the $GABA_AR$ α1β3γ2 exposed to increasing concentrations of modulator with constant concentration of GABA EC_3 of 0.1μM. The maximum DMSO concentration was 1%.

and DMSO solutions of the compounds were prepared and the assay was repeated. This

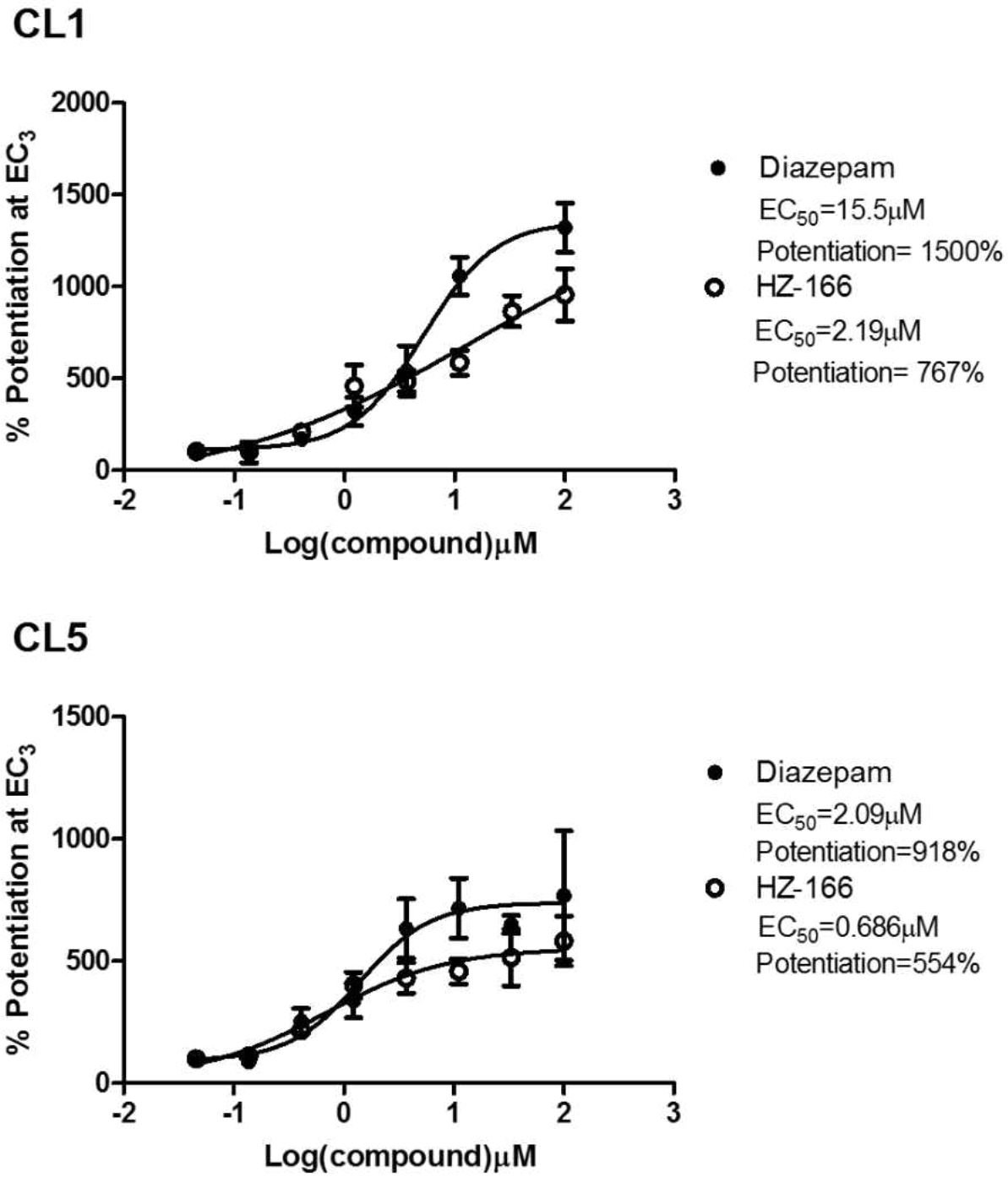


Figure 58. Stable cell lines 1 and 5 containing the GABAAR α1β3γ2 with new solutions exposed to increasing concentrations of diazepam and HZ-166 in combination with a constant concentration of GABA EC_{20} or 0.1μM. Compounds were solubilized in 1% max DMSO. N=6

modification had significant effects on the both the potency and efficacy of the compounds, Figure 58, with the current sweeps shown in Figure 59. The calculated potentiation for cell line 1 rose to

a range in the thousands, with cell line 5 not far behind. The efficacy of diazepam rose

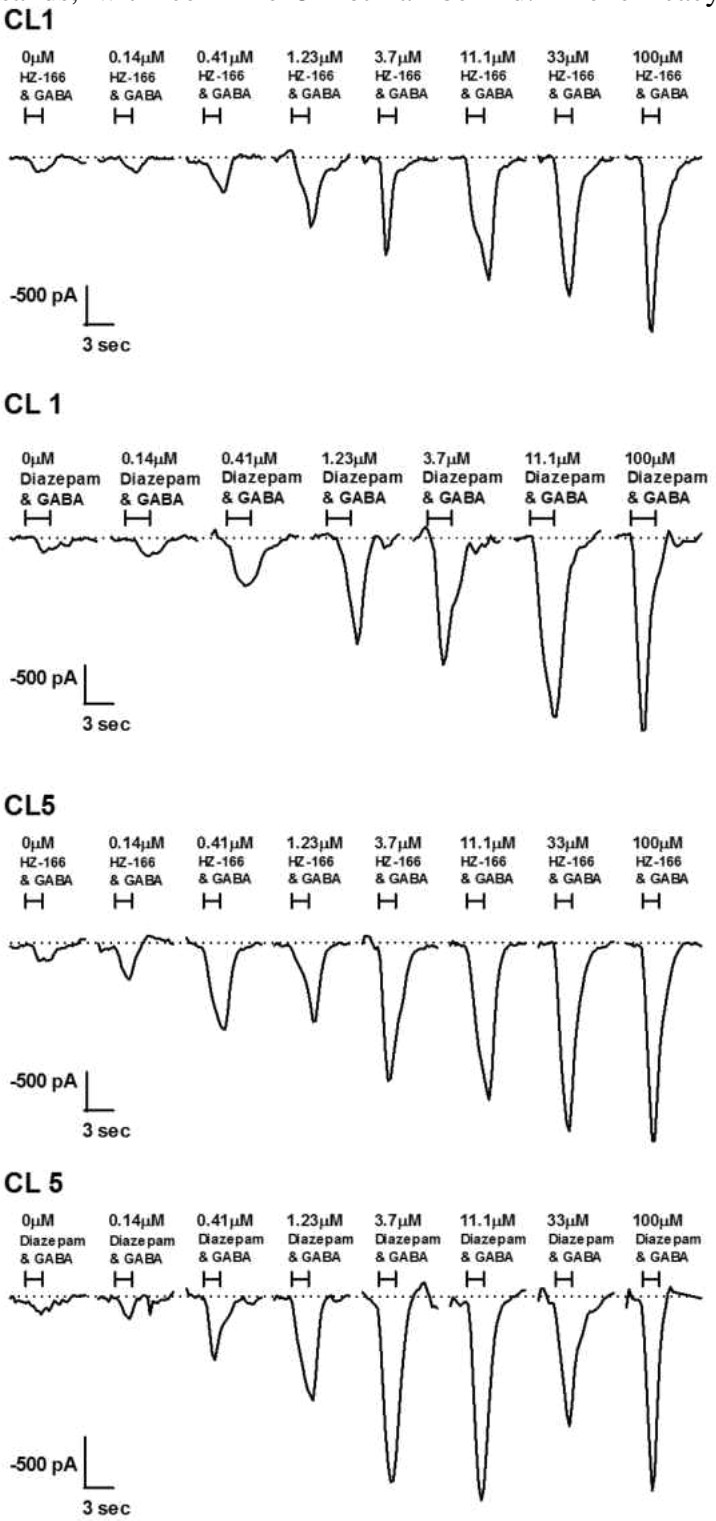


Figure 59. Current sweeps of recombinant stable cell lines (CL) expressing the $GABA_AR$ α1β3γ2 exposed to increasing concentrations of modulator with constant concentration of GABA EC_3 of 0.1μM. The maximum DMSO concentration was 1%.

significantly, surpassing that of HZ-166 in both cell lines. Whether this was the result of

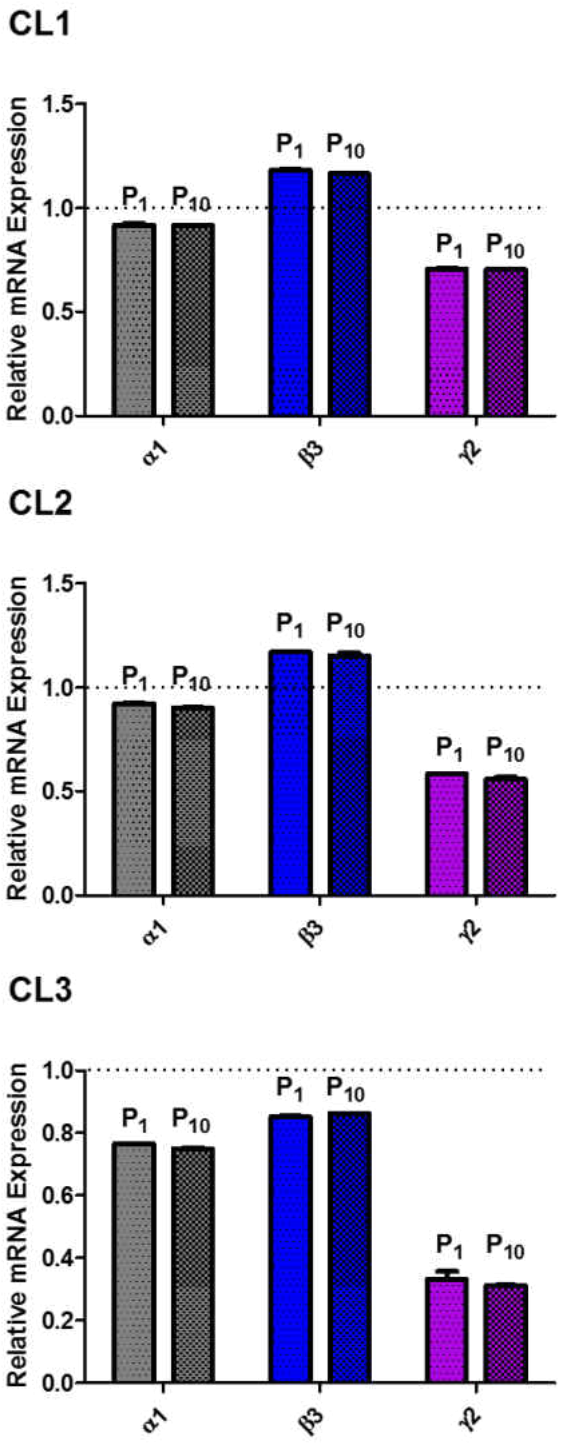


Figure 60. Retention of $GABA_AR$ subunit gene transcripts in the α1β3γ2 expressing cell lines (CL1-3) over a series of ten passages. Passage 1 (P1) to passage 10 (P10) mRNA levels were assessed via qRT-PCR. N=3

degradation of the active compound over time or change in the buffer solutions (evaporation or mold growth) is unknown. The former is more likely as latter as diazepam was far more effected than HZ-166. As far as comparison of cell lines, it would appear that the dose response of cell line 1 had not reached saturation by the final concentration of 100μM. In contrast, the response of cell line 5 plateaued for both compounds at around 1-3μM. It would almost appear as if the dose response curve for cell line 5 was offset and shifted towards the right to produce the response seen for cell line 1. This shift in potency was also observed in the GABA $EC_{50,}$ Figure 54, but not nearly to the same degree as seen here in Figure 56.

From this data it would appear that even among different clonal cell lines, the reproducibility has improved greatly from what was previously seen with transient transfections.

After selection, all clones were expanded and frozen down to preserve their expression characterization. It has often been reported that clones have been known to be unstable and lose expression or experience transgene silencing after several passages, particularly genes that utilize CMV promotor [229]. In order to address this concern, the cell line was characterized both immediately after generation and then after 10 passages which would be the equivalent of a month in culture. The results for these qRT-PCR experiments, Figure 60, exhibit the near negligible change in mRNA levels of the $GABA_AR$ subunits in the stably expressing clones.

In addition, although the HEK293T cell line had not exhibited DMSO sensitivity, problems were observed with the Jurkat E6-1 cell line. So the cell lines were tested at a constant at a constant

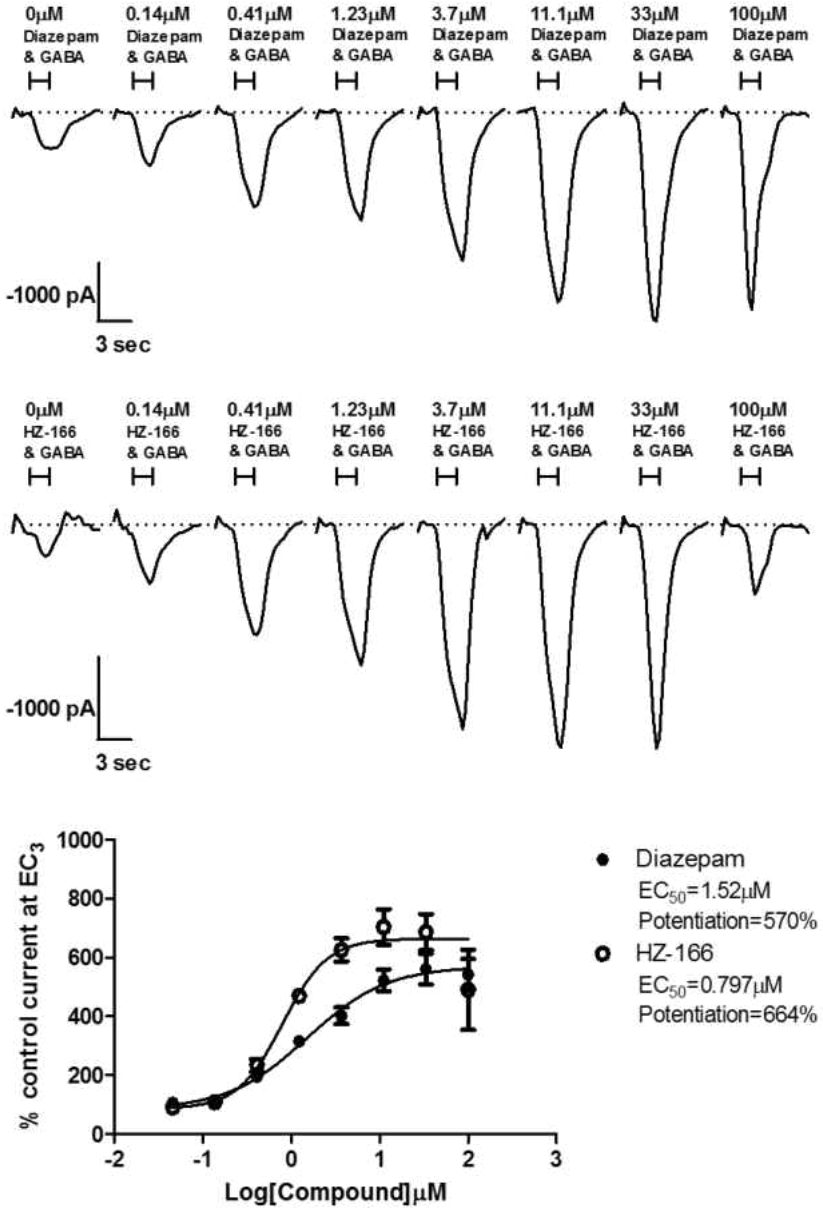


Figure 61. Current responses in CL5 isolated from HEK293T stably expressing the α1β3γ2. Modulators were tested with a constant concentration of GABA EC_3 0.18μM and DMSO concentrations of 0.3%. N=8

concentration of DMSO at 0.3%. The α1β3γ2 CL5 was recharacterized at constant DMSO and GABA EC_3. The results of this experiment can be seen in Figure 61. Though the potency of the compounds (EC_{50}) exhibited comparable results diazepam exhibited a vast reduction in efficacy.

The lowering in response of the cells to 100 μM HZ-166 and diazepam as well as the reduced total potentiation of diaazepam may be the result of solubility issues of the compounds in 0.3% DMSO.

To combat this issue, the maximum concentration of the compounds were lowered from 100 μM to 20 μM with the DMSO concentration kept at 0.3% DMSO. Additionally, it has also been reported that the diazepam produces a biphasic potentiation with distinct components in the nanomolar and micromolar [215]. The two components become more distinct in the presence of low GABA concentrations from EC_3 to EC_{10}, thus we determined that testing with the modulators should be performed at GABA EC_{20} so as not to overestimate the potentiation of the compounds. The results of this experiment can be seen in Figure 62.

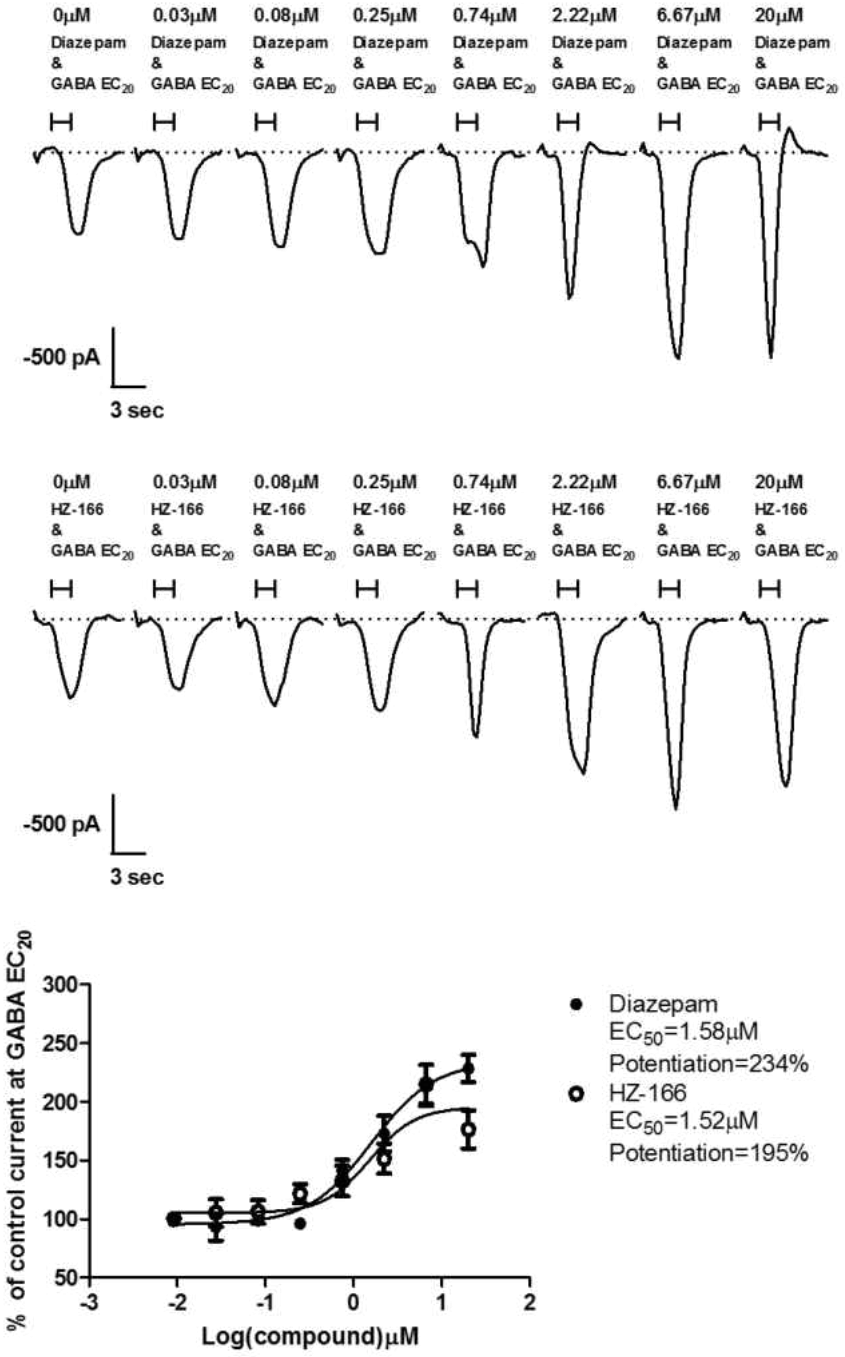


Figure 62. Current responses in CL5 isolated from HEK293T stably expressing the α1β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 1μM and DMSO concentrations of 0.3%. N=8

Additionally, the competitive antagonist bicuculline was used in the cell line characterization. The antagonist successfully inhibited the influx of chloride ions, Figure 63. 4μM

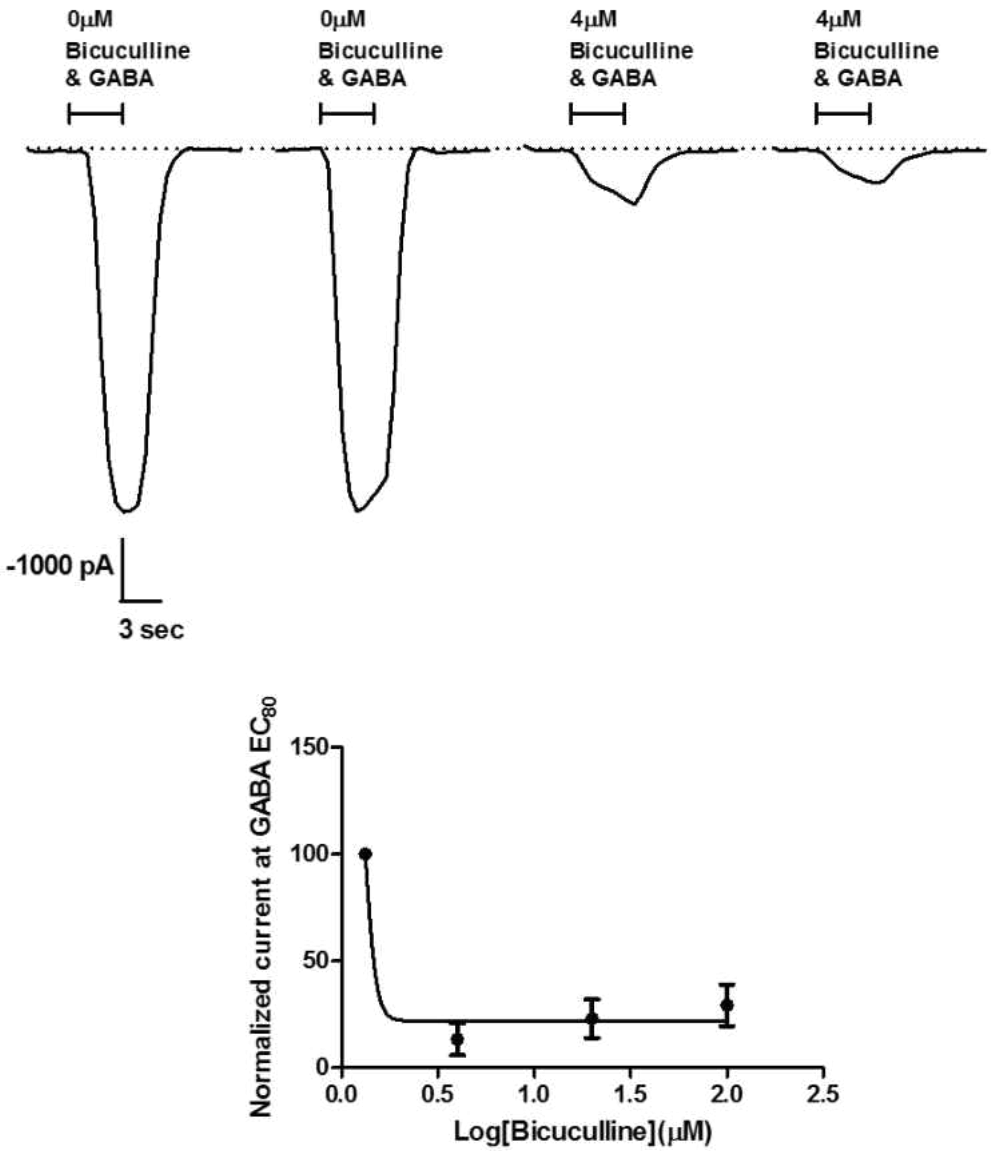


Figure 63. Cells stably expressing the α1β3γ2 $GABA_AR$ exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. Cells were preincubated with the antagonist for three minutes prior to activation with GABA. N=6

of bicuculline led to nearly complete inhibtion of the receptor ion channels. To ascertain whether the % DMSO effects the cell response over time, cells were dosed with GABA before and after three minute incubations with DMSO, Figure 64. The signal slightly increases overtime, with the response increasing 200pA from the first sweep to the last sweep. However this is a very slight change that should not interfere with analysis of modulators.

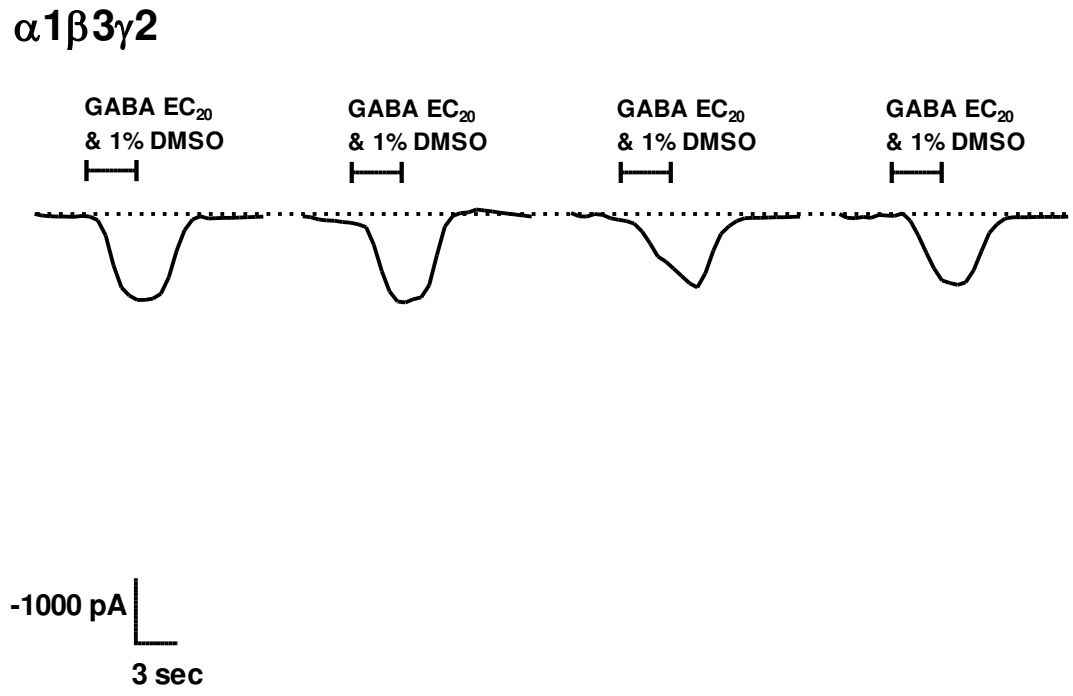


Figure 64. Response of cells after three minute incubations with 1% DMSO.

3.1.4.4 Conclusions

The creation of the recombinant stable cell lines expressing the $GABA_AR$ α1β3γ2 subtype resulted in multiple clonal lines with high expression of the receptor. Electrophysiological characterization of the cell lines showed the potential for high reproducibility of results even among different clone lines. In addition, the CMV promotor leads to a stable expression after integration. The retention of the genes of interest has shown long-term stability in the cell lines with minimal change after 10 passages (month in culture). The methods that we have described for creating stable cell lines could be applied to any genes and can easily be used to a manufacturing level on industry size scales.

3.2 αXβ3γ2 $GABA_AR$ Recombinant Cell Line

The three fragment constructs were planned to be completed in the following order: α4β3γ2, α5β3γ2, α3β3γ2, α2β3γ2, and α6β3γ2.

3.2.1 α4β3γ2 $GABA_AR$ RECOMBINANT CELL LINE

3.2.1.1 Molecular Cloning

3.2.1.1.2 Experimental

Cloning protocols

One Shot Mach1 T1 Phage-Resistant chemically competent cells (ThermoFisher, C862003) were used to generate DONR plasmids. PCR fragments were generated with flanking attB sites seen in Table 9 and ordered from Integrated DNA Technologies (IDT). The template DNA was combined with 1 µL of 20 µM of each of the primers, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM $MgSO_4$, 5 µL of 10X High Fidelity PCR Buffer, add 0.2 µL Platinum Taq DNA Polymerase High Fidelity (ThermoFisher, 11304-011) and the reaction mixture was diluted to 50 µL reaction. The thermal cycler (Eppendorf, Mastercycler RealPlex 4) was set for 30 PCR cycles with an initial denaturation step 94°C for 2 minutes, denaturing step at 94°C for 15 secs, annealing step at 55°C, and an extension phase at 68°C for 2 minutes.

PCR fragments were created using Q5 polymerase (NEB, M0491). The reaction mixture contained 10 µL of 5X Q5 reaction buffer, 1 µL of the 10 mM dNTPs, 1 µL each of 20µM forward and reverse primers, template DNA, 0.5 µL of Q5 High-Fidelity DNA Polymerase, 10 µL of 5X Q5 High GC Enhancer, and nuclease-free water to 50 µL. The PCR tubes were transferred to the thermocycler (Eppendorf, Mastercycler RealPlex 4) with an initial denaturation step at 98°C for 30 secs, with 30 cycles containing a denaturing step at 98° for 10secs, annealing phase at 55°C for

25secs, and extension step at 72°C for 45secs with a final extension at 72° for 2min. PCR products were purified by use of Diffinity Genomics RapidTip2 (Diffinity Genomics, RR050) which removed dNTPs, primers, primer-dimers and DNA polymerase from the sample. These pipette tips are added to a 100 µL Eppendorf Pipettor. The PCR products is aspirated into the pipette tip and mixed by pipetting up and down for one minute. The purified sample is dispensed into a PCR grade tube.

Antarctic Phosphatase (NEB, M0289) prevents self-ligation. Dephosphorylation of the 5'-ends involves 5µg of DNA in 1/10 volume of 10X AnP reaction buffer. 5 units or 1 µL of Antarctic Phosphatase was added before being incubated for 60min at 37°C. The mixture was heat inactivated for 5min at 70°C before moving to ligation.

T4 DNA Ligase (ThermoFisher, EL001) catalyzed the formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl. Linearized vector DNA at 50ng was combined with insert DNA at 150ng. 2 µL of 10X T4 DNA ligase buffer with 1 µL ligase was added and nuclease-free water was added to a total reaction volume of 20 µL. The reaction mixture was incubated for 10min at 22°C and 5 µL of the mixture was used to transform One Shot Mach1 T1 Phage-Resistant chemically competent cells (ThermoFisher, C862003). The cells were incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42°C without shaking and immediately transferred to ice for 2 min. SOC medium, 250 µL, was then added and incubated at room temperature. The tube was secured and shaken horizontally at 225 rpm at 37°C for 1 hr before being spread on pre-warmed

Restriction enzyme digestion was performed with 50 ng/µL of DNA with 4 µL of CutSmart buffer and 2 µL of ApaI. The reaction was incubated at 25°C for 60 min. 2 µL of BbvCI was added before the temperature was raised to 37°C for 60 min.

A series of control experiments involving multireactions were performed: reaction 1: pENTR L1-pLac-LacZa-L4/R4-pLac-Spec-R3/L3-pLac-Tet-L2/Dest, reaction 2: pENTR **Alpha2/**R4-pLac-Spec-R3/L3-pLac-Tet-L2/Dest, reaction 3: pENTR L1-pLac-LacZa-L4/**Beta3/**L3-pLac-Tet-L2/Dest, reaction 4: pENTR L1-pLac-LacZa-L4/R4-pLac-Spec-R3/**Gamma2/**Dest, reaction 5: pENTR **Alpha2/Beta3/Gamma2/**Dest. LR Clonase II Plus enzyme was added to the reaction mixture and incubated for 16 h at room temperature. Proteinase K was added for a 10min incubation at 37°C. 2 µL of the reaction mixture were transformed into Mach1 T1 cells and plated onto carbenicillin selection plates.

Refer to 3.1.2.2 Experimental for remaining cloning protocols.

3.2.1.1.3 Results and Discussion

The attB PCR products of all remaining alpha subunits was generated and BP recombination was performed with the BP clonase enzyme for insert into the DONRs for creation of the respective Entry Clone vectors. All BP recombination mixtures were transformed into competent One Shot Mach1 T1 cells. Growth on Kanamycin selection plates resulted in <100 colonies on each plate. The Entry Clones plasmids for the α3, α4, and α5 were created within two attempts, the first attempt failing due to quality of the PCR product. The third attempt yielded Entry Clones plasmids for the α2 and α6 without much difficulty.

The LR recombination reaction was performed with all completed Entry Clone plasmids in unison. Most plates yielded >10 colonies each with the exception of the α4 recombination which

resulted in growth of <20 colonies on ampicillin resistance plates. These colonies were all isolated and purified, sequencing confirmed the insertion of the α4 in one of the samples. However, though insertion of the β3 was successful, the γ2 insert was not present. When the recombination was repeated, this happened twice more with the α4. Since many of the colonies appeared not to contain any insert, antibiotic concentration was increased by two times from 100 µg/mL to 200 µg/mL In addition the temperature of the plate incubator was lowered from 37°C to 30°C. This would slow the rate of growth and in effect decrease the chances of plasmid loss or mutation. Despite this modification, the gamma 2 gene did not insert successfully.

The fact that the gamma 2 gene alone had a propensity to not recombine with the final construct could be due to instability of the insert as well as the large construct size. The γ2 Entry Clone quality, verified through diagnostic digest and 260/280 absorbance ratio above 1.8, did not appear to be the issue. To determine if a particular entry clone was flawed, control reactions were

Table 11. Troubleshooting reactions for the LR recombination reaction.

Reaction	Entry clone 1	Entry clone 2	Entry clone 3	Destination vector
1	L1-pLac-LacZa-L4	R4-pLac-Spec-R3	L3-pLac-Tet-L2	DEST vector
2	**Alpha2**	R4-pLac-Spec-R3	L3-pLac-Tet-L2	DEST vector
3	L1-pLac-LacZa-L4	**Beta3**	L3-pLac-Tet-L2	DEST vector
4	L1-pLac-LacZa-L4	R4-pLac-Spec-R3	**Gamma2**	DEST vector
5	**Alpha2**	**Beta3**	**Gamma2**	DEST vector

performed to analyze the number of colonies produced compared to the expected number in a given LR reaction. Control experiments with pENTR plasmids, Table 11, exhibited 30-50 colony growth except for reaction 5 containing all three of the inserts which had only 3 colonies. This may indicate that the combination of all three inserts resulted in toxicity or the accumulation of all three inserts is instable. When the insertion sites for the gamma 2 were examined, sequencing showed that they were undamaged. MAX Efficiency Stbl2 competent cells, suitable for cloning

large and unstable inserts, were purchased to offset instability of the plasmid. Due to the high background growth of colonies with an absence of entry clone, the destination vector was restored. A high background could be due to deletions, full or partial, of the ccdB gene in the destination vector so to maintain the integrity of the destination vector, the plasmid was propagated in ccdB Survival T1 E. coli strain in media containing carb and chloramphenicol.

DNA quantities, duration times, temperatures, plasmid qualities, etc were all examined for problems but did not overtly present any reason as to the difficulties experienced. Genetic

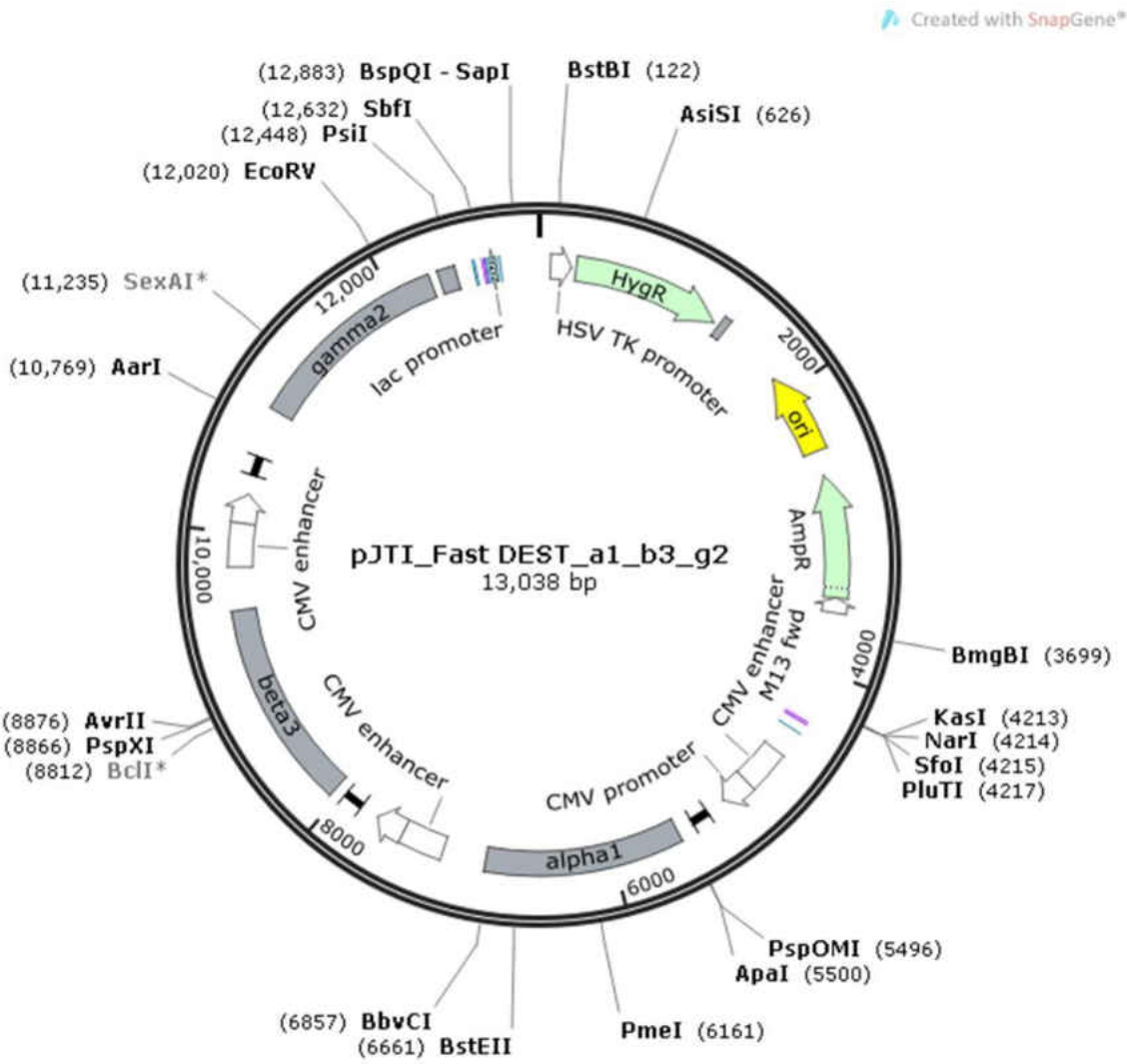


Figure 65. The completed α1β3γ2 construct with restriction enzyme sites.

sequencing, Clonase reagents, competent cells, and qRT-PCR reagents as well as other consumables were being repetitively and most times led to the creation of the empty DEST vector without recombination. As an alternative, the completed α1β3γ2 construct was used instead, shown in Figure 65. The finished construct contains all three fragments flanked with restriction enzymes. The alpha subunit is surrounded by the ApaI and the BbvCI which can be cut out and exchanged with alternative alpha subunits. PCR products were generated with ApaI and BbvCI sites for the α2 subunit (Primers ordered from IDT: AlphaApaI: 5'-TAAGCAGGGCCCGTGTCCACTCCCAGTTCAAT-3'; AlphaBbvCI: 5'-TGCTTAGCTGAGGATGTATCTTATCATGTCTGCTCGAAG-3') and were ligated into the pJTI_Fast DEST_a1_b3_g2 backbone with the α1 removed. However, this resulted in the recircularization of the backbone with the removed alpha1 fragment. Antarctic Phosphatase was used to dephosphorylate the 5' ends to prevent this phenomenon but resulted in no growth. Since the ApaI site is predominantly GC, Q5 polymerase was also tested since it has lower error rates and is renowned for its robust amplification with high GC content. Identical results were observed regardless.

Due to the time-consuming nature of these experiments, the costliness of the cells and enzymes, and the lack of molecular cloning equipment in the lab; it was decided that the most prudent decision was to contract out the remaining molecular cloning steps to another lab. Science exchange was used as an intermediary to hire Integrated Technology Enterprise, Inc based in Marietta, Georgia. The α1β3γ2 construct was sent to the independent contractor and the alpha 1 subunit was exchanged with α2, 3, 4, 5, and 6 to produce the remaining constructs. This lab utilized the same flanking ApaI and BbvCI sites with Gibson assembly to produce the plasmids and production of each one generally took one to two months for completion at an average cost of $350

per construct. The plasmid samples were amplified, purified, and analyzed upon receipt with no outstanding problems.

3.2.1.1.4 Conclusions

Though research has been published stating that recombination of constructs as large as 15-20kb is possible[223] the success rate and efficiency was lower than expected. There are many forums (Research Gate) discussing these issues with Gateway Cloning but the problem could have stemmed from multiple areas. Since Integrated Technology Enterprise had also stated difficulties in successfully growing the plasmids and had to move to lower temperatures for growth, this could be indicative that the γ2 DNA fragment of interest is toxic to the cells. In addition, the size of the construct may have necessitated use of electroporation for transformation. Incidentally, the reported construction of plasmids at 15-20kb using Gateway Cloning had electroporated into all competent cells[223].

In the future, in the creation of large constructs (>8,000bp) using Gateway Cloning, it may be fortuitous to invest in electroporation equipment as well as in electrocompetent cells. In addition, the linearization of the destination vector before LR recombination may be beneficial as the supercoiling of the vector may be preventing insertion.

3.2.1.2 Transfection and Clone isolation

3.2.1.2.2 Experimental

qRT-PCR Protocol

Refer to 3.1.3.2 Experimental. PCR primers used are listed in Table 12. Primers were ordered from Integrated DNA Technologies as 25nmole DNA oligos with standard desalting.

Table 12. qRT-PCR primers for $GABA_AR$ expressing stable recombinant cell lines

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
α2 (gabra2)	AAGAGAAAGGCTCCGTCATG	GCTTCTTGTTTGGTTCTGGAGTAG
α3 (gabra3)	ACAAGCACCACCTTCAACATAG	AGGTCTTGGTCTCAGCAGGA
α4 (gabra4)	GATGTCAACAGCAGAACTGAGGTG	TTGTGCCAGATCCAGAAGGTGGTG
α5 (gabra5)	GCCTTGGAAGCAGCTAAAATC	GAAGTCTTCTCCTCAGATGCTCT
α6 (gabra6)	CACTCTGACTCCAAGTACCATCTG	GTACACAAGGTTGAATCCTG
β3 (gabrb3)	CCTACTAGCACCGATGGATGTT	GATGCTTCTGTCTCCCATGTAC
γ2 (gabrg2)	CGCTCTACCCAGGCTTCACTAGC	TCGGGCCGAAGTTTGTTGTCGT
GAPDH (gapdh)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Automated Patch-Clamp Electrophysiology

Refer to 3.1.3.2 Experimental.

3.2.1.2.3 Results and Discussion

The α4β3γ2 construct was transiently transfected into HEK293T cells to ensure expression and function of the receptors. There is very little work published on the α4β3γ2 assemblies besides its hightened sensitivty to GABA (in comparison to other αXβ3γ2 subtypes) and it's inadequate expression levels in recombinant systems [211]. It has been reported that in frog oocytes, a cRNA

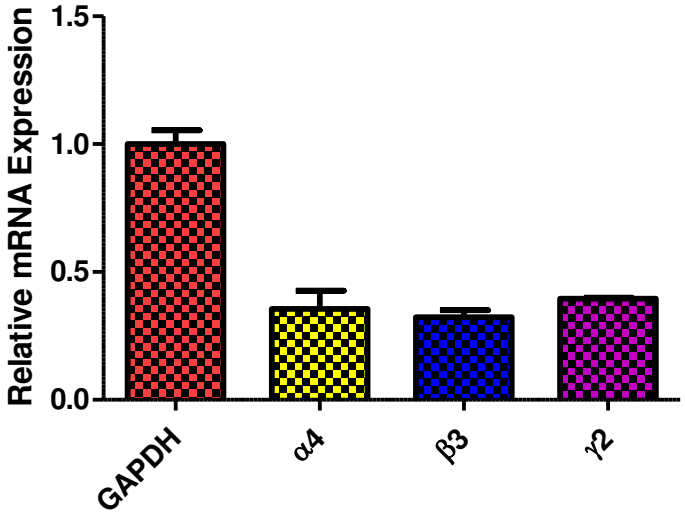


Figure 66. qRT-PCR of transiently transfected HEK293T cells containing the α4β3γ2 plasmid construct.

ratio of 5:1:1 for transient transfection for α4β3γ2 was required to observe consistent levels of expression. One publication has observed small increases in current with diazepam and GABA EC_{10} on frog oocytes expressing the α4β2γ2 and large increases in current for the β2γ2 assembly. Thus it was a concern using the plasmid as this would be a 1:1:1 ratio of subunit gene transcripts and may result in a herterogenous mixture of receptors, namely the α4β3 or the β3γ2. Both of these combinations respond to GABA while the β3γ2 responds strongly to diazepam. The expression of α4β3 has been reported to have condiderably low expression levels [230] so the major interfering assembly would be the β3γ2 [211]. The mRNA expression levels after transient transfection of the α4β3γ2 construct appeared to suggest there was expression of all subunits, though with lower expression of the alpha subunit, Figure 66. The GABA response did not reach saturation, Figure 67, which may have been indicative of a heterogenous population of receptors.

When diazepam was tested, Figure 68, the compound elicited a small change in current despite being regarded as a benzodiazepine-insensitive receptor. However, the GABA EC_3 was

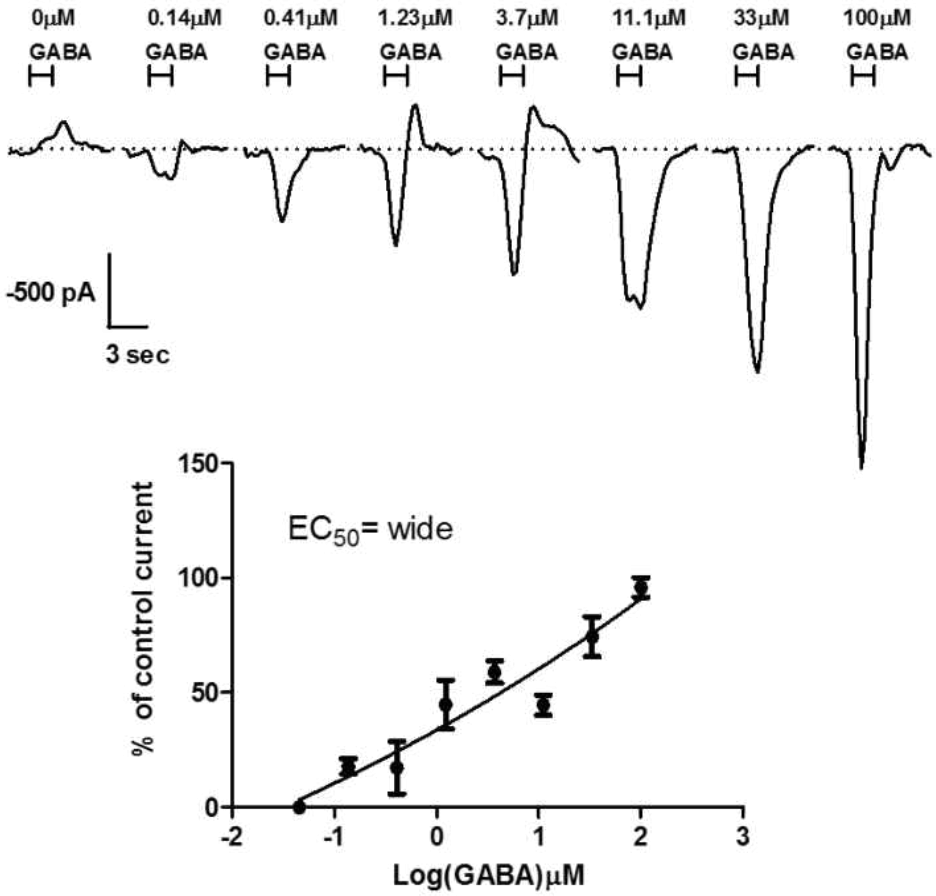


Figure 67. Cells transiently transfected with the α4β3γ2 construct exposed to increasing concentrations of GABA. N=4

estimated to be 0.1μM which may not have been sufficient, as evidenced by the GABA dose response in Figure 67 and the lack of initial negative current when the cells were dosed with GABA EC_3 prior to application of the compound in Figure 68.

This same current response was generated with the application of the imidazobenzodiazepine HZ-166, Figure 69. The large positive response to GABA EC_3 was

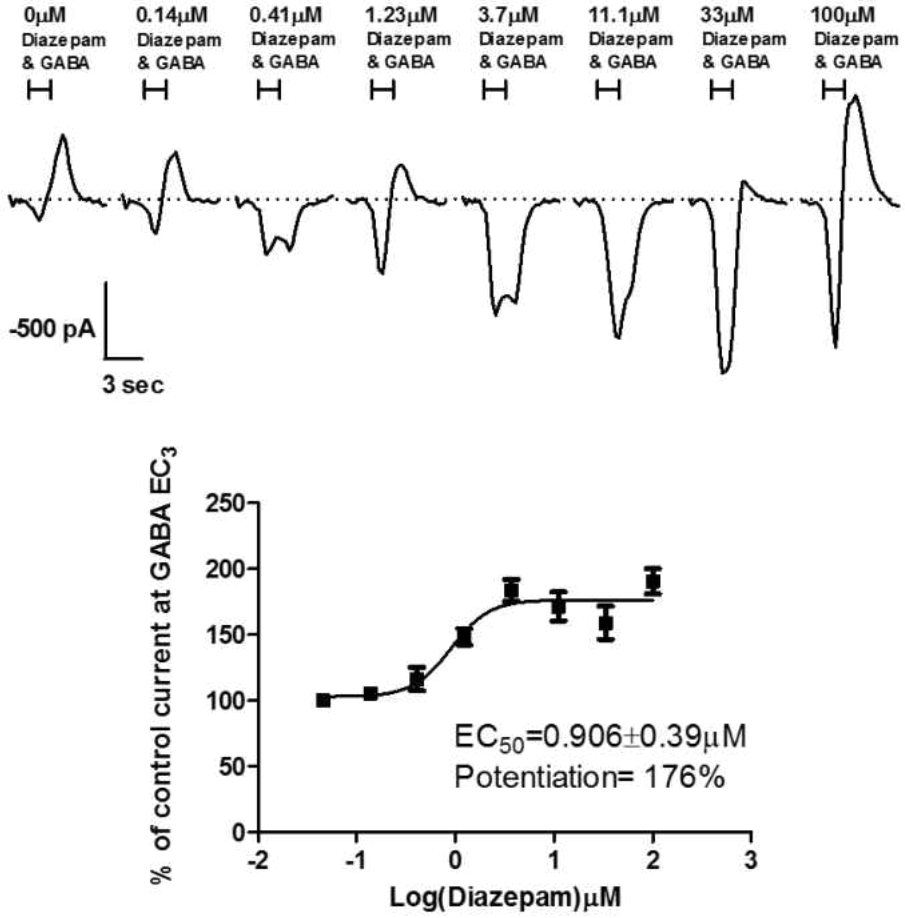


Figure 68. Cells transiently transfected with the α4β3γ2 construct exposed to increasing concentrations of diazepam in combination with the estimated GABA EC_3. The maximum DMSO concentration was 1%. N=4

transformed to negative current at 1.23μM HZ-166 and the signal increased further at 33 to 100μM.

In addition, the modulator XHE-III-74 Ethyl Ester (XHE-III-74EE) was tested. These compounds have been shown to cause a greater change in current for oocytes expressing the α4

and α6 [231]. The response of the transiently transfected cells to XHE-III-74EE was similar to both diazepam and HZ-166.

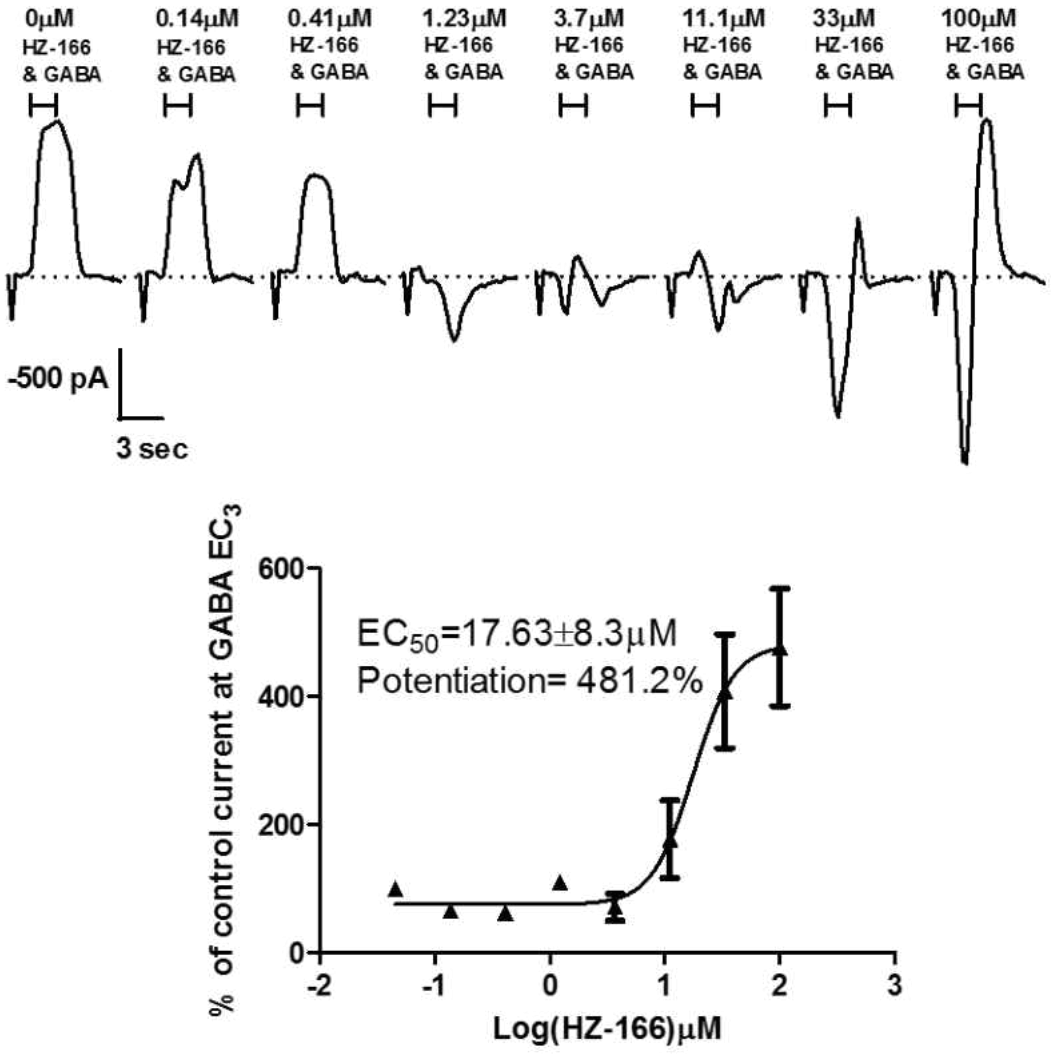


Figure 69. Cells transiently transfected with the α4β3γ2 construct exposed to increasing concentrations of HZ-166 in combination with the estimated GABA EC_3. The maximum DMSO concentration was 1%. N=4

The seemingly large initial positive current response to only extracellular solution and GABA may be the result of the small current changes near a noisy baseline. The changes in current reached a maximum response in a range of -500-1000pA. The reduction in the size of the current

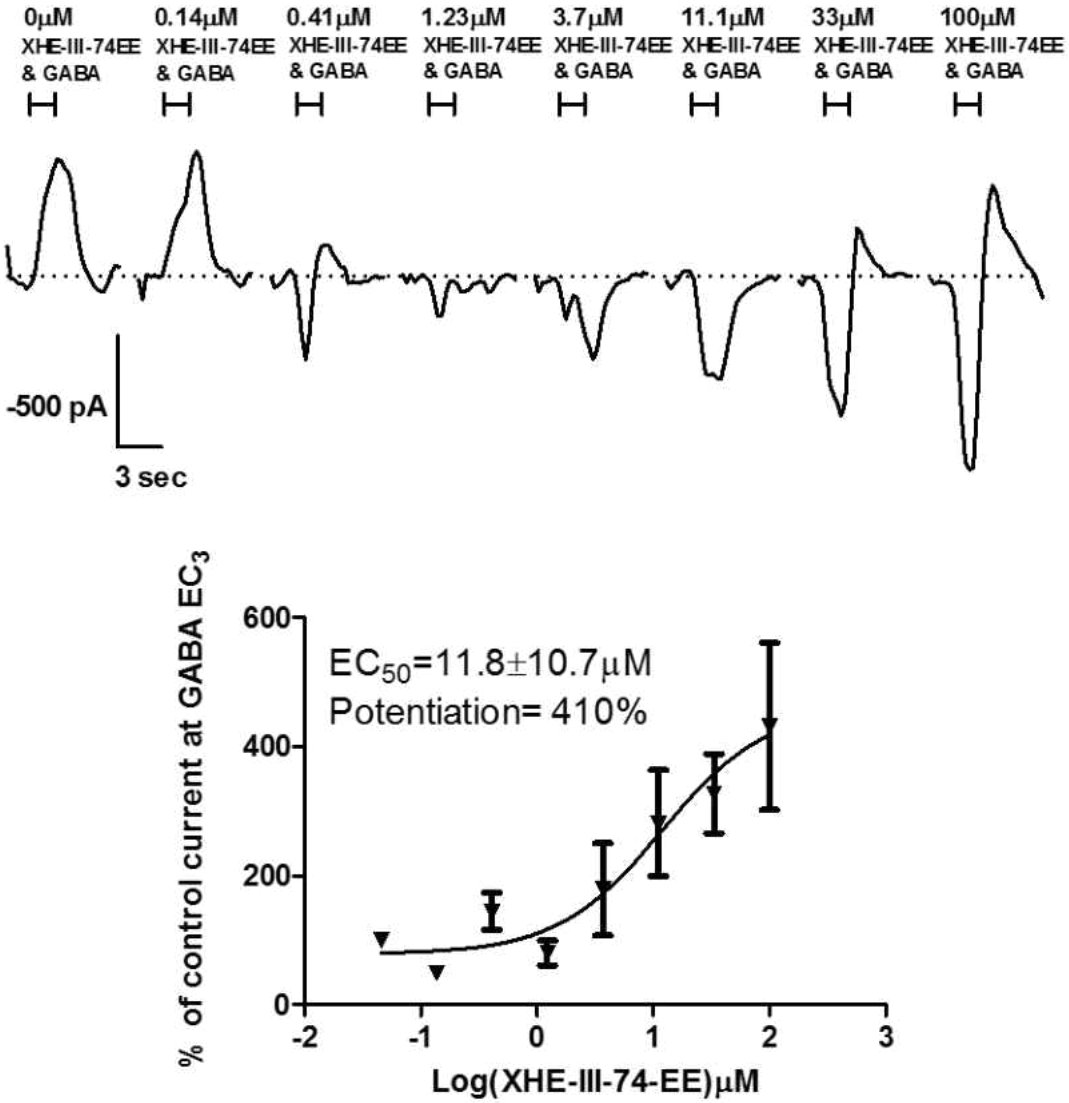


Figure 70. Cells transiently transfected with the α4β3γ2 construct exposed to increasing concentrations of XHE-III-74EE in combination with the estimated GABA EC3. The maximum DMSO concentration was 1%. N=4.

changes could be the result of poor transfection efficiences. These anomalous rectifying properties have been observed for both α4 and α6 containing receptors expressed in HEK293 cells previously

[232]. However, the signal can be improved by altering the intracellular and extracellular buffer composition. This problem was confronted with the isolation of the first α4β3γ2 clones, discussion of this can be found in 3.2.1.3.3 Results and Discussion.

3.2.1.2.4 Conclusions

Transfection with the α4β3γ2 resulted in both mRNA expression of the subunits and a recordable electrophysiological response to application of GABA. This confirms the result of the previous work performed with the α1β3γ2. Transfection with these large three fragment constructs results in successful expression of the receptor subunits. Although mRNA expression levels and the overall current signal was low (<1000pA), isolation of homogenously pure clones with high expression of the receptors would theoretically resolve this problem.

3.2.1.3 Characterization of Clone

3.2.1.3.2 Experimental

Automated Patch-Clamp Electrophysiology

The buffers were made from NaCl (Fisher, BP358-1), KCl (Fisher, BP366-1), $MgCl_2$ (Sigma, M8266), $CaCl_2$ (Acros Org, 123350025), Glucose (Sigma, G0350500), HEPES (Fisher, BP410-500), CsCl (Sigma, 203025), and EGTA (Tocris, 28-071-G). The extracellular solution contains: 238mM NaCl, 4mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 5.6mM Glucose, and 10mM HEPES at pH 7.4. The intracellular solution contains: 60mM KCl, 15mM NaCl, 70mM KF, 5mM HEPES, and 5mM EGTA at pH 7.25.

Optimization of the solutions not only changed the concentrations of chloride ions but also included the addition of cesium ions which blocks potassium leak current[233]. The extracellular solution contains 140 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose monohydrate, 10

mM HEPES, and pH 7.4 with NaOH. The intracellular solution contains 140 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, and pH 7.2 with CsOH. Compounds were diluted to 10 mM in DMSO.

To record $GABA_A$ currents, cell arrays were voltage clamped at a hyperpolarizing holding potential of -80 mV. Cells were centrifuged at 380g for 2 min and gently resuspended in ECS. This was repeated two more times before the cells were dispensed into the plate.

3.2.1.3.3 Results and Discussion

Transfection of the α4β3γ2 led to change in the morphology of the HEK293T cell line, Figure 71A-B. This was not observed after tranfection with any of the other $GABA_A$ receptor subtypes. Many of the cells appeared to exhibit loss of adherence, Figure 71B. Such changes in morphology of the HEK293T after transfection has been reported before [234]. In addition, antibiotic selection led to nearly complete elimination of all cells, Figure 71C. Cells which were isolated into 384 well plates immediately after the ten day selection perished soon after. However, if the cells were left to recover on the original selection plate for five more days (Figure 71D) and then isolated, clone survival increased and expansion of the cells could proceed.

This morphological change and low survival rates of transfected cells may suggest that the α4β3γ2 is toxic to the cells as well as confirming low expression and integration levels of the gene. Since the selection and isolation of the α4β3γ2 was more time-consuming and inefficient than the

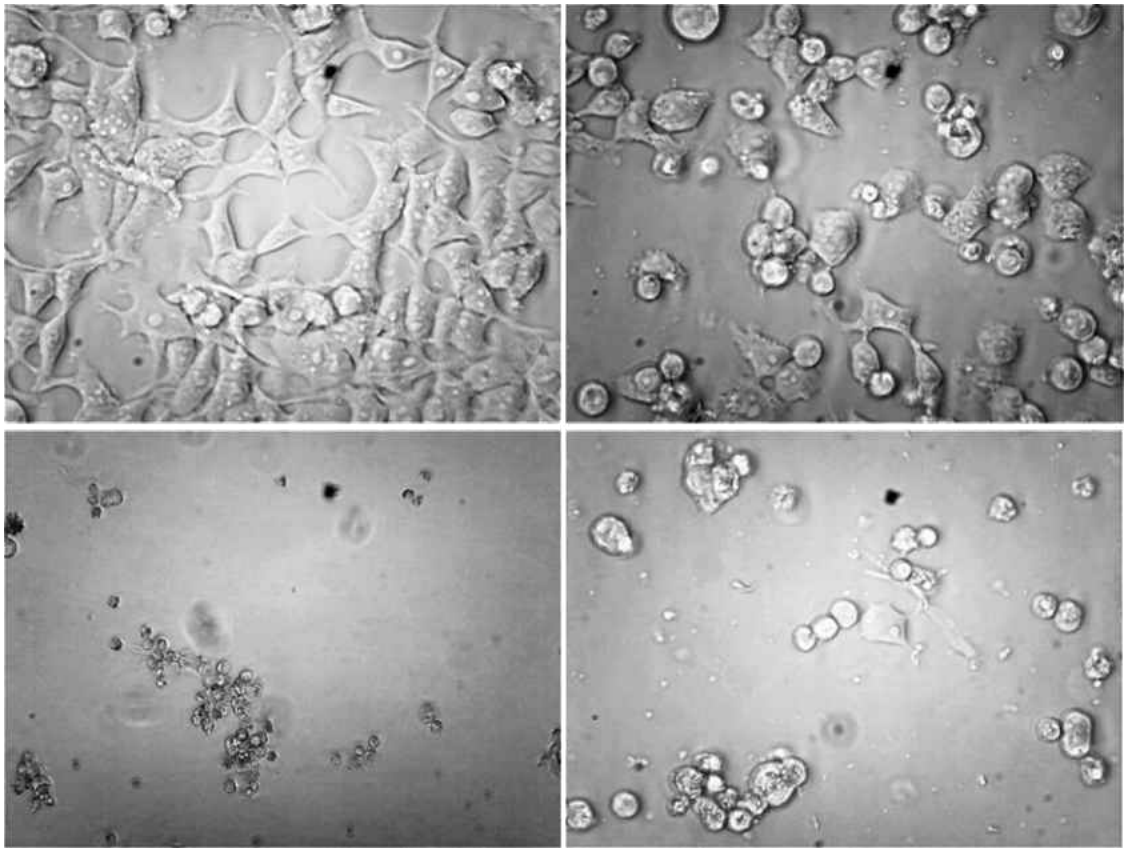


Figure 71. Images of the HEK293T cells during clonal isolation of the α4β3γ2 expressing cell lines. A) Image of uhealthy HEK293T cell line. B) Image of HEK293T cells after transfection using the α4β3γ2 construct. C) Cells after ten day exposure to Hygromycin B. D) Cells after five day recovery following antibiotic selection.

α1β3γ2, many more of the clones were isolated and expanded for analysis. The mRNA levels of 35 clonal cell lines were evaluated, results seen in Figure 72. Cell lines 1, 5, 17, and 27, expressing

varying levels of subunit mRNA, were examined further using electrophysiology. Experiments

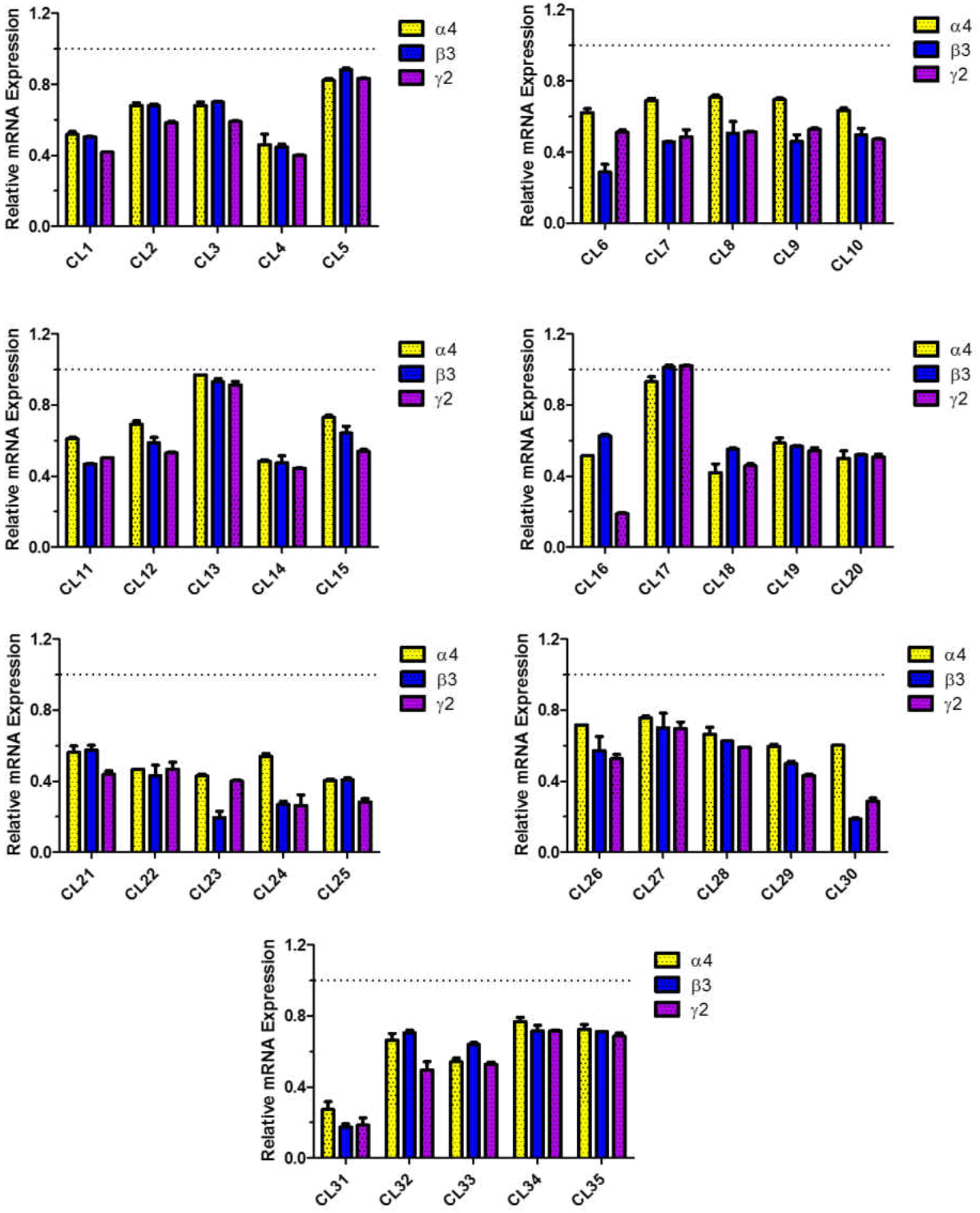


Figure 72. qRT-PCR of clonal cell lines (CL) of HEK293T cells stably expressing the α4β3γ2 plasmid construct. GAPDH is normalized to 1.0, designated with a dashed line. N=3 for each bar.

with these cell lines, Figure 73, exhibited an initial positive current which was previously observed in transiently transfected cells. All of the stable cell lines had a current response of <1500pA. Thus

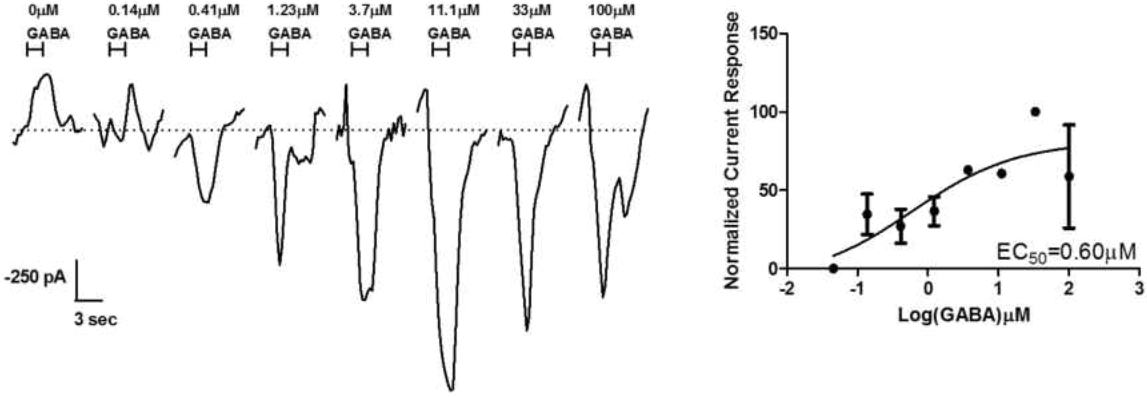


Figure 73. Cell line 1 isolated from HEK293T stably expressing the α4β3γ2. N=2

these low currents, despite stable and homogenous expression, must be the result of an inherent characteristic of the α4β3γ2.

The pipette solution (intracellular solution) is often designed to mimic the chloride concentrations in the natuaral physiological level. However, delieberately using high chloride concentrations can increase the chloride driving force. This can facilitate the detection of small inhibtory postsynaptic currents [235]. To test this, we switched to a cesium and chloride rich intracellular solution to block potassium leak currents as well as enhance small iPSCs.

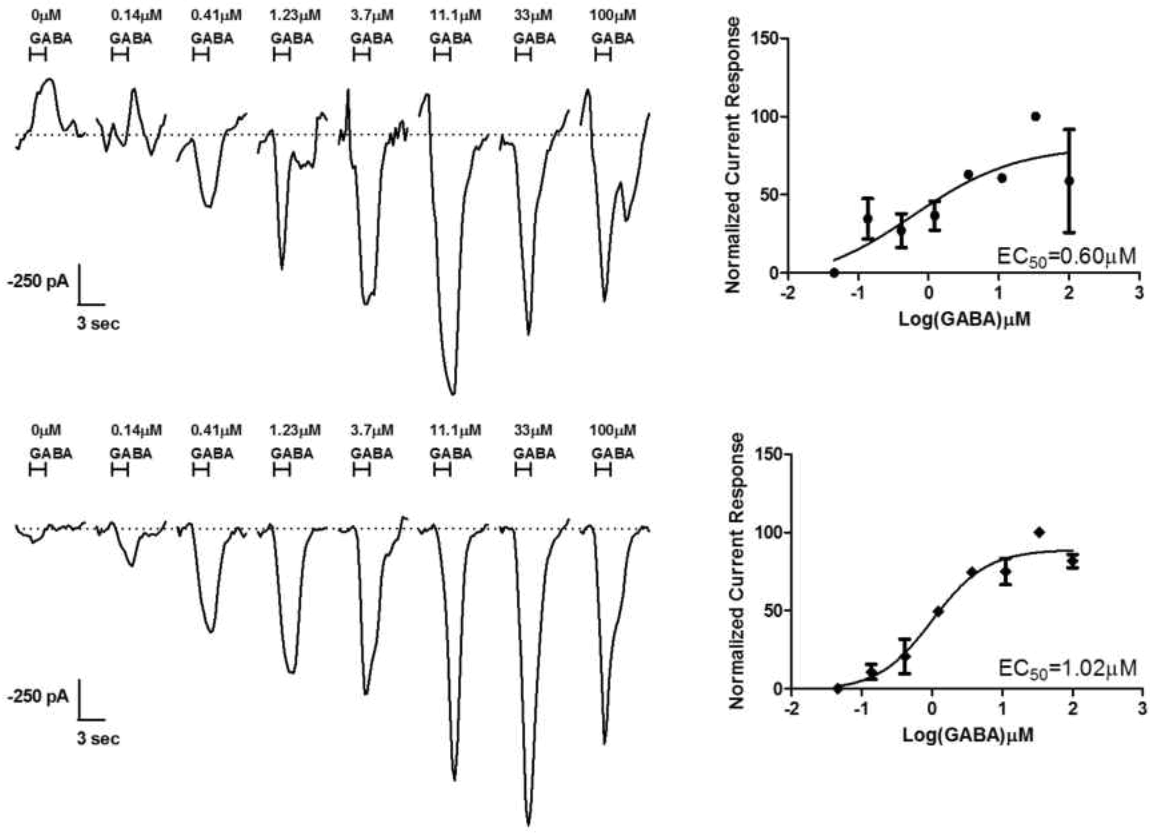


Figure 74. Comparison of current responses in Cell line 1 isolated from HEK293T stably expressing the α4β3γ2. Top: The typical IonFlux solutions elicited a noisy baseline. Bottom: A cesium and chloride rich buffer reduced the positive current changes. N=2

Figure 74 exhibits the differences in current between using the two buffer formulations. Though the maximum current response of the cells remained the same (~1200pA), the noise at the baseline changed signifantly. This was true for all of the α4β3γ2 stable cell lines tested, Figure 74-Figure 77. Despite the fact that CL5 and CL17 have the highest overall expression of the $GABA_AR$ mRNA, they exhibited the same overall current response to GABA. The maximum current response in the first solutions averaged 925 pA while after changing the buffer it averaged 1375 pA. To keep the results of the $GABA_AR$ cell lines uniform across all subtypes, the formulas of the cell solutions were permanently changed to the cesium and chloride rich buffers.

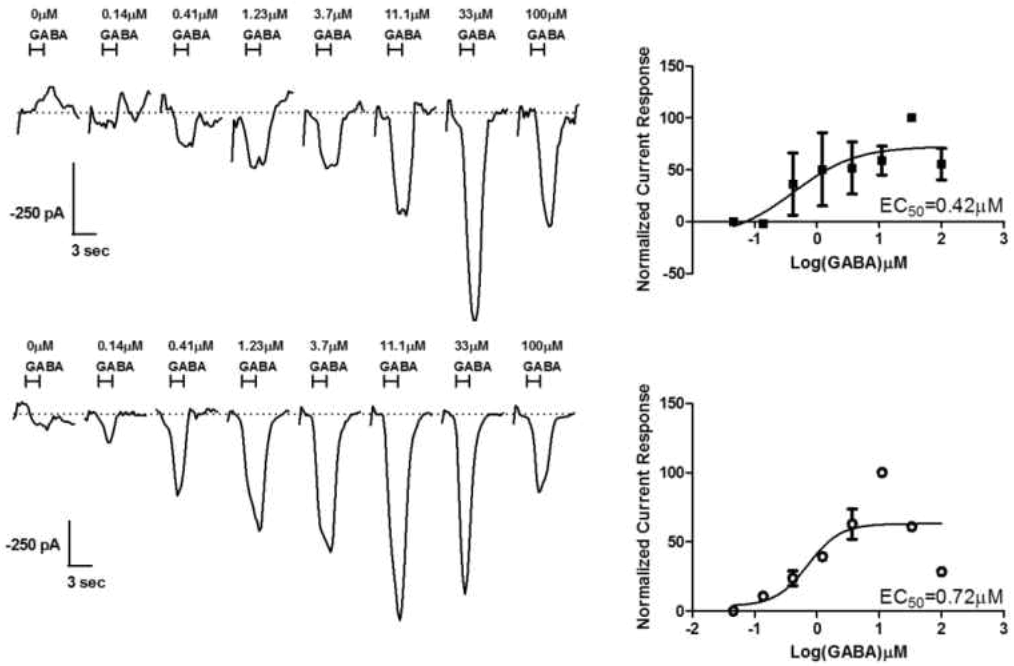


Figure 76. Comparison of current responses in Cell line 5 isolated from HEK293T stably expressing the α4β3γ2. Top: The typical IonFlux solutions elicited a noisy baseline. Bottom: A cesium and chloride rich buffer reduced the positive current changes. N=2

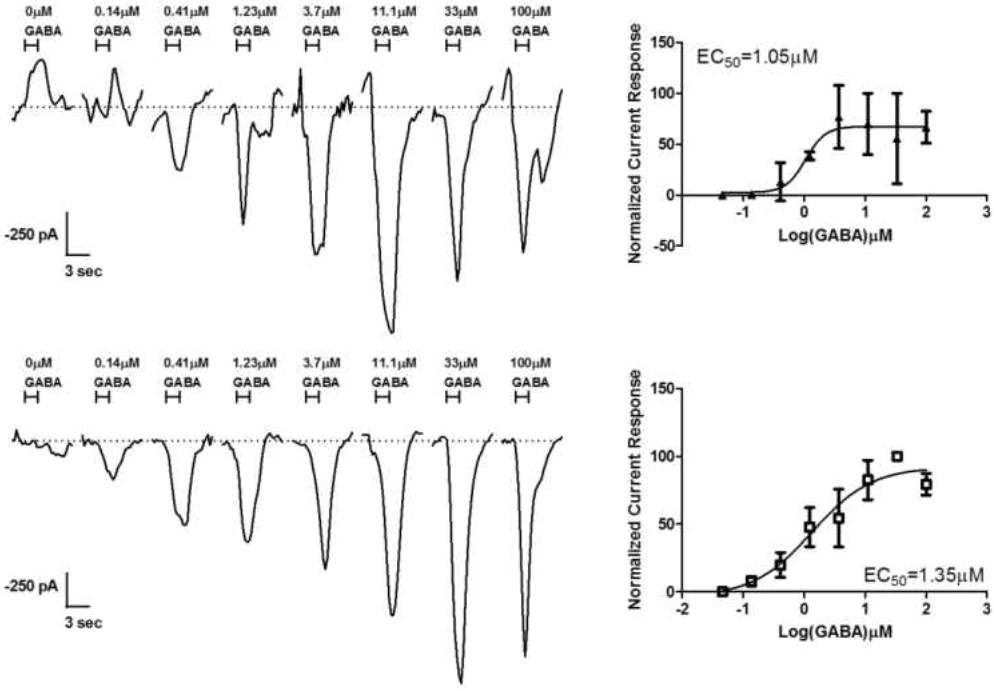


Figure 75. Comparison of current responses in Cell line 17 isolated from HEK293T stably expressing the α4β3γ2. Top: The typical IonFlux solutions elicited a noisy baseline. Bottom: A cesium and chloride rich buffer reduced the positive current changes. N=2

The GABA affinity for α4β3γ2 is 2-3 fold higher for the α4 than the α1. In addition, the α4

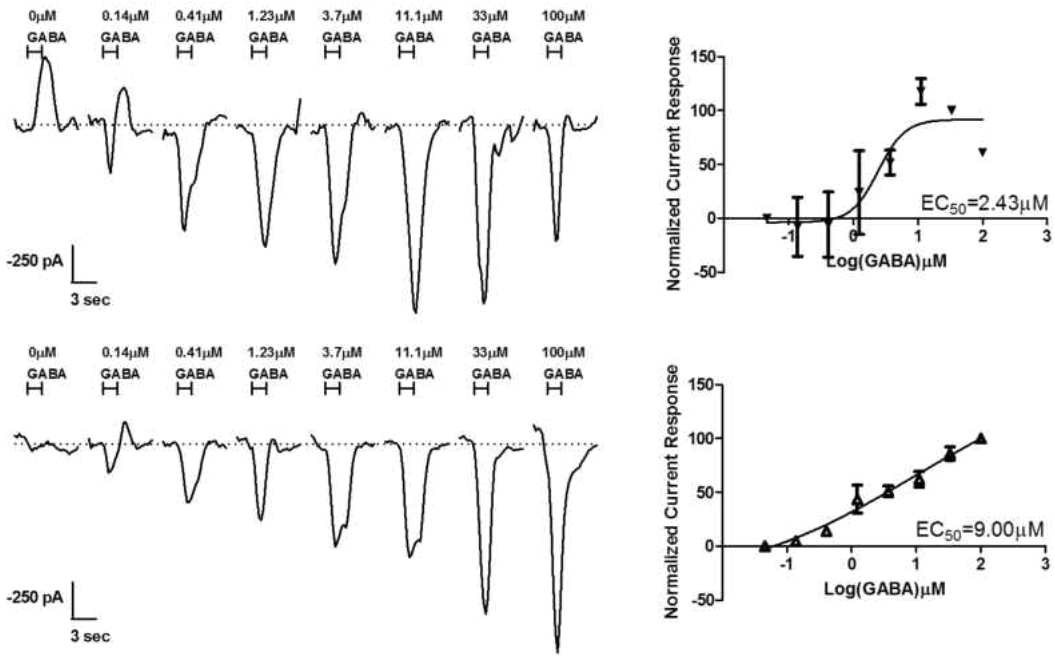


Figure 77. Comparison of current responses in Cell line 27 isolated from HEK293T stably expressing the α4β3γ2. Top: The typical IonFlux solutions elicited a noisy baseline. Bottom: A cesium and chloride rich buffer reduced the positive current changes. N=2

is easily oversaturated during prolonged low levels of GABA to suppress the overall response and desensitizes rapidly to repetitive GABA application.

In addition, since it has been reported that the diazepam produces a biphasic potentiation with distinct components in the nanomolar and micromolar [215]. The two components become more distinct in the presence of low GABA concentrations from EC_3 to EC_{10}, thus we determined that testing with the modulators should be performed at GABA EC_{20} so as not to overestimate the potentiation of the compounds.

As CL1 exhibited a high current response as well as the least deviation, this cell line was further analyzed using BZDs diazepam and HZ-166.

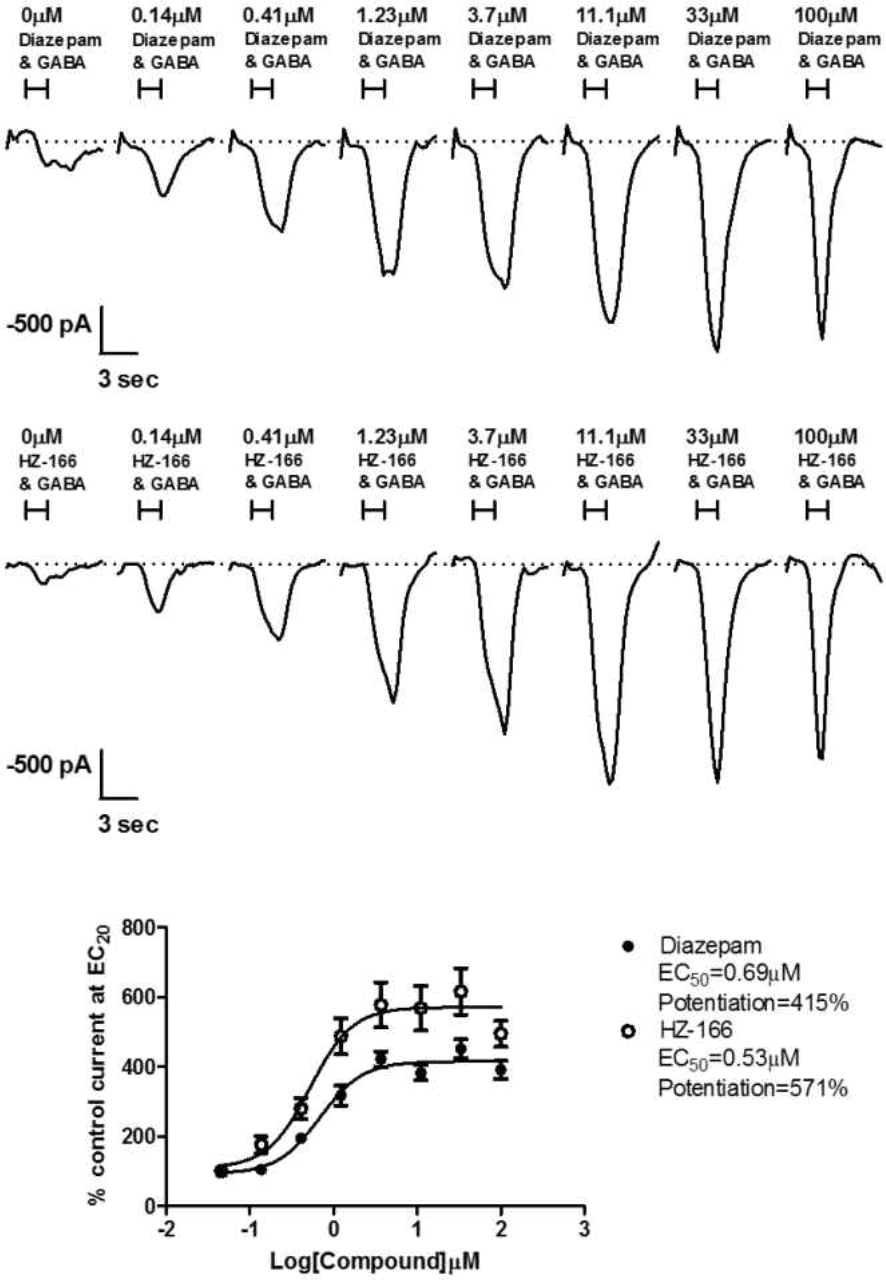


Figure 78. Current responses in CL1 isolated from HEK293T stably expressing the α4β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 0.3μM and DMSO concentrations of 1%. N=8

Extensive experiments were performed with these two modulators, using a constant concentration of GABA EC_{20} 0.3μM and a high concentration of 100μM compound, the current elicited resulted in high potentiation and current response of around -2000pA.

As this receptor is traditionally termed a BZD insensitive $GABA_AR$ subtype, it was theorized that the concentrations of the compounds was too high possibly creating a problem with

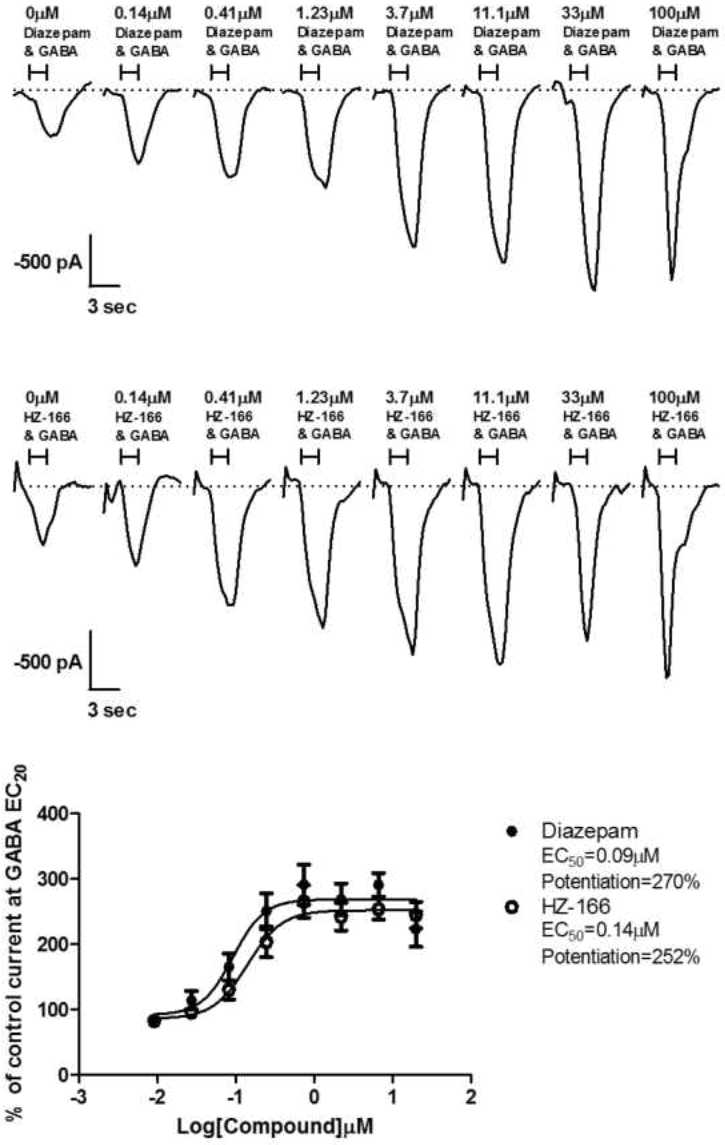


Figure 79. Current responses in CL1 isolated from HEK293T stably expressing the α4β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 0.3μM and DMSO concentrations of 0.3%. N=8

the solubility, or the percentages of DMSO was too high. Thus the concentrations of compound was lowered to 20μM and the DMSO percentages lowered to 0.3%. This experiment resulted in

Figure 79. The cells had the same maximum current response of -2000pA with the same general curve shape of the dose response.

These findings are in agreement with previous research published on the rat $GABA_AR$ α4β3γ2 expressed in *Xenopus* oocytes [236]. This study observed that both diazepam and flunitrazepam significantly potentiated GABA-gated currents in α4β3γ2 expressing oocytes but not for α4β2γ2 receptors. Indicating that β3 plays some role in the observed effect. Though this publication attempted to express α4β3γ2 in HEK293 cells, low expression levels forced them to substitute the signal peptide sequence of the β3 with the β2. The resulting curves in HEK293 cells showed little potentiation of GABA-evoked currents. A majority of studies exhibiting the α4/6 containing receptors insensitivity to benzodiazepines are performed in combination with the β2γ2 [211,237-240]. Taking our own measurements into consideration, this may suggest that the signal peptide of the β3 subunit is critical in the benzodiazepine sensitivity of these assemblies and previous findings observing that the receptors were benzodiazepine insensitive. However, we cannot discount the possibility that this binding is a result of binding at the α4β3 interface, rather than the α6γ2 interface. Binding at the α1β3 interface [241] as well as the α6β3 has been observed previously.

In addition to testing the positive modulators diazepam and HZ-166, antagonist bicuculline was used on all the created cell lines, Figure 80. Cells were pre-incubated with bicuculline for three minutes prior to the application of GABA. Most cell lines were inhibited with 0.4μM

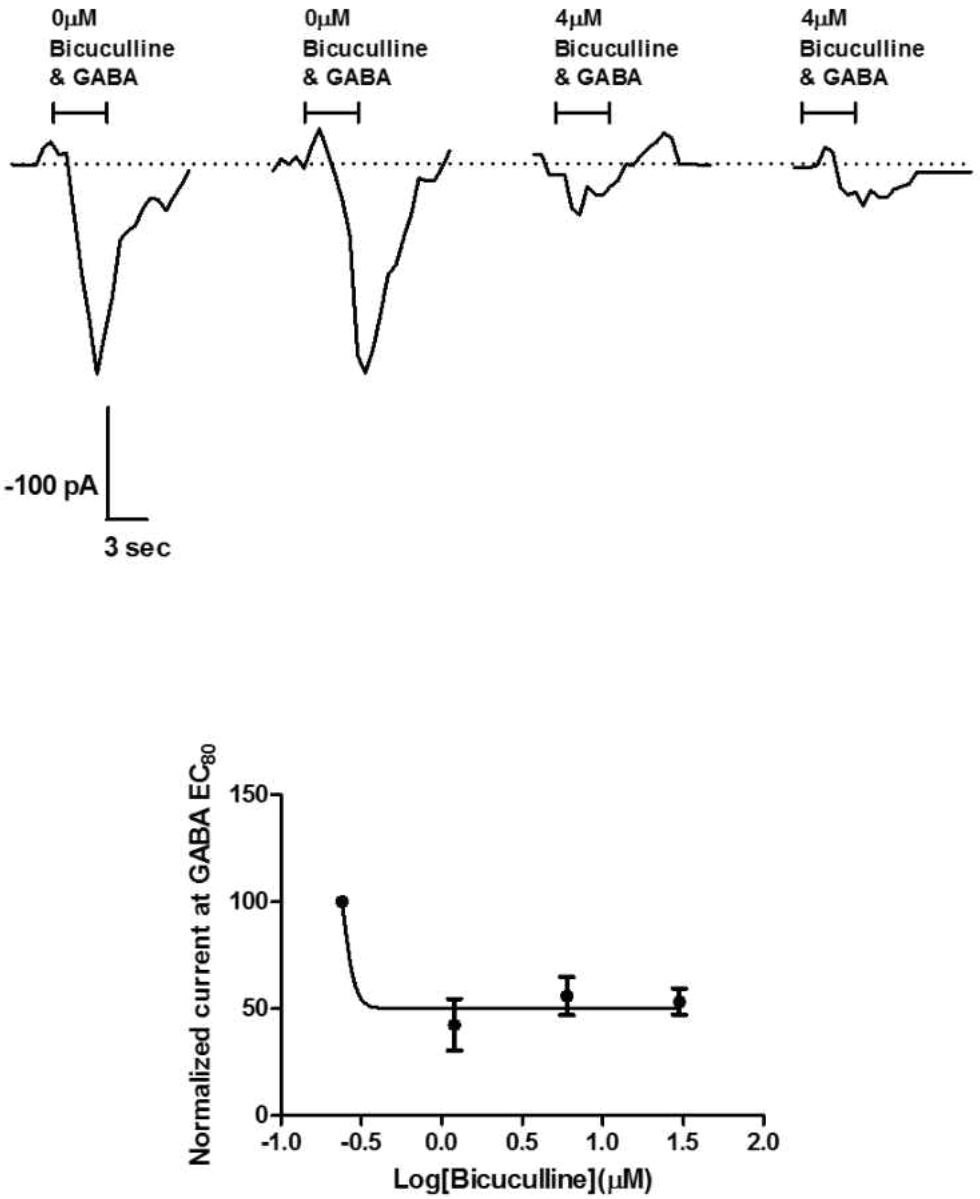


Figure 80. Cell line 1 stably expressing the α4β3γ2 $GABA_AR$ exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. Cells were preincubated with the antagonist for three minutes prior to activation with GABA. N=6

bicuculline which was the smallest concentration of the compound. Functional studies on recombinant and native $GABA_AR$ have reported that subunit composition does not affect the

antagonist action of bicuculline [242]. Unlike agonists and positive modulators, it would appear that antagonist activity is independent of the composition [243]. However, receptors which contain the α6 subunit have been observed to be less sensitive to bicuculline [244].

To ascertain whether the % DMSO effects the cell response over time, cells were dosed with GABA before and after three minute incubations with DMSO, Figure 81. The signal did not vary significantly over time, ranging from -148pA to -184pA.

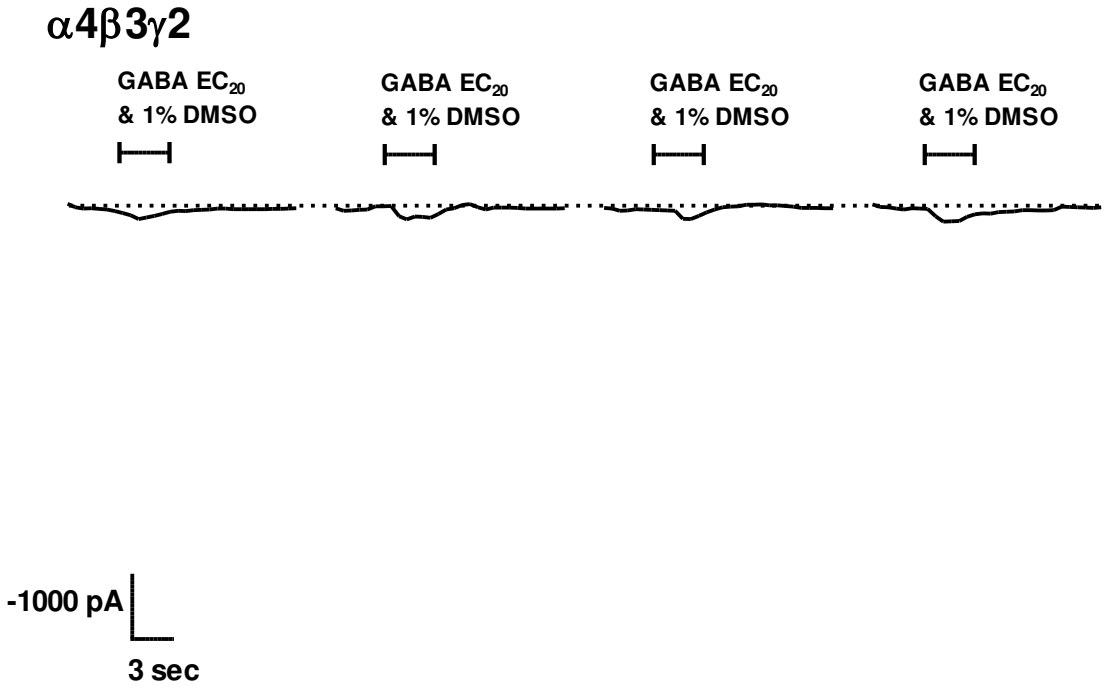


Figure 81. Response of cells after three minute incubations with 1% DMSO.

3.2.2 α5β3γ2 $GABA_AR$ RECOMBINANT CELL LINE

3.2.2.1 Transfection and Characterization of Clones

The α5β3γ2 construct, which was received during the clonal cell line isolation of the α4β3γ2, was also transiently transfected into the HEK293T cell line. The mRNA expression (primers listed in Table 12) confirms that transfection was successful, seen in Figure 82. The cells were also electrophysiologically characterized after transient transfection. This experiment was performed

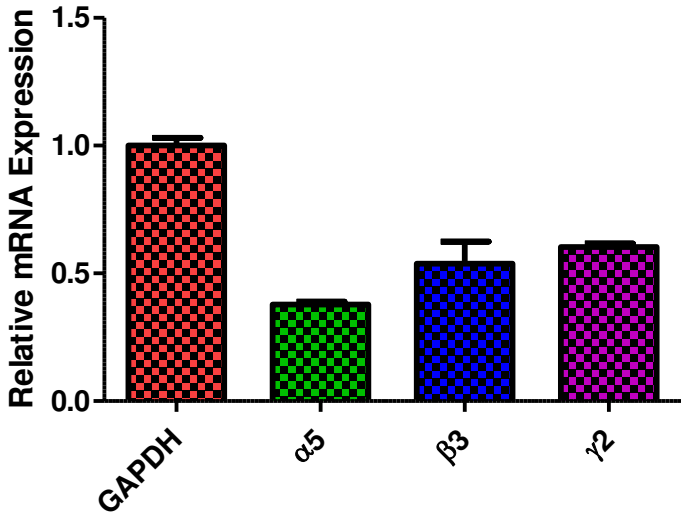


Figure 82. qRT-PCR of transiently transfected HEK293T cells containing the α5β3γ2 plasmid construct. N=3

with the initial formulation of low chloride, no cesium, intracellular solution. The average current response was around -1000pA for these transiently transfected cells, Figure 83.

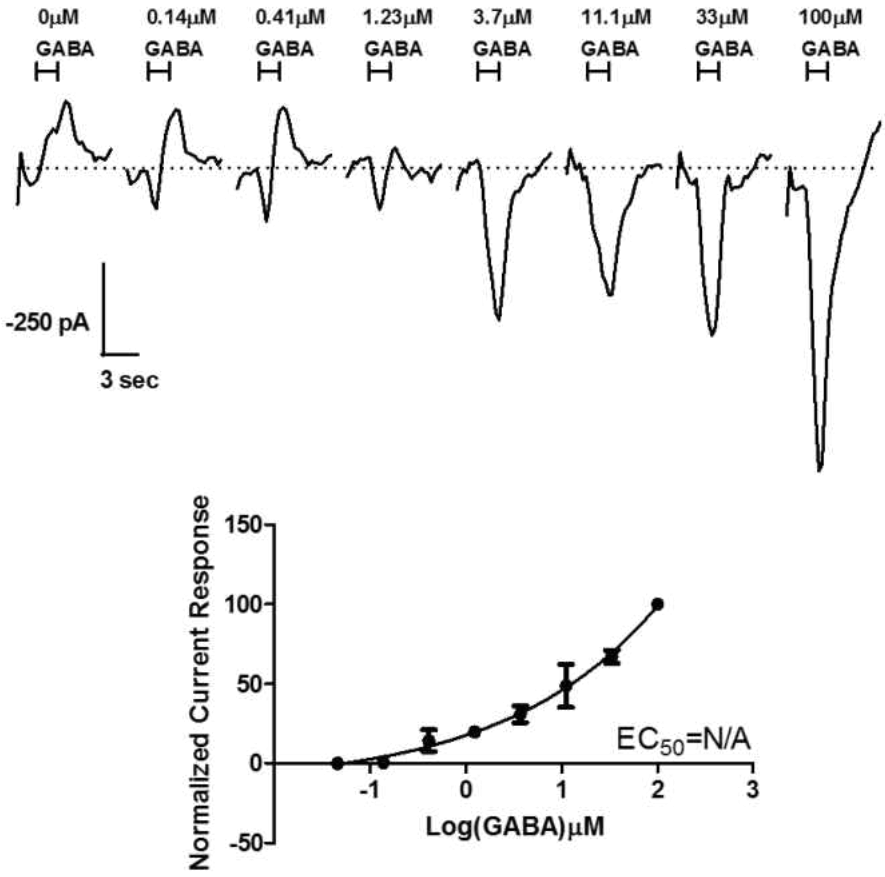


Figure 83. Cells transiently transfected with the α5β3γ2 construct exposed to increasing concentrations of GABA. N=4.

Transfection, antibiotic selection, and clone isolation of the α5β3γ2 into HEK293T did not present any serious concerns on morphology or survival. Eleven cell lines were isolated and their mRNA levels were characterized, Figure 84. Cell lines 5 and 7 were among the clones having the highest expression. These two cell lines were characterized further by automated patch clamp using the IonFlux.

Both cell line 5 and 7 both achieved high inhibitory current responses to the application of GABA. Cell line 5 had the largest current response, reaching -14,000pA as seen in Figure 86. Cell

line 7 reached a maximum negative current of -10,000pA, as seen in Figure 85. These dose response curves exhibit the α5β3γ2's low GABA sensitivity, as was previously reported [83].

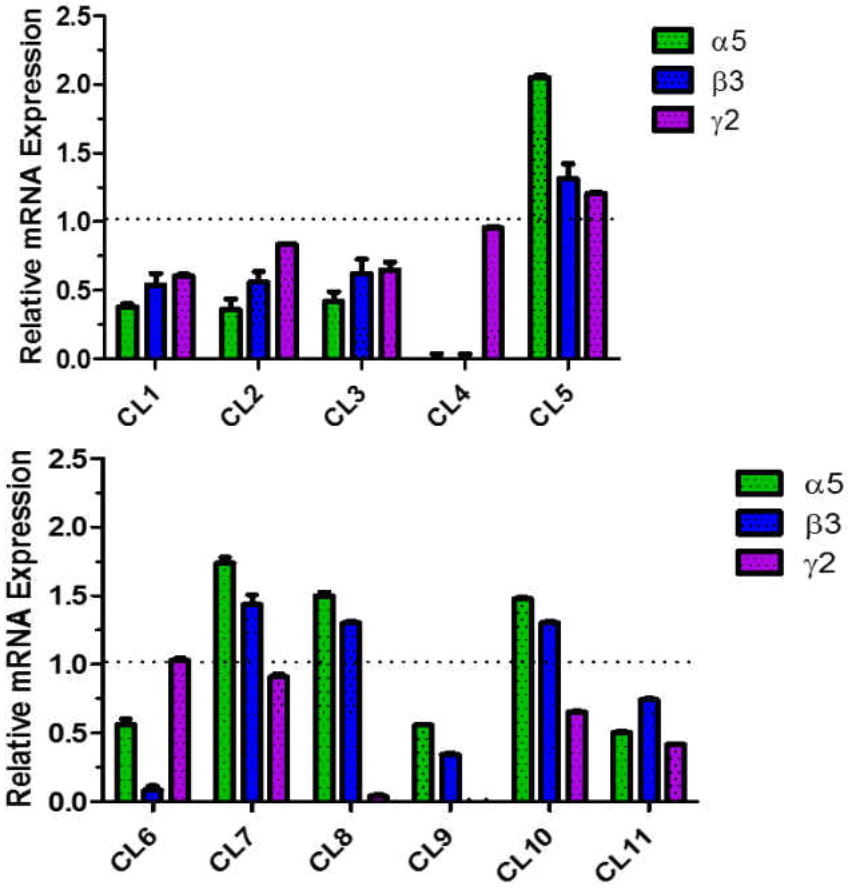


Figure 84. qRT-PCR of clonal cell lines (CL) of HEK293T cells stably expressing the α5β3γ2 plasmid construct. GAPDH is normalized to 1.0, designated with a dashed line. N=3 for each bar.

The response of the cells to positive modulators diazepam, HZ-166, and MP-III-004 were initially evaluated at GABA EC_3 with a serial dilution of DMSO. This resulted in the curves seen in Figure 88, the sweeps for these curves are shown in Figure 87. Tests with MP-III-004 reported an α5 selectivity, perfomed by collaborator Dr. Margot Ernst on frog oocytes. These experiments in oocytes yielded a potentiation of ~600% at maximum 10μM, This is in high agreement to our results on the automated patch clamp. This compound, when tested in parallel with HZ-166 and

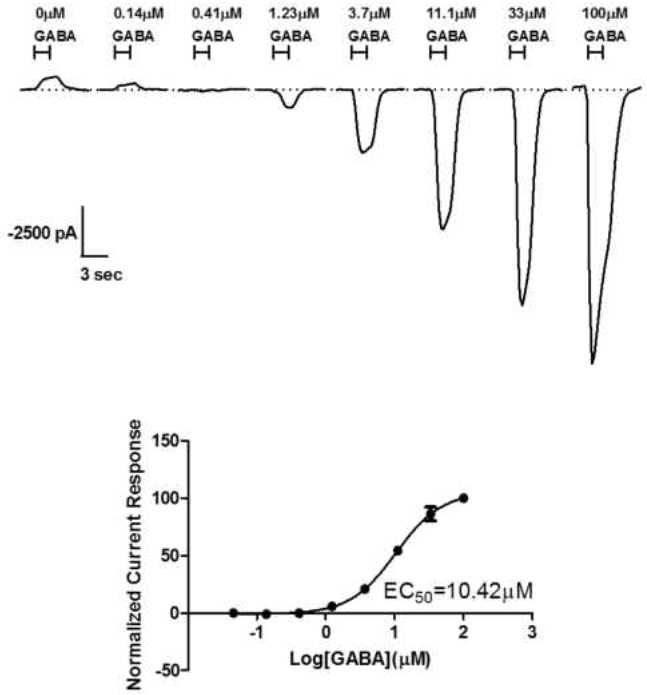


Figure 86. Current responses in Cell line 5 isolated from HEK293T stably expressing the α5β3γ2. N=2

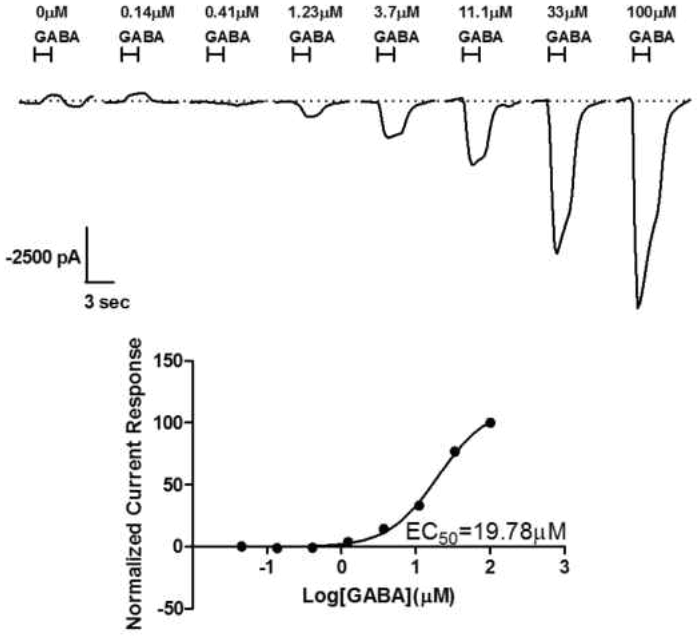


Figure 85. Current responses in Cell line 7 isolated from HEK293T stably expressing the α5β3γ2. N=2

diazepam, have nearly identical values for the maximum current response and calculated

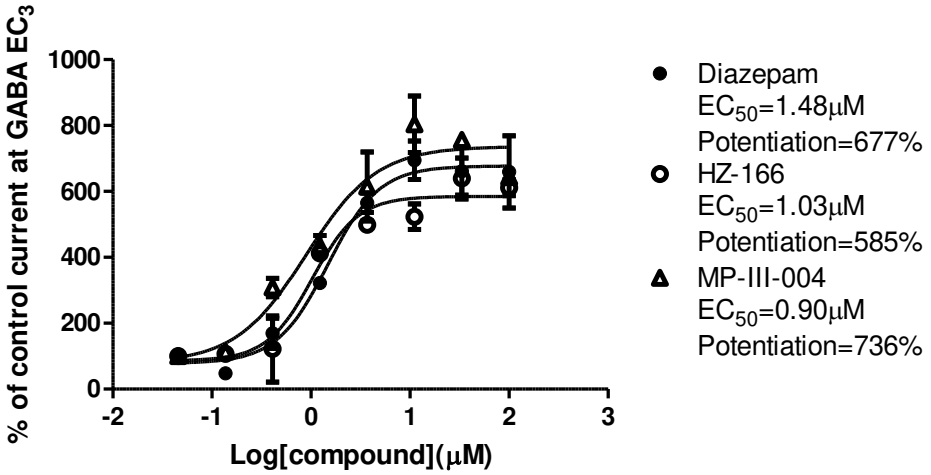


Figure 88. Current responses in CL5 isolated from HEK293T stably expressing the α5β3γ2. Modulators were tested with a constant concentration of GABA EC_3 0.7μM and maximum DMSO concentrations of 1%. N=2 for each curve.

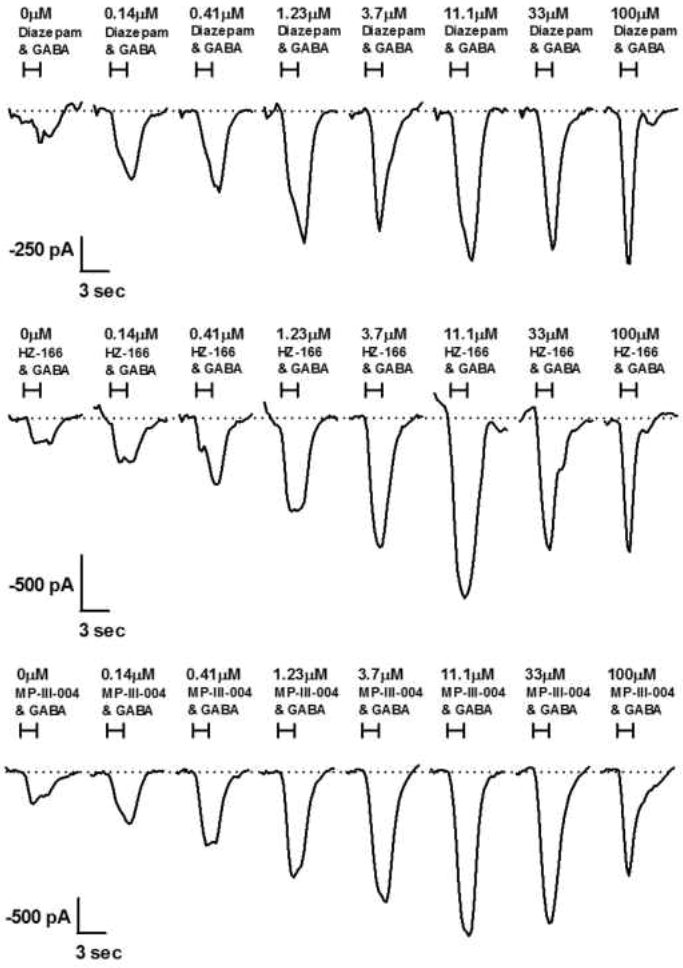


Figure 87. Current response sweeps of CL5 isolated from HEK293T stably expressing the α5β3γ2 exposed to increasing concentrations of modulator in combination with GABA EC_3.

potentiation.

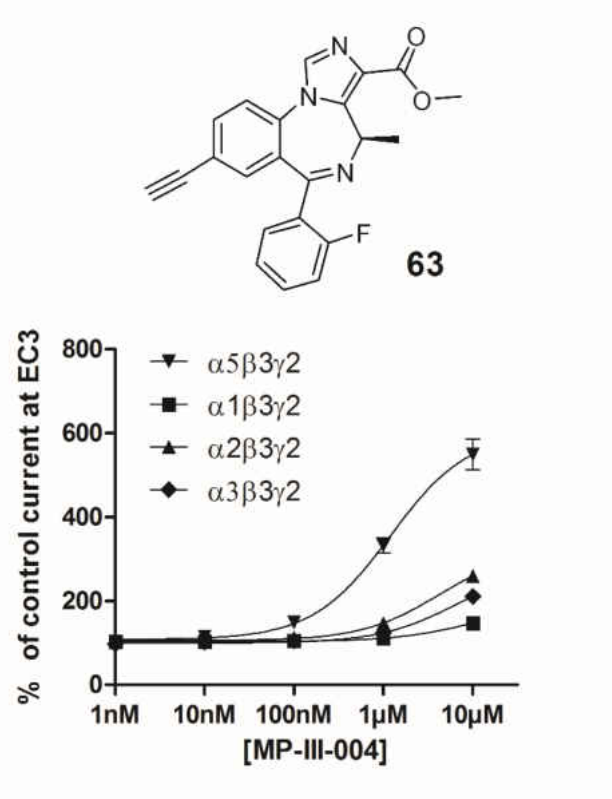


Figure 89. Structure and oocyte efficacy of MP-III-004 performed by Dr. Margot Ernst.

In order to evaluate the current without the characteristic biphasic components of BZD binding, we determined that testing with the modulators should be performed at GABA EC_{20} so

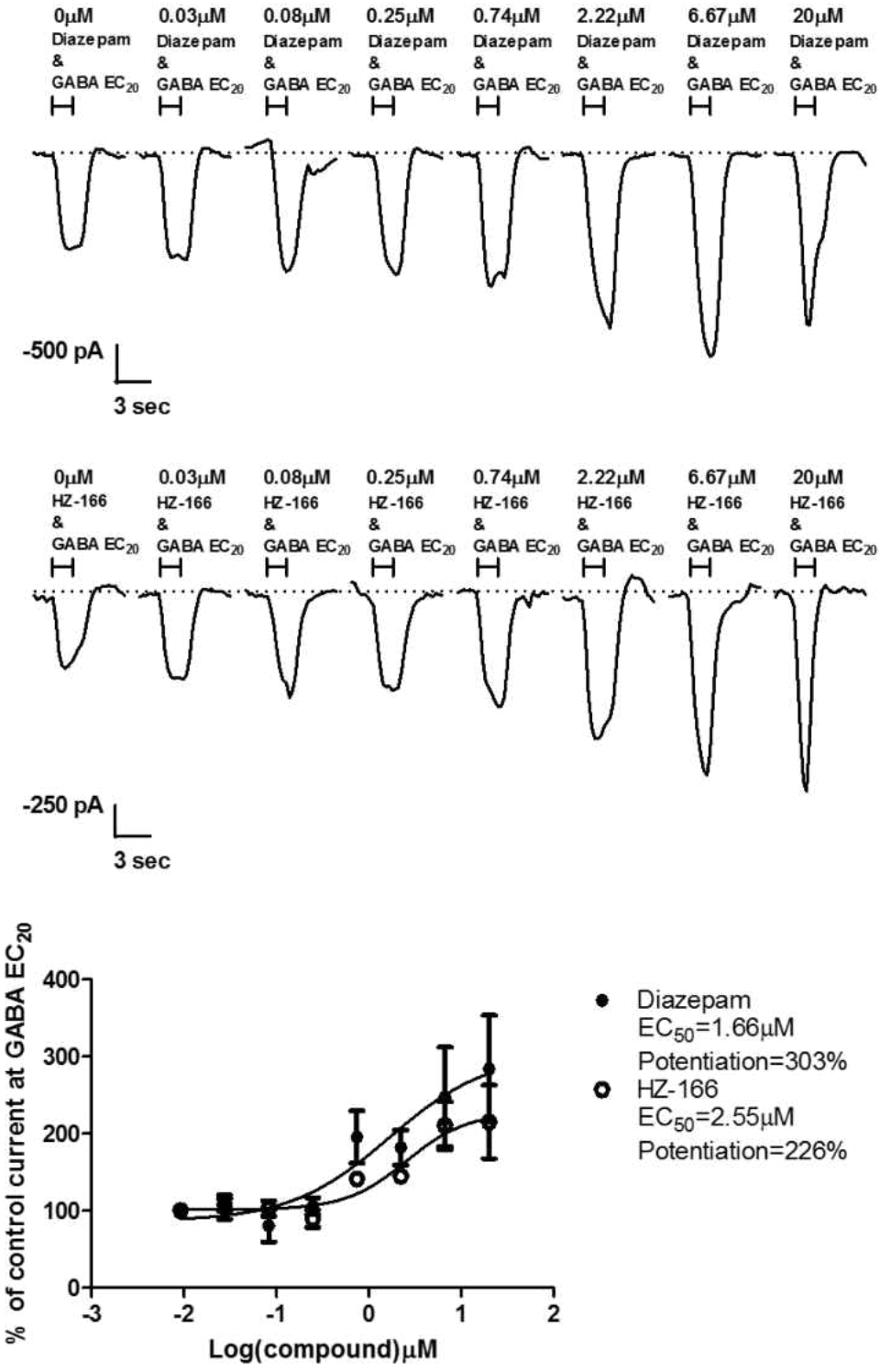


Figure 90. Current response of CL5 stably expressing the α5β3γ2 tested with a constant concentration of GABA EC_{20} 3μM and DMSO concentrations of 0.3%. N=4

as not to overestimate the potentiation of the compounds. These tests were performed with HZ-

166 and diazepam, Figure 90. The calculated potentiation was lower than previous results with GABA EC_{20}. However, this is in agreement with the results of HZ-166 tested using manual patch clamp on frog oocytes, Figure 26.

Lastly, the response of the cells to competitive antagonist bicuculline was evaluated for the α5β3γ2 containing receptors. In addition to testing the positive modulators diazepam and HZ-166, antagonist bicuculline was used on all the created cell lines, Figure 91. Cells were pre-incubated

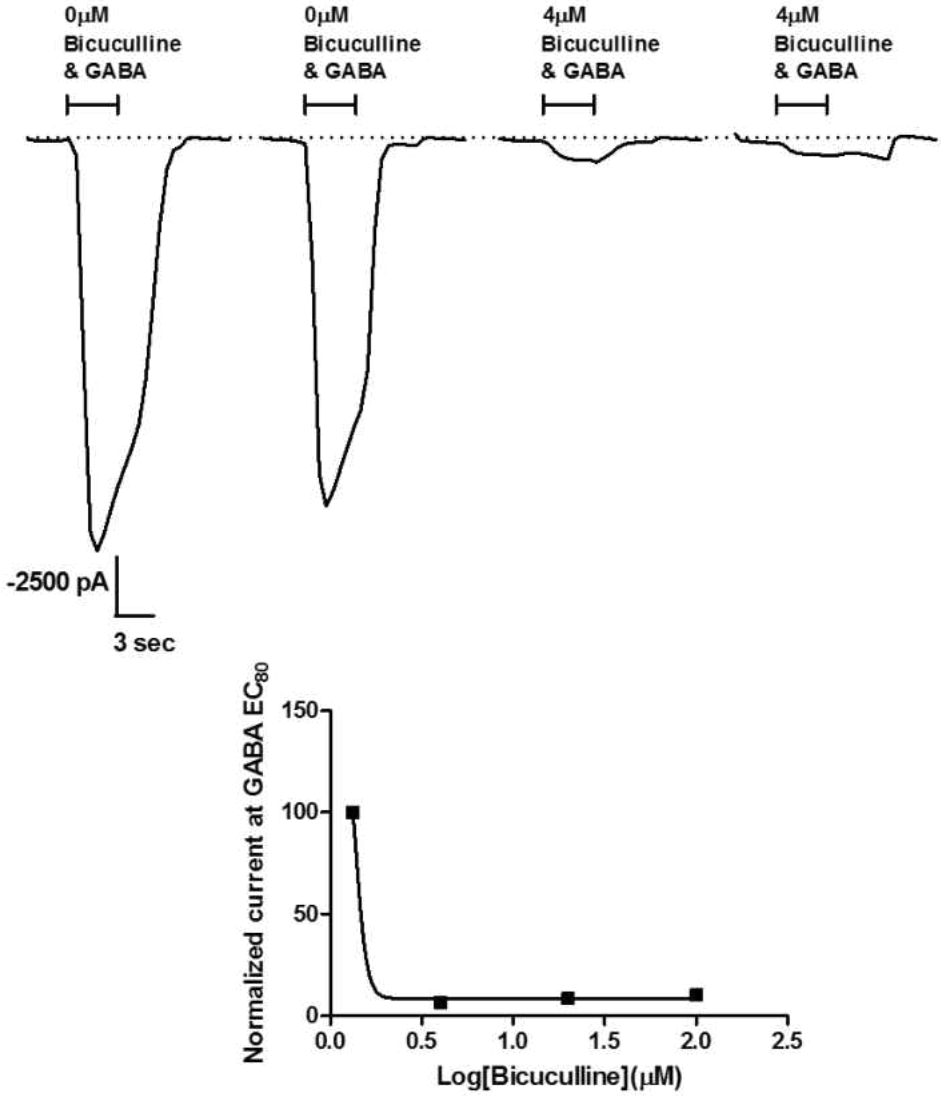


Figure 91. Cell line 5 stably expressing the α5β3γ2 $GABA_AR$ exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. N=6

with bicuculline for three minutes prior to the application of GABA. The cell line exhibited almost complete inhibition at the lowest concentration of 4 μm.

To ascertain whether the % DMSO effects the cell response over time, cells were dosed

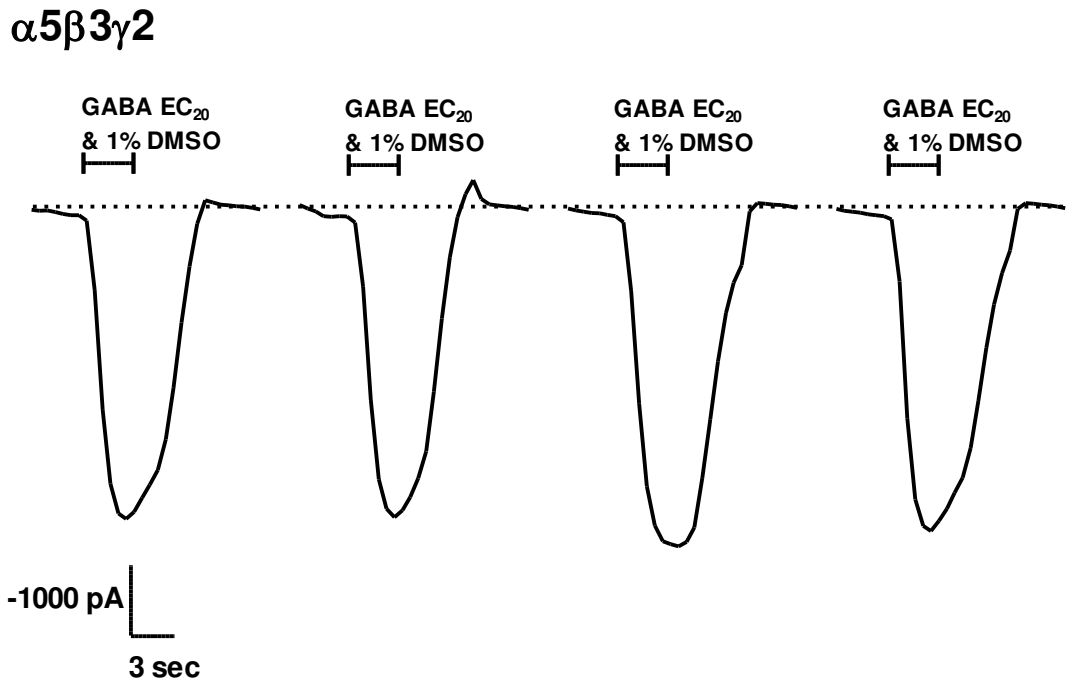


Figure 92. Response of cell line 5 stably expressing the α5β3γ2 $GABA_AR$ after three minute incubations with 1% DMSO.

with GABA before and after three minute incubations with DMSO, Figure 92. The signal did not vary significantly over time, ranging from -4307pA to -4473pA.

3.2.3 α2β3γ2 $GABA_AR$ RECOMBINANT CELL LINE

3.2.3.1 Characterization of Clones

The α2β3γ2 was the next plasmid to be stably expressed. Transfection, antibiotic selection, and clone isolation did not present any serious concerns on morphology or survival. Of the many clones that were isolated and expanded in 384 well plate, the ones presenting the fastest growth and healthiest morphological appearance were analyzed via qRT-PCR, Figure 93. The mRNA

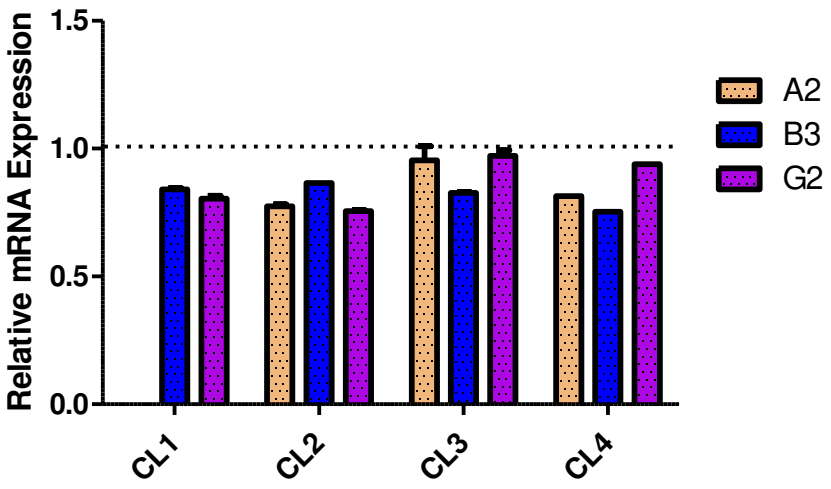


Figure 93. qRT-PCR of clonal cell lines (CL) of HEK293T cells stably expressing the α2β3γ2 plasmid construct. GAPDH is normalized to 1.0, designated with a dashed line. N=3 for each bar.

levels for CL2-4 were around the same level of expression for all three subunits of the alpha 2, beta3, and gamma2. Interestingly, CL1 exhibited no insertion of the α2 gene. This cell line was the slowest growing out of the selected cells with a turnover of 4-5 days instead of 2-3 as is normally observed for HEK293T cells. All of the cell lines were characterized

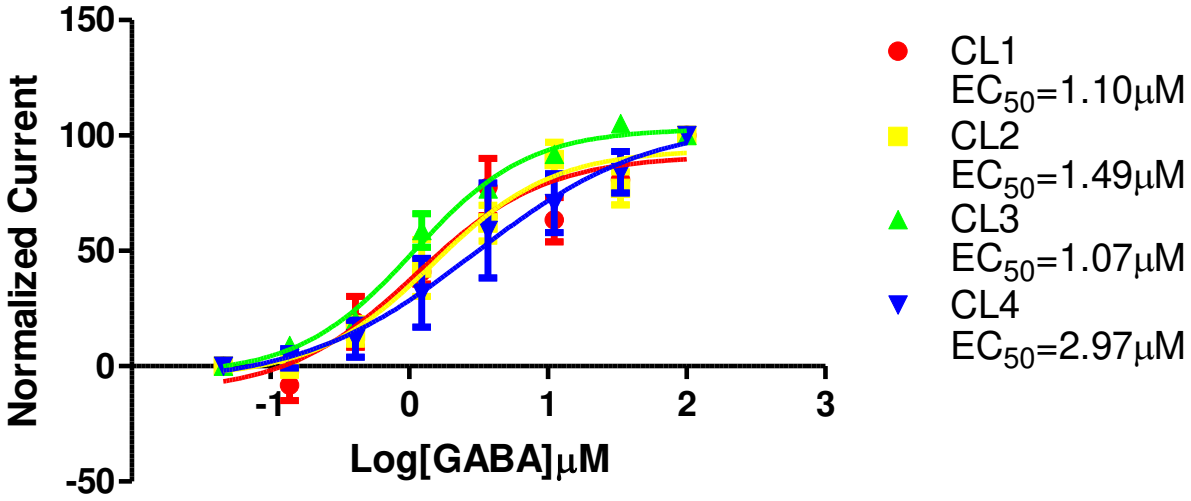


Figure 94. Current responses from HEK293T stably expressing the α2β3γ2 exposed to increasing concentrations of GABA. N=4 for each curve.

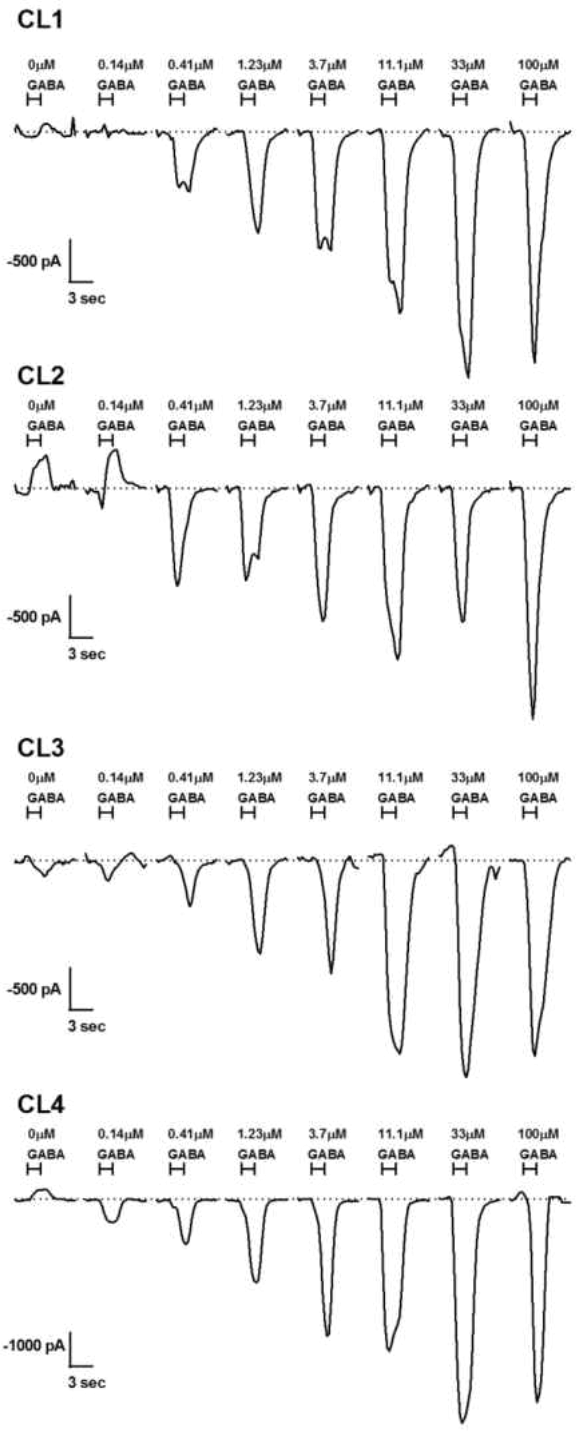


Figure 95. Current response sweeps of clonal cell lines isolated from HEK293T stably expressing the α2β3γ2 exposed to increasing concentrations of GABA.

electrophysiologically, seen in Figure 94. The current sweeps for these GABA dose response

curves can be seen in Figure 95. Despite only containing the β3γ2 subunits, the assembled receptors were still responsive to GABA. This has been described before for recombinant β3γ2 combinations [211].

Clonal cell lines were assessed using diazepam, Figure 96. The β3γ2 combinations were also slightly responsive to the exposure of diazepam as was previously described [211] in

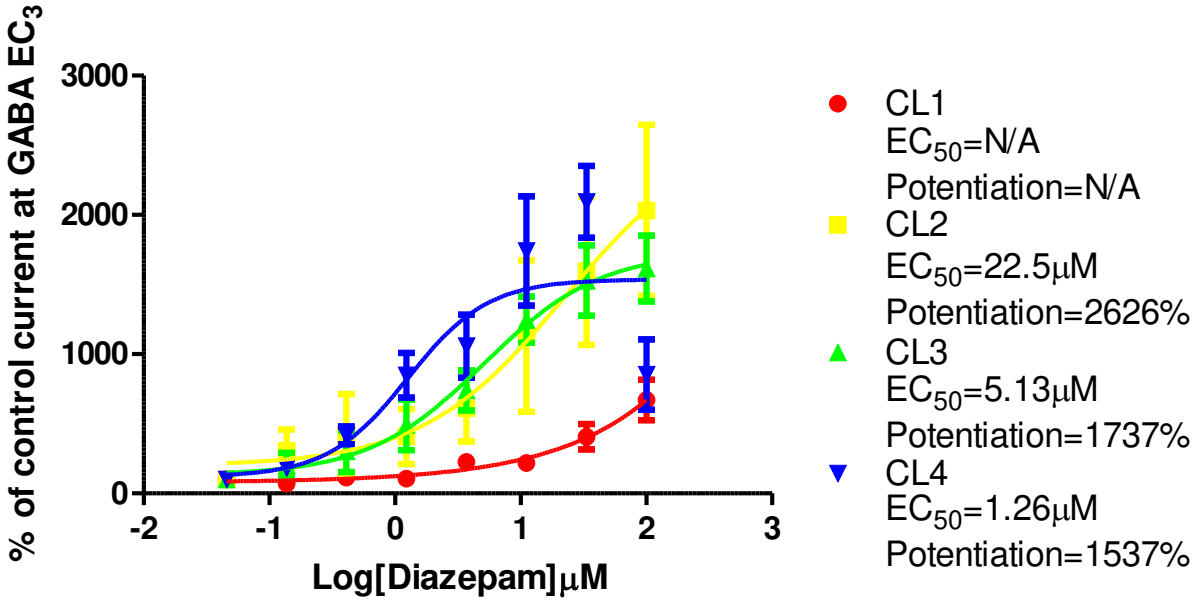


Figure 96. Current responses in cell lines isolated from HEK293T stably expressing the α2β3γ2. Modulators were tested with a constant concentration of GABA EC_3 0.7μM and maximum DMSO concentrations of 1%. N=2 for each curve.

recombinant systems in frog oocytes. The current sweeps for these curves can be seen in Figure 97.

The maximum current response averaged around -2500pA for all the clonal cell lines. The small amount of total current and the positive current elicited by GABA EC_3 could have been the

result of low expression of the receptor or a characteristic of the α2β3γ2 subtype. Cell line 3 of the

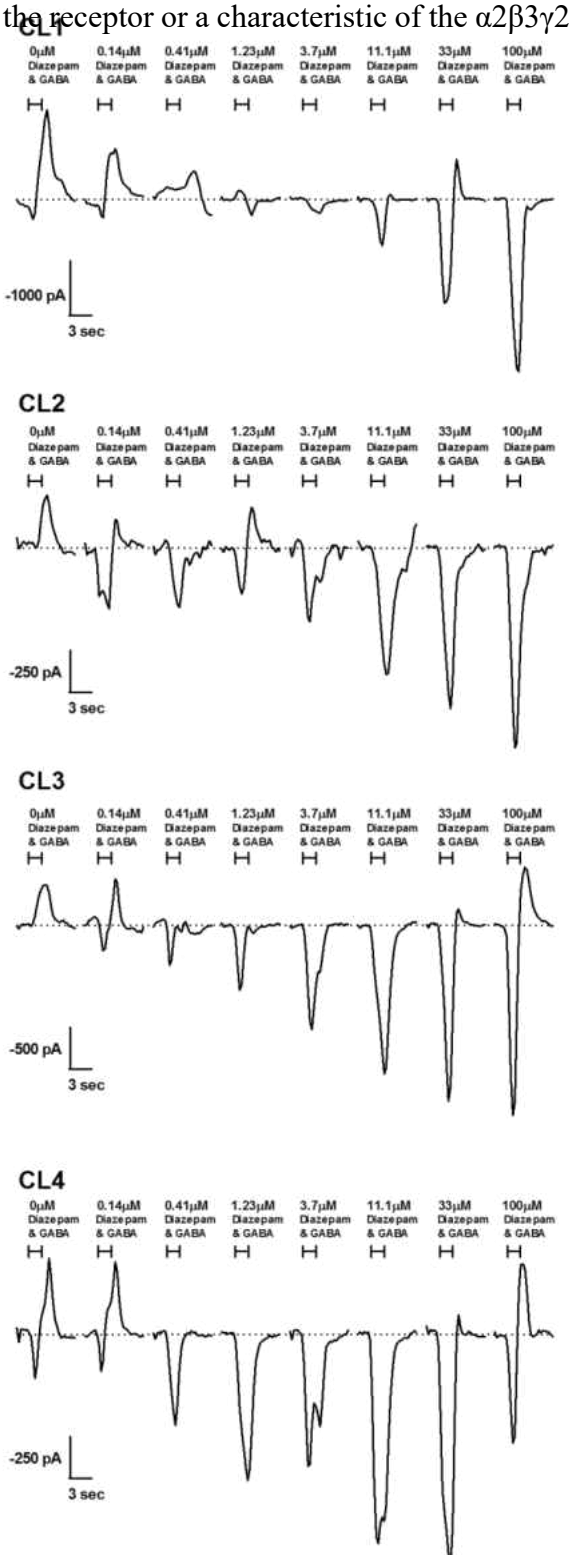


Figure 97. Current response sweeps of cell lines isolated from HEK293T stably expressing the α2β3γ2 exposed to increasing concentrations of modulator in combination with GABA EC3.

clones was chosen for further study as it appeared to reach saturation with GABA and diazepam.

As with the other receptor subtypes, the GABA concentration was raised from EC_3 to EC_{20}

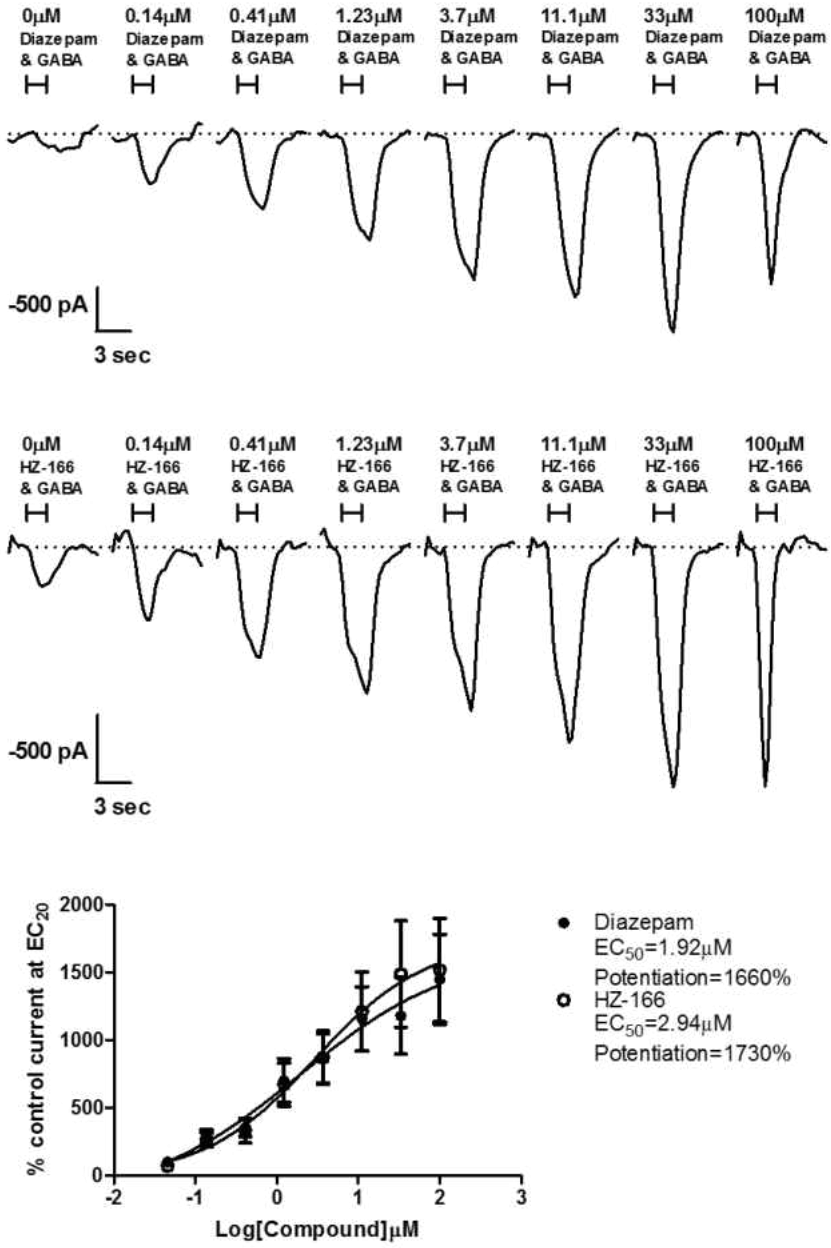


Figure 98. Current responses in CL3 isolated from HEK293T stably expressing the α2β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 0.3μM and DMSO concentrations of 0.3%. N=8

to eliminate any biphasic mechanism of binding. In addition, the DMSO percentage was kept at

0.3% consistently. The results of this experiment can be seen in Figure 98. The two curves suggest

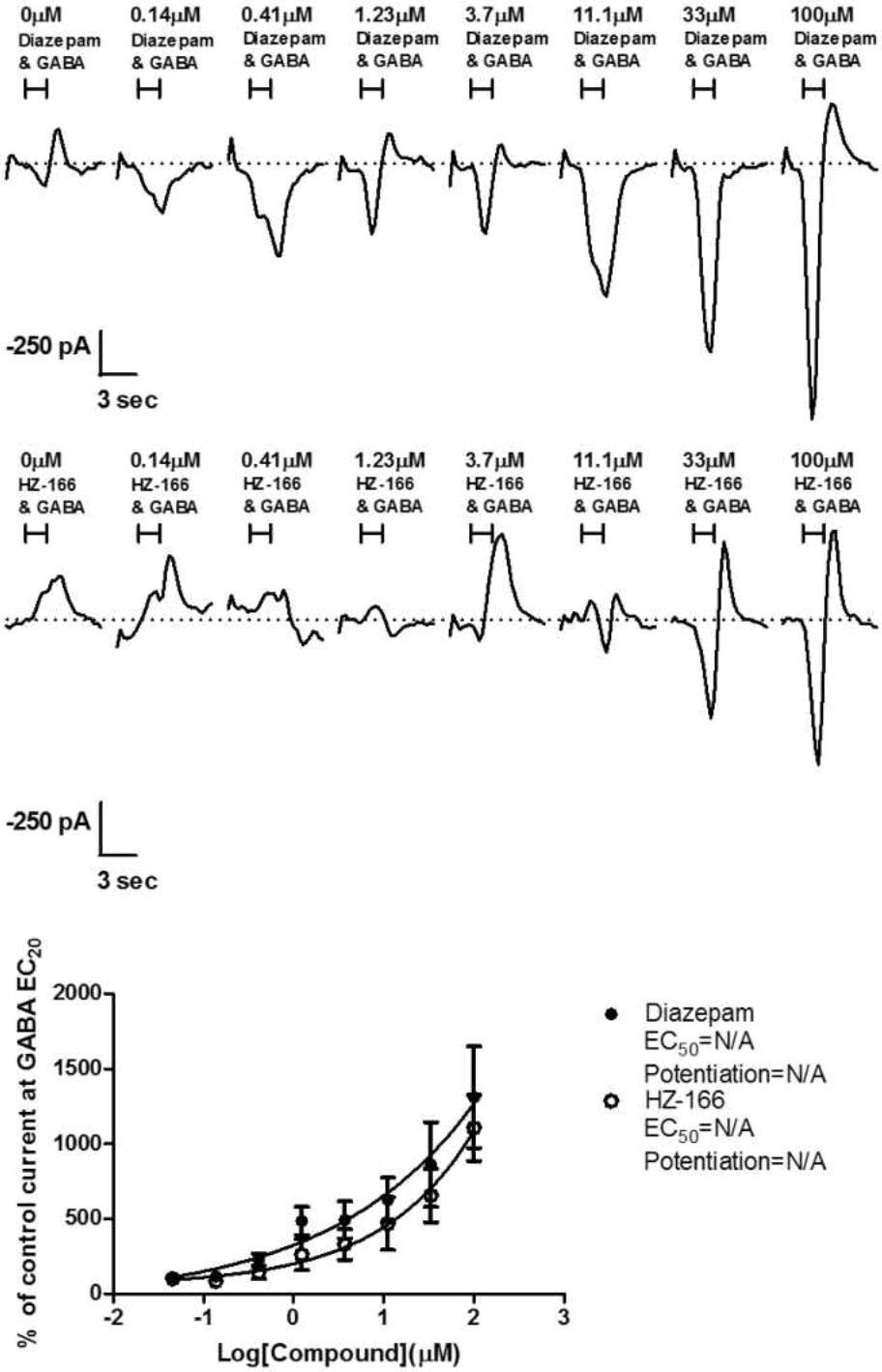


Figure 99. Current responses in CL1 isolated from HEK293T stably expressing the β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 0.3μM and DMSO concentrations of 0.3%. N=8

that there is very little to no difference in the effect of both diazepam and HZ-166 on the α2β3γ2 subtype. In addition to the α2β3γ2 containing CL3, the β3γ2 containing CL1 was also characterized as a control. The response of β3γ2 cell line is seen in Figure 99.

Lastly, the response of the cells to competitive antagonist bicuculline was evaluated for the α2β3γ2 containing receptors. In addition to testing the positive modulators diazepam and HZ-166, antagonist bicuculline was used on all the created cell lines, Figure 100. Cells were pre-incubated

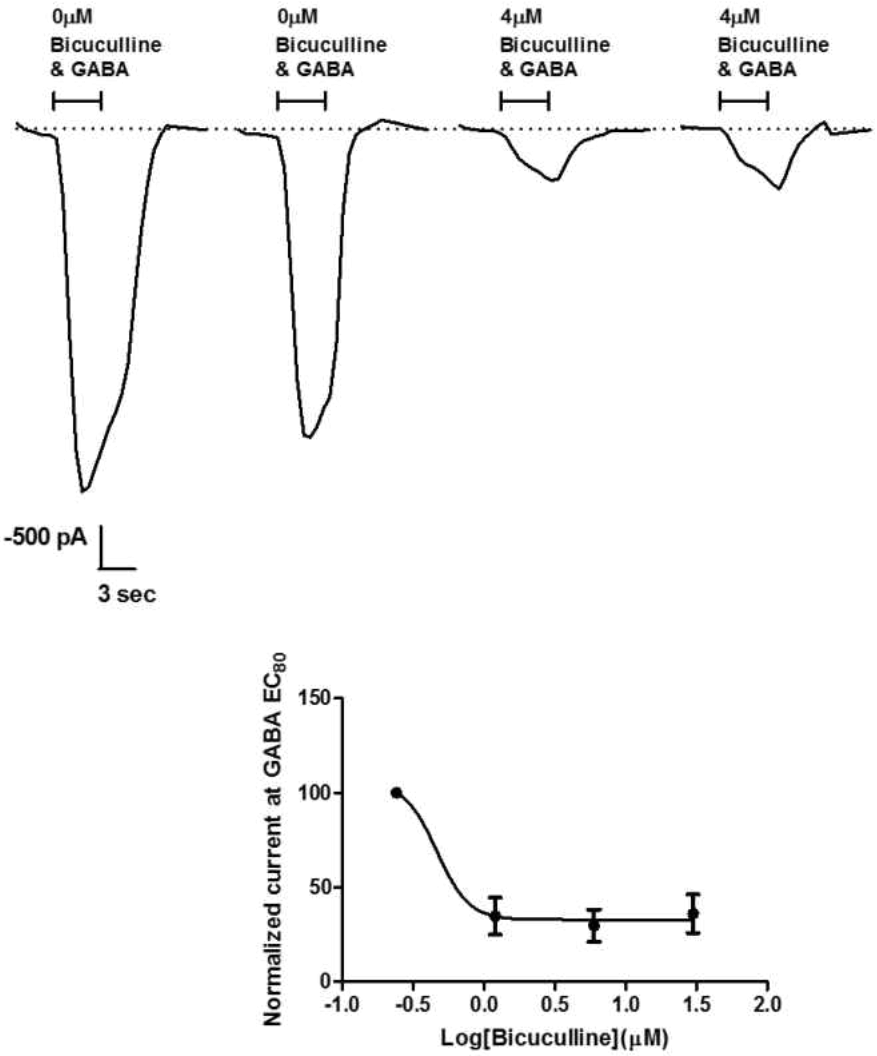


Figure 100. Cell line 3 stably expressing the α2β3γ2 $GABA_AR$ exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. N=6

with bicuculline for three minutes prior to the application of GABA. The cell line exhibited almost complete inhibition at the lowest concentration of 4 µm.

To ascertain whether the % DMSO effects the cell response over time, cells were dosed with GABA before and after three minute incubations with DMSO, Figure 101. The signal

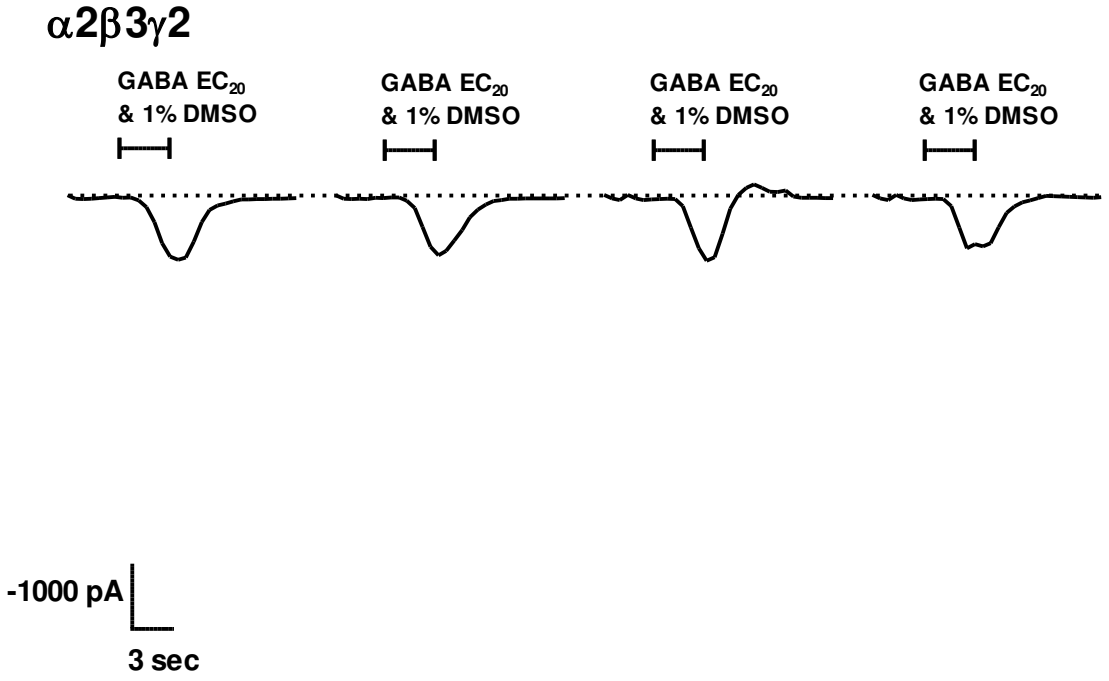


Figure 101. Response of cell line 3 stably expressing the α5β3γ2 GABAAR after three minute incubations with 1% DMSO.

slightly diminished over time, from -855pA to -698pA from the first to the last sweep. However, this small decrease should not interfere with the analysis of data for modulators.

3.2.4 α3β3γ2 $GABA_AR$ RECOMBINANT CELL LINE

3.2.4.1 Characterization of Clones

Cells transfected with the α3β3γ2 construct posed little problem. The cells remained healthy and isolation after antibiotic selection produced eleven very health cell lines. Interestingly,

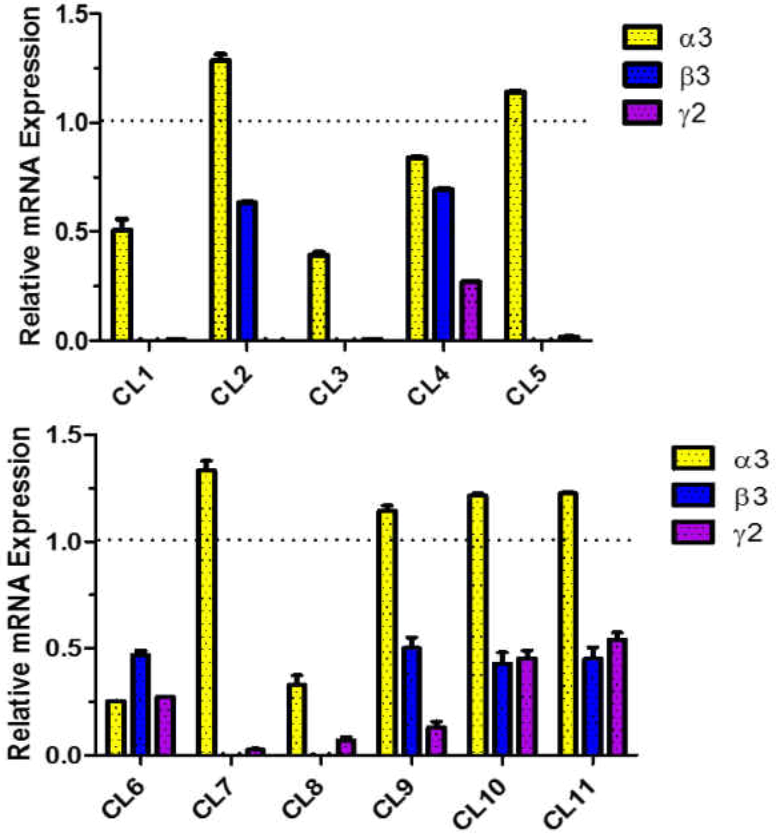


Figure 102. qRT-PCR of clonal cell lines (CL) of HEK293T cells stably expressing the α3β3γ2 plasmid construct. GAPDH is normalized to 1.0, designated with a dashed line. N=3 for each bar.

many of the cells did not successfully incorporate the β3 or γ2 gene. However, the α3 appeared to have a higher expression than was previously observed for the recombinant cell lines. Both CL10 and CL11 had the highest expression of the β3 and γ2 mRNA. Cell line 10 was taken further for electrophysiological characterization. The GABA dose response curve, seen in Figure 103, found that the GABA EC_{20} was 0.43 μM. Modulators were tested at this concentration with a constant percentage of DMSO 0.3%. Testing the two modulators diazepam and HZ-166, seen in Figure

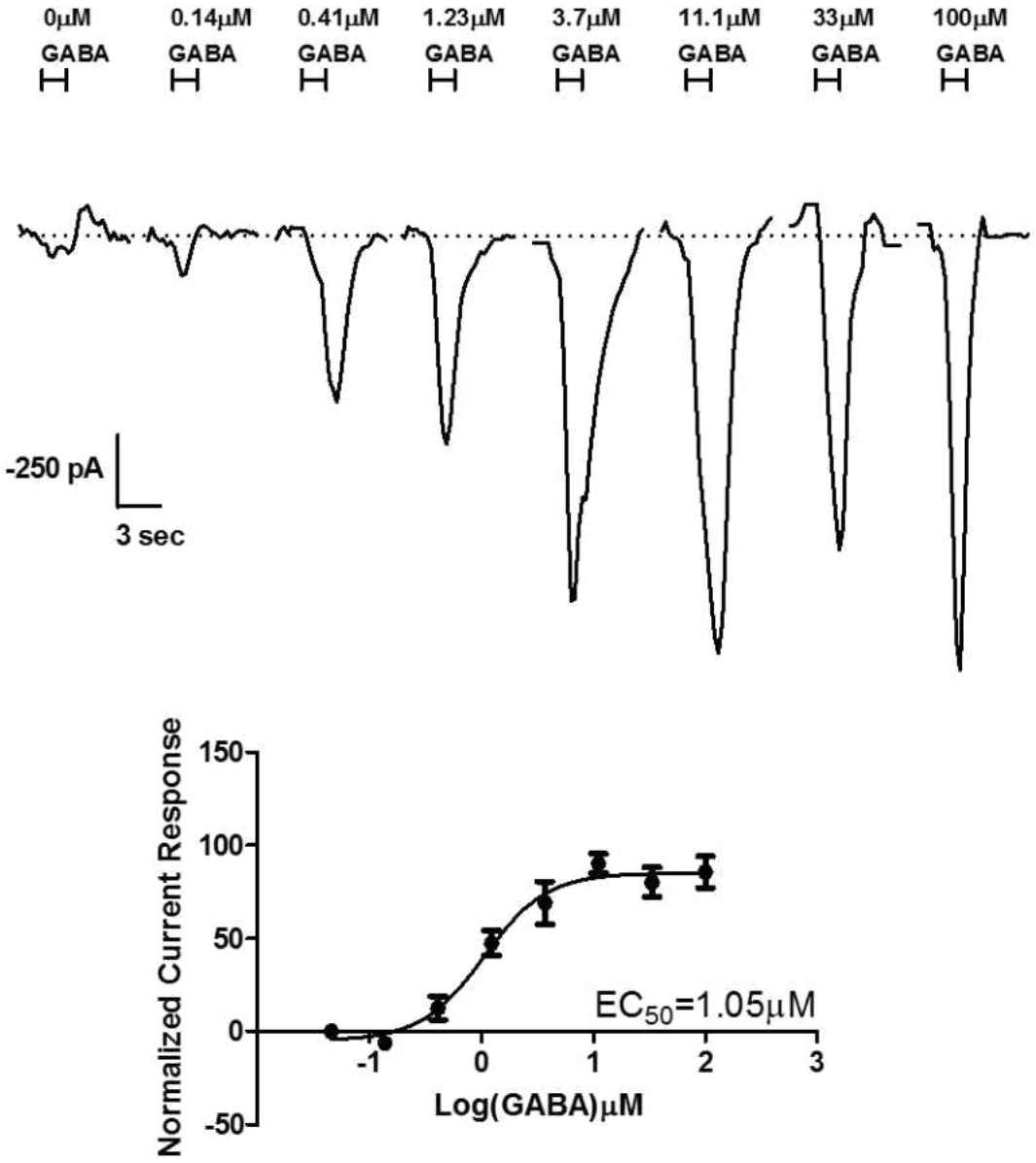


Figure 103. Current responses from cell line 10 stably expressing the α3β3γ2 exposed to increasing concentrations of GABA. N=4 for each curve.

104, it was found that this receptor subtype exhibited the largest difference in efficacies. HZ-166 had a significantly higher potentiation than for diazepam. This is in agreement with the reported selectivity of HZ-166 (ligand 2) for the α3 in frog oocytes[210], though the concentration utilized in

our study was 10X the maximum amount used in the oocytes, was performed at GABA EC_{20}

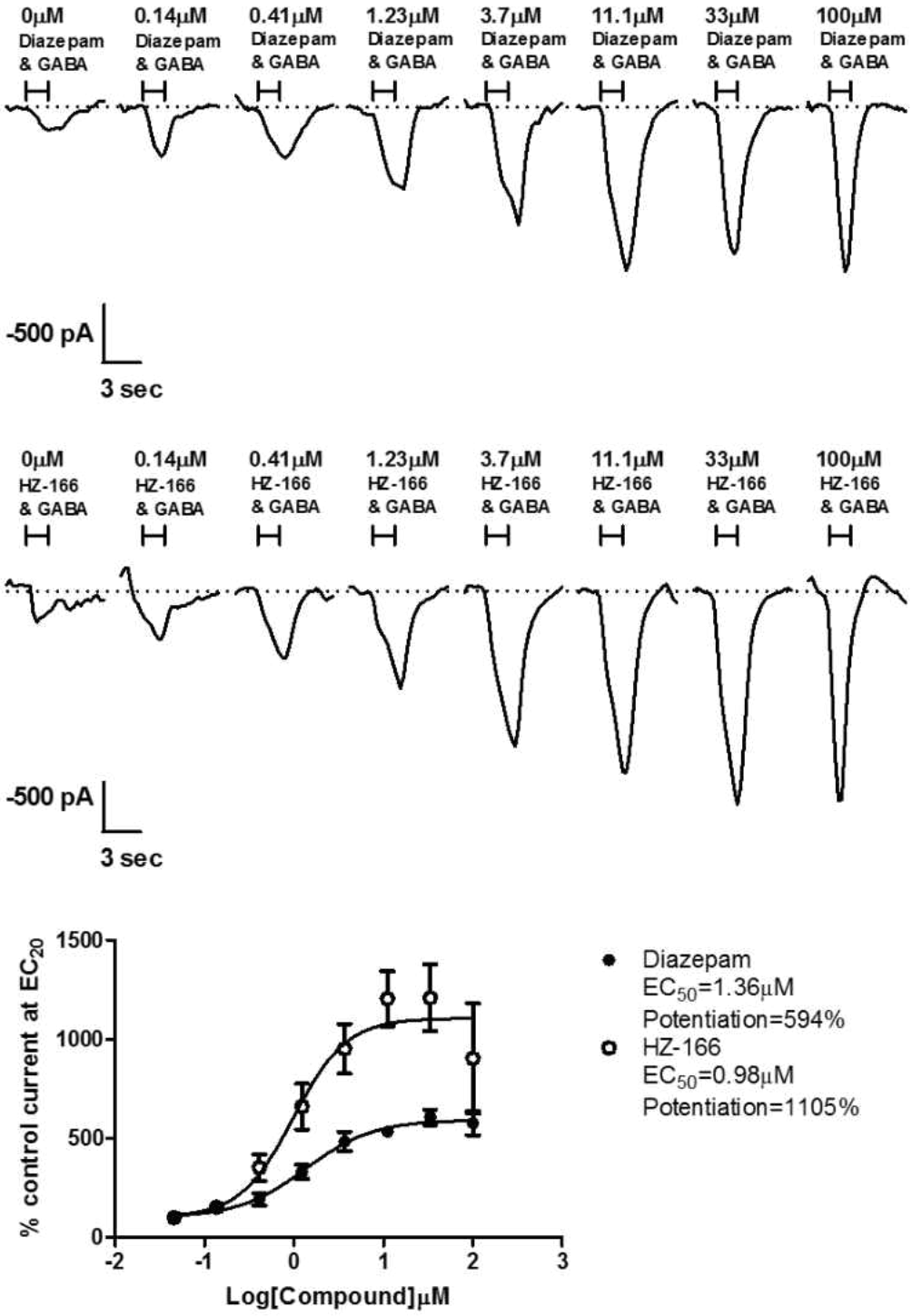


Figure 104. Current responses in CL10 isolated from HEK293T stably expressing the α3β3γ2. Modulators were tested with a constant concentration of GABA EC_{20} 0.43μM and DMSO concentrations of 0.3%. N=8

instead of $EC_{3,}$ and was recorded using automated rather than manual patch clamp.

Lastly, the response of the cells to competitive antagonist bicuculline was evaluated for the α3β3γ2 containing receptors. In addition to testing the positive modulators diazepam and HZ-166, antagonist bicuculline was used on all the created cell lines, Figure 105. Cells were pre-incubated

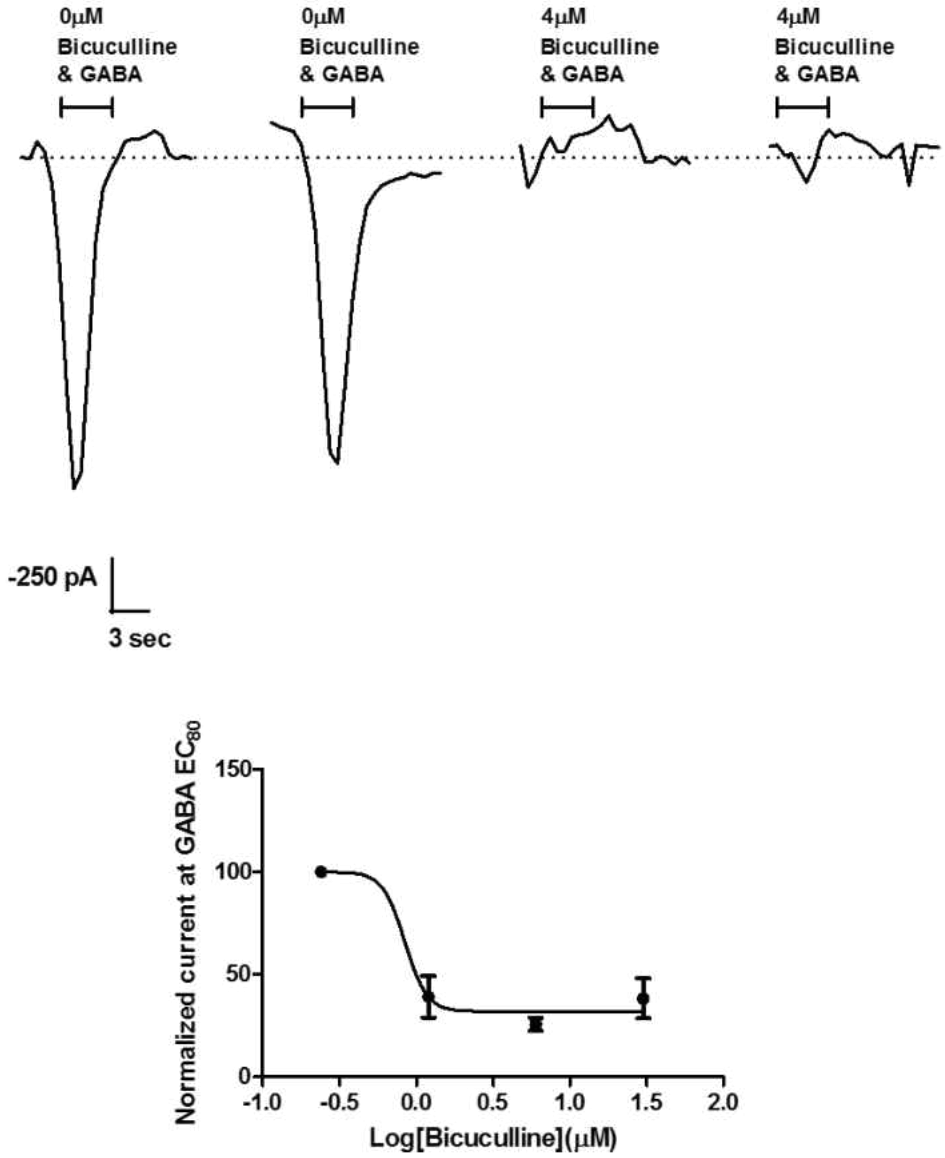


Figure 105. Cell line 10 stably expressing the α3β3γ2 $GABA_AR$ exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. N=6

with bicuculline for three minutes prior to the application of GABA. The cell line exhibited almost complete inhibition at the lowest concentration of 4µm.

To ascertain whether the % DMSO effects the cell response over time, cells were dosed with GABA before and after three minute incubations with DMSO, Figure 106. The signal did not

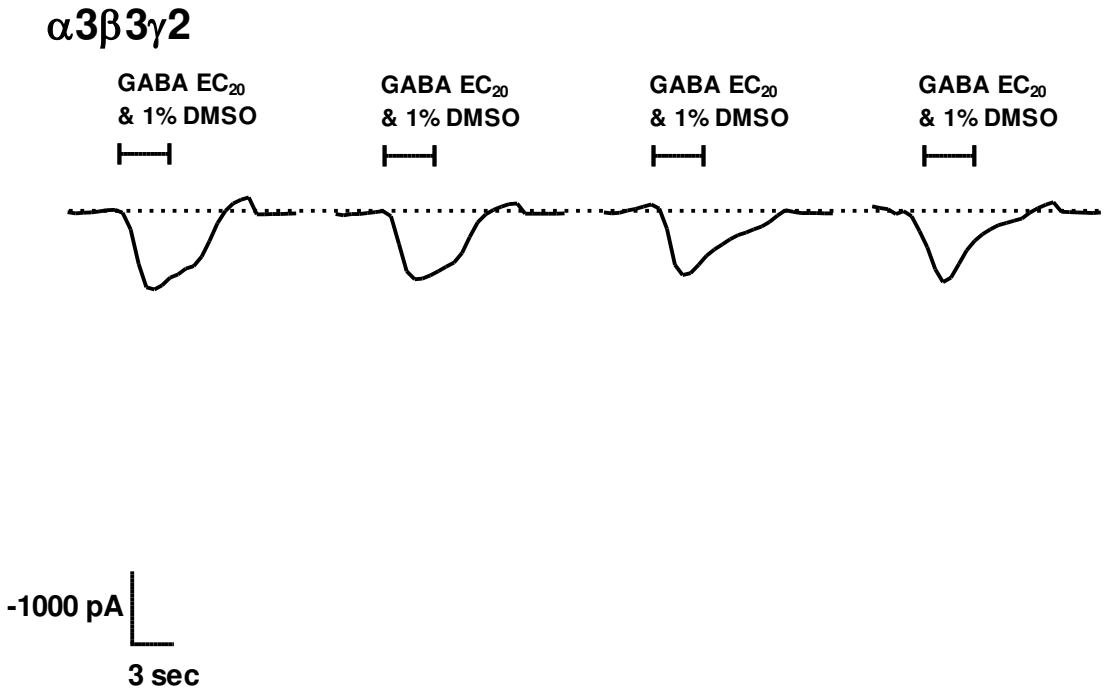


Figure 106. Response of cell line 3 stably expressing the α5β3γ2 GABAAR after three minute incubations with 1% DMSO.

vary significantly over time, ranging from -1057pA to -952pA from the first to the last sweep. However, this small decrease should not interfere with the analysis of data for modulators.

3.2.5 α6β3γ2 $GABA_AR$ RECOMBINANT CELL LINE

3.2.5.1 Characterization of Clones

The last cell line created was the α6β3γ2 which, like the α4β3γ2 containing receptors, are traditionally known as BZD insensitive. Like the α4β3γ2, the α6 $GABA_AR$ subtype containing cell line also presented low survival of expanded cell lines after antibiotic selection. Six of the healthiest cell lines were evaluated for $GABA_AR$ α6β3γ2 transcripts, Figure 107. Four cell lines were evaluated further on the IonFlux: CL1, CL2, CL3, and CL4. Of these, CL2 and CL4 had the

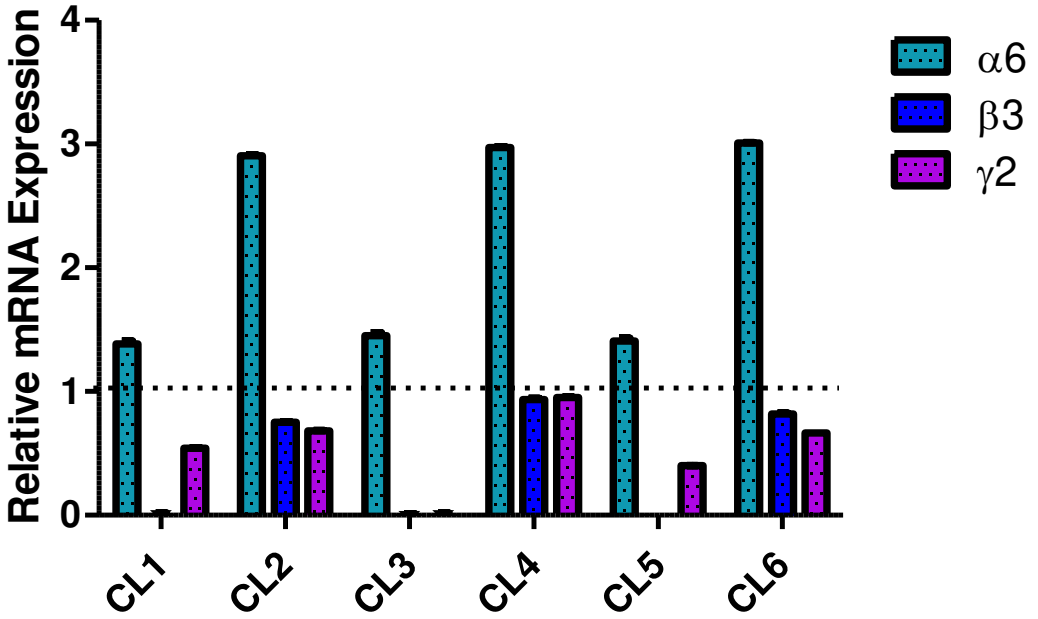


Figure 107. qRT-PCR of clonal cell lines (CL) of HEK293T cells stably expressing the α6β3γ2 plasmid construct. GAPDH is normalized to 1.0, designated with a dashed line. N=3 for each bar.

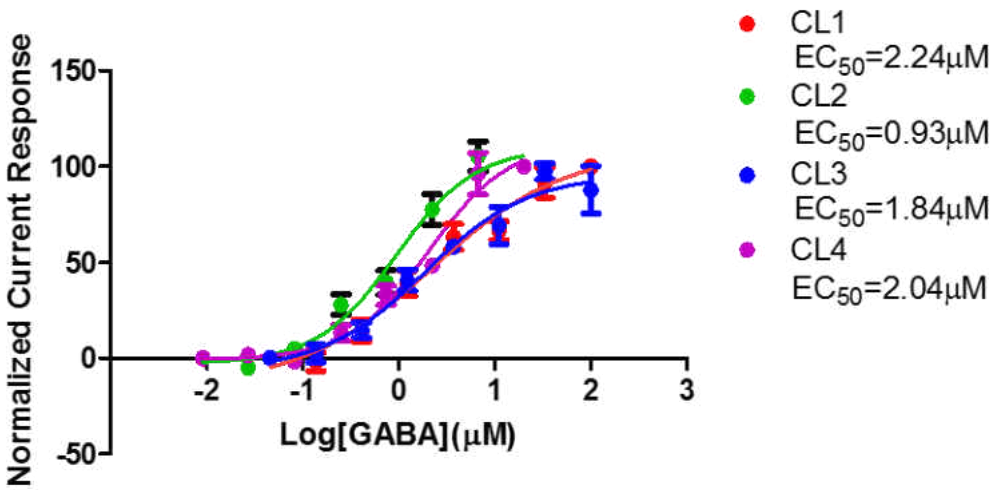


Figure 108. Current responses in cell lines isolated from HEK293T stably expressing the α6β3γ2 exposed to increasing concentrations of GABA. CL1 and CL3 were exposed to a maximum concentration of 100μM GABA while CL2 and CL4 were exposed to a maximum concentration of 20μM GABA. N=8 for each curve.

highest expression of all three receptor subtypes while CL1 and CL3 primarily expressed the alpha 6 subunit. The GABA dose response for these four distinctive clones are shown in Figure 108. The

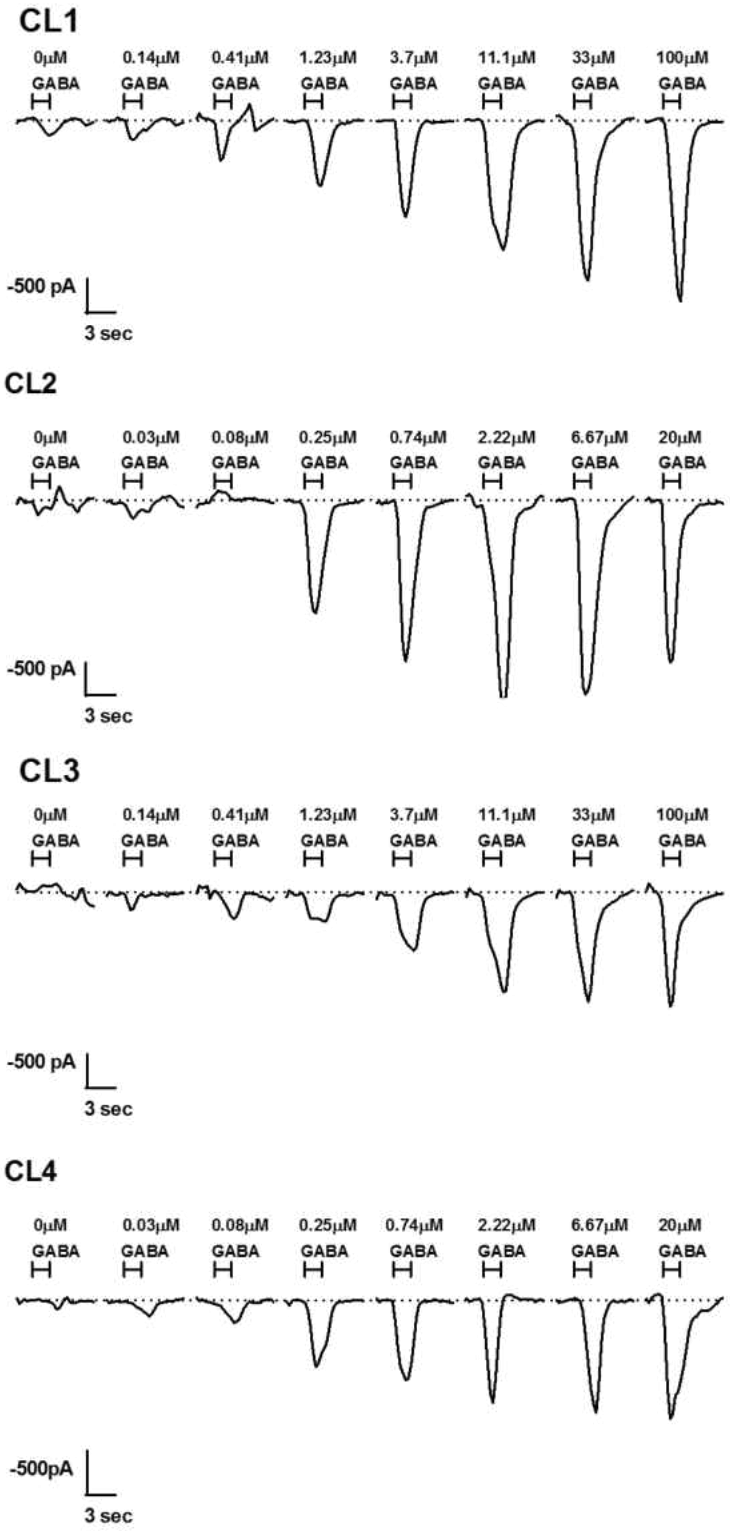


Figure 109. Current response sweeps of clonal cell lines isolated from HEK293T stably expressing the α6β3γ2 exposed to increasing concentrations of GABA.

current sweeps for these curves can be seen in Figure 109. The average maximum inhibitory

current from CL1 was 2100pA, CL2 had currents of 1760pA, while CL3 reached 1970pA, and CL4 had an average response of 1,550pA. The current response for CL1 and CL3 was higher than those of CL2 and CL4, despite the relative absence of the β3 subunit for both CL1 and CL3 as well as the γ2 subunit of CL3. However, the higher current response could be a result of the overall higher concentration of GABA used in the dose response. The near identical dose response curves for the two cell lines suggests that the α6 subunit is capable of forming homomeric receptors which respond to the binding of GABA.

Similarly to the α4β3γ2, the α6β3γ2 is traditionally known as a benzodiazepine insensitive receptor. Extensive experiments were performed with these two modulators, using a constant concentration of GABA EC_{20} 0.3μM and a high concentration of 100μM compound, the current elicited resulted in high potentiation and current response of around Thus the concentrations of

compound was lowered to 20µM and the DMSO percentages lowered to constant concentration of

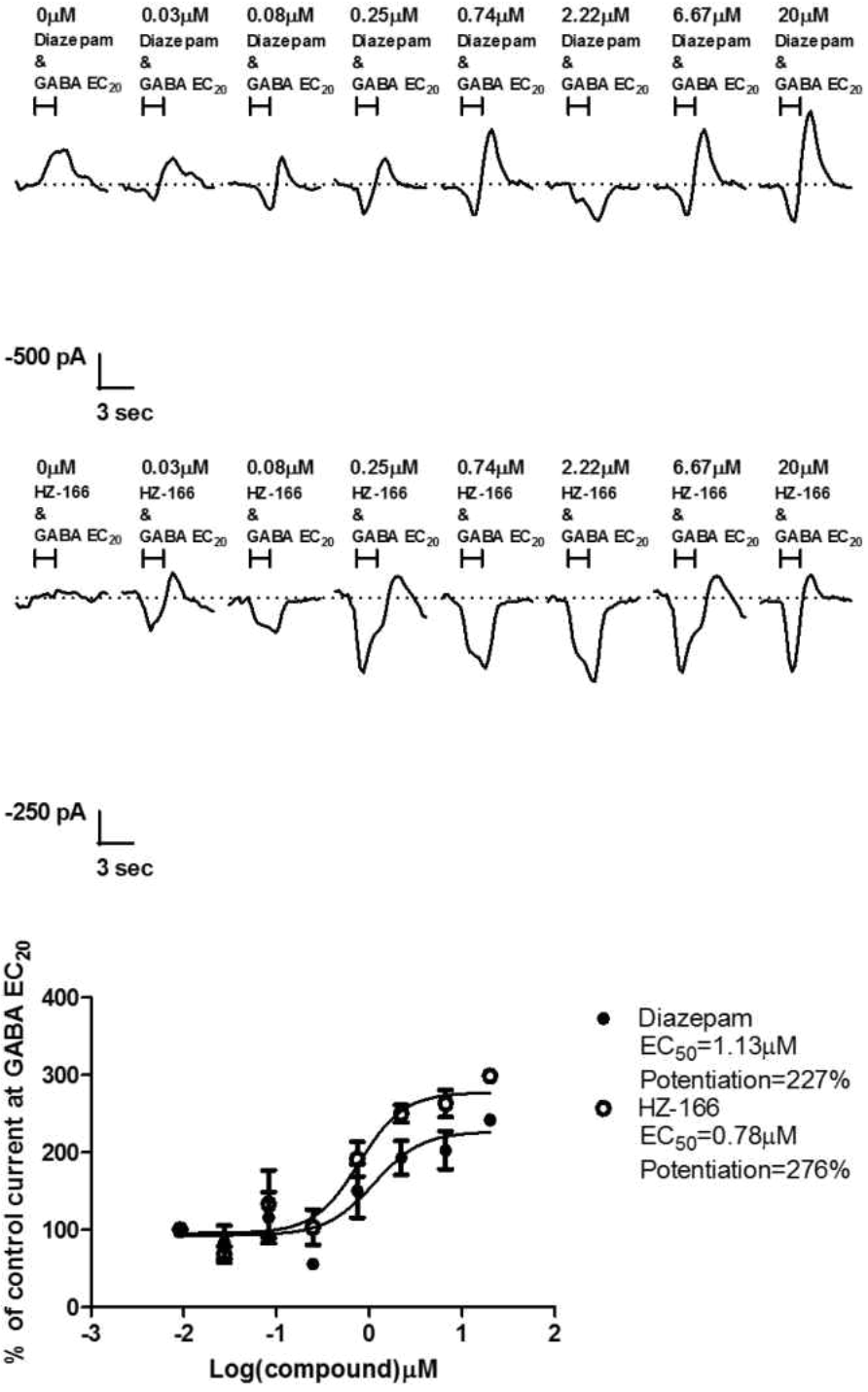


Figure 110. Current response of CL2 stably expressing the α6β3γ2 tested with a constant concentration of GABA EC_{20} 0.5µM and constant DMSO concentrations of 0.3%. N=4

0.3%. The resulting current response, seen in Figure 110, trended close to baseline readings with

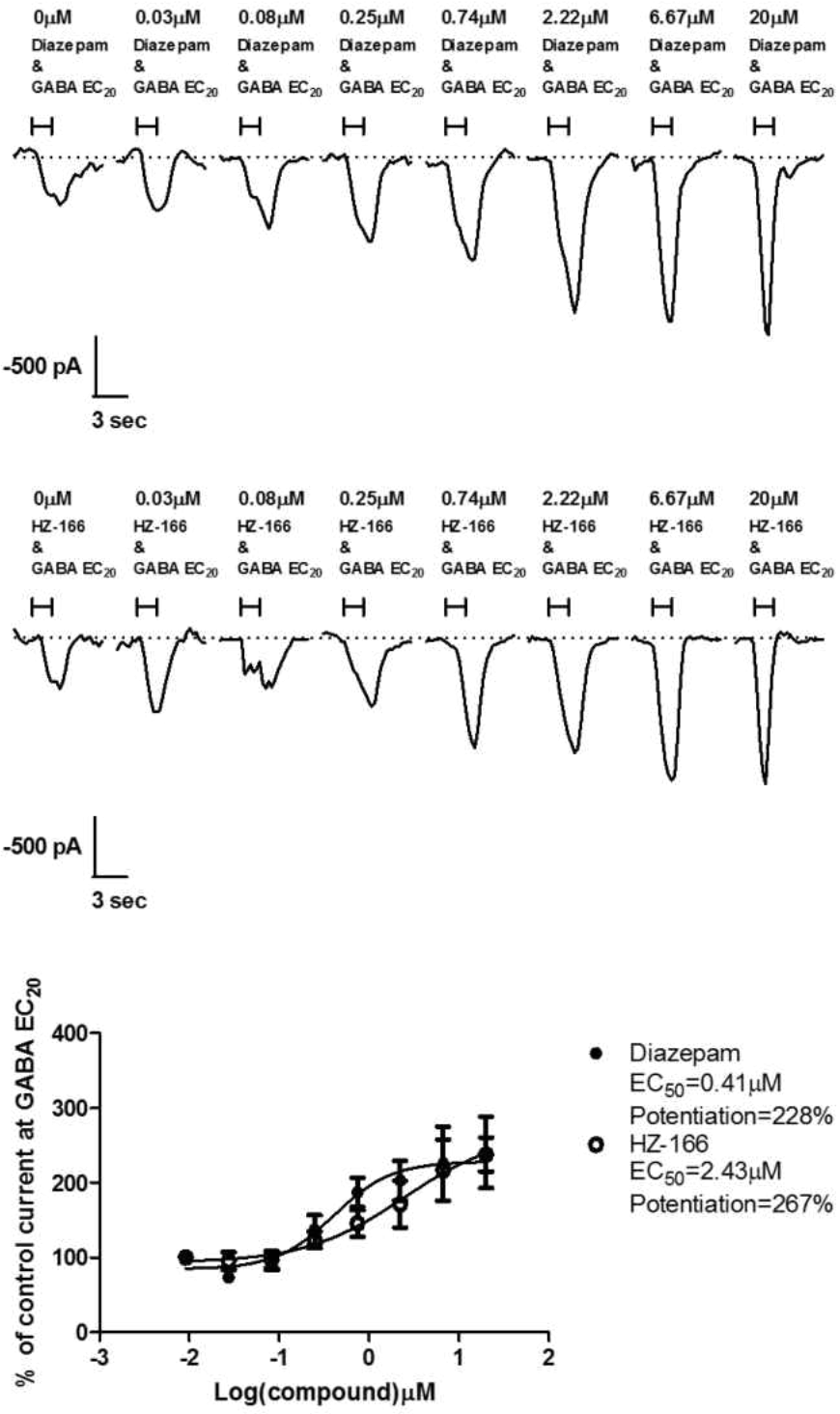


Figure 111. Current response of CL2 stably expressing the α6β3γ2 tested with a constant concentration of GABA EC_{20} 1μM and constant DMSO concentrations of 0.3%. N=8

a maximum response of -500pA for both compounds. The initial positive response in the diazepam sweep appears to be the result of small total current change. The GABA EC_{20} of 0.5 μM failed to elicit a response. Increasing the GABA concentration to 1 μM, which was within the standard deviation of the calculated EC_{20}, was able to elicit a negative response. Despite the α6 being described as benzodiazepine insensitive, these conditions resulted in a potentiation. Similar to the α4, studies on the α6 receptor are most often performed with the β2 [232,245,246] and the potentiation

could be due to the expression in combination with the β3. Refer to 3.2.1.3.3 Results and Discussion.

In addition to testing the positive modulators diazepam and HZ-166, antagonist bicuculline was used on all the created cell lines, Figure 112. Cells were pre-incubated with

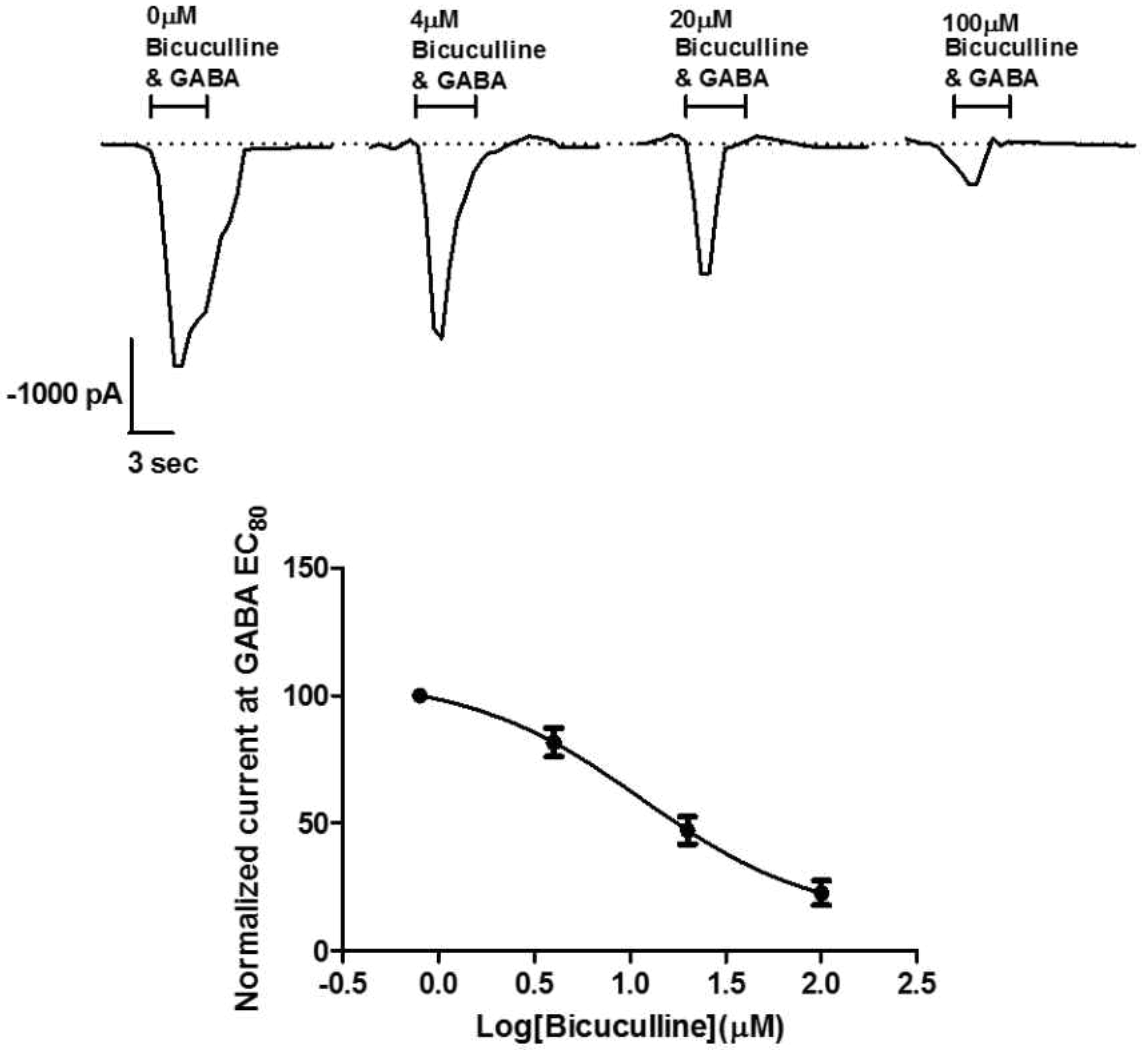


Figure 112. Cell line 2 stably expressing the α6β3γ2 GABAAR exposed to an increasing concentration of bicuculline in combination with a constant concentration of GABA EC_{80}. N=10.

bicuculline for three minutes prior to the application of GABA. Unlike the other cell lines which

exhibited almost complete inhibition at 4μM bicuculline, the α6β3γ2 required higher concentrations of the antagonist to inhibit the GABA-evoked current. This is in line with the observation that receptors which contain the α6 subunit have been observed to be less sensitive to bicuculline [244]. Unlike agonists and positive modulators, antagonist activity independent of the subunit composition with the exception of the α6 containing receptors [242,243].

To ascertain whether the % DMSO effects the cell response over time, cells were dosed with GABA before and after three minute incubations with DMSO, Figure 113. The signal did

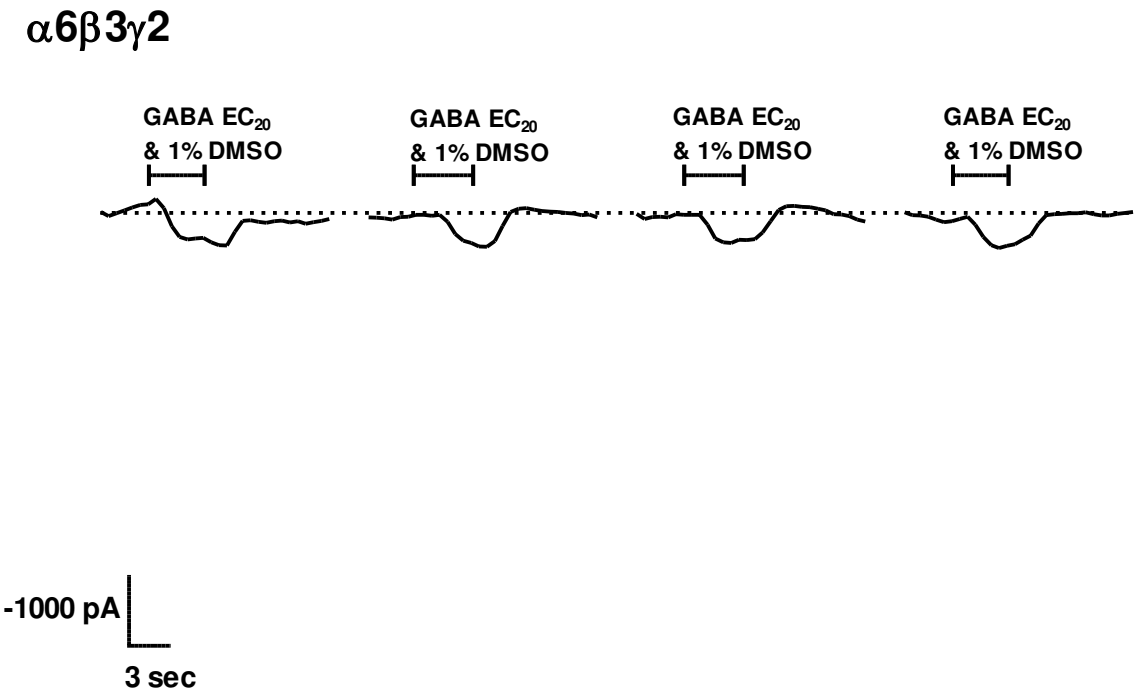


Figure 113. Response of cell line 2 stably expressing the α6β3γ2 $GABA_AR$ after three minute incubations with 1% DMSO.

not vary significantly over time, ranging from -415pA to -453pA.

3.2.6 CONCLUSIONS

From our findings, it would appear that the plasmid constructs are able to successfully integrate the genes of interest into HEK293T cells. The integration leads to generally high levels

of $GABA_AR$ subunit expression. The clones chosen all had around 50% or more mRNA expression as the housekeeping gene GAPDH. All of the cell lines were responsive to the binding of the agonist GABA, the antagonist bicuculline, as well as to modulation by diazepam and HZ-166.

The cells transfected with the α4β3γ2 and α6β3γ2 exhibited slow recovery and low levels of overall transfection levels, as evidenced by the widespread cell death during antibiotic selection. However, longer recovery times on the transfection plate allowed the cells to recuperate from the antibiotic exposure prior to isolation in the multi well plates.

By altering the formulation of the intracellular and extracellular solutions, small iPSCs could be enhanced and potassium leak could be inhibited with high levels of cesium. This becomes particularly important for receptor subtypes that do not exhibit current changes of more than -1,500pA. The background noise at baseline can create a positive current change from resting potential, resulting in a rise in current rather than a lowering. Attempts to calculate a potentiation from these values could be a misleading representation of the data.

Interestingly, we have detected measurable potentiation of GABA-evoked current in the HEK293T cells stably expressing the $GABA_AR$ α4β3γ2 and α6β3γ2 to diazepam and HZ-166. Whether this is the result of the β3γ2 subunit composition or binding at a site alternative to the traditional αγ2 site, such as the αβ interface, has yet to be determined.

The clonal cell lines among stable transfections had very similar changes in current despite having very differing levels of mRNA expression. In general, the α5β3γ2 cells had the greatest current responses in the range around -10,000pA while α3β3γ2 and α4β3γ2 had a much more diminished current signal around -2,000pA. This would suggest that the maximum change in current is a characteristic of the receptor subtype rather than the extent of mRNA expression levels.

This recombinant stable cells system can be used to assess the selectivity of any $GABA_AR$ modulator. By comparing the efficacies and potencies of novel BZDs with pre-existing and FDA approved $GABA_AR$ modulators the alpha subtype selectivity can be determined. This method of comparing results is necessarily performed on a batch to batch basis for transient expression systems as the observed potentiation has been known to flux [247,248], however this repetitive step may be able to be reduced in the future with the reproducibility and clonal purity of these recombinant stable cell lines. From this data, structure activity relationship can be assessed and the compounds can be improved to create future subtype selective $GABA_AR$ modulators with any desired specificity.

CHAPTER 4: ICELL NEURONS

4.1 Introduction

In his famous literary work *Heart of Darkness,* author Joseph Conrad perhaps best describes the evolution of the brain and humankind: "The mind of man is capable of anything—because everything is in it, all the past as well as all the future". The human brain is not exceptional in its cellular composition, containing as many neuronal and non-neuronal cells as any primate brain of its size. Thus it appears unexpectedly surprising that the cognitive abilities between humans and other great apes differ so greatly. The answer to this question may be found in the composition of neurons, neuroanatomical structure, and the function of the cerebral cortex and cerebellum [249].

The central nervous system is primarily composed of two kinds of cells: neurons and glia. A much quoted and wide held belief was that the human brain contains 100 billion neurons and for every neuron there were 10 glial cells, derived from the Greek word for "glue", which provides the scaffolding for neuronal architecture [250,251]. However, new studies have shown that this number is an overestimation and that the human brain actually contains 86 billion neurons and 85 billion non-neuronal (glial) cells [252]. Since each neuron can form connections with more than 1,000 other neurons, the adult human brain has been conservatively estimated to have more than 60 trillion neuronal connections [253]. These connections are called neuronal synapses, as previously discussed in **2.1 Introduction**.

When GABA is released from vesicles, it rapidly activates the postsynaptic $GABA_A$ family of receptors. This elicits a transient inhibitory postsynaptic current known as a phasic response. After release into the synaptic cleft, GABA is rapidly removed from the intercellular space by

specific transporters (GAT). The majority of the GABA is transported back into the synapse [254]. If multiple vesicles from several terminals release GABA, a 'spillover' occurs that activates postsynaptic receptors as well as extrasynaptic receptors. As discussed in 1.1 History of GABAA Receptor, $GABA_AR$ is differentially expressed in certain subcellular regions and $GABA_A$ receptors composed of the α1, α2, and α3 subunits are primarily located postsynaptically and mediate phasic inhibition [45-47] with rapid desensitization and millimolar sensitivity while the α5, α4, and α6 subunits form extrasynaptic receptors which mediate tonic inhibition [47] and displaying slow kinetics with nanomalor sensitivity [48]. GABA transporters such as GAT1 and GAT3 work to remove the excess neurotransmitter from the synapse but if a low concentration of ambient GABA persists then this presence can tonically activate the high-affinity extrasynaptic receptors [255,256].

Most neurons can be divided into four distinct parts: the cell body called the soma, the processes which receive a signal called the dendrites, the processes on which the signal flows called the axon and at the end of the axon are the axon terminals which house the neurotransmitters. There are two major types of neurotransmission: chemical or electrical. Electrical synapses occur when two neurons are physically connected to one another through protein structures called gap channels while chemical synapses occur between an axon and a dendrite that are physically separated by the fluid-filled synaptic cleft [257]. Unlike electrical synapses, chemical synapses require neurotransmitters to act as chemical messengers.

$GABA_AR$ expression occurs on the surface of neurons in chemical synapses and regulation of the receptor is highly dynamic. Receptors are synthesized and assembled into pentameric structures in the endoplasmic reticulum (ER) [258]. Exit into the Golgi network as well as subsequent trafficking to the plasma membrane is facilitated by multiple associated proteins [255]. Subcellular

localization, ie: synaptic versus extrasynaptic, appears to be orchestrated by a complex act involving diffusion and trapping of select assemblies [259]. When $GABA_AR$ diffuse into the membrane, the widely expressed microtubule-binding protein, gephyrin can transiently capture and anchor the receptors to the membrane [260]. Receptors are recycled and can leave from or be added into the synapse by lateral diffusion and endo- or exo-cytosis. Faulty GABA trafficking has been implicated in multiple diseases such as epilepsy [261], anxiety [256], Huntington's [262], Angelman syndrome [263], fragile X syndrome [264], schizophrenia [265], and drug abuse [266]. Just a single cell can

contain a highly complex population of mixed $GABA_AR$. For example, the hippocampal pyramidal cell is covered with GABAergic terminals, receiving around 1700 synapses [260]. An illustration depicting such a GABAergic synapse can be seen in Figure 114.

When attempting to screen or design a subtype selective BZD, it is critical to keep in mind that selective affinity does not necessarily correspond with selective efficacy. For example, a compound with a high affinity but a low efficacy for a particular subtype could have a high efficacy

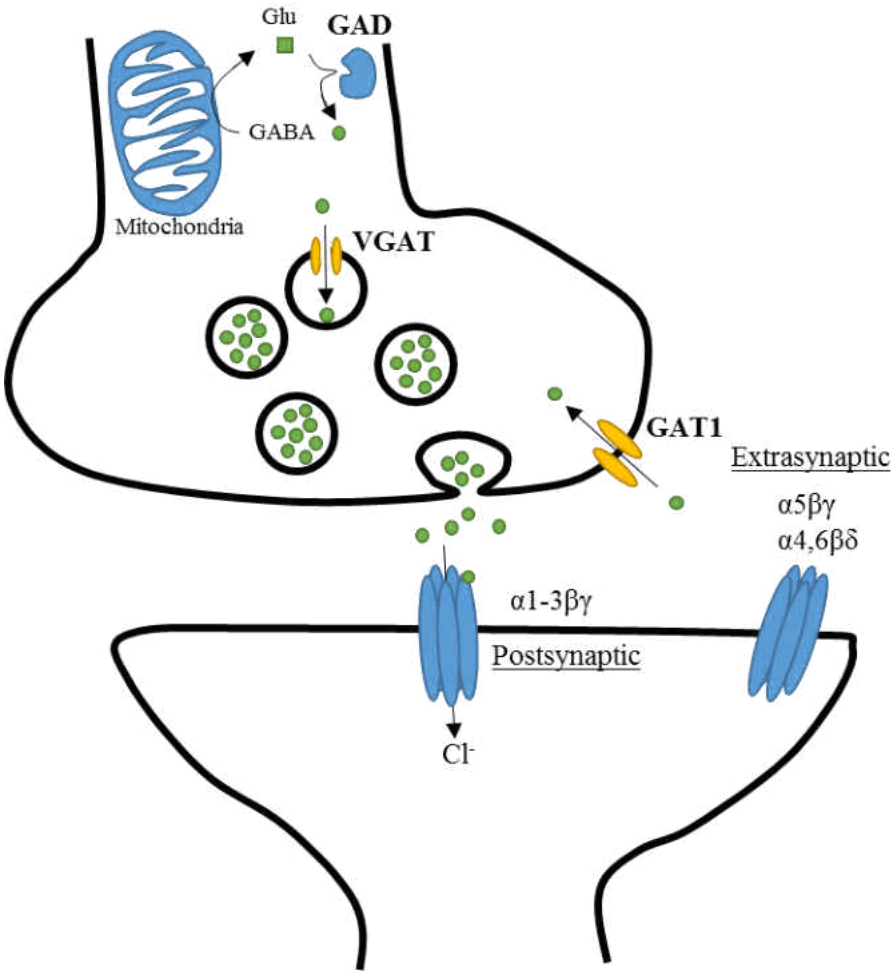


Figure 114. GABA and $GABA_A$ receptor at the synaptic cleft. GABA is synthesized in the cytoplasm of the neuron by glutamic acid decarboxylase (GAD) and transported into synaptic vesicles by vesicular GABA transporter (VGAT). The GABA releases from vesicles into the synapse where it can bind to both postsynaptic (α1-3) or extrasynaptic (α4-6) receptors. GABA is cleared from the cleft by the plasma membrane associated GABA transporter (GAT1).

and low affinity for another subtype, leading to a summation of response in a mixed population of subtypes [83,267,268]. One must take into account the overall affects that a compound will make once exposed to a mixture of subtypes as they will be expressed in the brain.

Taking into consideration these concerns, we propose that coupling together studies of small molecules in respect to recombinant subtype selectivity along with characterization with stem cell derived neurons would provide a more complete pharmacological profile and better reflect observations performed *in vivo*. Human-induced pluripotent stem cells (hiPSCs) provide a novel method when paired with an automated patch clamp instrument to allow for high-throughput study of benzodiazepines in a more physiologically relevant environment of mixed GABA-ergic receptors.

The neurons used in this study were human-induced pluripotent stem cells obtained from Cellular Dynamics International (CDI, Madison, WI) termed iCell Neurons™ and assayed with plate format automated patch clamp. These cells represent a highly pure population composed primarily of GABAergic and glutamatergic neurons. The instrument used was the IonFlux from Molecular Devices (Sunnyvale, CA) which utilizes microfluidic compound delivery on timescales below 100ms, facilitating the recording of fast activating ligand gated ion channels. Using this platform, a large number of cells (20 per ensemble) can be under voltage clamp and exposed to a series of concentrations of compound within a short time period in parallel across a plate. Continuous recording coupled with fast solution exchange enables high-throughput screening of the $GABA_A$ receptor subtypes. To our knowledge, iCell Neurons have yet to be used on an automated "plate reader" patch-clamp platform but have been studied using conventional patch clamp as well as a chip-based, automated patch clamp [269].

The nonselective BZD diazepam and the α2/3 favoring imidizobenzodiazepine HZ-166 were tested on the iCell neurons. In addition, CW-04-020, a drug that binds at a novel site between the α and β interface and is strongly influenced by the α subunit was used [241].

4.2 Genomic Characterization via qRT-PCR

4.2.1 INTRODUCTION

Human induced pluripotent cells (hiPSCs) are a type of pluripotent stem cell that is able to be generated directly from adult cells. This technique bypasses the controversial use of embryonic stem cells. iPSCs were first derived from mice in 2006 from the lab of Shinya Yamanaka in Kyoto, Japan. With the introduction of four specific genes encoding transcription factors, adult cells were converted to pluripotent stem cells [270]. Following this, in 2007, human iPSCs were generated from adult human cells through the use of four genes: Oct3/4, Sox2, Klf4, and c-Myc using a retroviral system [271]. For this pioneering work, Yamanaka was awarded the Nobel Prize in 2012.

Generation of iPSCs is typically time consuming and inefficient. Furthermore, cells have a restricted proliferative potential meaning that new cells must be generated and characterized for each experiment. Commercially available iPSCs provide a convenient source of highly pure populations of cells at industrial quantities. However, the characteristics of the expressed receptors in the iCell neurons has been reported to fluctuate over time while in culture [272] so characterization prior to electrophysiological assay becomes necessary.

4.2.2 EXPERIMENTAL

Cell Culture Reagents and Instrumentation

iCell Neurons (Cellular Dynamics, NRC-100-10-001) were provided via cryopreserved single-cell suspensions in 1.5 mL cryovials containing 2.5 million plateable cells per vial. iCell

Neuron Maintenance Medium (Cellular Dynamics, NRM-100-121-001) and iCell neuron medium supplement (Cellular Dynamics, NRM-100-031-001) were thawed and combined to create the complete media. Poly-L-ornithine (PLO) and laminin were used to provide the base layer and coating for cell attachment to the 6-well plate (Costar). 1 mL of 0.01% PLO (Sigma-Aldrich) was added to one well of a 6 well-plate and incubated at room temperature for 1 hr. The PLO was aspirated and 3 mL of 3.3µg/mL laminin (Sigma-Aldrich, L2020) was added and incubated at 37°C for 1 hr. Cells were removed from liquid nitrogen and placed in a 37°C water bath for exactly 3min before being sterilized and moved to the cell culture hood. The contents were transferred to a sterile 50 mL centrifuge tube. The cryo-tube was rinsed with 1 mL of room temperature complete medium which was pipetted into the 50 mL centrifuge tube dropwise to prevent osmotic shock to the cells. 10 mL of room temperature complete medium was then added to the 50 mL tube at 1-2 drops/sec while gently swirling the tube. Cells were kept in a 37°C and 5% CO_2 and maintained for 10 days with exchange of 50% of the media every 3 days. Cells were washed with Dulbecco's phosphate-buffered saline, without Ca^{2+} and Mg^{2+} (Life Technologies) and dissociation of the cells was performed using 1X TrypLE Select (Invitrogen). Prior to use on the automated patch clamp, cells were centrifuged at 380 x g for 5 minutes and gently resuspended in extracellular solution. This was repeated two more times before dispensing the cells into the plate ($8x10^5$ cells/ml).

qRT-PCR Reagents and Instrumentation

iCells were homogenized using the QIAshredder (Qiagen) and RNA isolated via the RNeasy kit (Qiagen). Total RNA was quantified via a Tecan Infinite M1000 plate reader (Tecan) in a UV-Star 384-well plate (Greiner Bio-One). A QuantiFast SYBR Green RT-PCR Kit (Qiagen) was used for the real time PCR following manufacturer's recommendations. Primers used in these studies are as follows in Table 13. Real-time RT-PCR was carried out on a Mastercycler (Eppendorf). The ΔCt method was used to measure the relative expression of target gene, GAPDH. Standard errors of mean were calculated from two biological independent experiments performed in triplicates. Significance was calculated using Dunnett ANOVA in GraphPad Prism. IonFlux software (IonFlux App) was used for data acquisition and exported to an Excel (Microsoft, Redmond, WA) for organization. The data was loaded onto GraphPad Prism 5 (GraphPad Software, San Diego, CA) for automated analysis of concentration dose-response curves.

Table 13. Human primers list for quantitative RT-PCR of iCell Neurons.

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
α1 (GABRA1)	GGATTGGGAGAGCGTGTAACC	TGAAACGGGTCCGAAACTG
α2 (GABRA2)	GTTCAAGCTGAATGCCCAAT	ACCTAGAGCCATCAGGAGCA
α3 (GABRA3)	CAACTTGTTTCAGTTCATTCATCCTT	CTTGTTTGTGTGATTATCATCTTCTTAGG
α4 (GABRA4)	TTGGGGGTCCTGTTACAGAAG	TCTGCCTGAAGAACACATCCA
α5 (GABRA5)	CTTCTCGGCGCTGATAGAGT	CGCTTTTTCTTGATCTTGGC
α6 (GABRA6)	ACCCACAGTGACAATATCAAAAGC	GGAGTCAGGATGCAAAACAATCT
β3 (GABRB3)	CCGTTCAAAGAGCGAAAGCAACCG	TCGCCAATGCCGCCTGAGAC
γ2 (GABRG2)	CACAGAAAATGACGGTGTGG	TCACCCTCAGGAACTTTTGG
GAPDH (GAPDH)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

qRT-PCR Protocol

Cells were harvested once 80% confluent using 0.05% Trypsin or Detachin solution and pelleted by centrifuging at 1000 rpm for 2 minutes. Media was aspirated and the cell pellet was resuspended in RTL buffer. The mixture was pipetted into the QIAshredder spin column and spun

for 2 minutes at max rpm. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The solution was then transferred to an RNAeasy spin column and spun down for 15 sec at 10,000 rpm. Then washed with buffers to purify the bound RNA with the flow through discarded. After the column is washed and dried, RNA is eluted by addition of 30-50 µL of RNase-free water. Total RNA concentration was determined by UV at 260 nm and protein contamination was assessed at 280 nm. The RNA was then diluted and used for qRT-PCR with the QuantiFast SYBR Green RT-PCR Kit (Qiagen). The cycling conditions used was 10 min at 50°C (reverse transcriptase), 5 minutes at 95°C (PCR initial activation step), 10 sec at 95°C (denaturation), and 30 sec at 55°C (annealing and extension) for a total of 50 cycles. Data was taken in triplicate and the relative mRNA expression levels were calculated using wells containing no template and normalizing to housekeeping gene GAPDH.

4.2.3 RESULTS AND DISCUSSION

Visual observation of the iCell Neuron growth confirmed cell viability after thawing. Morphological inspection of developing branched networks with dendritic processes and active synapses corresponded with the expected appearance, Figure 115, as described by the manufacturer.

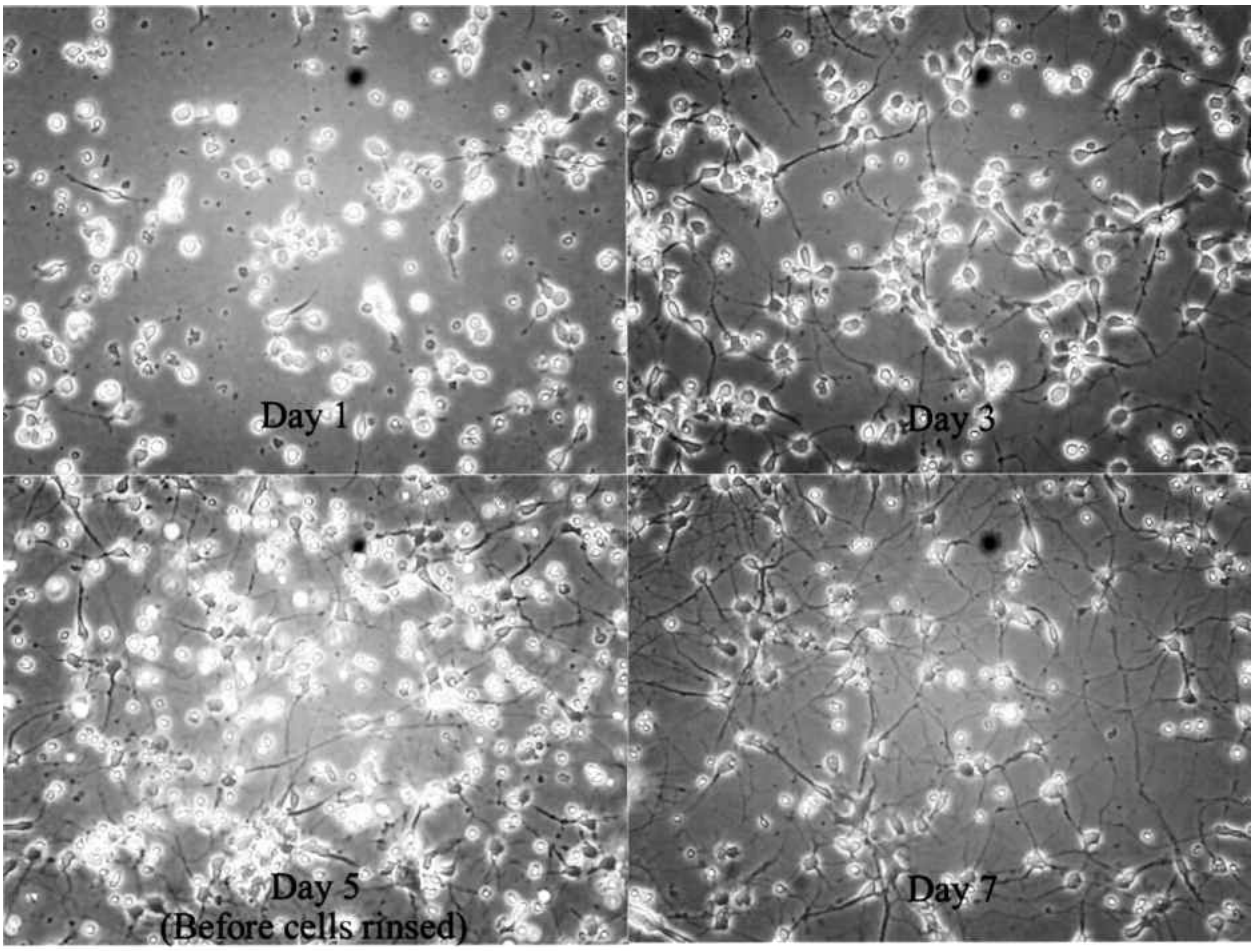


Figure 115. Cultured iCell Neurons exhibited typical neuronal morphology with development of branched networks. These images were obtained using phase contrast microscopy at 20X. iCell Neurons at days 1, 3, 5, and 7 post-plating.

The expression of $GABA_AR$ subunits in iCell Neurons was determined by qRT-PCR, . A moderate mRNA amount of γ2 was identified, which forms part of the binding site for benzodiazepines together with α $GABA_AR$ subunits. In addition, a high level of β3 mRNA was detected. Among the different $GABA_AR$ alpha subunits, high expression of α5 and unexpectedly

lower expression of α1 makes for an uncommon neuronal subtype distribution. It is estimated that

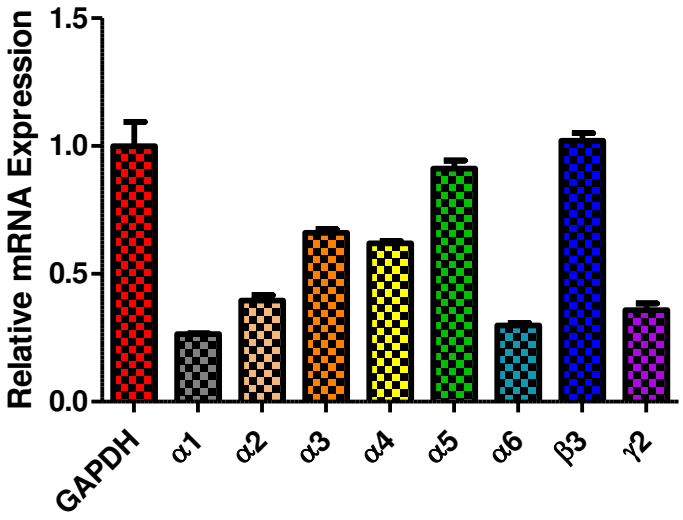


Figure 116. The relative mRNA expression of $GABA_AR$ in iCell Neurons quantified using qRT-PCR. Cells were in culture for 10 days prior to collection. Data includes results in triplicate from two independent experiments, N=6.

α5 is part of less than 5% of all the $GABA_AR$ in the brain while α1 is part of nearly 60% [37]. Furthermore, we observed lower but similar mRNA levels for α3 and α4 subunits and even lower expression of α2 and α6 subunits.

4.2.4 CONCLUSIONS

Quantification of receptor subtype expressions levels revealed a moderate amount of the γ2 which is necessary for benzodiazepine sensitivity. The high degree of α5 subunit mRNA expression as well as the unexpectedly low degree of α1 mRNA makes for uncommon subtype distribution. The expression of the α5 subytpe is mainly localized in the cerebral cortex, hippocampus, and olfactory bulb and serve as extrasynaptic receptors [273]. iCell neurons represent a population with largely a forebrain identity which encompasses the cerebral cortex, we believe

this is contributing to the high level of expression of α5 in the cells as it has been studied that these receptors play a role in learning and memory [38,274] associated with the cerebral cortex.

The $GABA_A$ receptor subtype distribution in iCell Neurons may be of particular interest in the study of compounds that target α5 containing $GABA_A$ receptors. In humans, α5 has been identified as a susceptibility locus for schizophrenia [178] and depression [275]. This receptor subtype appears to be regulated heavily by stress hormones and changes in expression are often associated with stress-related disorders [198] and follow traumatic brain injury [50]. In addition, α5 has also been implicated to impede learning and memory, and development of a selective inhibitor of α5-containing receptors may serve as a cognitive enhancer in Alzheimer's disease patients.

The expression of $GABA_A$Rs in iCell Neurons have been compared to their expression in the human brain at various stages of neurodevelopment [272]. Interestingly, lower expression of α1 and γ2 were observed in brain samples of human neonates in comparison to adults. In addition, β3, α4, α5, α2, and α3 subunits were expressed at higher levels during the first twelve months, but less in the adult brain. Our results are consistent with the observation that iCell Neurons, when cultured for 10 days, are more similar to the $GABA_A$R expression profile of a neonatal than a human adult.

Thus, iCell Neurons may offer an excellent representation of the $GABA_A$ receptor distribution to test pharmaceuticals targeting neurodevelopmental disorders, depression, cognitive deficiencies, and severe brain injury.

4.3 Proteomic Characterization via Electrophysiology

4.3.1 INTRODUCTION

As with planar patch clamping, cells are randomly chosen from suspension by application of suction and good cell quality and viability are mandatory for obtaining good results. Earlier

work[276] has observed successful recording of the response of GABA and antagonist bicuculline on an Nanion chip-based NPC-16 Patchliner Octo which traps 8 cells. Besides this work, to the best of our knowledge, there has been no other publications reporting use of iPS neurons on automated patch clamp.

4.3.2 EXPERIMENTAL

Electrophysiological Reagents and Instrumentation

The buffers were made from NaCl (Fisher, BP358-1), KCl (Fisher, BP366-1), KF (ACROS, 212602500), $MgCl_2$ (Sigma, M8266), 5 D-glucose monohydrate (Sigma, G0350500), HEPES (Fisher, BP410-500), CsCl (Sigma, 203025), and EGTA (Tocris, 28-071-G). The intracellular solution (ICS) contained (mM): 50 KCl, 10 NaCl, 60 KF, 20 EGTA, 10 HEPES, pH 7.2 with KOH. The extracellular solution (ECS) contained (mM): 140 NaCl, 4 KCl, 1 MgCl, 2 $CaCl_2$, 5 D-glucose monohydrate, and 10 HEPES, pH 7.4 with NaOH.

GABA (Sigma-Aldrich, A2129) was made into a 10mM stock solution and diluted in ECS to appropriate concentrations for use. HZ-166 [277] and CW-04-020 (PZ-II-029) [278] was provided by the lab of Dr. James Cook, diluted to a 10mM DMSO stock. Diazepam (Sigma-Aldrich, D0899) was made into a 10mM DMSO stock and diluted to appropriate concentrations in ECS for use.

4.3.3 RESULTS AND DISCUSSION

Four vials of iCell neurons were received and individual experiments were performed on each vial separately. In each plate, 320 cells are captured for whole cell recording. Once cells were trapped, compound addition wells were programmed to open with 3 second duration intervals. The lapse between compound additions ranged from 60-120 seconds, dependent on the degree of expected saturation of the cells at increased concentrations of modulator. All traps yielded an acceptable seal resistance, Figure 117. The success rate of the assays, defined as detectable current

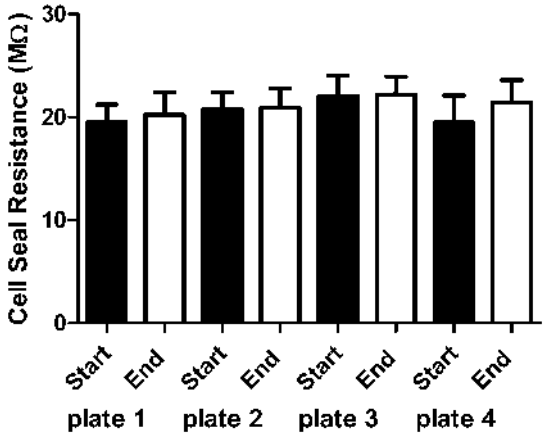


Figure 117. Seal resistance of patched clamped icells Neurons. Twenty individual cells are patched clamped in a trap channel. Seal resistance for the cells remained stable throughout the experiment.

(>1000pA) was 100% for all experiments.

Seven increasing concentrations of GABA were used to determine the electrophysiological EC_{50} value of GABA. The agonistic effect of GABA was measured at a maximum concentration

of 100µM on IonFlux ensemble plate and the GigaOhm Seal plate, Figure 118. Each GABA application occurred for 3000 ms. Negative current increased during that time period and reached saturation followed by desensitization. A rapid decrease of negative current was observed once GABA was washed away by ECS by closing the GABA-containing microfluidic channel. A change of negative current in respect to the baseline was observed at a concentration of 0.14 µM GABA. The maximum negative current was achieved at concentrations higher than 1 µM GABA. The success rate of the assays, defined as detectable negative current of more than -500 pA for the highest concentration of GABA, was 100%.

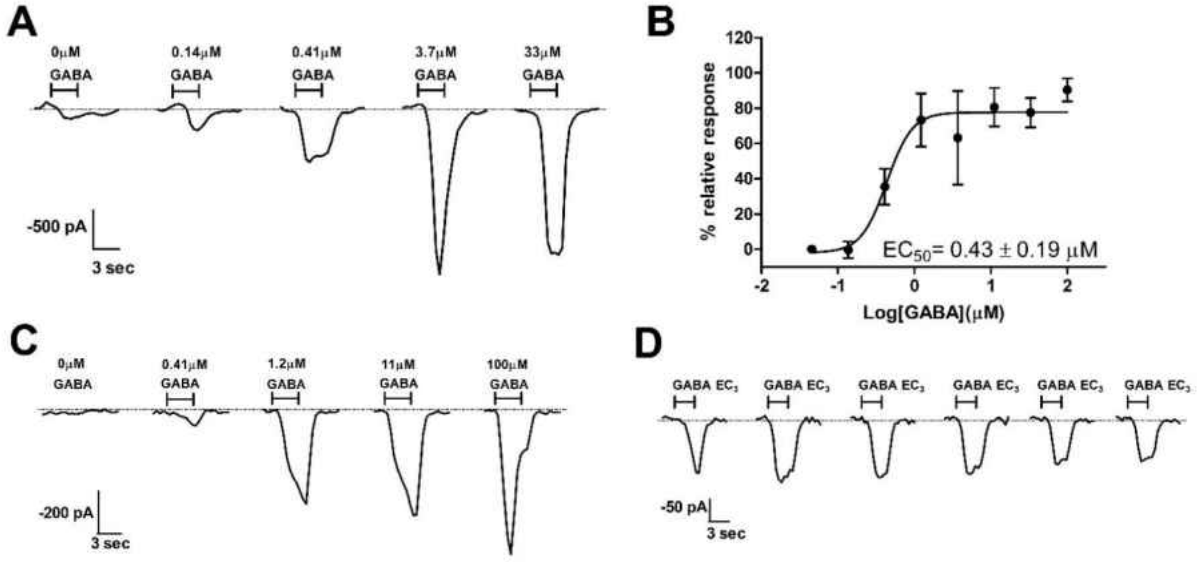


Figure 118. GABA-induced current responses of iCell Neurons measured with the IonFlux instrument. A) Current recordings of different concentrations of GABA applied for 3 seconds using a trap channel that has twenty patched clamped iCell Neurons; B) Concentration-dependent current response curve of GABA using trap channels that have twenty patched clamped iCell Neurons (N = 16); C) Current recordings of different concentrations of GABA applied for 3 seconds using a single cell recording with a trap channel that has one patched clamped iCell Neuron; D) Current recordings of 100 nM GABA (EC3) applied repeatedly for 3 seconds using a trap channel that has twenty patched clamped iCell Neurons.

The experiment was carried out with sixteen independent ensemble trap channels, Figure 118A. The EC_{50} value of 0.43 ± 0.19 µM was determined using non-linear regression. Furthermore, a single cell electrophysiology experiment was carried out using a GigaOhm Seal

Plate in combination with the IonFlux. The success rate for this plate dropped to 25%, however, the resistance for iCell Neurons increased to 100 MΩ. The individual current traces for different GABA concentrations are depicted in Figure 118C. Similar to the multi-cell experiments, negative current increased and saturated during three second application periods of GABA followed by rapid decrease of negative current during the washout period. The overall current changes were smaller than those recorded for multi-cell experiments, Figure 118A. The reproducibility of current change for 100 nM of GABA EC_3 was established to determine the electrophysiological effects of positive allosteric $GABA_AR$ modulators, Figure 118D. It was demonstrated that repeated three second applications of 100nM GABA had a consistent negative current change when applied six consecutive times.

In addition, repeated application of 100 nM GABA for three seconds preceded the application of increasing concentrations of positive allosteric $GABA_AR$ modulators in the presence of 100 nM of GABA as part of the automated patch clamp protocol to characterize the electrophysiological affinity and efficacy of $GABA_AR$ modulators for iCell Neurons. The average negative current change in the presence of 100 nM GABA equals 100% of control current at

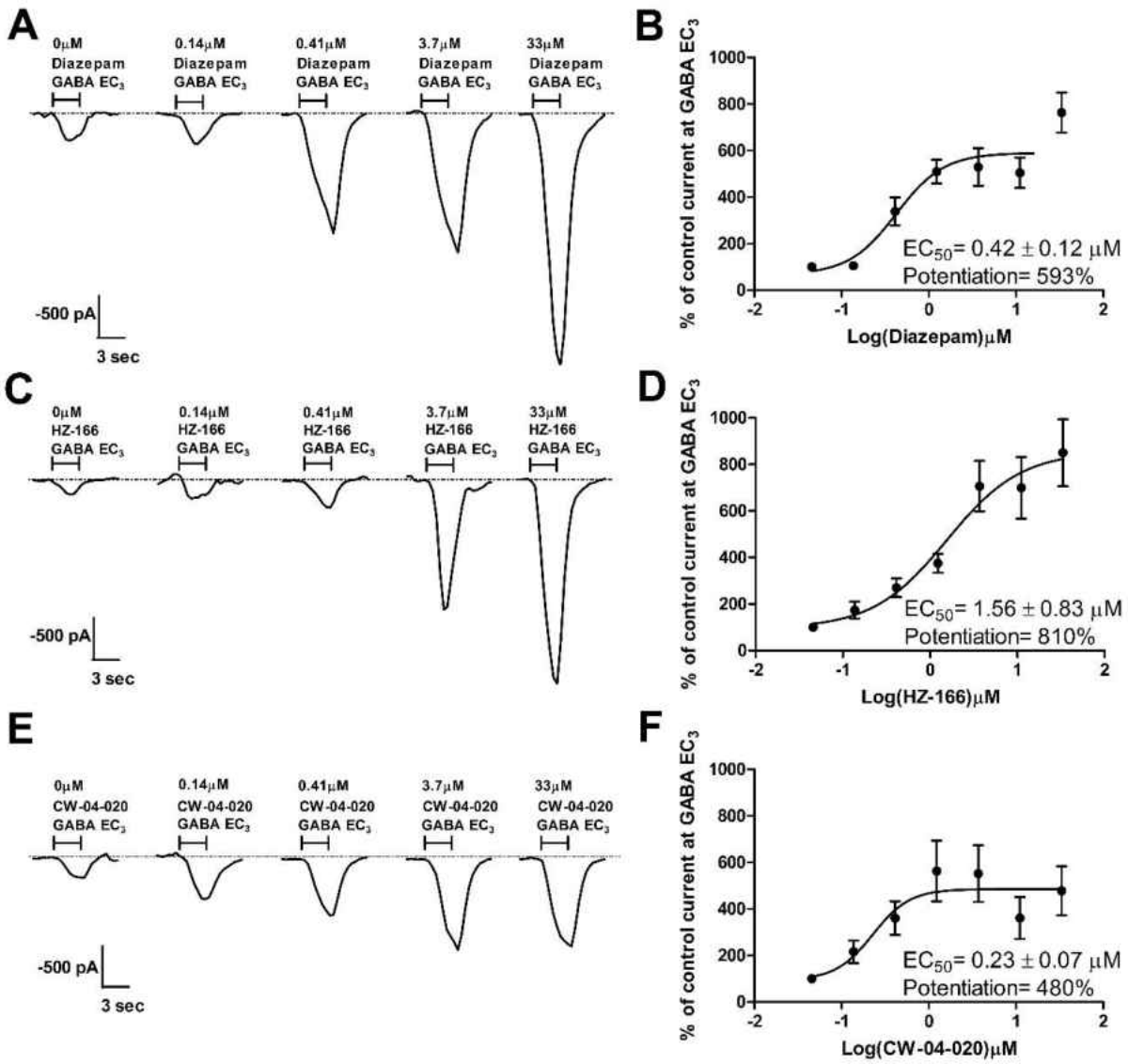


Figure 119. Current responses of twenty patched clamped iCell Neurons from a trap channel located on an IonFlux 16 plate in the presence of positive allosteric GABAAR modulators and GABA. A) Current recordings of iCells Neurons in the presence of different concentrations of diazepam and 100 nM GABA applied for 3 seconds; B) Concentration-dependent current response curve of iCell Neurons in the presence of diazepam and 100 nM GABA (N = 20); C) Current recordings of iCells Neurons in the presence of different concentrations of HZ-166 and 100 nM GABA applied for 3 seconds; D) Concentration-dependent current response curve of iCell Neurons in the presence HZ-166 and 100 nM GABA (N = 20); E) Current recordings of iCells Neurons in the presence of different concentrations of CW-04-020 and 100 nM GABA applied for 3 seconds; F) Concentration-dependent current response curve of iCell Neurons in the presence CW-04-020.

GABA EC_3 for Figure 119. For the compound application, $GABA_AR$ modulators were dissolved in ECS with a maximum of 0.3% DMSO. At a concentration of 0.41 μM diazepam in the presence of GABA a significant negative current change was observed, Figure 119A. Diazepam

concentrations between 1.23 µM and 11.1 µM resulted in a higher but similar negative current change that enabled affinity and efficacy determination. The EC_{50} value calculated for diazepam for iCell Neurons was EC_{50} of 0.42 ± 0.12 µM with a potentiation of 593% in respect to the current observed for 100 nM GABA. The affinity of diazepam among $GABA_A$ receptors bearing different α-subunits using HEK293 cells transfected with $GABA_AR$ subunits decreased from α1>α5>α2>α3, whereas the efficacy, although very similar, decreased from α2>α1>α5>α3 [279]. Interestingly, a concentration of 33 µM diazepam induced a second component of potentiation, which was earlier reported by Walters et al for α1β2γ2 transfected oocytes due to the presence of two components of potentiation in $GABA_A$ receptors, namely binding of α1β2γ2 and α1β2.[215] HZ-166 is a novel anxiolytic compound with less efficacy toward the α1 subtype. The selectivity profile of this compound is α5>α2>α1>α3 for affinity and α3>α2>α5>α1 for efficacy. [280] Investigations with iCell Neurons using the IonFlux showed a slow but steady increase of negative current starting at 0.14 µM of HZ-166, Figure 119C. Saturation of negative current signals were less pronounced at higher concentrations of HZ-166, which might be caused by occupation of different populations of $GABA_A$Rs like diazepam. The calculated EC_{50} value for the most sensitive $GABA_A$ receptor was 1.56 ± 0.83 µM at a potentiation of 810% in respect to the current change observed for 100 nM GABA Figure 119D. Finally a recently discovered α6-selective $GABA_AR$ modulator CW-04-020 (PZ-II-029) was investigated [278]. At a concentration of 0.14 µM a significant increase of negative current was observed Figure 119E. Negative current changes did not significantly change for concentrations higher than 1.23 µM and resulted in an EC_{50} calculation of 0.23 ± 0.07 µM, Figure 119F. The potentiation based on the negative current observed at 100 nM GABA was 480%.

Positive modulators were tested at a maximum concentration of 100μM and serially diluted in a solution of ECS containing a GABA concentration of anticipated EC_3 estimated at 0.1μM, Figure 118: B-D. This established the baseline of 100% to determine % potentiation due to the addition of a positive modulator. The highest DMSO concentration was <1%. Vehicle control measured at the same DMSO concentration showed no significant response.

4.3.4 CONCLUSIONS

The presented preliminary results indicate that iCell neurons present an excellent means of testing a compounds overall efficacy on a mixed population of human $GABA_A$ receptors. The IonFlux microfluidic automated patch clamp platform necessitates that the cells are in suspension when read. The high success rate despite the nonoptimal morphology of the cells in suspension showed great promise. It has been a continuing struggle to develop a method to reliably screen compounds for $GABA_A$ subtype selectivity.

In order to characterize a compound's functional effects, measurements are taken via electrophysiological techniques expressing recombinant $GABA_A$ receptor subtypes to gain a better understanding of their effects in vivo. However, it is very difficult to liken a subtype occurring from recombinant co-expression with an in vivo subtype. This is due to the complexity and inaccessibility of the CNS. The co-occurrence of multiple subtypes of GABA receptors in small regions, or even on a single neuron gives rise to further difficulties. Therefore, analysis of a compound cannot be solely based on the response that comes from recombinant systems. The physiological and behavioral effects observed in clinical trials are a result of control by an amalgamation of GABA-ergic networks, not a singular subtype. In addition, in the event that receptor subtype selectivity is achieved and shown through study with recombinantly expressed

receptors, this effect could be negated by its additional interaction with a second site that is present in a broader range of receptor subtypes [281].

Bi-phasic effect of midazolam has been noted when rat sacral dorsal commissural nucleus (SDCN) neurons are exposed to higher concentrations (30uM-300uM). Wherein current will be inhibited and the dose-response will have a bell-shaped curve [282]. A similar effect is seen here. This biphasic effect is also present in recombinantly expressed receptors in the application of diazepam and appears only in the presence of very low GABA concentrations. In addition, the low affinity site does not require the presence of the γ2 subunit as it is present on the α1β1 receptors. However this effect appears not to have clinical relevance since most physiological effects of BZDs can be blocked by flumazenil [215].

Screening on recombinant cell lines ignores the complexity which arises when multiple subtypes are expressed on a single cell. Screening compounds on specific receptor subtypes in parallel with induced pluripotent stem cells provides a valuable and unique perspective on the effects a $GABA_A$ receptor ligand will have in humans. Based on our findings, iCell neurons are capable of being used on the "plate reader" based automated patch clamp and provide an all-encompassing view of a compounds total efficacy and potency. These readings may provide a more biologically relevant reflection of the effects the compound will exhibit in vivo when exposed to a diverse GABA-ergic network. Creating subtype selective imidizobenzodiazepines that target small subpopulations of the $GABA_A$ receptors should generate modulators that lack the major side effects associated with classic benzodiazepines. Animal models are invaluable in elucidating disease mechanisms and functional roles of specific genes but have been known to fall short when it comes to translating findings into human therapeutics [283]. It is well known that the

expression levels of $GABA_A$ receptor subunits and their localized cellular distribution in rodents do not necessarily correspond with the human brain [50,52]. hiPSCs capture the genetic makeup of a patient, making it possible to study human neurons containing a specific sets of mutations. Most neuropsychiatric disorders, such as autism, schizophrenia, and depression, have a strong genetic component. High-throughput screening of hiPSCs suggests the possibility of attaining cells with specific attributes that mirror the neuronal makeup of patients and could allow for more efficient and accurate screening to treat neuronal disease.

The $GABA_A$ receptor subtype distribution in iCells may be of particular interest in the study of compounds that selectively target the α5 containing $GABA_A$ receptors. In humans, the α5 has been identified as a susceptibility locus for schizophrenia [178] and depression [275]. This receptor subtype appears to be heavily regulated by stress hormones and changes in expression are often associated with stress-related disorders. Autopsy studies of those who suffered from SMD upregulation of α5 with a down regulation of α1 in suicides who died during an episode of major depression [198]. In addition, the α5 has also been implicated to impede learning and memory and that development of a selective inhibitor of the α5-containing receptors may serve as a cognitive enhancer in Alzheimer's disease patients. Similarly, after traumatic brain injury there is an observed upregulation to α5 in the hippocampus which provides enhanced tonic inhibition for an acute neuroprotective measure [50,284]. However, this enhanced tonic inhibition can impede functional plasticity [188]. Thus, modulation of the extrasynaptic α5 that mediates tonic inhibition may prove to be a novel strategy to aid and enhance recovery after a stroke or devastating brain injury. In brief, it can be concluded that these cells may offer an excellent representation of the $GABA_A$ receptor distribution to test pharmaceuticals targeting depression, cognitive deficiencies,

and severe brain injury. These assays may provide an even better reflection of the effects in humans than animal models are currently capable of.

CHAPTER 5: DEVELOPMENT OF FLUORESCENCE-BASED HIGH THROUGHPUT SCREENING ASSAY FOR MODULATORS OF THE $GABA_A$ RECEPTOR

5.1 Introduction

Automated patch-clamp improves the throughput of manual patch-clamp, however microfluidic plates are expensive and yield only a limited number of data points.

Green fluorescent protein (GFP) was first isolated from the Aequorea jellyfish [285]. The wild type protein, crystal structure seen in Figure 120, has a complex absorption spectrum with a

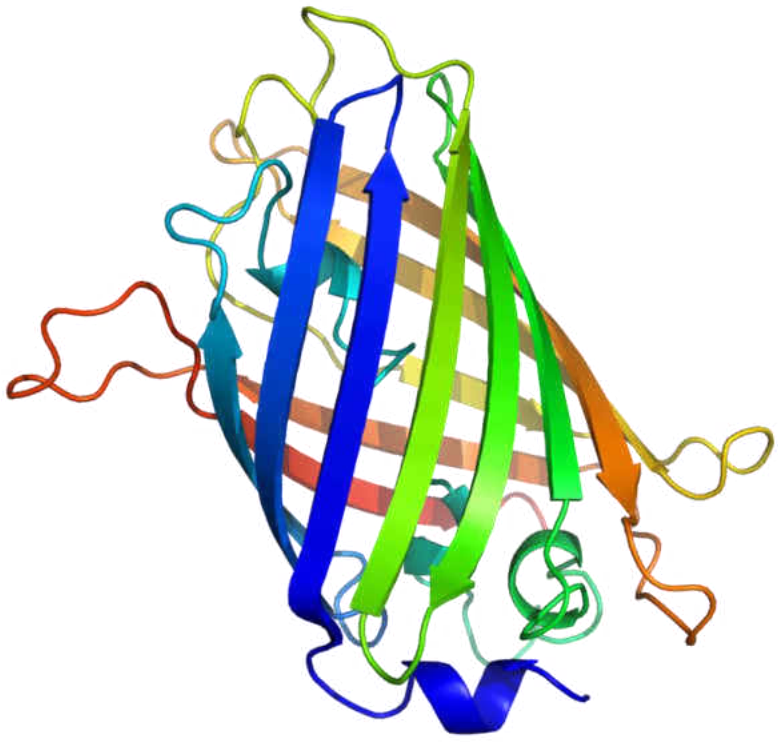


Figure 120. Crystal structure of the *Aequorea Victoria* green fluorescent protein. PDB: 1EMA

maximal excitation at 395 nm and a minor peak at 475 nm [286]. Fluorescence results from deprotonation of the Tyr66 residue in the chromophore. The resulting ionic species exhibited

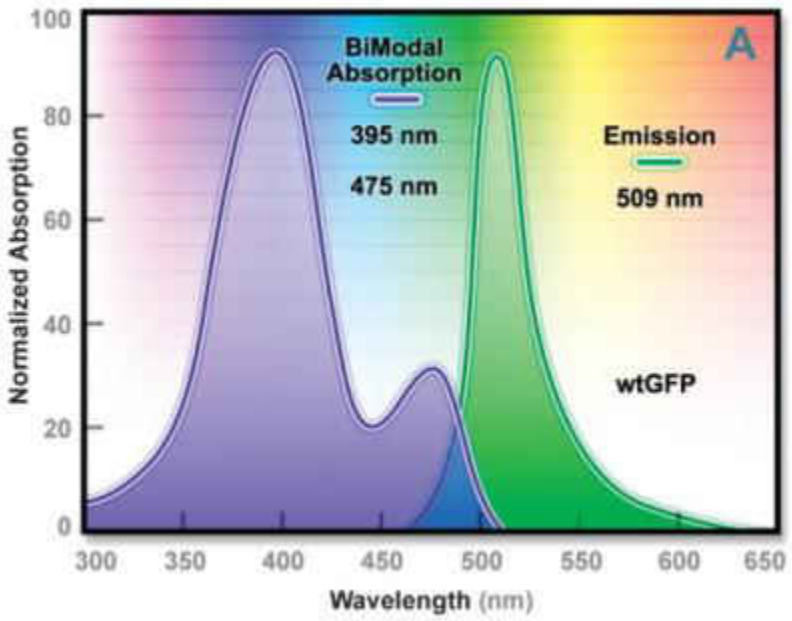


Figure 121. The absorption and emission spectral profiled of wild-type *A. victoria* GFP. Used with permission of the Royal Society of Chemistry, reference cited in text.

excitation at 476 nm. The protonated Tyr66 reside is responsible for the major absorption peak at 476 nm. Mutagenesis studies to fine-tune the spectral qualities of the protein has generated a wide variety of fluorescent proteins that range from blue to yellow [286] including substitution of the Thr203 in the beta-barrel near the chromophore with tyrosine (T203Y). This resulted in a 20nm red-shift, generating a new fluorescent protein with yellow-green emission, called yellow fluorescent protein (YFP). The enhanced version (eYFP) is engineered further with three more point mutations (S65G, V68L, S72A) to improve the efficiency of protein maturation and expression. The excitation and emission of this eYFP are 514 nm and 527 nm respectively, seen in Figure 122 [286]. These mutations led to proteins with brighter fluorescence but also to heightened sensitivity to acidic pH and to the quench of anions. A unique feature is that eYFP is sensitive to chloride ions. Random mutation have identified that H148Q and I152L increases halide sensitivity [287,288]. I^-, NO_3^-, Br^-, and Cl^- anions can quench the YFP-mediated fluorescence. Chloride-channels such as the $GABA_AR$, mediate an increase of chloride ions in the cell when exposed to GABA,

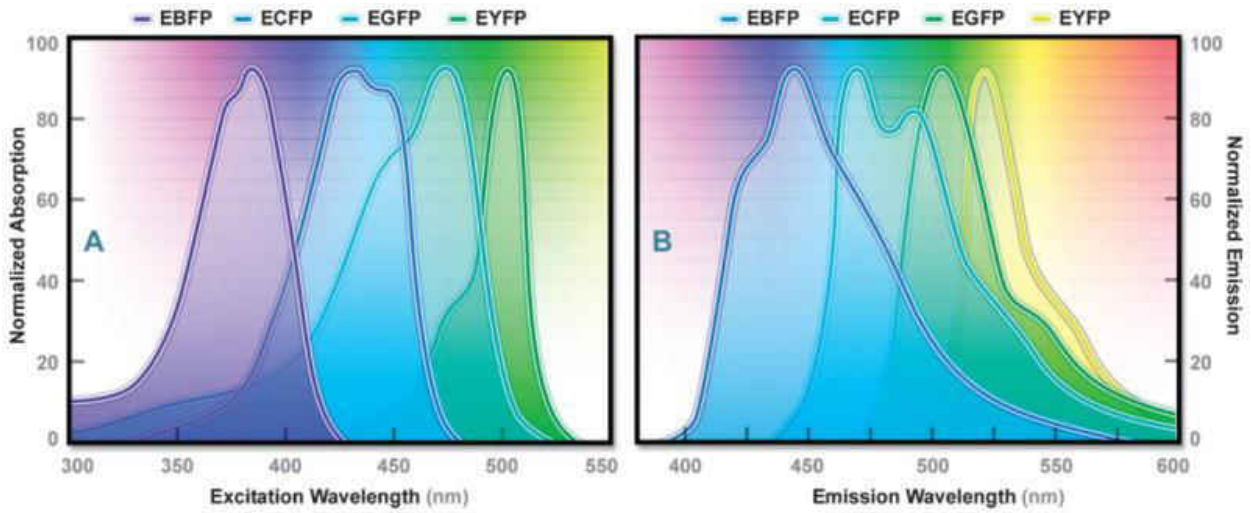


Figure 122. The absorption (A) and emission (B) spectral profiles of the enhanced Aequorea-GFP derivatives: enhanced blue fluorescent protein (eBFP), enhanced cyan fluorescent protein (eCFP), enhanced green fluorescent protein (eGFP), and enhanced yellow fluorescent protein (eYFP). Used with permission of the Royal Society of Chemistry, reference cited in text.

which in turn can quench the eYFP when expressed in the cytosol of a cell. An assay with transiently expressed $GABA_AR$ with α1β1 subunits and eYFP has been developed and verified with GABA as well as with channel blocker picrotoxin [289]. The anion selectivity for quenching is ClO_4^- ~ I^- > SCN^- > NO_3^- > Cl^- > Br^- > formate >acetate. Recently a similar assay was developed that applied α2β3γ2 transiently transfected in CHO-K1 cells and determined the response to agonist GABA, six positive modulators, and channel blocker bicuculline [290].

5.2 Assay Optimization

5.2.1 INTRODUCTION

It should be noted that in the course of studying the potential of eYFP to design an assay, a 2013 publication reported the development a YFP-based assay using CHO-K1 cells that were transiently transfected with the $GABA_A$ α2β3γ2 with the halide sensing eYFP H148Q/I152 using electroporation [290]. However, this assay was performed using a serial dilution of GABA with a fixed concentration of known significant positive allosteric modulators such as diazepam,

lorazepam, clobazam, and alpidem. The EC_{50} shifts for these modulators were estimated at 1.4 times shift in the GABA EC_{50} or a difference in pEC_{50} or 0.15 compared to GABA. This was calculated to have statistical significance when averaging across 3 occasions. There was no detection of significant allosteric effect using weak modulators such as TPA-023 or L-838417. Hence it would appear that this method would not be suitable to distinguish small changes in efficacy among similar strong or weak modulators and thus would not be a good screening tool for structure activity relationship (SAR) studies. However, we continued our experimentation as to whether a serial dilution of compound with a constant concentration of GABA would elicit any discernable difference between similar modulators.

5.2.2 EXPERIMENTAL

Plasmid Propagation Reagents

The eYFP-H148Q-I152L was purchased from AddGene provided the lab of Peter Haggie [287]. The plasmids arrived on paper disks and were eluted with RNase Free Water (Fisher, BP24701). 1 µL of the elution was added to a tube of NEB 5-alpha competent E. coli cells (New England BioLabs, C2987H) and flicked to mix. The mixture was placed on ice for 2 minutes, undisturbed and immediately heat shocked at exactly 42°C for 30 secs. The tube was moved to ice for 2 min, after which 950 µL of SOC was added to the mixture. 50 µL and 100 µL were spread onto a 100 µg/mL carbenicillin (GoldBio, C10325) plate and grown overnight at 37°C. A colony was chosen and used to inoculate LB broth (Fisher, BP9733-500) containing carbenicillin. The resulting culture is centrifuged at 6,000 x g for 15 min at 4°C to pellet the bacteria and the supernatant removed. The plasmid DNA is then extracted using a gravity-flow anion-exchange HiSpeed Plasmid Maxi Kit (Qiagen, 12663). The pellet was resuspended in buffer and an alkaline

lysis is performed before the lysate is cleared by filtration. The lysate is then added to a primed HiSpeed tip to bind DNA, wash, and finally elute. Isopropanol was added to the elution to precipitate the DNA and collected using the QIAprecipitator. The final elution from the QIAprecipitator yields ultrapure plasmid DNA. The DNA concentration was determined by UV at 260 nm using the Tecan Infinite M1000 plate reader. Protein impurities were minimal by comparison of the 260/280 nm ratio.

Cloning Reagents and Instrumentation

The eYFP gene was removed from the vector via enzymatic digestion using XhoI (NEB, R0146) and SmaI (NEB, R0141) and treated with Antarctic phosphatase (NEB, M0289) which adds phosphate groups to exposed 3'OH groups to prevent self-ligation of sticky ends. 2 µL of digested eYFP, 4 µL of water, 0.7 µL of AnP buffer and 1 µL of Antarctic phosphatase were incubated at 37°C for 15 min. The mixture was heat inactivated at 65°C for 5 min. The alpha1-Lab-pCI underwent digestion with the same enzymes and the eYFP was inserted using 1 µL T4 ligase (NEB, M0202S), 10 µL of 2x ligation buffer, 3 µL of the fragment (purified by gel) and 1 µL of the pCI vector (purified by gel) and incubated at room temperature for 15 min. 1 µL of the reaction was added to a tube of NEB 5-alpha competent E. coli cells (New England BioLabs, C2987H). The cells were cultured onto a 100 µg/mL carbenicillin (GoldBio, C10325) plate and grown overnight at 37°C. A colony was chosen and used to inoculate LB broth (Fisher, BP9733-500) containing carbenicillin and purified using the HiSpeed Plasmid Maxi Kit (Qiagen, 12663).

EcoRI digestion was performed using 1 µL of EcoRI (NEB, R0101S), 1 µg of DNA, 5 µL of CutSmart buffer and nuclease-free water to the total reaction volume of 50 µL. The reaction was incubated at 37°C for 1 hr. The digest was run on a 1% agarose gel with 0.5 g of agarose

(Fisher, BP160-500) with 50 mL of 1xTBE buffer, microwaved in bursts of 30 secs until the agarose was dissolved. 5 µL of an ethidium bromide replacement, Bullseye DNA Safestain (MidSci, C138) was added to visualize the DNA under ultraviolet light. The Bullseye 1 Kb DNA ladder (MidSci, BEDNA1KB) was used to measure band migration on the gel. The agarose was poured into a small gel tray with a well comb. The gel was allowed to solidify at room temperature for 20-30 min. The gel was run at 80-150 V for around 30 min until the dye line was 50-75% down the gel.

Fluorescence Microscopy

Initial imaging was performed with the Nikon Eclipse TE2000-U was connected to mercury-100W, Prior ProScan II, Sutter Lambda 10-3. Later imaging was performed on a Nikon Ti-E inverted fluorescence microscope using a 20X and 40X/NA 1.4 objective.

Measurements of total fluorescence were taken with the Tecan Infinite M1000 plate reader with at 470 nm excitation, 550 nm emission, bandwidth 5 nm, and gain optimized at 245. The buffer used was composed of 137 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 at pH 7.4.

Cell Culture Reagents and Instrumentation

A commercially available, human embryonic kidney (HEK 293T) cell containing the simian vacuolating (SV) virus 40 T-antigen origin of replication [207] was used in all the stable cell lines. HEK 293T cells were purchased (ATCC) and cultured in 75 cm^2 flasks (CellStar) coated in matrigel (BD Bioscience, #354234), a gelatinous protein secreted by mouse sarcoma that facilitates cell adhesion to the flask. Cells are grown in DMEM/High Glucose (Hyclone, SH3024301) media to which non-essential amino acids (Hyclone, SH30238.01), 10 mM HEPES

(Hyclone, SH302237.01), 5 x 10^6 units of penicillin and streptomycin (Hyclone, SV30010), and 10% of heat-inactivated premium US-sourced fetal bovine serum (FBS) (Biowest, SO1520HI) were added. Cells are harvested using 0.05% Trypsin (Hyclone, SH3023601) or Detachin (Genlantis T100100) which both disrupts the cell monolayer and proteolytically cleaves the bonds between the cells and flask; the latter more gently for patch-clamp study. The media utilized in transient transfections contains the same components only the FBS was heat-inactivated and dialyzed FBS (Atlanta Bio, S12650H), then cells were rinsed and shaken in Serum Free Media (Hyclone, SH30521.01).

Cell transfection was conducted by lipid-based methods using Lipofectamine with PLUS reagent (Life Technologies, #15338020). 5 µg of each of the plasmids was combined with 25 µL of Plus reagent and incubated for 5 min, 75 µL of Lipofectamine was then added to the mixture and incubated for 30 min before being added to a 60-80% confluency 75cm^2 flask. Cells were counted on a hemocytometer, 20 µL of cell suspension are aliquoted onto the slide and 3 counting areas whose volume is 100 nL are averaged and multiplied by $1x10^4$ to give a concentration of cells in cells/mL When measured with the Tecan plate reader, cells were transferred to the 96-well plate (ThermoSci, 12-566-71) cultured with 100 µL of media while a 384-well plate (ThermoSci, 12-566-1) contained 25 µL of media. Since the plates are clear on the bottom to provide optical access of the cells, an adhesive bottom seal should be applied prior to reading the plate from the top to reduce scattering (Perkin Elmer, 6005199). Initial buffer composition was 200 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES at pH 7.3. Compounds were dissolved as a 10 mM DMSO solution and final DMSO concentration in the assay was 1%. DMSO only controls were included in every experiment with no detectable effect of DMSO on the quench.

5.3.3 RESULTS AND DISCUSSION

Initial transfections with the eYFP-H148Q-I152L plasmid exhibited low fluorescence signals after a 24 hour recovery. We surmised the low signal was the result of unmatured eYFP

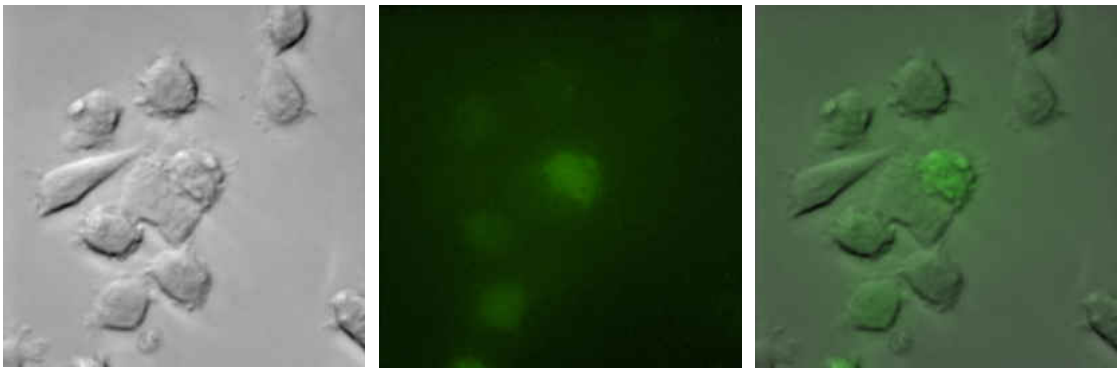


Figure 125. Image of eYFP in HEK293T cells after 24hr transfection (20X objective)

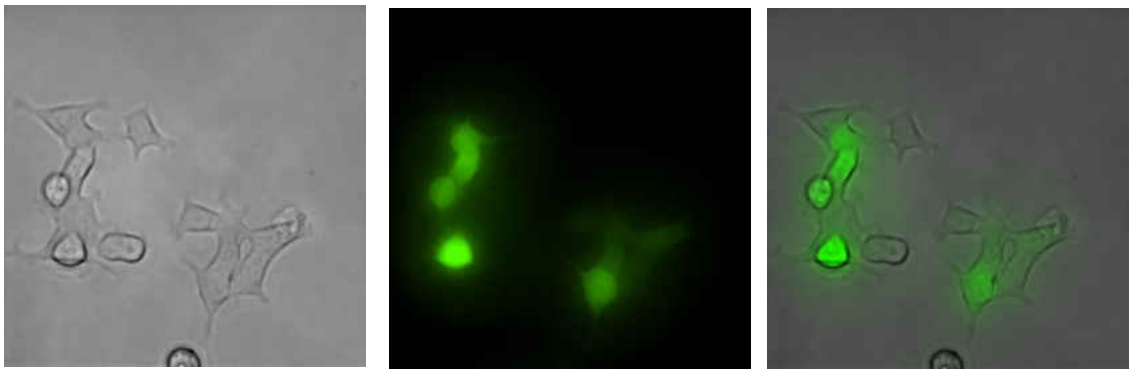


Figure 123. Image of eYFP in HEK293T cells after 48hr transfection (40X objective)

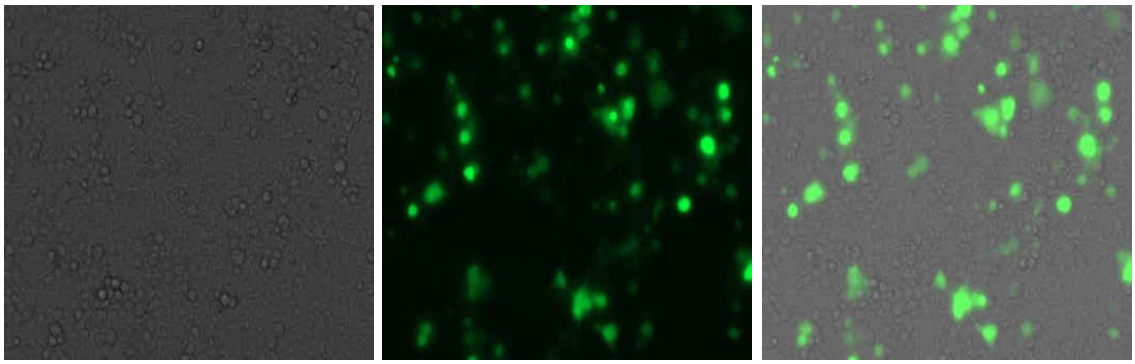


Figure 124. Overall efficiency of eYFP transfection after 48 hours (20X objective)

rather than a low percentage of successful transfection. Fluorescence imaging of individual cells

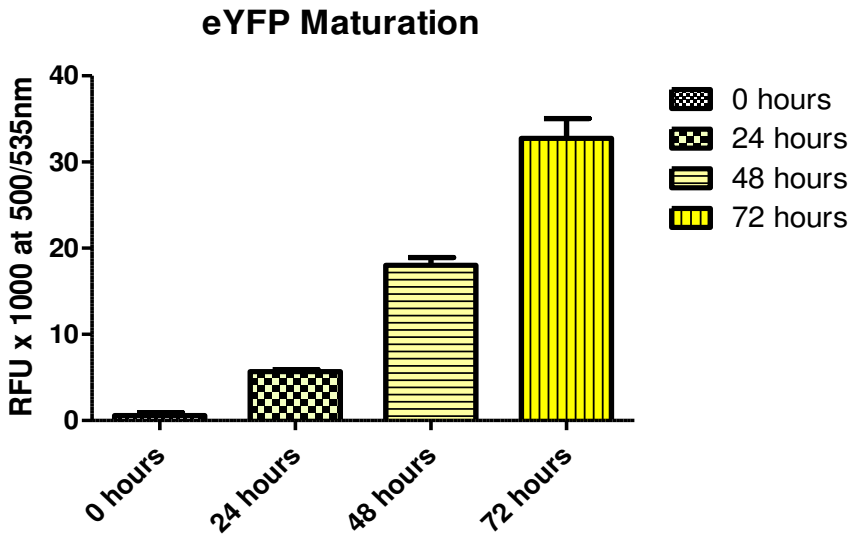


Figure 126. eYFP-H148Q-I152L length of protein maturation after transient transfection, N=12, reading performed on the Tecan M1000.

confirmed this, seen in Figure 125 after 24 hours and Figure 123 after 48 hours. The overall transfection efficiency was also imaged, seen in Figure 124. Using a fluorescence plate reader, cell fluorescence was assessed over time and it was determined that optimal maturation for eYFP required up to 48-72 hours to attain a high level of translation and correct folding in HEK293T cells at 37°C, seen in Figure 126. The fluorescent protein, once expressed, requires several steps before becoming functional. The protein first must be folded correctly, then a torsional rearrangement of the active site of the fluorophore occurs which allows a nucleophilic attack that results in the formation of a ring system, finally oxidation of the ring system forms the final system of conjugated bonds causing the formation of the mature fluorophore. This entire process is known as maturation [291].

Another dynamic feature of these fluorophores is the photobleaching effect. That is, when the fluorophore is exposed to continuous illumination for several tens of seconds, the fluorescent intensity will decay. The photobleaching effect over multiple readings was assessed and was

determined to be nonsignificant within one to three readings, Figure 127, sufficient enough to record the change in quench.

In order to achieve maximal quench of the eYFP, a plasmid combining the α1 $GABA_AR$ (Figure 128) and the eYFP (Figure 129) was created using molecular cloning. The backbone containing the eYFP had an EcoRI site after the gene that could accommodate the α1 $GABA_AR$

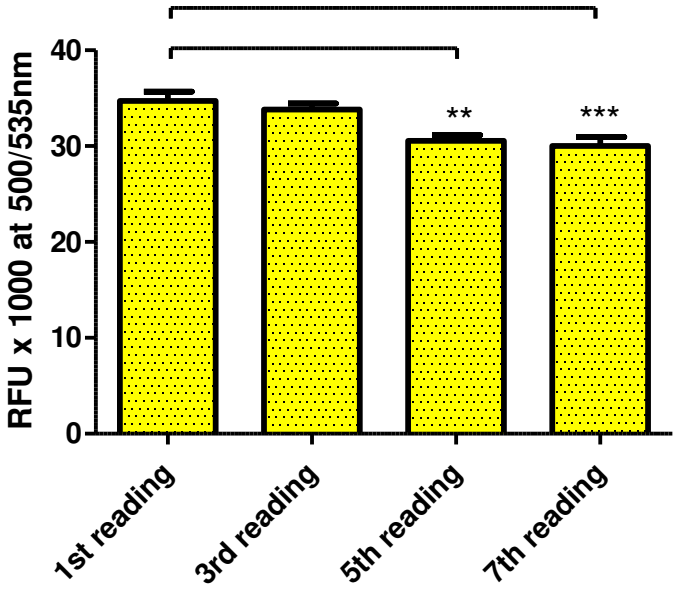


Figure 127. Photobleaching of the eYFP over seven measurements with the Tecan M1000, 50 flashes each reading. N=24. Significance (*) calculated using One-way ANOVA and Dunnett's Multiple Comparison Test.

gene which was flanked by two EcoRI sites. Multiple colonies were isolated from these experiments but always resulted in the eYFP backbone re-ligated back into the original sequence containing no insert. Two other restriction enzyme sites that both plasmids had in common were an XhoI and XbaI. Again the ligation involved the insertion of the α1 $GABA_AR$ sequence into the eYFP plasmid. These experiments also resulted in the original eYFP plasmid. So instead of using the eYFP backbone, the vector containing the α1 $GABA_AR$ gene was used as the receiver of the eYFP gene. This was achieved using restriction enzymes SacI and ApaI. This strategy quickly led

to the successful expression of the eYFP and α1 $GABA_AR$ gene. However, the two proteins were not fused and the eYFP contained a stop codon. Thus a site directed mutagenesis or a PCR with specifically designed primers would need to be performed to enable the generation of a fusion

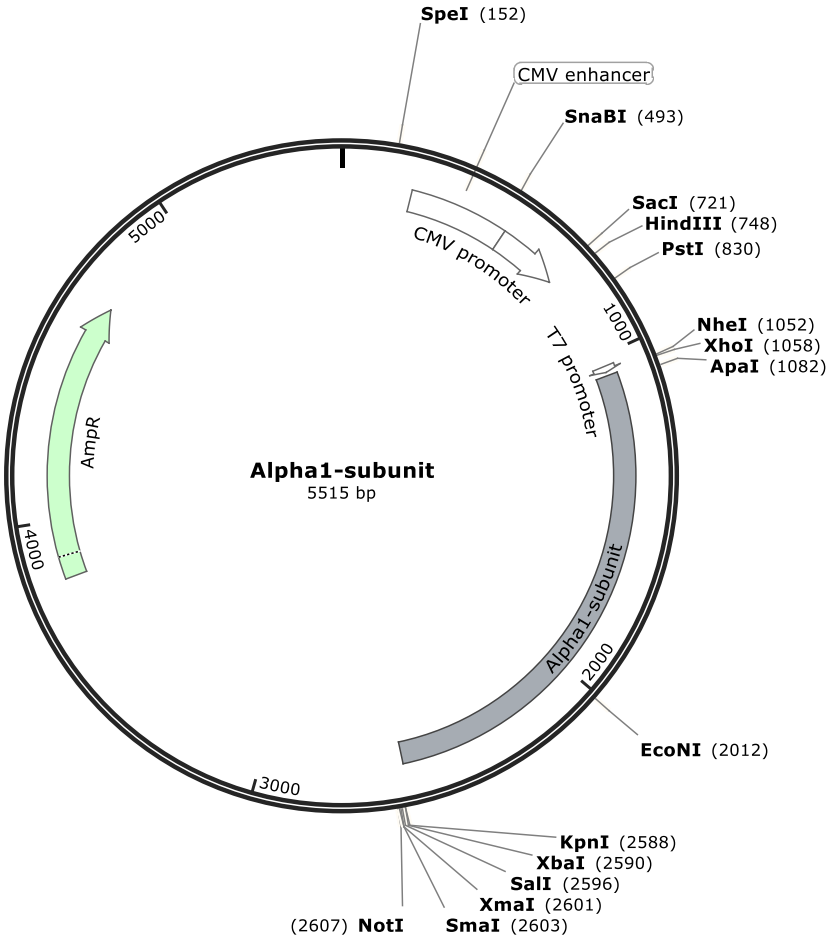


Figure 128. The plasmid containing the alpha1 $GABA_AR$ subunit with restriction enzyme sites.

protein. Thus assay development was performed using an unfused eYFP and $GABA_AR$ α1 subunit. A Gaussia Luciferase (AddGene, pCMB-GLuc_2) was also purchased for possible creation of fusing a subunit with luciferase to create a bioluminescence resonance energy transfer (BRET) assay.

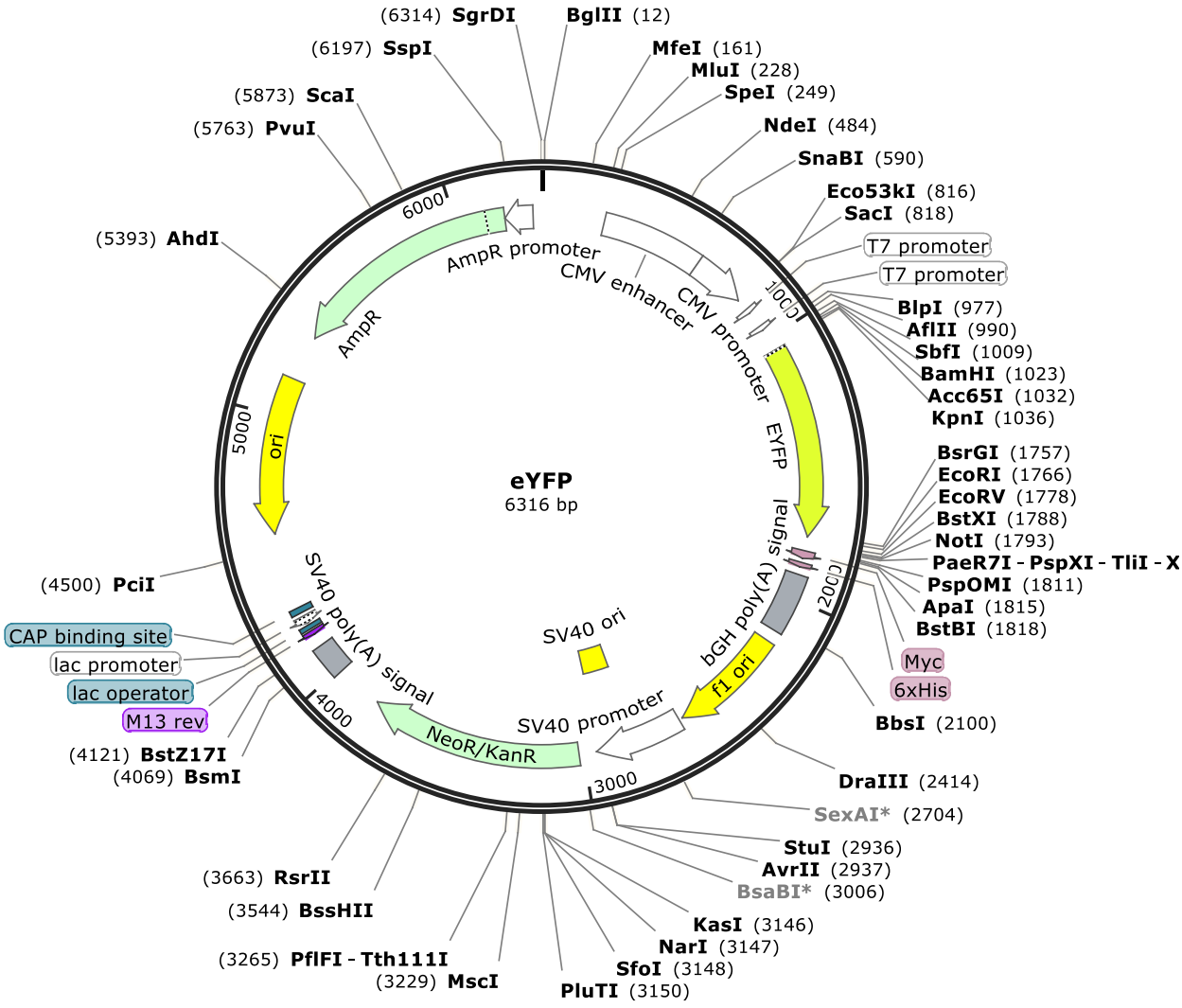


Figure 129. The plasmid containing the eYFP gene with restriction enzyme sites

Based on earlier findings that the iodide concentration greatly affected the basal quench of the fluorophore as well as the potency of GABA, we modified our solution. The NaI buffer was composed of 130mM NaCl, 10mM NaI, 20mM Hepes, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$ at pH 7.3.

The agonist GABA elicited a clear dose response curve with high concentrations achieving

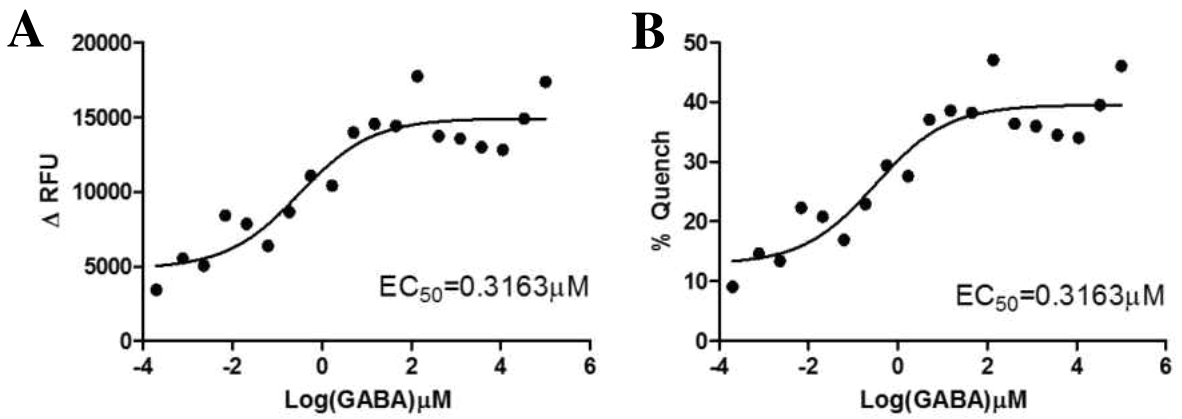


Figure 130. eYFP quench induced by GABA in 10mM I^- buffer. A) The change in the relative fluorescence units was calculated before and after addition of GABA in 10mM I^- buffer. B) The percent quench was calculated as the average of the maximum signal achieved subtracted from the average of the background.

as much as 40% quench with low concentrations retaining a 10% quench from the addition of the NaI buffer, Figure 130. This corresponds with what was previously published [290] despite having different transfection methods and mammalian cell lines.

The paper that was previously published perfomed a serial dilution of GABA with a

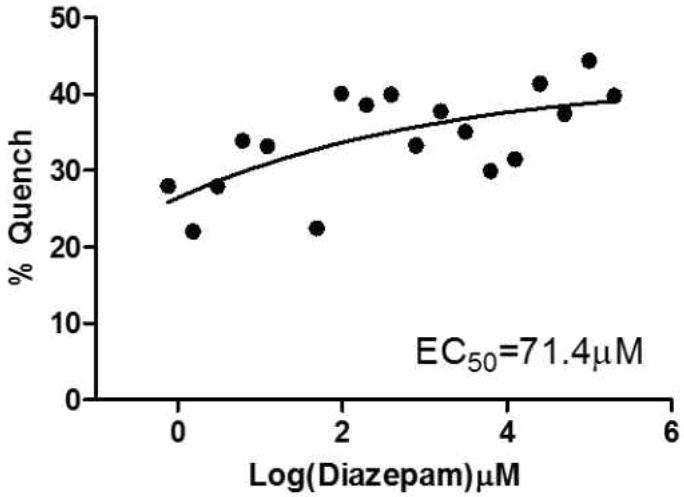


Figure 131. Cells transfected with the α1 and eYFP were exposed to a mixture of serially diluted Diazepam with 0.1μM GABA in NaI buffer and fluorescence was immediately recorded.

constant concentration of 1μM modulator, citing slight differences in the shift. Instead of

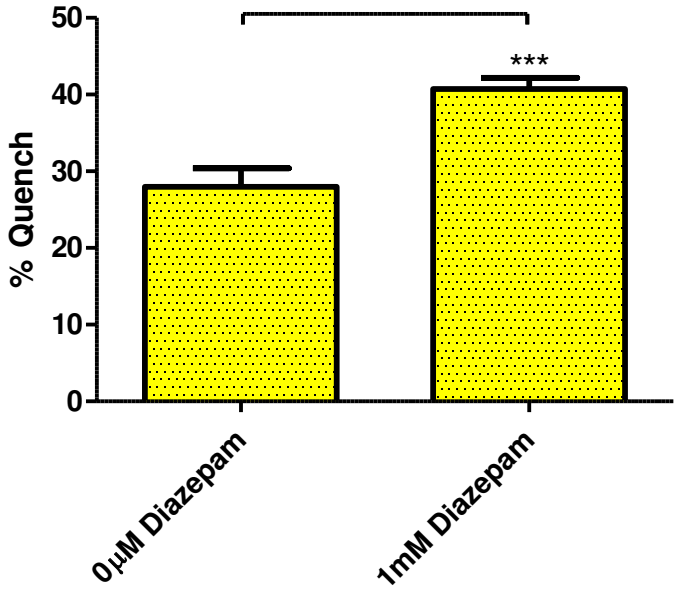


Figure 132. Comparison of quench between the DMSO control and 1mM of Diazepam. Statically significant or non-significant at $p<0.05$

recapitulating this EC_{50} shift, a serial dilution of compound with constant concentration of GABA was attempted. This method resulted in plots resembling Figure 131.

Though the overall graph does not appear to show a significant increase in quench, a comparison of average values achieved using 1mM Diazepam vs DMSO after the addition of an EC_{20} concentration of 0.1μM GABA showed statiscal significance with 95% confidence, when averaging across 4 occasions, Figure 132.

In order to maximize the quench, the assay was optimized in varying ways. Firstly, the assay necessitated buffer and liquid exchange. The method of exchanging the liquid greatly effected the quench. For example, emptying the plate via flipping the plate or via aspiration using a vacuum resulted in varying changes in signal, Figure 134. We determined that emptying the plate via a flip resulted in lower change in fluorescence as well as a lower varience or deviation in

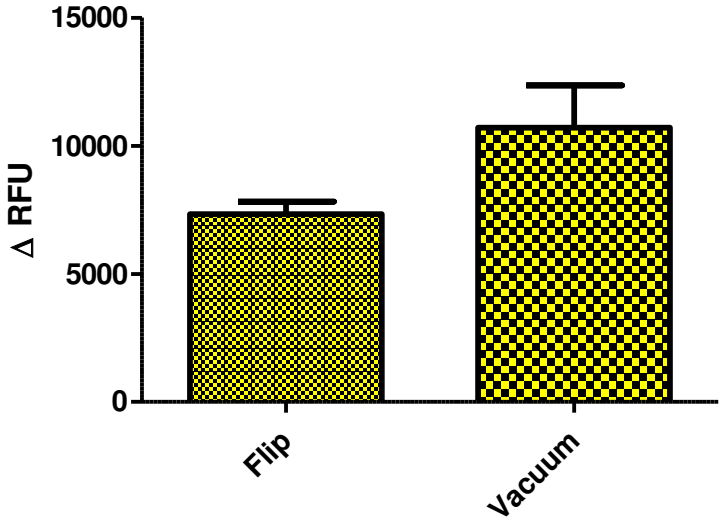


Figure 134. Comparison of plate emptying methods for liquid exchange in the eYFP assay.

results. A visual assessment of the cells after liquid exchange revealed that the loss in fluorescence was due to loss of the adherent cells, Figure 133. Previously, cells had been plated at 20,000 cells

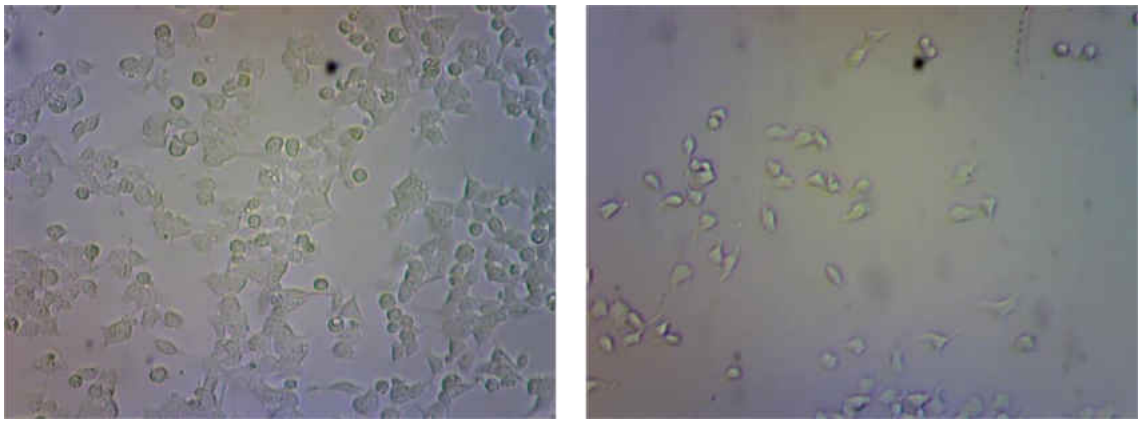


Figure 133. Light microscope image of a well in the 96 well plate with cells plated at 20,000 cells per well with 48 hours of maturation time before and after two exchanges in buffer including the 15 minute preincubation with DMSO or compound.

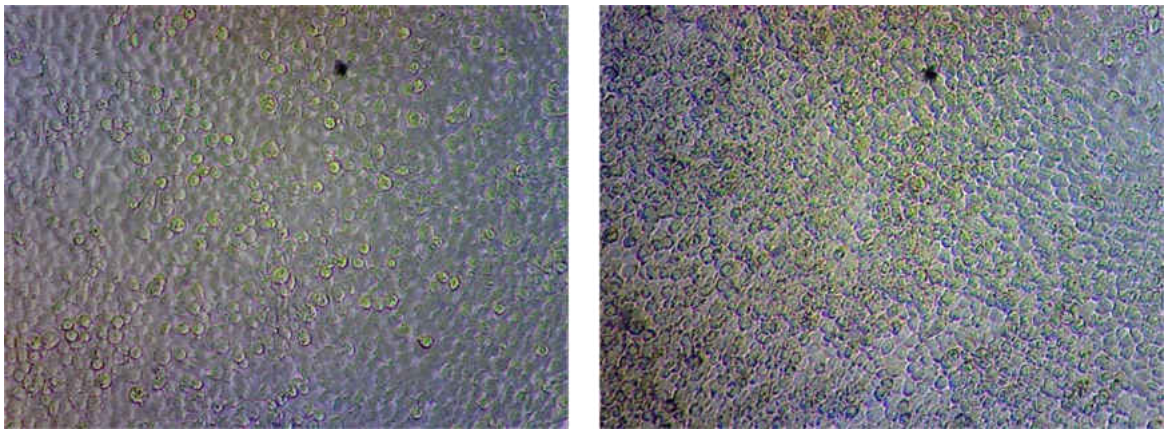


Figure 135. Light microscope image of a well in the 96 well plate with cells plated at 50,000 cells per well with 48 hours of maturation time before and after two exchanges in buffer including the 15 minute preincubation with DMSO or compound.

per well in a 96 well plate which resulted in a roughly 50% confluency after the 48 hour eYFP maturation time. In order to cell loss, 50,000 cells were plated per well which resulted in a 100% confluency after the fluorophore maturation time. This high seeding density prevented the loss of cells, Figure 135. In addition, we changed to directional reading to a bottom read fluorescence

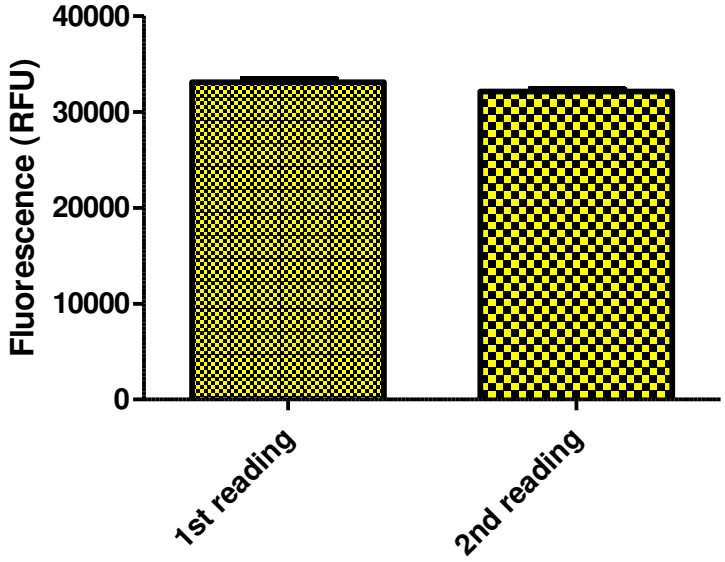


Figure 136. Cells plated at 50,000 cells per well at 100% confluency at the time of reading saw negligible change in the fluorescence signal after liquid exchange. N=7 in a 96 well plate.

confirming that the signal was not lowered after liquid exchange, Figure 136.

Further optimization brought attention to the differential expression that may be due to the vector backbone of the plasmid. It was observed that the plasmid containing the eYFP as well as the α1 gene led to higher levels of fluorescence suggesting that the backbone containing the α1 led to higher expression of the protein after transfection. A simple EcoRI digestion, gel separation

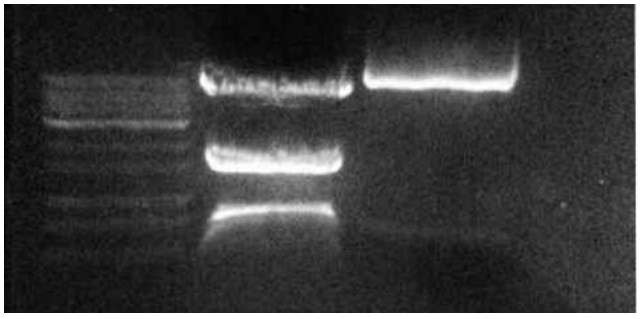


Figure 138. Agarose gel of the eYFP-$GABA_AR$ α1 construct. Left column is Bullseye 1Kb DNA Ladder, middle is the EcoRI digest of the eYFP-$GABA_AR$ α1 construct, right is the undigested eYFP-$GABA_AR$ α1 construct.

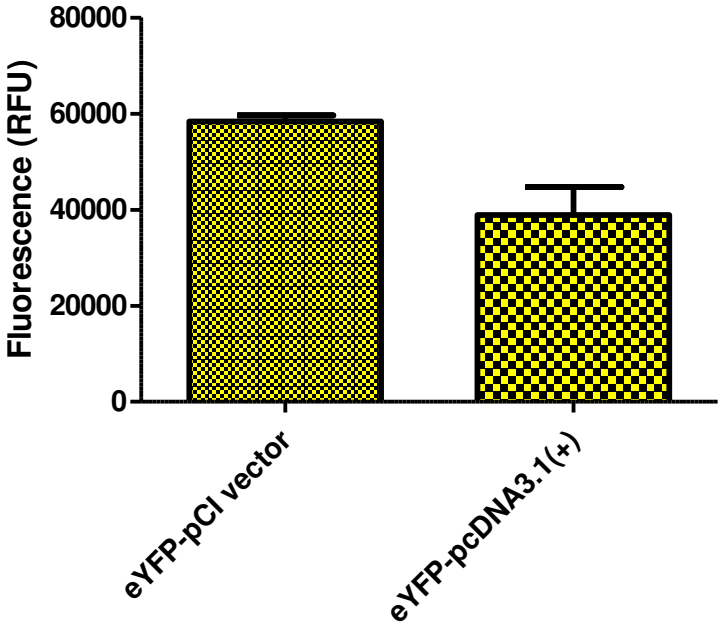


Figure 137. Cells transfected with the eYFP contained in the pCI vector vs the pcDNA3.1(+) vector. N=24 in a 96 well format.

(Figure 138), and ligation led to the creation of the pCI-eYFP plasmid with excised α1 gene. The difference between fluorescense signals is seen in Figure 137.

As a result of these changes, the GABA dose response at low concentration of agonist was close to baseline with negligible quench instead of the 10% quench observed previously published by Johansson et al. [290], Figure 139. It should be noted that the GABA dose response curves can be

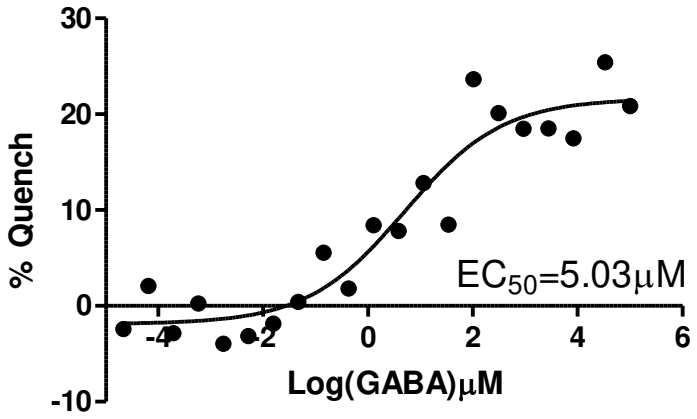


Figure 139. Cells containing the eYFP and the α1β3γ2 subtype quenched by a serial dilution of agonist GABA in NaI buffer.

performed with only two exchanges with buffer and the recording is immediate. In contrast, the testing of modulators required long incubation times in 1% DMSO and three exchanges with varying buffer solutions. For example, the first exchange is to get a reading with only buffer, the second exchange is the incubation of modulator, finally the third exchange is the a mixture of modulator with GABA. In addition to the liquid exchanges, it should be considered that the plate is read four times: after the first exchange, after the second exchange, after the incubation with the second exchange, and finally after the third exchange. This can alter the calculated change in fluorescence signal to above the baseline due to loss of cells.

The first modulator tested was the negative modulator bicuculline, Figure 140. Transfected

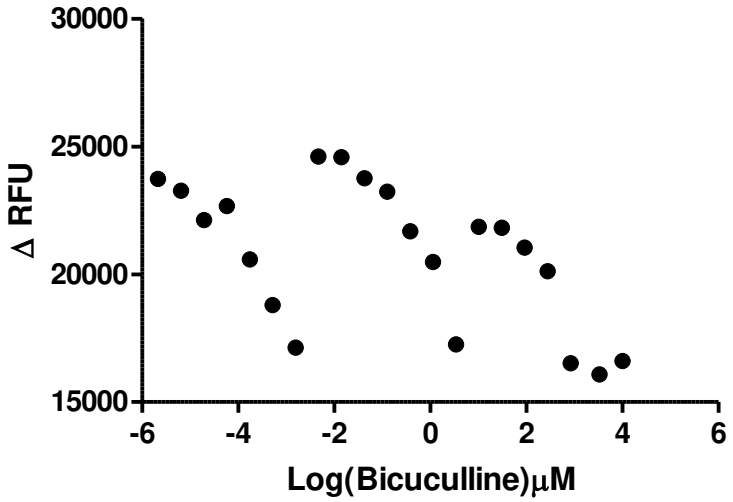


Figure 140. Cells transfected with the eYFP and $GABA_AR$ α1 subtype exposed to an increasing concentration of Bicuculline. Cells were in a 96 well plate and serial diluted from top to bottom with the first row devoted to DMSO control.

cells were preincubated for fifteen minutes prior to activation with GABA in NaI buffer. Plotting the change in fluoresence, the cells appeared to exhibit a dramatic edge effect as every seven points represent a column on the plate since the serial dilution ran top to bottom. To combat this, liquid was added into the spaces between the plate wells. It was also estimated that the highest concentration range of bicuculline, from 1 μM to 1 mM, was necessary to exhibit complete quench of the fluorophore as exhibited by the bottom of the dose response curve. The quench at this range were investigated further and exhibted a clear inhibition of the $GABA_AR$, Figure 142. It should be noted that the total quench was around 10%, half of what was observed with the agonist.

Tests with the strong positive modulator diazepam exhibited very little if any observable trend in quench from the range of nanomolar to millimolar concentrations, Figure 141. Initial

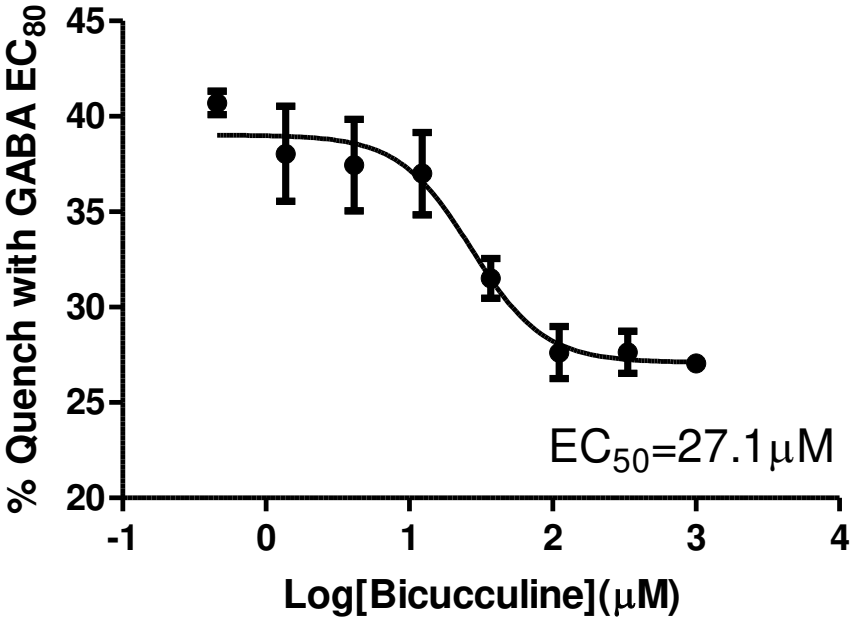


Figure 142. Cells containing the eYFP and the α1β3γ2 subtype. The quench is lowered as the bicuculline inhibits the influx of chloride ions to quench the fluorophore. N=3.

experiments using diazepam appeared to exhibit a slight trend but had been performed without

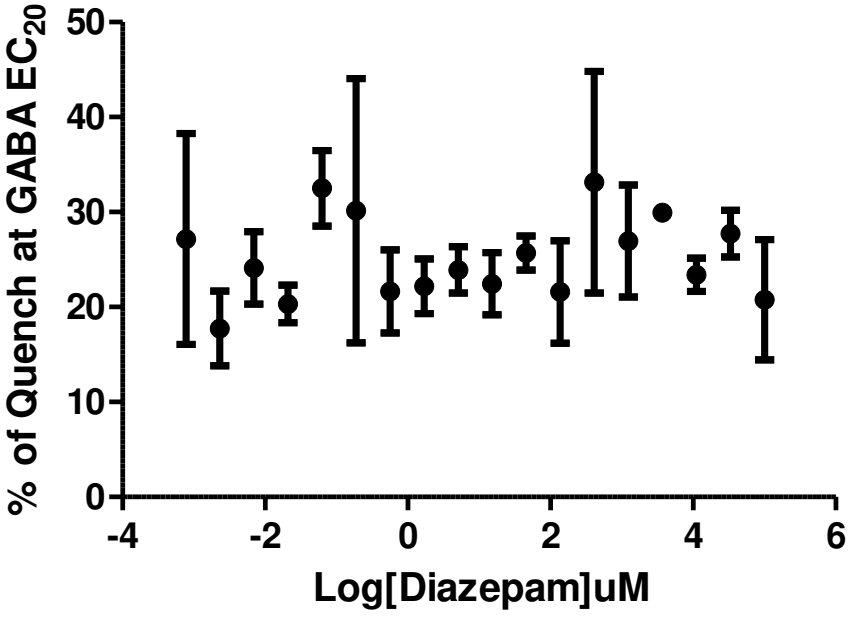


Figure 141. Cells transfected with the α1 and eYFP were pre incubated with Diazepam 15 minutes prior to addition of 0.1μM GABA in NaI buffer.

preincubation with the compound. To test whether this modification caused an effect, the experiment was repeated with the same large range in concentrations, Figure 143. This plot

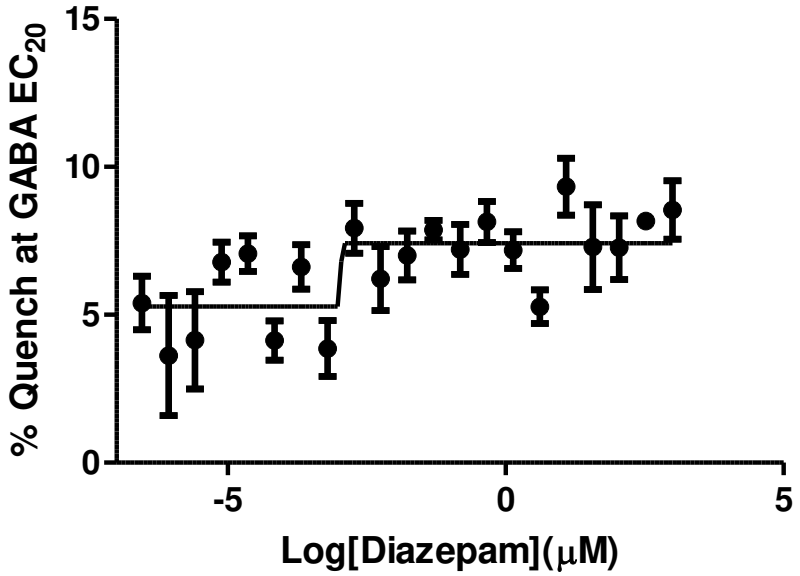


Figure 143. Cells transfected with the α1 and eYFP were exposed to a mixture of serially diluted Diazepam with 0.1μM GABA in NaI buffer and fluorescence was immediately recorded.

exhibited a general trend upwards but an overall change in quench of 3%. Though this again does not appear to show a significant increase in quench, a comparison of average values achieved using

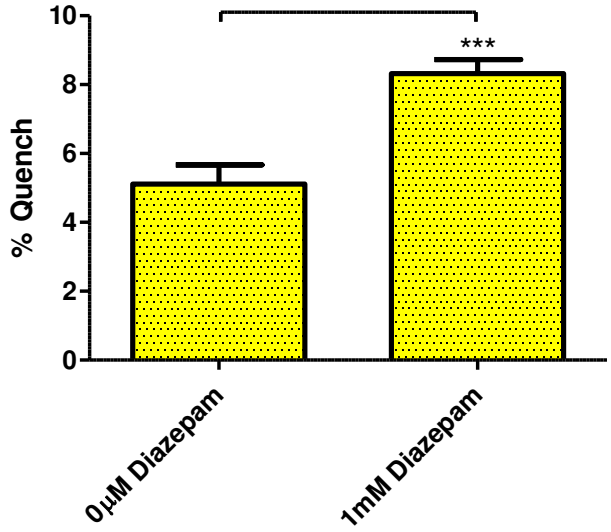


Figure 144. Comparison of quench between the DMSO control and 1mM of Diazepam. Statically significant or non-significant at $p<0.05$

1mM Diazepam vs DMSO after the addition of an EC_{20} concentration of 0.1µM GABA showed statistical significance with 95% confidence, when averaging across 7 occasions, Figure 144.

5.3.4 CONCLUSIONS

Previous study of diazepam in CHO-K1 cells were performed with a constant amount of diazepam and an increasing amount of GABA [290]. Though it was initially unclear why investigators chose this atypical arrangement, observations of the inability of positive modulator and constant GABA to sufficiently cause dose-response quench appears to be the likely reason. However, this assay could be optimized further by the creation of fusion proteins. A prime location to test would be the intracellular loop located between the TM3 and TM4 of the subunits. Though functional testing to determine if the correct folding of the subunit protein would be critical as the assembly of the pentameric receptor may be compromised as well as the agonist and modulator binding sites. Incorporation of a luminescent protein may result in further sensitivity and might allow for BRET signal capture. However, the same complications as seen with a fluorescent fusion protein might occur with a luminescent fusion protein. The steric hindrance caused by two fluorophores attached to the two alpha subunits as well as the bioluminescent protein attached to the gamma subunit may prevent assembly and function of the receptor.

Though there are limitations, the eYFP assay would be well-suited to screening initial hits for BZD compounds with significant modulatory effect. However, the insensitivity of the assay would suggest that it cannot reliably produce an EC_{50}. In the work previously published, researchers observed no shift for weak modulators and could not distinguish strong modulators such as diazepam and lorazepam from each other. However, as an initial screening tool, the eYFP assay is a fast and inexpensive method that does not necessitate dye loading, commercial kits, or

expensive reagents; it has the potential to be used in the high-throughput identification of novel modulators of the $GABA_AR$. In addition, certain challenges such as low assay reproducibility, also noted in studies with the CHO-K1 cells [290], can be overcome by use of the stable recombinant cell lines previously generated.

CHAPTER 6: IMMUNOLOGICAL ROLE OF $GABA_A$ RECEPTOR ON T-LYMPHOCYTES

6.1 Introduction

An effective immune response is a crucial part of organism survival and discrete inflammatory response to localized areas can prevent most pathogenic invasions from spreading. The magnitude of the response must be well-balanced: too little and the immunodeficiency can cause infection and cancer, too much and morbidity and diseases such as rheumatoid arthritis, Crohn's, atherosclerosis, diabetes, Alzheimer's, MS, cerebral and myocardial ischemia can develop [292]. If the inflammation happens to leak over to the bloodstream: sepsis, meningitis, and severe trauma can result and can be more dangerous than the initial stimulus [293,294].

The immune system is known to employ ion channels in T lymphocytes and mast cells to mediate the cellular response against foreign pathogens. Some of the ion channels expressed are calcium release-activated calcium (CRAC) channels, P2X receptors, transient receptor potential (TRP) channels, potassium channels, chloride channels, and magnesium and zinc transporters. Among the expressed chloride channels are volume-regulated Cl^- channels (Cl_{swell}) which open when the T cells swell in hypotonic environment to allow the efflux of both chloride and water to return to a normal cell volume [295,296]. The cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-gated anion channel which opens and closes to allow the flow of chloride anions down their electrochemical gradient [297]. Lastly, the $GABA_AR$ has been found to express in human, mouse and rat T cells [298].

It was long believed that $GABA_AR$ would not have any activity outside the CNS since they would require millimolar concentrations of the neurotransmitter, unachievable without

concentrated synaptic release [299]. However it has since become clear that extrasynaptic channels assemblies can be saturated by nano to micromolar concentrations [300]. It may be that the majority of the $GABA_A$ receptors on the white blood cells are those of the extrasynaptic variety as there is little evidence for the large concentrations of GABA needed to achieve activation of synaptic channels within in the immune system. The GABA blood plasma levels in healthy individuals is around 100nM [301] with 99% of total body GABA and 95% of its synthesizing enzyme GAD being located in the brain and spinal cord [302]. Metabolism of the peripheral GABA takes place by GA BA-T in the liver, which reaches activity almost as high as in the brain [303]; platelets also actively uptake GABA and have GABA-T activity but their role in peripheral GABA homeostatis appears to be minor [304,305]. In addition, GABA does not readily cross the BBB [306]. The GABA concentration normally detected in the blood of healthy individuals is 100 nM. The reason for the presence of GABA in the blood is not well understood since the molecule does not penetrate the blood-brain barrier and thus would be unable to access the CNS. The finding that immune cells produce and secrete GABA would thus explain the concentration in plasma. Interestingly, the plasma GABA levels can be an index of brain GABA activity and a low concentration can be a biomarker for a psychiatric or mood disorder such as depression [307], suicide [308], bipolarism [309], PTSD [310], and schizophrenia [311] as well as anxiety [312-314].

The components of the GABA signalling network have been found to be present in the immune system. For example, GAD exists in two isoforms: GAD 67 and GAD 65, the numbers refering to the molecular mass of the enzyme. GAD 67 which is localized in the neuronal body and GAD 65 which is primarily in nerve terminals [315]. This localization suggests that GAD 65 plays an important role in synaptic neurotransmission and GAD 67 regulates GABA synthesis [316]. GAD 65 is present in dendritic cells and macrophages [317] of mice while GAD 67 has been detected

in human peripheral monocytes [318]. Intact GAD 65 and GAD 67 are also present in neutrophil granulocytes which may indicate that neurotphils can also produce GABA [319]. In addition, GABA secretion has been detected from mouse macrophages and T cells [317,320] as well as the extract from human peripheral blood macrophages [321]. Stimulation of macrophages and dendritic cells with lipopolysaccharide increased GAD 65 while the amount of secreted GABA. Stimulation of $CD4^+$ with anti-CD3 and anti-CD28 antibodies also had no effect on the GABA concentration in the medium. GABA-T has also been detected in macrophages, $CD4^+$ T cells, and peripheral human monocytes [317,318]. Stimulation increased the expression of GABA-T in T cells but did not ater expression in macrophages [317]. Finally, the transporter that transports GABA into synaptic vesicles, vesicular inhibitory amino acid transporter (VIAAT) was found to be expressed in peripheral human monocytes [318]. So it would appear that the cells of the immune system are able to both synthesize and release the neurotranmitter GABA. Given that the necessary components of GABA signalling are expressed in immune cells, it could be possible that GABA plays either an autocrine or paracrine signalling role in immune cells. Evidence for this signalling role is mounting. GABA application results in decreased cytokine secretion and T cell proliferation [322-324] as well as decreased the transient increase in intracellular calcium concentration that is associated with activation of the cells [325].

The discovery of $GABA_AR$, though recent, is not particularly surprising. T-cells express a variety of neurotransmitter receptors that appear to be upregulated and downregulated in some diseases. The state of the T-cell (naïve, activated, CD4/CD8/Th1/Th2/Teff/Treg) appears to be a deciding factor in whether the neurotransmitter will activate or suppress the cell. Macrophages also have a number of neurotransmitter receptors such as the nicotinic cholinergic receptor [326]. Vagal stimulation results in the release of acetylcholine which inhibits the production of pro-

inflammatory cytokines by macrophages. Macrophages and neutrophils can also produce adrenaline and noradrenaline when incubated with lipopolysaccharide [327]. Elevated plasma norepinephrine and epinephrine can effect lymphocyte and monocyte function [328]. It would appear that T-cells also inherently produce many of the neurotransmitters. This suggests that neurotransmitters play an important role in T-cell function and may even serve as the intermediary for communicating between the brain and the immune system.

T-cells producing acetylcholine have also been observed and appear to inhibit cytokine production by vagus nerve stimulation [329]. CD4+ T cells produce very high quantities of acetylcholine when compared to CD8+ T cells or B bells and mitogens stimulates the cells to produce and secrete even more of the neurotransmitter [330]. The excitatory neurotransmitter glutamate, for example, is Glutamate receptor is found, in order of lowest to highest quantities, on human peripheral T-cells, human T helper clone, and human T leukemia cell line. However, though they are present in the naïve cells, upon activation, the cells release granzyme B which cleaves the receptor from their surface. The receptor is completely eliminated from the surface of the cell for ~48 hours; after which, normal levels are restored [331]. This cleavage mechanism also operates in neurons [332]. In addition, it was found that the animal model for multiple sclerosis had high levels of expression for the glutamate receptor GluR3 [331] and that human MS patients have an upregulation of GluR3 expression during relapse [333]. This is reviewed by Ganor [334].

Dopamine has direct effects on immune cells and is also endogenously produced by T cells. The case of dopamine receptor expression on T-cells is particularly interesting. Dopamine receptors have five different seven-transmembrane G-protein-coupled receptors named D1-D5. The receptors D2-D5 have been found to express in T-cells and appear to be responsible for

specific roles such as triggering adhesion to fibronectin, which was found to be mediated specifically by D3R and D2R [335]. In addition, it has been found that dopamine induces specific cytokine secretion. D3R triggered secretion of TNFα, D2R of IL-10, and D1/D5R triggered both cytokines [336]. An abnormal expression or response of dopamine-receptors on T-cells has been observed in in schizophrenia [337-339], Parkinson's [340,341], Alzheimer's [342], Migraine [343], HIV [344,345], MS [346-351], inflammatory bowel disease (IBD) [352-354], Rheumatoid arthritis [355-358], systematic lupus erythematosus [359,360]. A thorough review has been published [361] on the subject.

It is still unknown what the exact function these CNS receptors play in the T cells. GABA administration inhibited T cell proliferation and induced the production of IL-2 and IFNγ. In addition, in vivo, GABA or GABAergic agents ameliorated disease outcome in several autoimmune animal models such as type 1 diabetes [322], rheumatoid arthritis [362], and multiple sclerosis [317]. Functional $GABA_AR$ act as inhibiters of antigen-specific T cell proliferation [299] and inhibits the production of interleukin (IL)-6, IL-12, inducible nitric oxide (iNOS), IL-1β, and tumor necrosis factor (TNF)-α [363,364]. Interestingly, $GABA_AR$ agonists in rodent models have been observed to accelerate cutaneous recovery and prevent epidermal hyperplasia in wounds [365] and suppress wound-induced cutaneous inflammation [363]. This would perhaps suggest that the biological role of the receptor expression on the T cell is to inhibit the activation of the T cells when a GABA-secreting cell is encountered. However, the mechanisms by which expression of the receptors is controlled as well as the production or release levels has yet to be determined.

It has also been found that specific subsets of the $GABA_AR$ have been identified in the human airway smooth muscle cells and the guinea pig tracheal smooth muscle model. The mRNA for α4, α5, β3, π, θ, and γ1-3 were found and immunoblots confirmed α4, α5, β3, and γ2. In

addition, the selective $GABA_A$ receptor agonist muscimol was able to relax a histamine induced contraction and appeared to facilitate relaxation in the airway, suggesting that this route may present novel therapeutic target for airway smooth muscle relaxation [366]. Further work has been performed to show that the both α4 and α5 selective imidazobenzodiazepines promote airway smooth muscle relaxation and affects cellular calcium [367,368].

TSPO, a protein localized primarily in the outer mitochondrial membrane, is present in multiple tissues and organs besides in the CNS, unlike the $GABA_AR$, and its primary role is to import cholesterol through the outer mitochondrial membrane to be made into neurosteroids as THDOC or allopregnanolone. These neurosteroids are known to enhance $GABA_AR$ function [369]. TSPO may also play a role in the observed electrophysiological response; forming a complex with voltage-dependent anion channel (VDAC) and the adenine nucleotide transporter (ANT) [370]. Though TSPO was originally termed a mitochondrial receptor, it was discovered that more than 50% of the TSPO receptors were not associated with mitochondria and antibody studies have found that expression is found on the plasma membrane as well as the mitochondria [371,372]. The association of these complexes forms a maxi-choride channel which has been successfully patch-clamped for electrophysiological study [373].

6.2 Electrophysiological Studies

6.2.1 INTRODUCTION

Circa 450 BC, Hippocrates observed a respiratory illness common in anglers, tailors and metalworkers. The illness was characterized by laborious breathing or ἄσθμα (aazein) in ancient greek. The earliest treatment prescribed was owl's blood in wine [374]. Asthma is estimated to effect 315 million persons globally [375] and is expected to increase to 400 million by 2025 [376]. It has been

extimated that 1 in every 250 deaths worldwide is due to asthma, resulting in 250,000 deaths each year worldwide [376] with nearly 25 million in the U.S. alone. In 2007, the health care cost burden to the US economy was estimated at $56 billion. With the increasing prevalence of obesity, a contributor to asthma severity, this number is only expected to rise. Although the morbidity is low, hospitalization and health care costs run upwards to $20 billion annually. Asthma is characterized by chronic and intermittent attacks of breathlessness, wheezing, and cough. This disease often presents in childhood but one in four may continue or have a recurrence of symptoms well into adulthood [377].

The causes of asthma have yet to be fully understood but it appears to be a complex combination of environmental and genetic factors [377]. Family and twin studies have suggested that the genetic contributions approach 60% but genome-wide analysis suggests that there is very small involvement from a great number of loci and no single gene has been identified with any degree of certainty [378,379]. Air quality and endotoxin exposure have been linked to asthma development and symptom severity [380,381]. Epidemiological studies have found that a severe viral upper respiratory infection occurring early in life can increase risk of developing the asthmatic phenotype, particularly if their families have a history of atopy [382,383]. The inability to find a singular gene responsible for the phenotype, combined with the heterogeneity in response to commercial therapeutics, may be indicative that distinct subtypes of the disease exist. There already exists evidence of this distinction with allergic and non-allergic asthma, also called atopic vs non-atopic, based on the presence or absence of allergen-specific antibodies, assessed by skin prick serological tests [383,384] . Whether atopic and nonatopic asthma are two distinct entities is unknown as the mechanism of pathogenesis is still not clear. However, both forms of asthma exhibit the presence of bronchial mucosal infiltrate with eosinophils and elevated expression of

eosinophil active chemokines and cytokines, indicating local T-cell activation. There are subtle differences between the two forms at the mucosal and submucosal levels, however the similarities seem to outweigh the differences [385]. The hallmark symptom of asthma is airway hyper-responsiveness [386] as well as the presence of chronic inflammation in the lower airways which prevents continuous airflow, resulting in episodes of coughing, wheezing, breathlessness, and chest tightness [387].

Chronic inflammation of the airway is the major marker of asthma and is caused in large part by Th2 cells, their cytokines, and eosinophils. Many animal models attempt to recreate these major symptoms [388]. Il-17 associated neutrophilic airway inflammation and pauci-granulocytic subtypes have also been studied and appear to play some role in adult-onset cases of asthma [389]. Frequent exposure and inhalation of allergens can cause a persistent inflammation; resulting in structural changes to the airway. These changes, collectively known as "airway remodeling" include increase in subepithelial matrix glycoproteins, smooth muscle hypertrophy, and epithelial mucus metaplasia. Airway remodeling is believed to be the major cause for poor airflow and airway hyper-responsiveness [390].

The classic model of asthma links the disease to reaginic immune globulin (IgE). These IgE antibodies bind to mast cells in the airway mucosa which causes a release of mediators stored in cell granules and begins a cascade of mediator synthesis and release. Acute bronchoconstriction involves the production of histamine, mast cell activating tryptase, inflammatory leukotrienes C4 and D4 which all accumulate to produce the "early asthmatic response". The "late asthmatic response", characterized by second sustained period of bronchial constriction caused by an influx of inflammatory cells in the bronchial mucosa which sensitizes bronchial reactivity for weeks after

the exposure to the allergen. Late phase response is believed to be a result of the cytokine population composed of IL-5, 9, and 13 produced by Th2 lymphocytes. Production of the IgE antibodies is genetically influenced [391].

Examination of Figure 145 from [392] illustrates the differentiation pattern of CD4 cells. T lymphocyte cells are a mixture of $CD4^+$, $CD4^-/CD8^-$, $CD8^+$, and $CD4^+/CD8^+$ cells [393] which, under normal growth conditions, produces little to no IL-2. Phorbol 12-myristate 13-acetate (PMA) is often used to activate protein kinase C and stimulate low-level production of IL-2. Phytohemagglutinin (PHA) can also be used and triggers low level T-cell activation and IL-2 production by binding to the cell surface receptor complex. The combination of PMA and PHA results in a strong enhancement in IL-2 production [394]. This technique is often used to stimulate T cell activation of cells in culture. The strong IL-2 production indicates that the cells are

differentiated into Th1 cells. Th1 cells produce IFN-γ, tumor necrosis factor-beta (TNF-β) and interleukin-10 (IL-10); mediating immune response against tumor cells, intracellular viruses and bacteria through the activation of macrophages and cytotoxic T-cells. Secretion of IL-4 induces naïve $CD4^+$ cells to become Th2, leading to the expression of transcription factor GATA3. The cascading production eventually leads to IL-4, IL-5, IL-13, IL-21, and IL-31 which also contribute

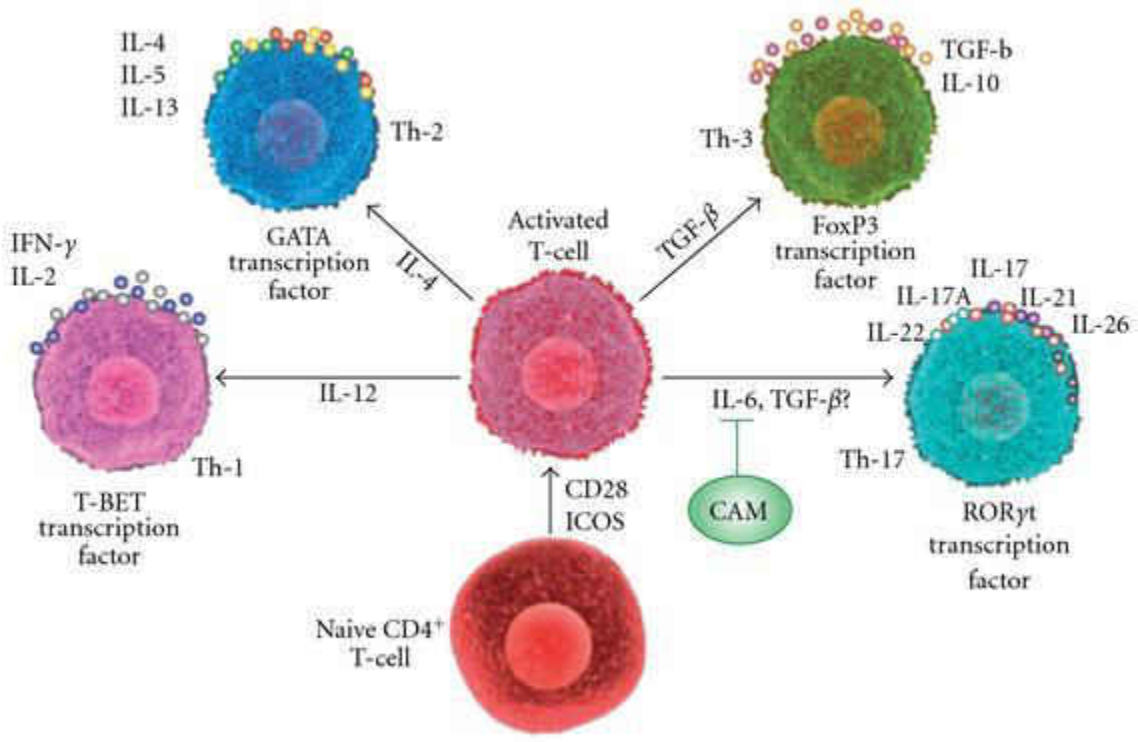


Figure 145. General scheme of T-helper cell differentiation. Naive T cells are activated and can differentiate into four effector T-helper cells: Th1, Th2, Th3, or Th17. These cells produce different cytokines for specialized immune function. Th-1 cells produces IFN-γ which regulates antigen presentation and cellular immunity. Th2 cells produce IL-4, IL-5, and IL-13 to regulate B-cell responses, important mediator of allergic diseases. Th3 cells produce TGF-β and IL-10 to regulate Th1 and Th2 cells. Th17 regulates inflammatory response by expressing IL-17, IL-21, IL-22, and IL-26. Open Access cited in text and used with permission.

to the pathogenesis of asthma and allergy [395-397]. Thus a more in-depth study of Th2 cells may be useful in the future. Th17 cells develop in response to IL-6, IL-23, and TGF-β. IL-6 and IL-23 then activate STAT3, increasing the expression of transcription factors RORγt and RORγ which promotes expression of IL-17A, IL-17F, IL-21, and IL-22. There has been evidence found that

Th17 cells plays an essential role for host defense against invasion by extracellular bacteria [398] and in mediating memory $CD4^+$ cells involved in autoimmune disease [399]. Th17 cells have also been connected to allergic inflammation, mediating the activation of inflammatory cells in the airways, so common in asthma pathogenesis [400,401]. Interestingly, IL-17 has been detected in the cerebrospinal fluid of patients with multiple sclerosis [402]. It has been observed that when the presence of Th17 outnumbers Th1 in the brain, or drops below a certain threshold in the spinal cord, the result is inflammation [403]. The recruitment of monocytes and macrophages to the inflammatory sites then leads to myelin and axonal damage [404,405]. The suggestion is that IL-17 and its producer Th17 cells, are an important regulator of CNS autoimmunity.

Because the exact biochemical mechanism of asthma development has yet to be fully understood, finding an accurate animal model is difficult. In general, mice do not naturally develop asthma however it is possible to induce the symptoms using ovalbumin (OVA). OVA derived from chicken egg is commonly used to induce an allergic pulmonary inflammation in rodents. Though this produces robust results, OVA is not implicated in human asthma so there has been work in sensitizing mice to house dust mite (HDM) and cockroach extract [406,407].

Acute sensitization requires exposure to the allergen with an adjuvant. The most commonly used adjuvant is $AlOH_3$ or alum which promotes the Th2 phenotype in the immune system [408]. This model reproduces the elevated levels of IgE, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy, airway hyper-responsiveness and some bronchio-constriction to allergen challenge. However, the chronic inflammation of the airway wall with persistent AHR and remodeling changes do not occur due to the short-lived nature of the acute model. Another problem

with the acute model is that some drugs have shown efficacy in acute challenge but fail to exhibit anything in the clinical studies such as VLA4 antagonists [409], PAF antagonists [410] and IL-4 antagonists [411,412].

The chronic allergen challenge model attempts to reproduce the airway remodeling and persistent AHR features of asthma. This model requires repeated exposure of low levels of allergen for up to 12 weeks and adjuvant is not always required. Long-term challenge often leads to development of tolerance and lowered inflammation and AHR [413]. However, there are still some characteristics that this model lacks. Chronic asthma typically results in significant increases in airway smooth muscle [414] and a lack of mast cells in the airway [415] which does not occur in the mouse model. However, it is most likely that invention of a mouse model that completely reflects the complex disease is impossible and cannot be achieved in a single model.

There is no cure for asthma but treatment is divided into two groups: short-term relievers for acute bronchoconstriction relief and long-term controllers to reduce symptoms and prevent an attack. Short-term relief agents, such as β-adrenoceptor stimulants, relax airway smooth muscle. Theophylline and anti-muscarinic agents can also reduce acute airway constriction. Long-term controllers such as inhaled corticosteroid, can reduce inflammation. Corticosteroids can have profound immunosuppressive effects on the lymphoreticular system, inhibiting the trafficking patterns and effector functions of lymphocytes and macrophages [416,417]. There are also alternative targeting approaches such as the leukotriene pathway antagonists or inhibitors of mast cell degranulation [418].

Currently, treatment guidelines recommend the usage of inhaled corticosteroids for long-term control of persistent asthma in combination with long-acting β2 adrenergic agonists [419].

Inhaled steroids help prevent asthma symptoms but do not relieve them during an attack and can take up to 3 months of daily use to reach the best results. Larger doses of steroids may affect hypothalamic-pituitary-adrenal function and bone turnover and long-term use can result in serious systemic adverse effects such as suppressed adrenal function, bone loss, skin thinning, growth impairment, and cataract formation [420,421]. In addition, unbiased cluster analysis indicates that 5-10% of asthmatics fall into the category of relative glucocorticoid insensitivity [422]. This reduced responsiveness to the effects of corticosteroids is a major barrier to the management of asthma in smokers, severe asthmatics, and those with chronic obstructive pulmonary disease (COPD) [423]. Bronchodilators are used as a quick relief rescue treatment for asthma however they are associated with side effects such as nervousness, increased heart rate, upset stomach, trouble sleeping, and muscle aches [420,424,425].

Taking these shortcomings into account, the need for alternative treatments exists despite the availability of current drug options. Targeting the $GABA_AR$ could, in theory, work dually to relax airway smooth muscle while also suppressing inflammatory cells. The expression of which has been observed on both routes.

6.2.2 EXPERIMENTAL

Cell Culture

Jurkat, Clone E6-1 cells (ATCC, TIB-152) which are a T lymphocyte from a 14 year old boy with acute T cell leukemia were used for the studies. Cells were grown in suspension with RPMI-1640 Medium (ATCC, 30-2001) with 10% heat-inactivated FBS (BioWest SO1520HI), non-essential amino acids (Hyclone, SH30238.01), 10 mM HEPES (Hyclone, SH302237.01), and 5 x 106 units of penicillin and streptomycin (Hyclone, SV30010) in 75 cm2 flasks (CellStar).

To activate the cells, 1µg/mL of phytohemagglutinin (PHA) (Sigma, L2646) and 50ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, P8139) were added to the cell suspension and incubated for 24 hours at 37°C at 5% CO_2.

Automated Patch-Clamp Electrophysiology

The buffers were made from NaCl (Fisher, BP358-1), KCl (Fisher, BP366-1), $MgCl_2$ (Sigma, M8266), $CaCl_2$ (Acros Org, 123350025), Glucose (Sigma, G0350500), HEPES (Fisher, BP410-500), CsCl (Sigma, 203025), and EGTA (Tocris, 28-071-G). The intracellular solution contains 140mM CsCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 11mM EGTA, 10mM HEPES, and pH 7.2 with CsOH. The extracellular solution contains 140mM NaCl, 5.4mM KCl, 1mM $CaCl_2$, 10mM D-glucose monohydrate, 10mM HEPES, and pH 7.4 with NaOH. Compounds were diluted to 10mM in DMSO.

To record $GABA_A$ currents, cell arrays were voltage clamped at a hyperpolarizing holding potential of -80mV. Cells were centrifuged at 380g for 2 min and gently resuspended in ECS. This was repeated two more times before the cells were dispensed into the plate.

Rotarod Studies

BALB/c mice were trained to maintain balance at a constant speed of 15 rpm on the rotarod instrument (Omnitech Electronics Inc) until mice were able to perform for 3 min at three consecutive time points. Three groups of mice containing eight mice each were divided into a vehicle group (10% DMSO, 40% propylene glycol, and 50% PBS), diazepam group, and test group. The volume of the injection was 100 µL. Ten minutes after the injection, peak time determined by pharmacokinetic data, mice were placed onto the rotarod for 3 min. A fail was assigned for each mouse that fell from the rotarod prior to 3 min. Mice were rested 2-3 days before

administration of another dose or a different compound. At 40mg/kg of XHE-III-74 Ethyl Ester, the i.p. injection volume was doubled (200 µL) due to solubility.

qRT-PCR Protocol

Cells were harvested once 80% confluent using 0.05% Trypsin or Detachin solution and pelleted by centrifuging at 1000rpm for 2 minutes. Media was aspirated and the cell pellet was resuspended in RLT buffer. The mixture was pipetted into the QIAshredder spin column and spun for 2 minutes at max rpm. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The solution was then transferred to an RNAeasy spin column and spun down for 15 sec at 10,000rpm. Then washed with buffers to purify the bound RNA with the flow through discarded. After the column is washed and dried, RNA is eluted by addition of 30-50 µL of RNase-free water. Total RNA concentration was determined by UV at 260nm and protein contamination was assessed at 280 nm. The RNA was then diluted and used for qRT-PCR with the QuantiFast SYBR Green RT-PCR Kit (Qiagen). The cycling conditions used was 10 min at 50°C (reverse transcriptase), 5 minutes at 95°C (PCR initial activation step), 10 sec at 95°C (denaturation), and 30 sec at 55°C (annealing and extension) for a total of 50 cycles. Data was

Table 15. Human primers list for quantitative real-time RT-PCR of immune cells.

$GABA_AR$ subunit	Forward Primer (5'-3')	Reverse Primer (5'-3')
α1 (GABRA1)	GGATTGGGAGAGCGTGTAACC	TGAAACGGGTCCGAAACTG
α2 (GABRA2)	GTTCAAGCTGAATGCCCAAT	ACCTAGAGCCATCAGGAGCA
α3 (GABRA3)	CAACTTGTTTCAGTTCATTCATCCTT	CTTGTTTGTGTGATTATCATCTTCTTAGG
α4 (GABRA4)	TTGGGGGTCCTGTTACAGAAG	TCTGCCTGAAGAACACATCCA
α5 (GABRA5)	CTTCTCGGCGCTGATAGAGT	CGCTTTTTCTTGATCTTGGC
α6 (GABRA6)	ACCCACAGTGACAATATCAAAAGC	GGAGTCAGGATGCAAAACAATCT
β3 (GABRB3)	CCGTTCAAAGAGCGAAAGCAACCG	TCGCCAATGCCGCCTGAGAC
γ2 (GABRG2)	CACAGAAAATGACGGTGTGG	TCACCCTCAGGAACTTTTGG
δ (GABRD)	ATGCTGGACCTGGAGAGCTA	GAGGACAATGGCGTTCCTCA
TSPO (TSPO)	GAGCTCCCCTGAACAGCAG	CCATGGTTGTCCCGCCATAC
VDAC1 (VDAC1)	CGAGTGACCCAGAGCAACTT	CTCCCCGAGTCTACCACTGA
GAPDH (GAPDH)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

taken in triplicate and the relative mRNA expression levels were calculated using wells containing

Table 14. Mouse primers list for quantitative real-time RT-PCR of immune cells.

$GABA_AR$ subunit	Forward Primer (5'-3')	Reverse Primer (5'-3')
α1 (gabra1)	CAAGAGCAGAAGTTGTCTATGAGT	GCACGGCAGATATGTTTGAATAAC
α2 (gabra2)	GCTACGCTTACACAACCTCAGA	GACTGGCCCAGCAAATCATACT
α3 (gabra3)	GCCGTCTGTTATGCCTTTGTATTT	TTCTTCATCTCCAGGGCCTCT
α4 (gabra4)	AGAACTCAAAGGACGAGAAATTGT	TTCACTTCTGTAACAGGACCCC
α5 (gabra5)	AAGTTCGCTCCGGCAGTATG	TGTTCTTGCCTCCAACTTGATCT
α6 (gabra6)	CTTGCTGGAAGGCTATGACAAC	AAGTCTGGCGGAAGAAAACATC
β3 (gabrb3)	CTTTGCGGGAGGAAGGCTTT	GGGGTCGTTTACGCTCTGAG
γ2 (gabrg2)	ACTTCTGGTGACTATGTGGTGAT	GGCAGGAACAGCATCCTTATTG
δ (gabrd)	TCAAATCGGCTGGCCAGTTCCC	GCACGGCTGCCTGGCTAATCC
TSPO (tspo)	GAGCTCCCCTGAACAGCAG	CCATGGTTGTCCCGCCATAC
VDAC1 (vdac1)	CGAGTGACCCAGAGCAACTT	CTCCCCGAGTCTACCACTGA
GAPDH (gapdh)	ACCACAGTCCATGCCATCAC	CACCACCCTGTTGCTGTAGCC

no template and normalizing to housekeeping gene GAPDH. Primer sets used are listed in Table 15 and Table 14.

Cell metabolite extraction

An excess of Jurkat E6-1 cells, 5mL of $5x10^6$ cells/mL, were centrifuged at 380 x g at room temperature. The media was aspirated and the cells were washed with 5mL of PBS (Hyclone, SH30256.01), centrifuged at 380 x g, and the buffer aspirated. This was repeated two more times to ensure that residual metabolites from the media did not contaminate the extract.

A three-phase methanol/chloroform/water extraction [426] was performed on the sample. Methanol/chloroform/water was added at a volume ratio of (6:6:5.4) at a total volume of 17.4 mL. The solution was vortex for 3 minutes and centrifuged at 10,000 x g for 10 minutes. This separates the mixture into three distinct phases: the upper aqueous layer contains water-soluble low-molecular weight endogenous metabolites while the non-polar metabolites such as lipid molecules were found in the bottom organic phase. Biological macromolecules and proteins were precipitated and trapped in the solvent layer between the aqueous and organic phases. Only the upper aqueous phase was kept. The sample was transferred to a rotary vacuum evaporator until nearly dry and then moved to dry under a gentle flow of N_2 gas. The sample can then be stored at -80°C until analysis.

Splenocyte isolation

Male Balb/c mice were sensitized three times with i.p. injections of 2mg/kg/d ovalbumin (Ova) (Sigma) emulsified in 2 mg of Alum (Thermo Sci) on days 0, 7, and 14 in a total volume of 100 µL. Mice were then challenge intranasally (i.n.) with 1mg/kg/d Ova for 5 days from days 23-27. Control mice were sensitized with Ova and challenged with saline. Mice were euthanized with a cocktail of ketamine (300mg/kg) and xylazine (30mg/kg) (Sigma) by i.p. injection at 100 µL. The spleen was extracted and two frosted-end glass slides were sterilized with ethanol before homogenizing the spleen between the frosted ends of the slides. A single cell suspension was

pelleted at 360g by centrifugation and the supernatant was aspirated. The pellet was resuspended in 5mL/spleen of 1X Lysis Buffer (Thermo Fisher, 420301). The mixture was incubated at room temperature for 5 minutes. The reaction was stopped by diluting the Lysis buffer with 30mL of 1X PBS (Hyclone, SH30256.01). Cells were centrifuged at 360g, the supernatant removed, and the pellet resuspended in DMEM (Hyclone, SH3024301) containing 50mL of fetal bovine serum (Biowest, SO1520HI) and 5mL of 100X penicillin/streptomycin (Hyclone, SV30010).

Cells were challenged in culture, ex vivo, with Ova at 10µg/mL with 2 x 10^6 cells per mL overnight at 37°C at 5% CO_2.

Whole Blood Fractionation

The author's blood was drawn by the Norris Health Center with the help of Clinical Services Director Dr. Aamir Siddiqi and lab supervisor Terry Karl. Whole blood, 5mL, was collected in an EDTA vacutainer (Fisher Sci, 368589). The sample was centrifuged at 1500 x g for 15 minutes at room temperature. This separates the blood into two layers: the upper yellow plasma layer and the lower red blood cell layer. At the interface of these two layers is a thin film of white blood cells known as the buffy coat. The plasma was layer was aspirated down to ~1mm from the red blood cell layer. Using a circular motion, the buffy coat was carefully aspirated into a tube. 5mL of 1X Lysis Buffer (Thermo Fisher, 420301) was added and the mixture was incubated at room temperature for 5 minutes. The reaction was stopped by diluting the Lysis buffer with 30mL of 1X PBS (Hyclone, SH30256.01). Cells were centrifuged at 360g, the supernatant removed, and the pellet was conserved for mRNA extraction. Taking into account the probable activity of the intracellular RNases that would be released in the lysis step, 1% of β-mercaptoethanol (β-ME) was added during the resuspension of the pellet with RLT buffer. The reducing agent irreversibly

denatures RNases by reducing disulfide bonds and disrupts the native confirmation of the enzyme. Though the RLT buffer contains guanidinium isothiocyanate which is a strong but temporary denaturant, addition of β-ME ensures that a majority of RNases are inactivated [427].

6.2.3 RESULTS AND DISCUSSION

Use of the Jurkat E6-1 cells on the automated patch clamp IonFlux was first established. Cells were harvested from suspension and the extracellular and intracellular solutions were prepared. The electrophysiological response of the cells to the agonist GABA from the first experiment can be seen in Figure 146. The imidazobenzodiazepine HZ-166 was also used in

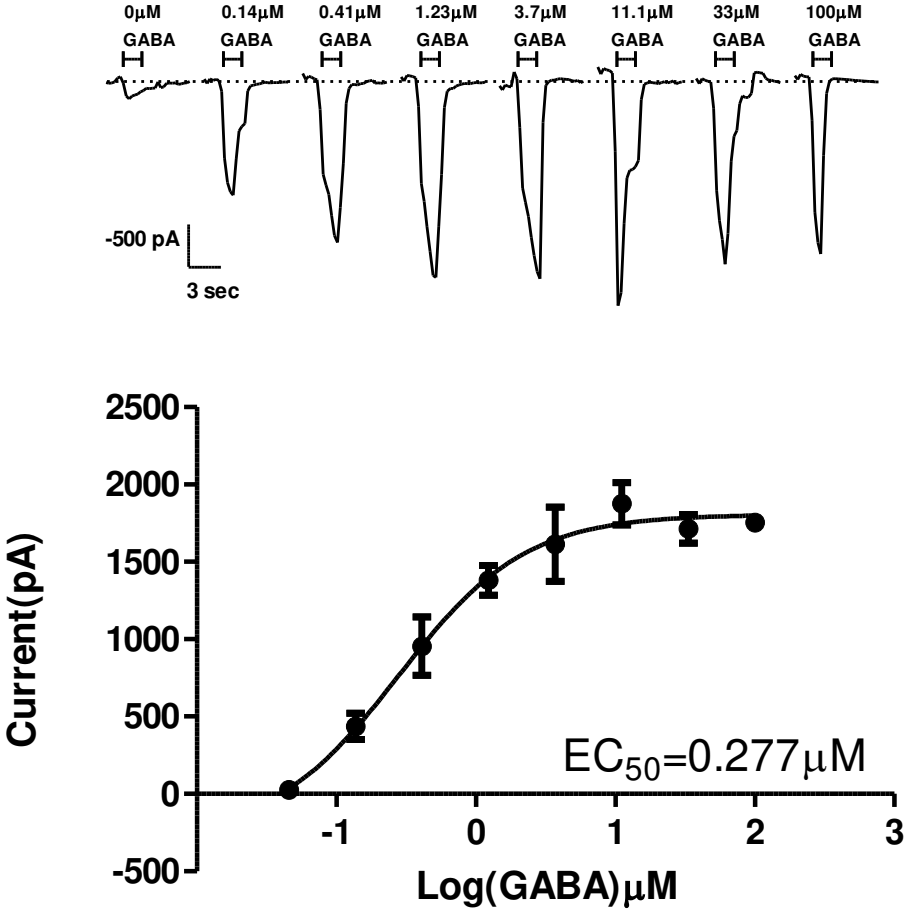


Figure 146. Jurkat E6-1 cells exposed to increasing concentrations of GABA in ECS buffer. N=4

combination with an EC_{20} concentration of GABA to determine if positive modulation with these compounds was possible, Figure 147. The results suggested that the subset of receptors present on the Jurkat E6-1 T lymphocytes were responsive to both the agonist GABA as well as the BZD

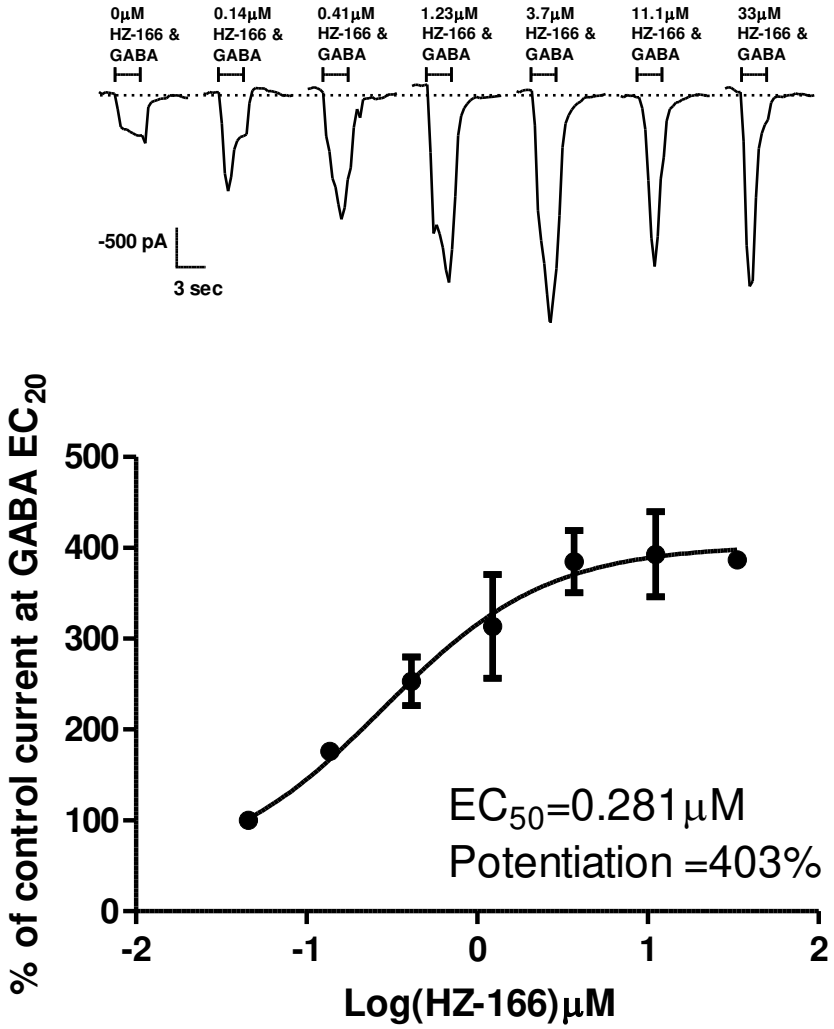


Figure 147. Jurkat E6-1 cells exposed to increasing concentrations of HZ-166 in combination with a constant concentration of GABA EC_{20} or 0.1μM. HZ-166 was solubilized in 0.3% max DMSO. N=4

positive modulator HZ-166. This would also indicate that the receptors contain a gamma2 subunit as this confers benzodiazepine sensitivity.

High quantities of the α4 were found in immunoblot using human airway smooth muscle cells and guinea pig tracheal cells. Novel $GABA_AR$ positive allosteric modulators with enhanced

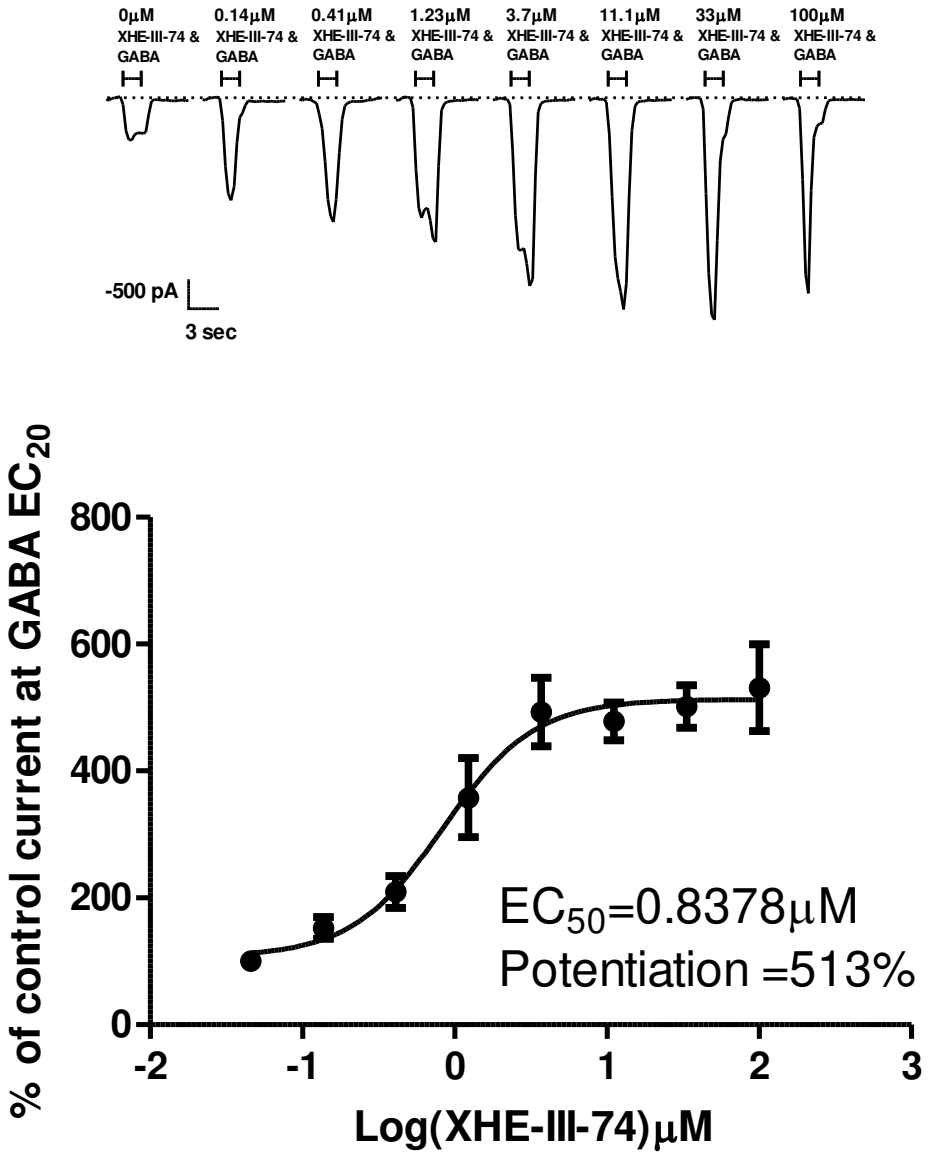


Figure 148. Jurkat E6-1 cells exposed to increasing concentrations of XHE-III-74 in combination with a constant concentration of GABA EC_{20} or 0.1μM. XHE-III-74 was solubilized in 0.3% max DMSO. N=4

α4/6 subunit selectivity were synthesized using iterative computational analyses. These compounds, CMD-45 and XHE-III-74, were observed to have higher induction of currents in frog oocytes expressing the α4/6 containing receptors [231]. It was also observed that pretreatment with these compounds inhibted histamine-induced increases in intracellular calcium concentrations which suggests that the inhibition of calcium influx plays a role in the mechanism of relaxation.

These α4/6 selective compounds primarily differ in the lack of a pendant phenyl. The results of the XHE-III-74 and CMD-45 application on Jurkat cells can be seen in Figure 148 and Figure 149. These results would imply that these drugs have a dual effect: 1) the airway smooth muscle relaxation and 2) the chloride influx and resulting inhibition of T cell function.

XHE-III-74 Etyl ester (XHE-III-74EE or L-655,708) and its metabolite XHE-III-74 Acid (XHE-III-74A) was found by a collaborator to display subtype selectivity specifically for the α4 and α5 subtype. To determine if the compounds had CNS effects due to penetration of the BBB, rotarod studies were performed. The rotarod test is a performance test that measures motor behavior [428] and is frequently used in early stages of drug development to screen out drugs that

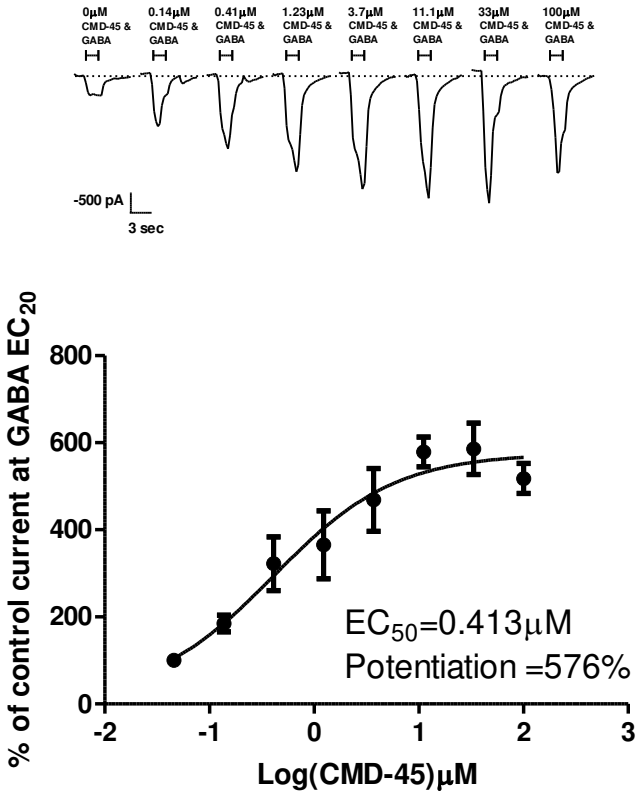


Figure 149. Jurkat E6-1 cells exposed to increasing concentrations of CMD-45in combination with a constant concentration of GABA EC_{20} or 0.1μM. CMD-45 was solubilized in 0.3% max DMSO. N=4

may cause impairments [429]. Compounds were delivered via i.p. injection to Male Balb/c mice, the results are as seen in Figure 150. Impairment was evaluated after 10 min by the ability to stay on a rotating rod moving at 15 rpm. Diazepam, the nonselective positive modulator, caused significant motor impairment as low as 5mg/kg while both XHE-III-74EE and XHE-III-74A exhibited no effect at these concentration. The XHE-III-74 did cause significant impairment at dosages of 40mg/kg but the XHE-III-74A was devoid of impairing effects, consistent with its inability to penetrate the BBB.

The electrophysiological effect of these compounds were evaluated using Jurkat E6-1 cells which were in the 30th passage (3 months in culture). Previously, experiments had been performed

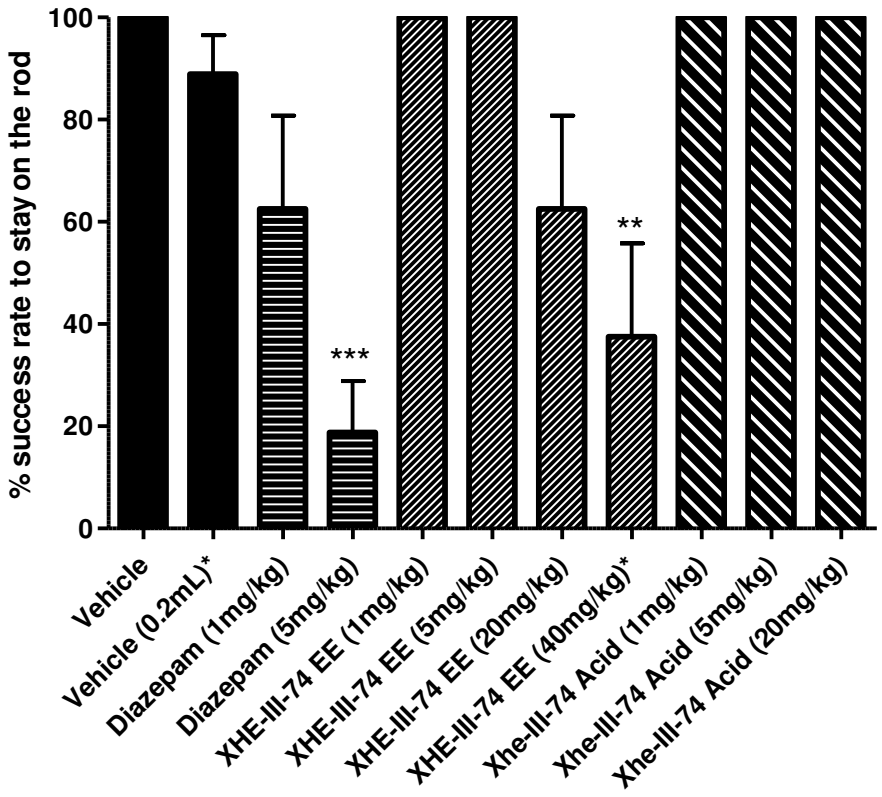


Figure 150. Effect of XHE-III-74EE and XHE-III-74A on sensorimotor coordination. BALB/c mice received a single i.p. injection and were tested 10 min after application. The % success rate is expressed as mean ± SEM, N=8. Significance calculated by $p < 0.05$ compared to vehicle-treated mice.

with cells prior to their 10th passage (1 month in culture). Interestingly, the GABA dose response curve for the high passage cells had shifted considerably downward, Figure 151. This experiment was repeated and produced the same down shifted curve, Figure 152.

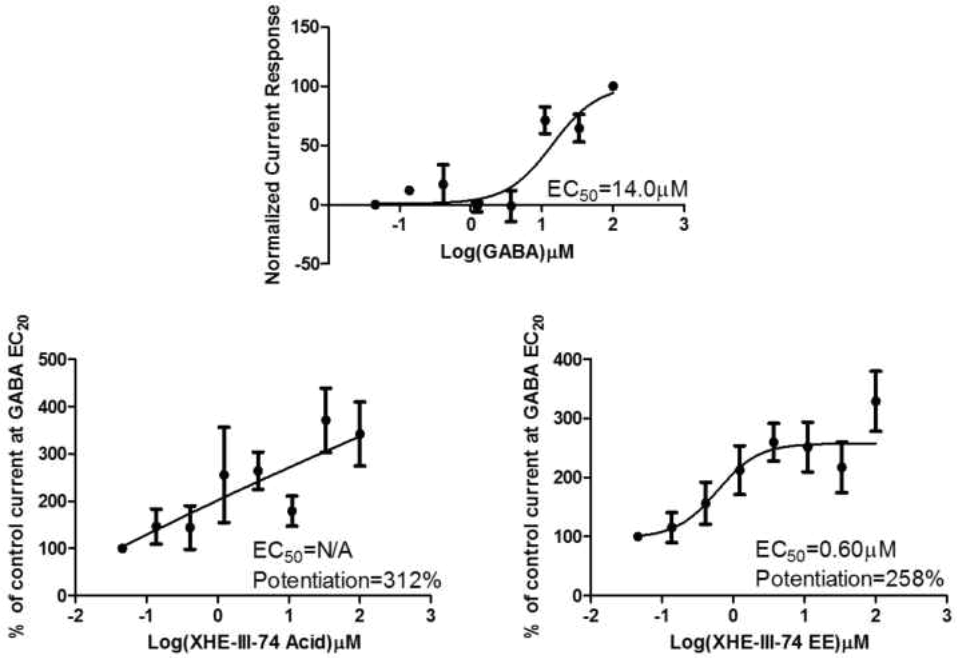


Figure 151. Dose response curves for Jurkat E6-1 cells in passage 30. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO, N=6

The passage number, that is the degree of subculturing a cell line has undergone or literally the number of times cells have been transferred from vessel-to-vessel, has been shown to have an affect on the cell line's characteristics over time [430-434]. These effects often emerge in changes in morphology, response to stimuli, growth rates, and protein expression.

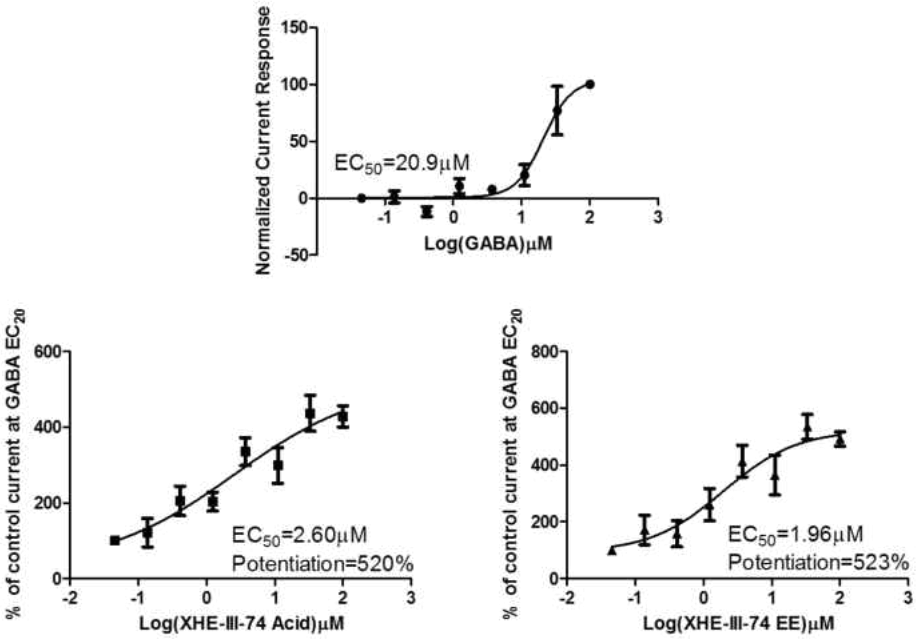


Figure 152. Recapitulation of dose response curves for Jurkat E6-1 cells in passage 30. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO, N=6

The change in the dose response curve for GABA can be contributed to this passage number-related effects. There is no straightforward method to determine how many passages are too many with papers reporting ranges from passage 18 to 40 for mouse insulinoma cells [435] (MIN-6) or passage 25 to 60 for prostate cancer cell line [436] (LNCaP).

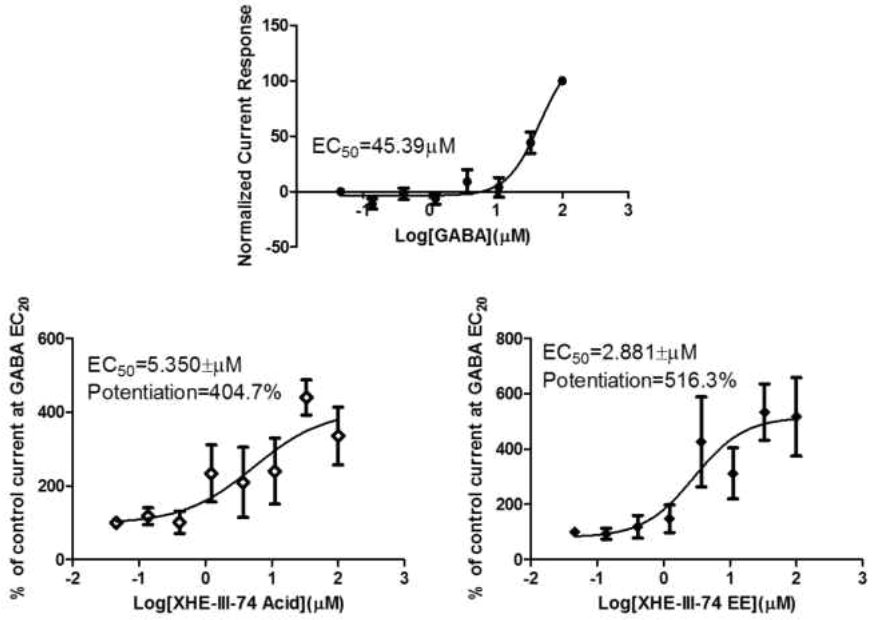


Figure 153. Dose response curves for Jurkat E6-1 cells in passage 5. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 1µM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE in combination with a constant concentration of GABA EC_{20} or 1µM. Compound was solubilized in 1% max DMSO, N=6

A cyropreserved vial of cells in low passage (3) were unthawed and allowed to adjust until passage 5. These cells were harvested and then exposed to the same tests, Figure 153. And again at passage 7, Figure 154, however the shifted dose response persisted.

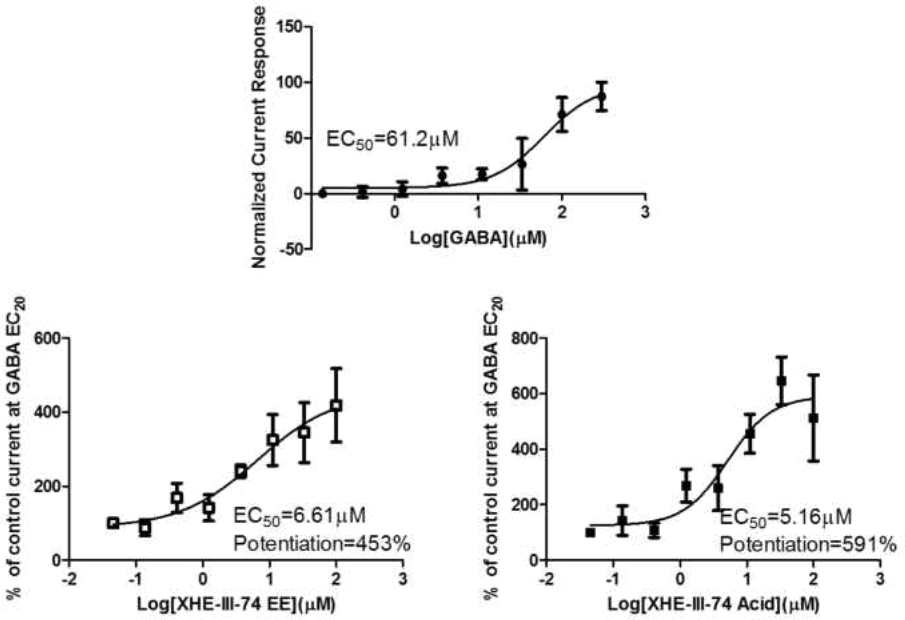


Figure 154. Recapitulation of dose response curves for Jurkat E6-1 cells in passage 5. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE in combination with a constant concentration of GABA EC_{20} or 1μM. Compound was solubilized in 1% max DMSO, N=6

Thus a new vial of Jurkat E6-1 cells were purchased from the manufacturer. These cells were put into culture and assayed in passage 2. The results, Figure 156, better reflect the initial GABA dose respone curves collected. The difference in GABA dose response may indicate some change in the expressed subytpe population over passage time, however this line of inquiry was not pursued further. The cumulative graphs for GABA dose response and modulator application for the experiments perfomed on the newly purchased Jurkat cells can be seen in Figure 155.

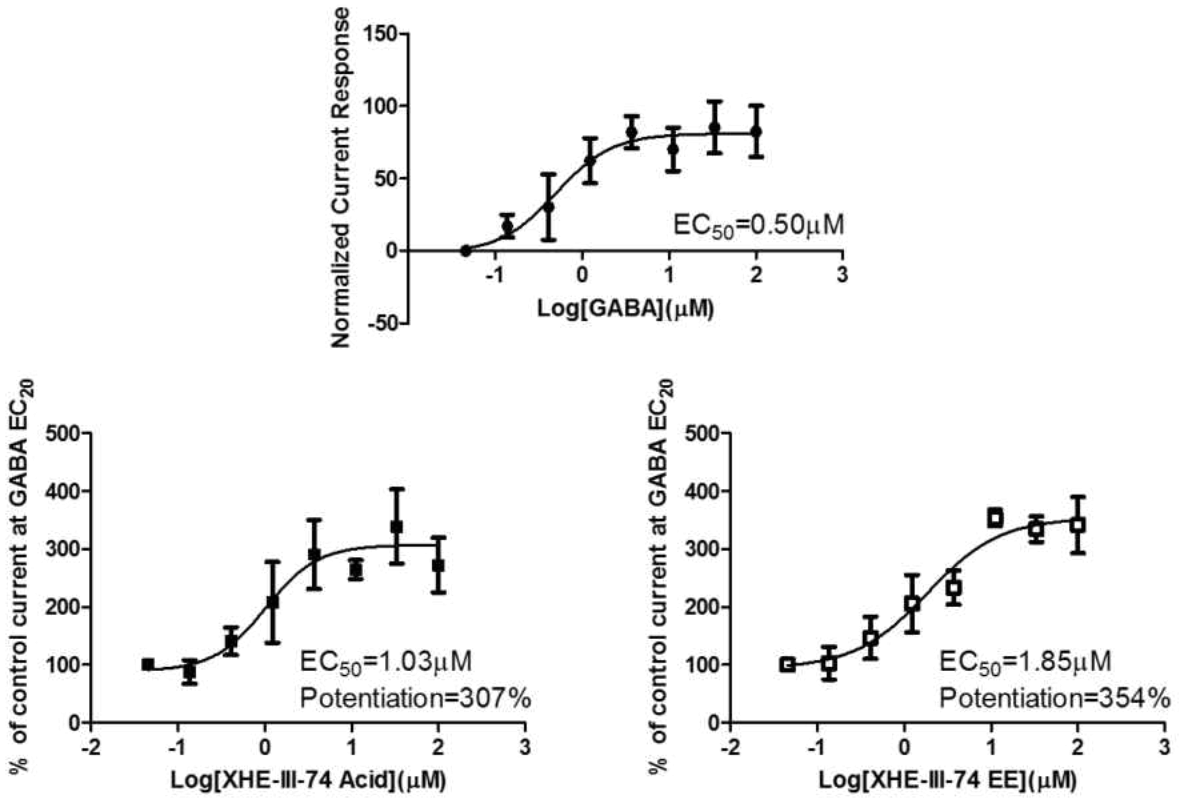


Figure 156. Dose response curves for Jurkat E6-1 cells in passage 2. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 0.1µM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE in combination with a constant concentration of GABA EC_{20} or 0.1µM. Compound was solubilized in 1% max DMSO, N=6

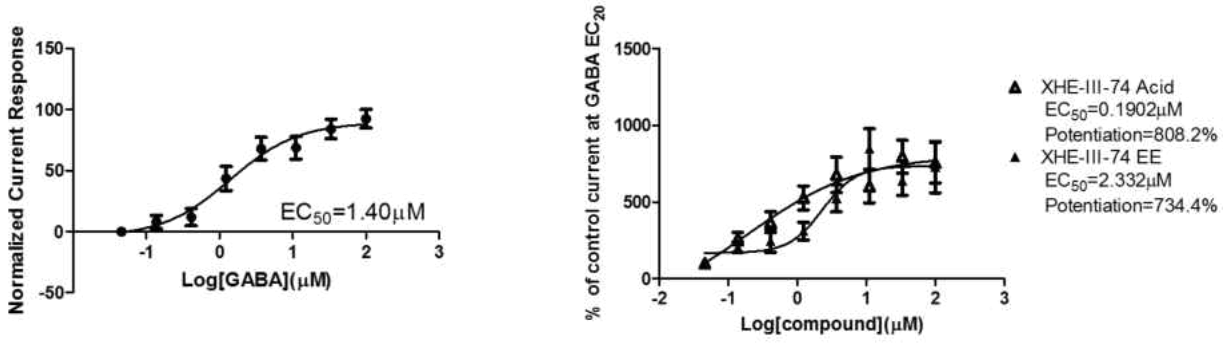


Figure 155. Cumulative data sets of dose response curve for Jurkat E6-1 cells in passage 2**.** A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=8, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 0.1µM. Compound was solubilized in 1% max DMSO N=12,

As the presence of TSPO could be intefering with the results; BZD compounds were assayed without the presence of GABA. Since TSPO does not bind GABA, any effect observed

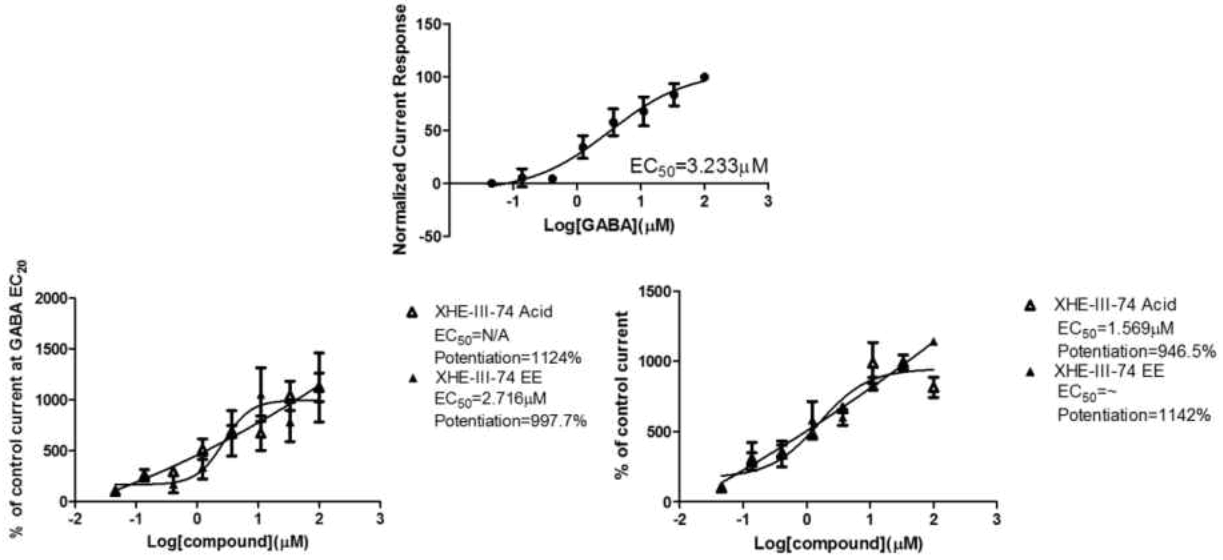


Figure 157. Dose response curves for Jurkat E6-1 cells in passage 2. A) Cells were exposed to increasing concentrations of GABA diluted in ECS buffer, N=4, B) cells exposed to increasing concentrations of XHE-III-74A in combination with a constant concentration of GABA EC_{20} or 0.1μM. Compound was solubilized in 1% max DMSO N=6, C) cells exposed to increasing concentrations of XHE-III-74EE without the presence of GABA. Compound was solubilized in 1% max DMSO, N=6

from BZD alone may be due to the peripheral receptor which oftentimes pairs with the anion channel VDAC. Thus the compounds were tested with and without the presence of the agonist GABA, results are seen in Figure 157. Suprisingly, it would appear that a majority, if not the entirety, of the potentiation achieved can be attributed to the application of positive modulator alone. To ensure that the modulation was the effect of $GABA_AR$, a battery of tests were performed using selective negative modulators of the $GABA_AR$. The GABA competitive antagonist

bicuculline, the GABA negative allosteric modulator picrotoxin, and the benzodiazepine receptor

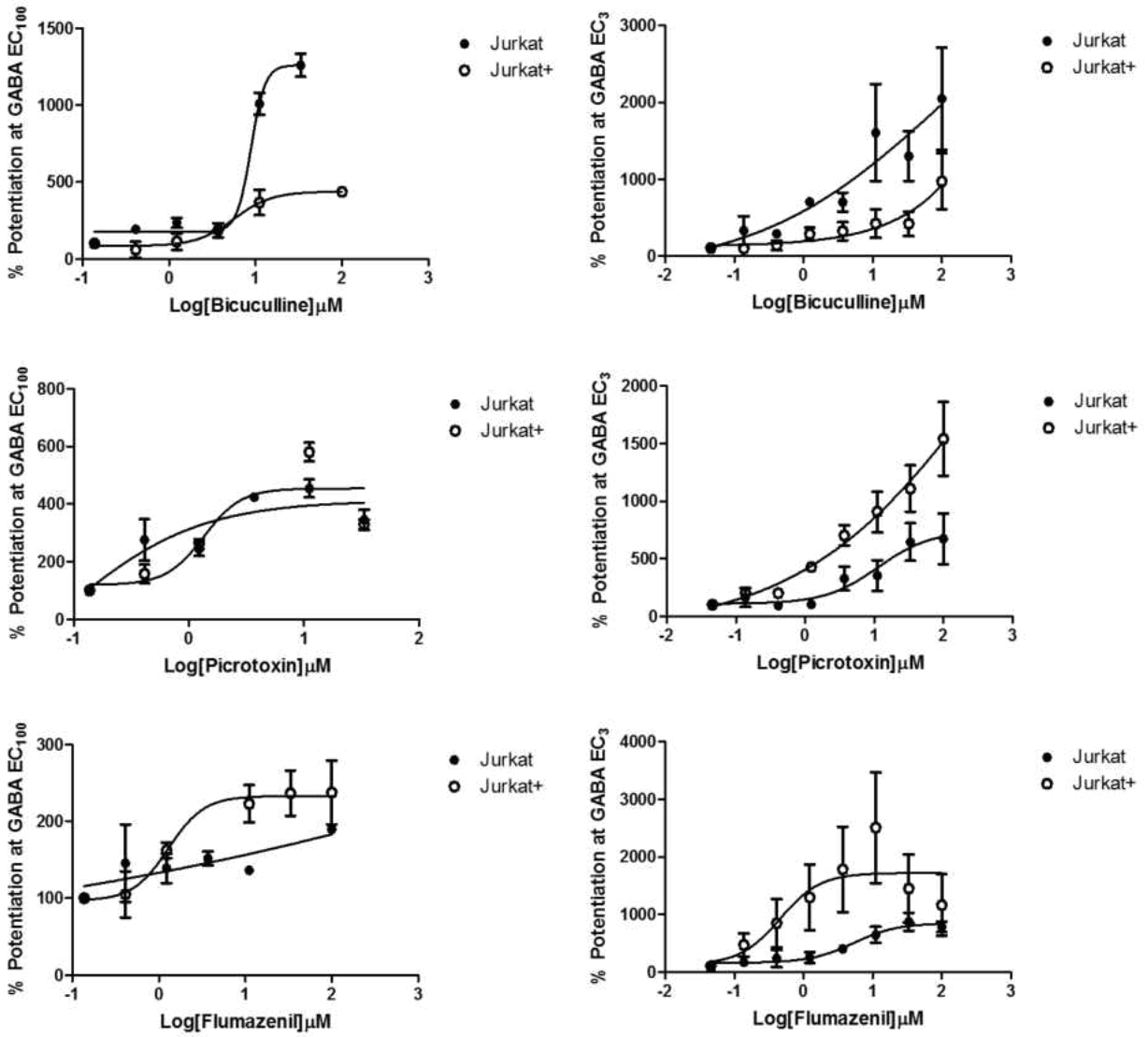


Figure 158. Dose response curves for Jurkat E6-1 cells to negative modulators. Cells were activated (Jurkat+) with 1µg/mL PHA and 50ng/mL PMA left overnight. 1a) Cells were exposed to increasing concentrations of bicuculline in combination with a constant concentration of GABA EC_{100} or 30µM, compound was solubilized in 1% max DMSO with N=2 per curve, 1b) Cells exposed to increasing concentrations of bicuculline in combination with a constant concentration of GABA EC_3 or 0.05µM, compound was solubilized in 1% max DMSO with N=2 per curve, 2a) Cells exposed to increasing concentrations of picrotoxin in combination with a constant concentration of GABA EC_{100} or 30µM, compound was solubilized in 1% max DMSO with N=2 per curve, 2b) Cells exposed to increasing concentrations of picrotoxin in combination with a constant concentration of GABA EC_3 or 0.05µM, compound was solubilized in 1% max DMSO with N=2 per curve, 3a) Cells exposed to increasing concentrations of flumazenil in combination with a constant concentration of GABA EC_{100} or 30 µL, compound was solubilized in 1% max DMSO with N=2 per curve, 3b) Cells exposed to increasing concentrations of flumazenil in combination with a constant concentration of GABA EC_3 or 0.05µM, compound was solubilized in 1% max DMSO with N=2 per curve.

antagonist flumazenil were all tested. The unusual response from these cells can be seen in Figure

158. These compounds were tested at both GABA EC_{100} as well as EC_3. From the results, it would appear that these compounds were potentiating the signal rather than inhibiting it.

It was hypothesized that this might be due to the slow onset of the negative modulators, which are often tested after long preincubation times. Thus a new protocol was programmed into the IonFlux to allow for 3 minute long incubations of the modulator compounds followed by application of the agonist GABA mixed with respective concentration of the modulator. This method however, only allows for the collection of data from 3 different concentrations of the compound. The results are seen in Figure 159.

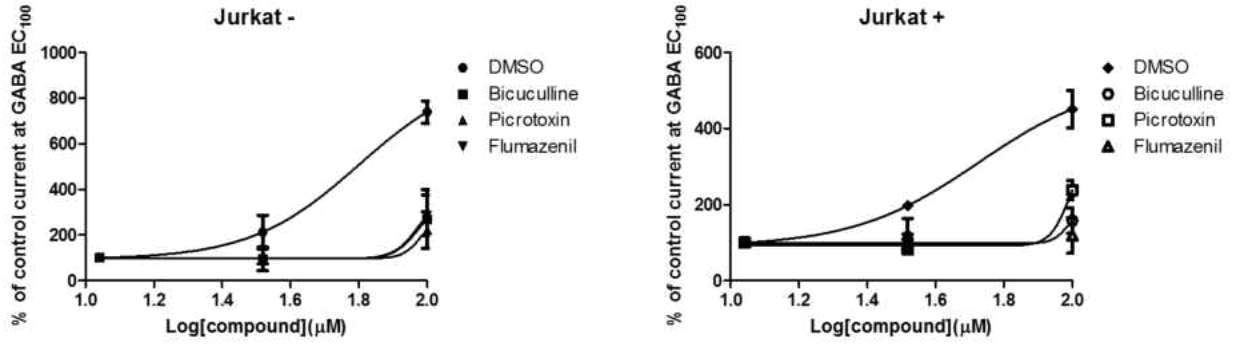


Figure 159. Jurkat E6-1 cells which were preincubated with compound for 180 seconds prior to activation with agonist GABA. A) Cells preincubated with increasing concentrations of compound before being exposed to a constant concentration of GABA EC_{100} or 30μM.

The response of the control, DMSO, was unexpectedly rising from baseline which could be indicative of two conditions: 1) DMSO is eliciting a dose response curve or 2) GABA has a slower than expected onset of action and successive doses of a constant concentration is required

to elicit the maximal current response. These two possiblities were tested and are shown in Figure

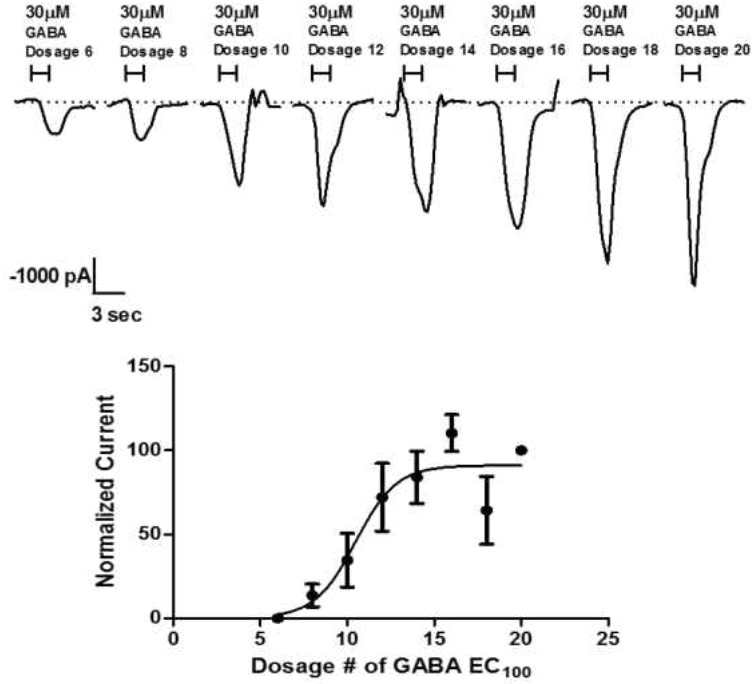


Figure 160. Jurkat E6-1 cells exposed to successive doses of a constant concentration of high GABA elicited a dose response curve. N=4

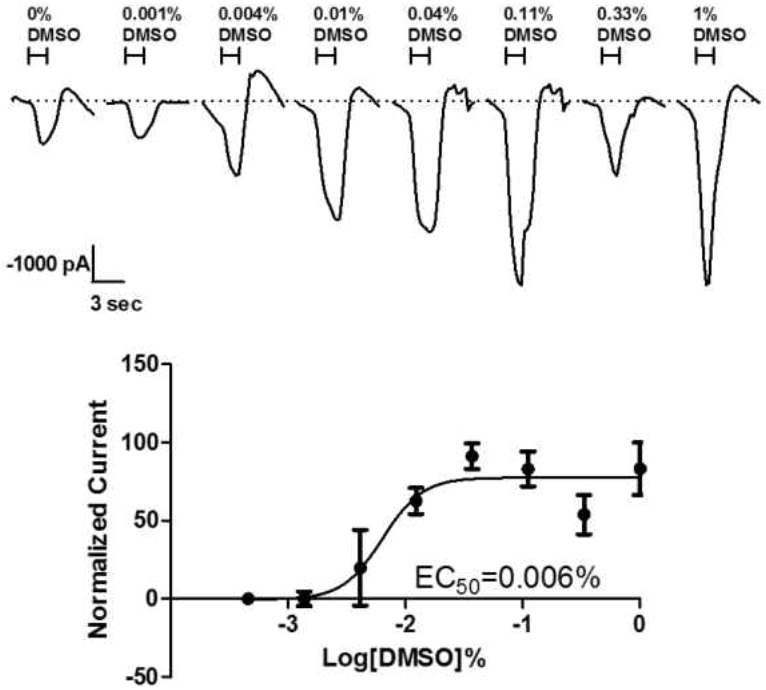


Figure 161. Jurkat E6-1 cells exposed to increasing percentages of DMSO elicit a dose response. N=4

160 and Figure 161. Figure 160 reveals that successive doses, upwards of 20, are required to bring out a maximum current response to the same concentration of GABA. This would suggest either an altered binding profile for these receptors, activation of present $GABA_B$ metabotropic receptors, or the requirement of surface associated GABA to reach the maximum response. In any case, this attribute means that using previous methods, the baseline canot be accurately established in order to measure the potentiation caused by positive modulators. Figure 161 reveals another problem and that is the sensitivity of the Jurkat cells to DMSO. It would appear that amounts as low as 0.004% DMSO is enough to potentiate a signal. This may be due to DMSO's ability to dissove

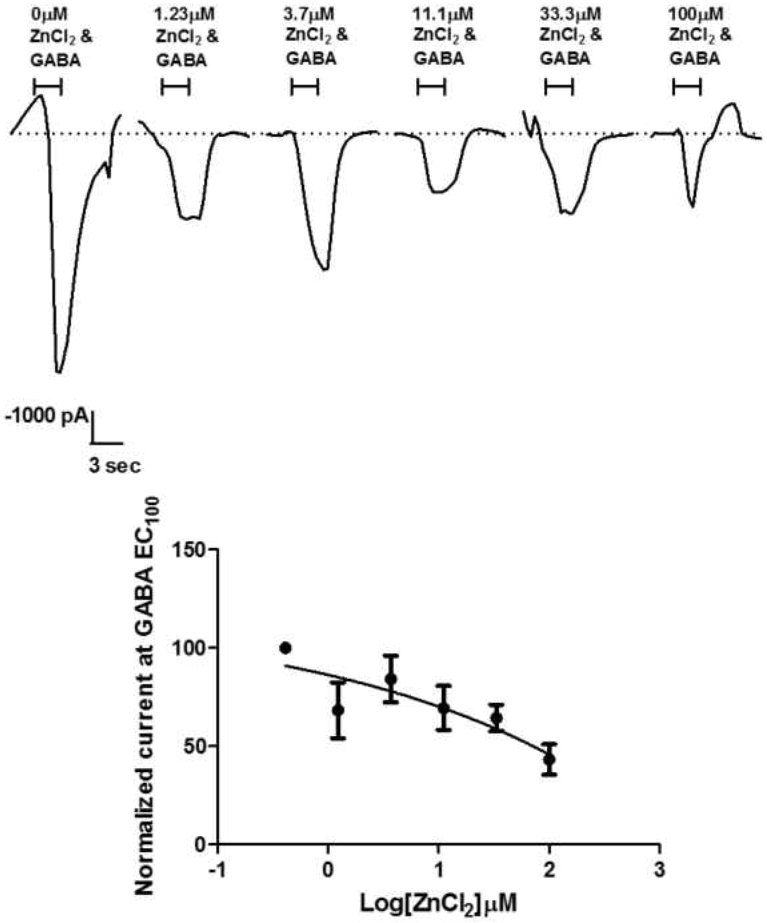


Figure 162. Jurkat E6-1 cells exposed to increasing concentrations of zinc chloride in constant concentration of GABA EC_{100} or 30μM. Salt was diluted in ECS buffer. N=7

and permeabilize cell membranes [437]. Another possibility is that the solvent increases the osmolarity, causing slight transient shrinkage, altering the current response. To determine whether the negative modulators would have an effect without DMSO; the water soluble, negative allosteric modulator zinc chloride was also tested, Figure 162. Without preincubation, the signal decreased from 100% to less than 50%.

The expression of receptor subtypes found in the Jurkat cells as well as the amount of TSPO and VDAC has yet to be reported with consistency [298,325,438]. It is also worthwhile to keep in mind that Jurkat E6-1 cells are a mixture of $CD4^+$, $CD8^+$, $CD4^+/CD^{8+}$, and $CD4^-/CD8^-$; all of which may express varying levels of $GABA_AR$ under differing conditions [393]. To determine the expression profile of the population of utilized cells, the mRNA was isolated for qRT-PCR. It would appear

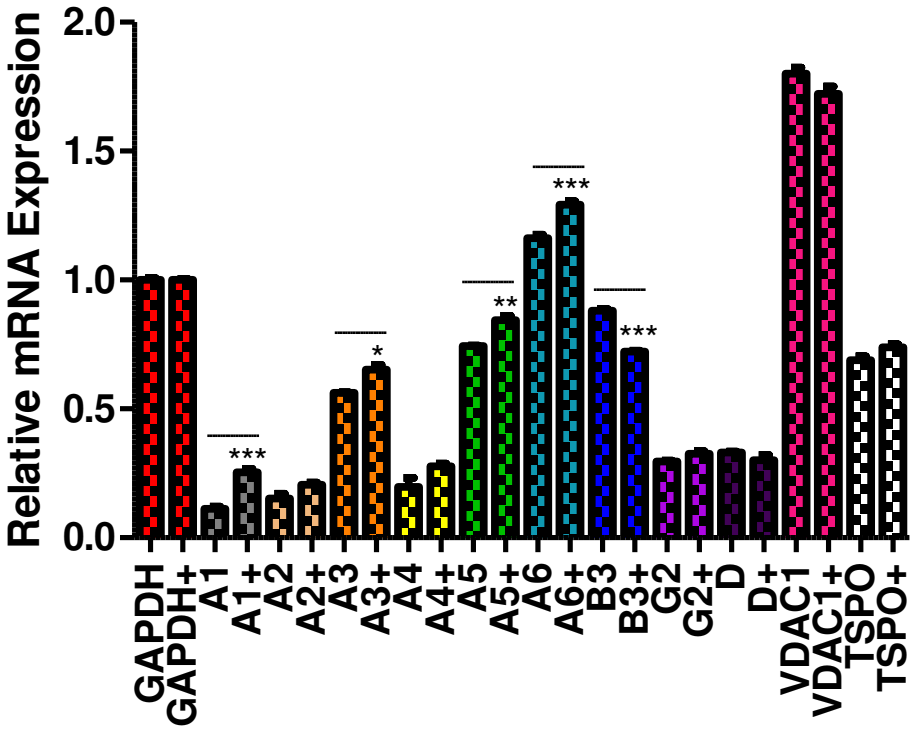


Figure 163. The relative mRNA expression of $GABA_AR$ and TSPO associated proteins in inactivated vs activated (+) Jurkat E6-1 cells. Data analyzed using one way ANOVA, $p>0.05$. Statistical significance evaluated with 95% confidence, N=3. Jurkat cells were activated (+) by incubation overnight with PMA and PHA.

that the most significant changes after activation occurred in the expression of the increased α1, increased α6, and decreased β3 subunits. In addition, to a less extent, α3 and α5 also exhibited an increase in expression after activation. The amount of TSPO mRNA is comparable to the amount of $GABA_AR$ subunit which, though nonideal, is more favorable than having quanitites that far surpass the $GABA_AR$ transcripts. The extremely high amount of α6, coupled with the very low amount of α1 makes the Jurkat E6-1 cells a poor model of human T lymphocytes which, despite having inconsistent findings for detection, should have low expression of the α6 subunit and the highest expression of the α1 [318,325]. It is well known that cell characteristics can change over time with immortalized cell lines and the high amount of α6 mRNA expression may be indicative of the status of these cells. The Jurkat E6-1 cells have undergone much criticism for defective expression of several enzymes and proteins normally found in T-cells [439].

GABA has been detected in human peripheral blood monocyte-derived macrophages [321] as well as in the macrophage and lymphocytes derived from human psoriatic skin [440]. GABA secretion has been detected from stimulated mouse macrophages and T cells [317,320]. However it has yet to be reported, to the extent of my knowledge, whether Jurkat cells endogenously produce the ligand GABA. By performing a three phase methanol/chloroform/water extraction, the water soluble metabolites of the Jurkat cell lysate were isolated. GABA was detected from this extraction, Figure 164. A next step would be to quantify the amounts of GABA produced and

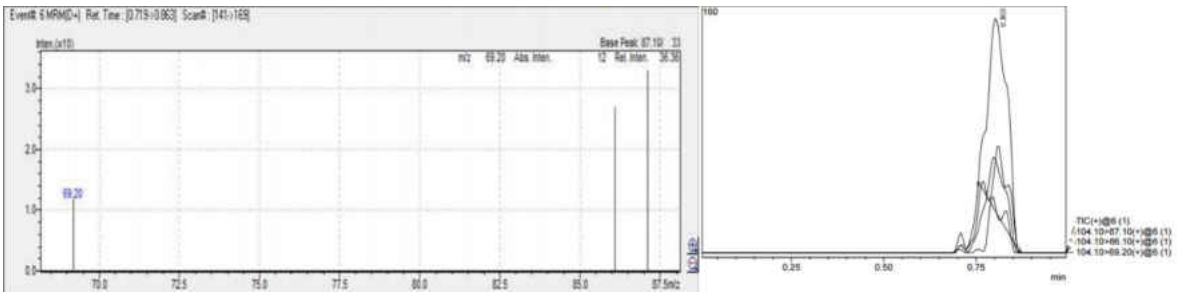


Figure 164. Detection of GABA in Jurkat E6-1 cell extract by triple quad LCMS-8040

under what conditions, such as activation of the cells or addition of a $GABA_AR$ modulator, in which production of the ligand can either be stimulated or inhibited. However, the method to create an LCMS protocol in order to quantify GABA was beyond the scope of this study.

Though it has then been found that GABA is endogenously produced by Jurkat cells, it has yet to be determined whether it is exogenously secreted. If GABA is also secreted by these cultured, leukemic human T-lymphocytes then this would further prove that the neurotransmitter serves a signaling role.

In the attempt to find a more suitable model, murine splenocytes isolated from Ova sensitized Balb/c mice were considered.

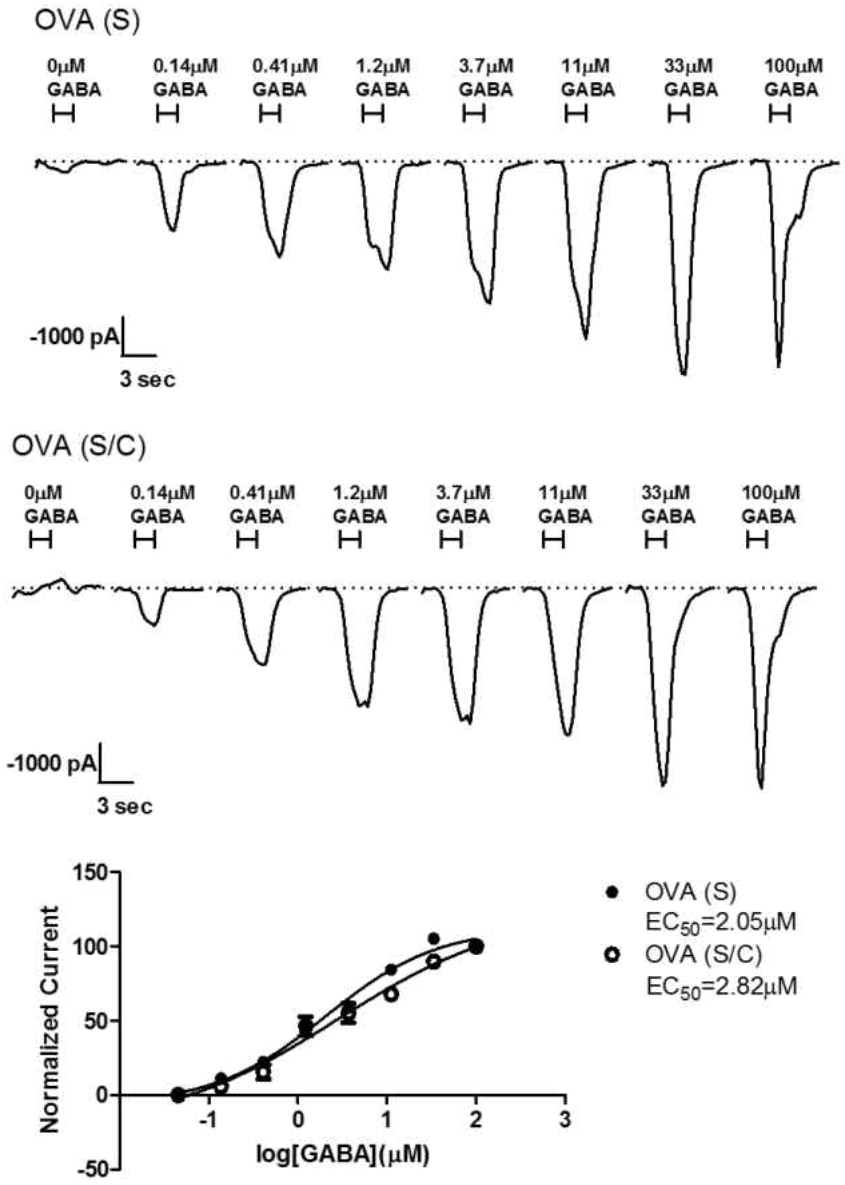


Figure 165. GABA current response of murine splenocytes isolated from male Balb/c mice. Mice were sensitized with OVA and were either unchallenged (S) or challenged in culture (S/C). N=4 per curve.

Spenocytes are a heterogenous mixture of different white blood cells purified from splenic tissue. The spleen has been described to have a similar structure to a large lymph node and acts as storage for red blood cells and lymphocytes. Thus cells isolated from the spleen can consist of T and B lymphocytes, dendritic cells, macrophages. The results of the automated patch clamp

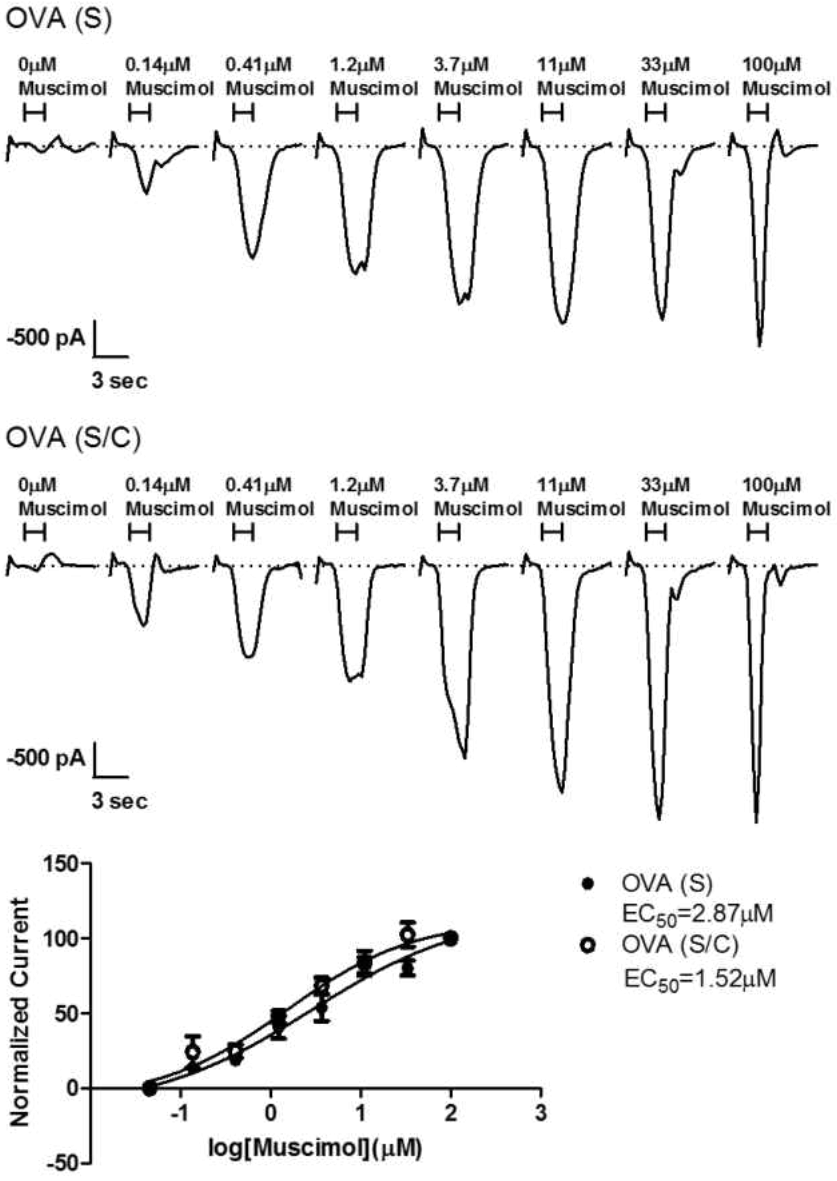


Figure 166. Musclimol current response of murine splenocytes isolated from male Balb/c mice. Mice were sensitized with OVA and were either unchallenged (S) or challenged in culture (S/C). N=4 per curve.

experiment on the mixture is seen in Figure 165. Cells were also exposed to the GABA analog muscimol, Figure 166. Muscimol is a non-addictive psychoactive constituent of *Amanita muscaria*

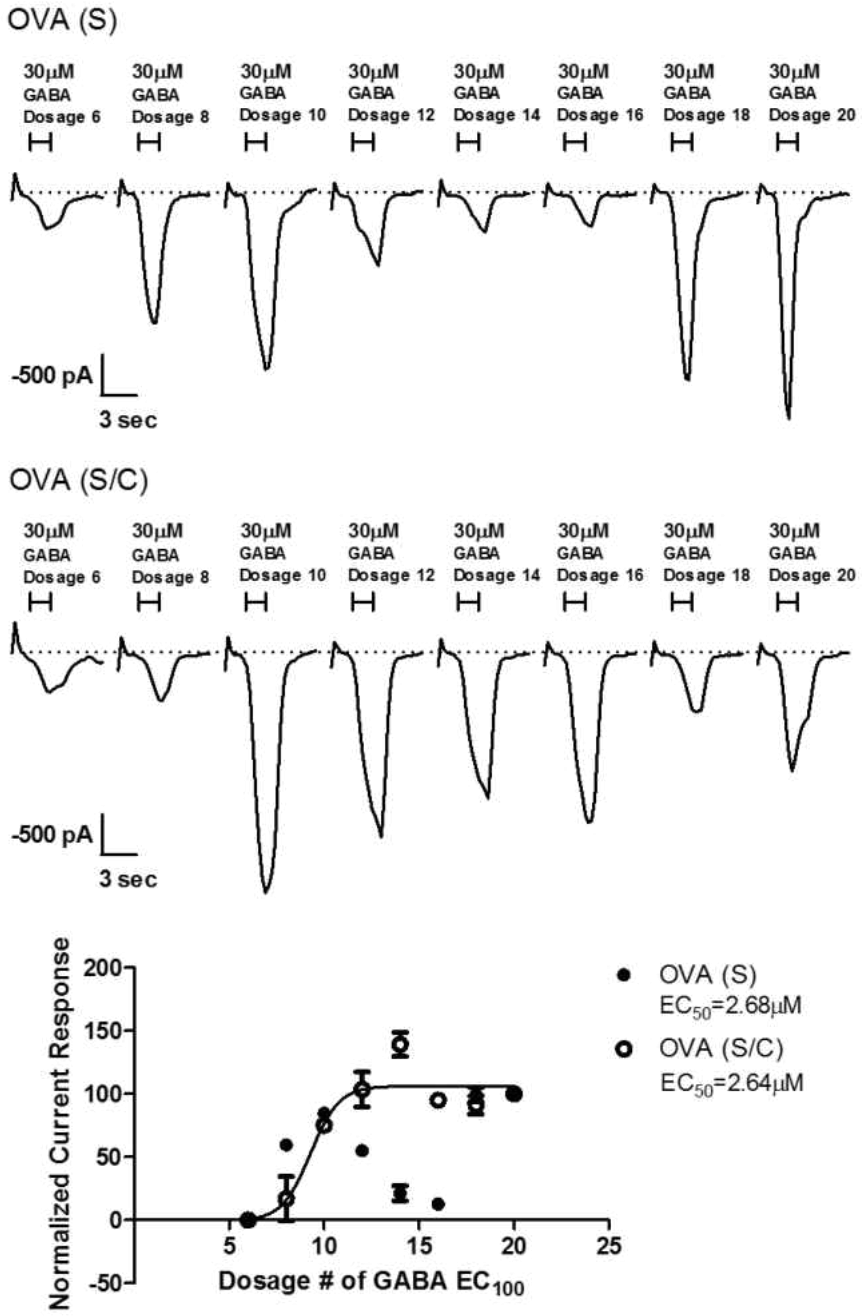


Figure 167. Successive doses of GABA on murine splenocytes isolated from male Balb/c mice. GABA EC_{100} of 30µM. Mice were sensitized with OVA and were either unchallenged (S) or challenged in culture (S/C). N=2 per curve.

mushroom and is a potent $GABA_AR$ agonist that displays sedative-hypnotic and dissociative psychoactivity when taken and binds to the same site as GABA.

It should be noted that since the splenocytes are heterogenous mixtures of leukocytes and the cells were not separated further, population density and morphological characteristics could favor a particular cell type. For example, the high population of lymphocytes makes it more likely that the cells trapped are either T or B cells; however, macrophages are large and though the popultion is low- the cells may be trapped more easily; at the same time, the morphology of dendritic cells could prevent them from being easily trapped and sealed with high resistance. Electrophysiological characterization of the separated homogenous cell types would be a worthwhile endeavor for the future.

As evidence in Figure 165 and Figure 166, both GABA and muscimol elicited clear dose response currents. The current response following successive doses of GABA as well as the response of the cells to DMSO were also a concern. The results for these experiments can be seen in Figure 167. The murine splenocytes had an inconsistent response to repetive doses of GABA EC_{100}. Whether this was a result of the mixed population of trapped cells, changes in receptor expression after the OVA challenge, or change in the population of cells after OVA challenge; it would appear that the cells require up to 10 repetive doses to reach a maximum response to GABA. However this signal rapidly diminishes and can slowly rise back (S/C) or promptly spike back (S) Figure 167. In any case, it would appear that the murine splenocytes suffer from the same problem as the Jurkat cells as a baseline cannot be accurately established to measure positive modulation.

The DMSO sensitivity of the murine splenocytes was also evaluated, Figure 168. DMSO quantities as low as 0.001% elicit a signficant current response. Confirming the splenocytes

sensitivity for GABA, zinc chloride was utilized again as a water soluble negative allosteric modulator, Figure 169. Zinc inhibited channel activity, decreasing the signal from 100% to 50%.

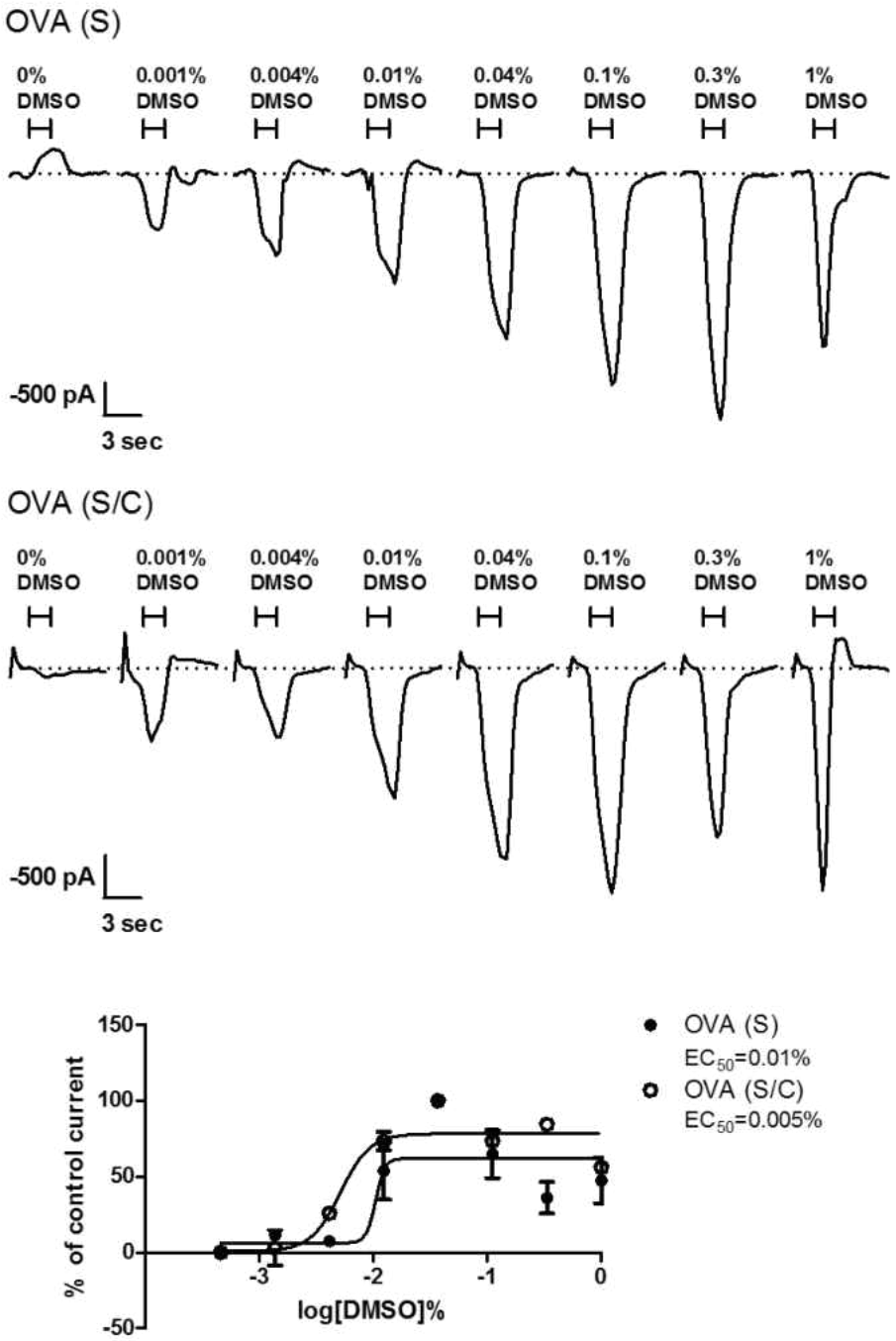


Figure 168. Increasing concentrations of DMSO on murine splenocytes isolated from male Balb/c mice. Mice were sensitized with OVA and were either unchallenged (S) or challenged in culture (S/C). N=2 per curve.

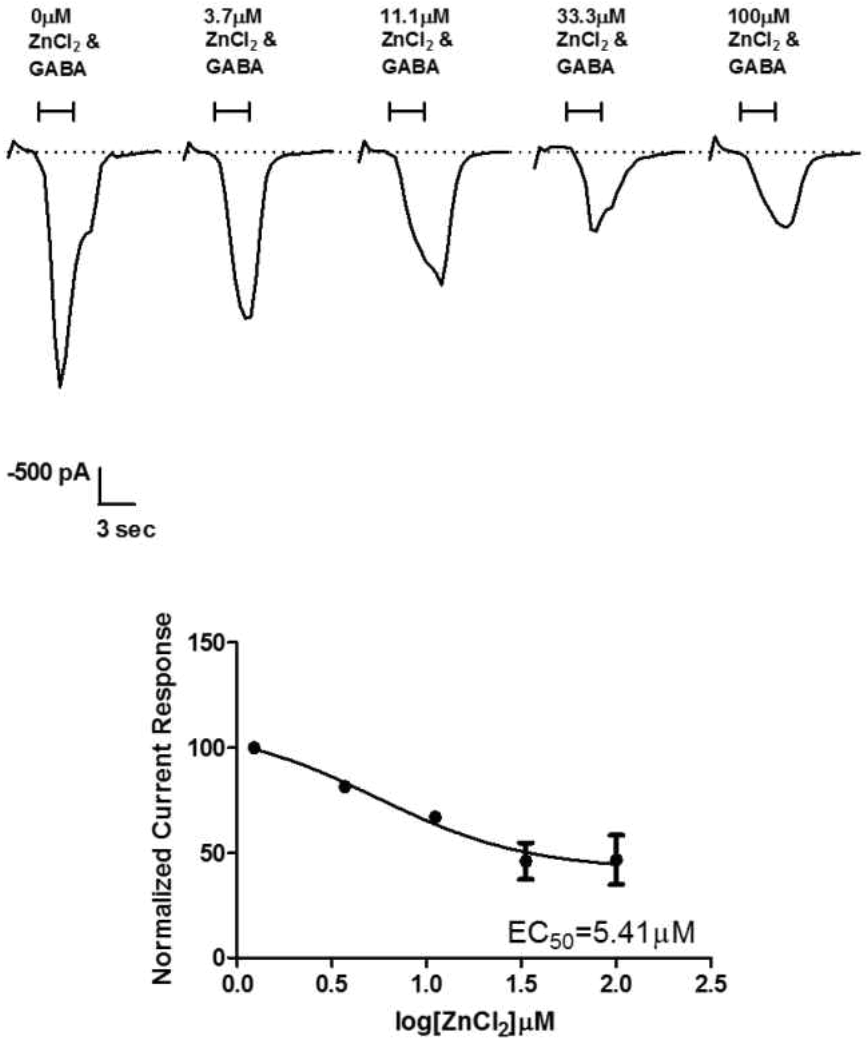


Figure 169. Murine splenocytes from male Balb/c mice exposed to increasing concentrations of zinc chloride. Mice were sensitized using Ova. N=2.

The expression of $GABA_AR$ in spleen cells from Balb/c mice has, to the best of my knowledge, not been found or published. However, the splenocytes from NOD mice have shown functional $GABA_AR$[299] and the spleen cells of $GAT1^{+/+}$ and $^{-/-}$ mice have exhibited expression of α1, α2, α5, β1, β2, δ, γ1, γ3 mRNA [2]. Thus a qRT-PCR for the murine splenocytes of male Balb/c mice was performed, Figure 170. The results had a striking absence of the α1 receptor. The low quantities of the β3 subunit as well as the high expression of α2 and α5 corresponds well with the results previously published for the spleen cells of $GAT1^{+/+}$ and $^{-/-}$ mice [2]. The quantitites of α3,

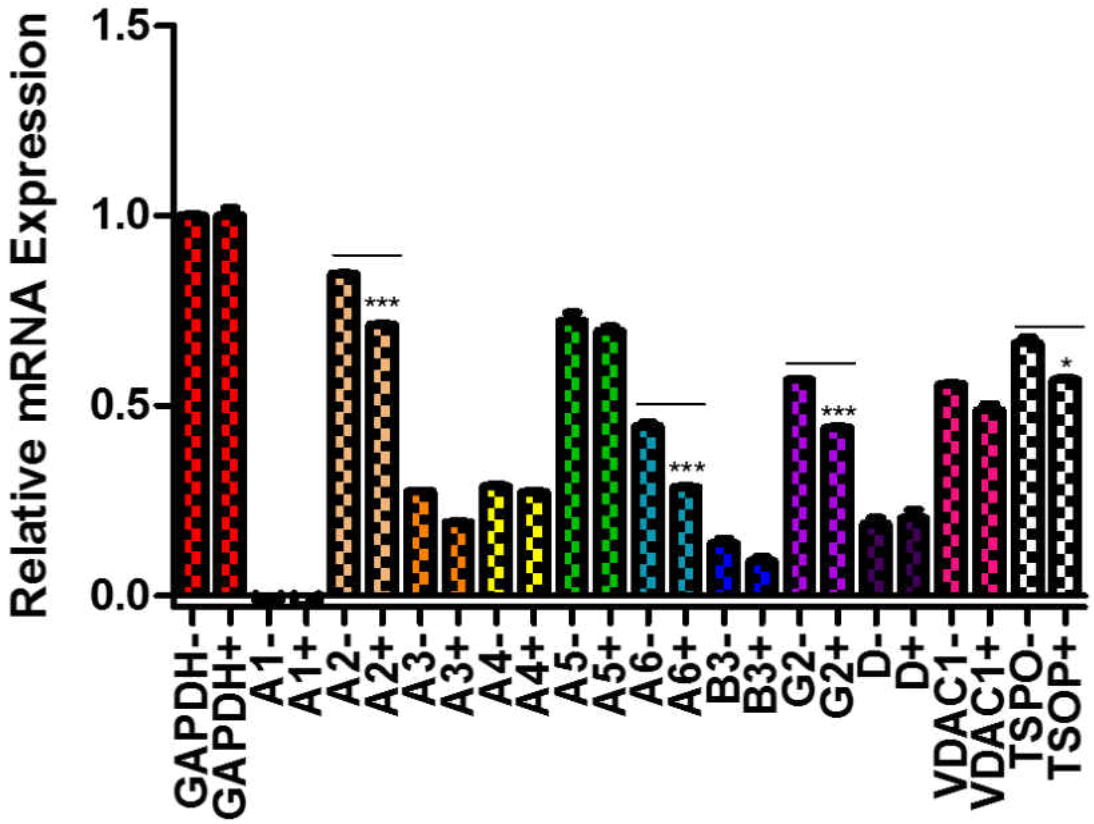


Figure 170. The relative mRNA expression of $GABA_AR$ and TSPO associated proteins in Ova sensitized and Ova sensitized and challenged (+) murine splenocytes. Spleen cells were isolated from male Balb/c mice which were Ova sensitized. Data analyzed using one way ANOVA, $p>0.05$. Statistical significance evaluated with 95% confidence, N=3. Cells were challenged (+) in culture overnight ex vivo with Ova.

α4, α6, γ2, VDAC1 and TSPO were not reported previously in any murine splenocytes. The high amount of expressed γ2 subunit may represent that the mice may be a misleading animal model for studying the immunosuppressive effect of BZDs as the high quanities will provide further BZD binding sites than what is found expressed in humans. Furthermore, unlike what was observed in the Jurkat cells wherein most mRNA levels rose after PMA/PHA activation- the Ova challenged splenocyte cells trended downwards in $GABA_AR$ subunit mRNA expression. The α2, α6, and γ2 subunit mRNA significantly decreased upon Ova challenge of the cells. Whether this is a species dependent characteristic for mRNA regulation or that the upregulation observed in Jurkat cells were the result of the immortalized status of the cell line is yet unknown. However, these two

cellular models had significant differences in subunit expression. Most concerning is the lack of the α1 mRNA.

Due to the relative inconsistency of reporting for mRNA quantification in immune cells; it was adventageous to perform a qRT-PCR on primary human cells. To do this, human peripheral blood mononuclear cells (hPBMCs) were extracted from whole blood. The blood was fractionated and the mRNA of the white blood cells collected in the buffy coat was quantified, Figure 171. The

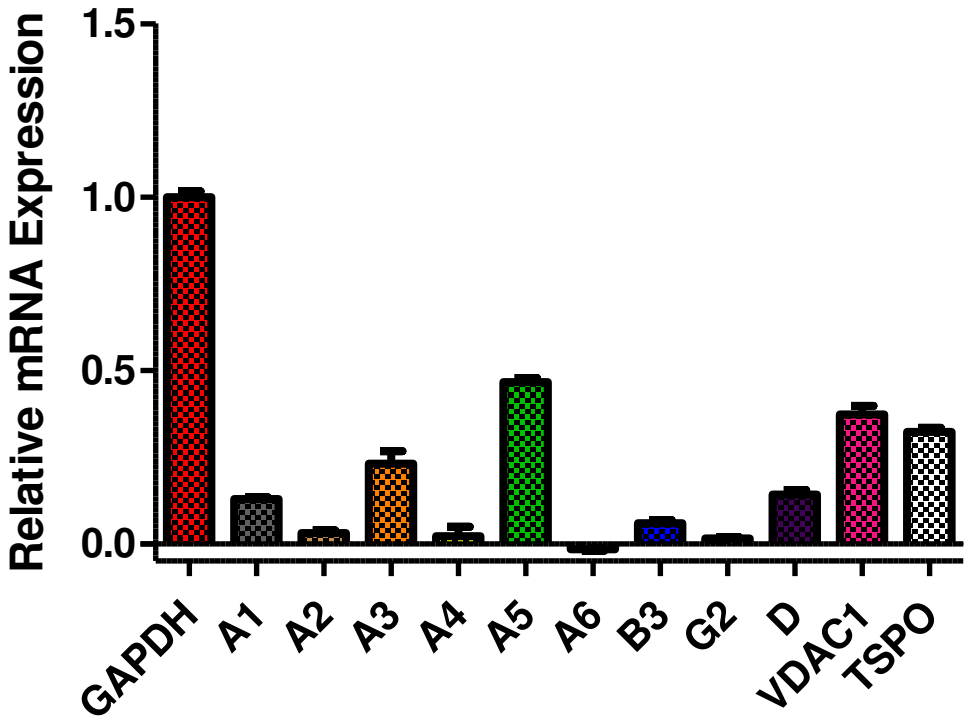


Figure 171. The relative mRNA expression of $GABA_AR$ and TSPO associated proteins in human peripheral blood mononuclear cells. Cells were isolated from whole blood extracted from a human female, Asiatic, 28 years of age. N=3.

overall yield of mRNA for 5mL of whole blood was low (40 µL of 102 µg/mL), however the quantity was sufficient to perform qRT-PCR. The results coorespond well with previously published PCR cDNA band intensity studies with the exception of the high expression of the α5. The two previously published studies utilized identical primers with one reporting the prescence

of α1, α3, α4, β2, β3, γ2, δ, ε[325] and the other study reporting consistent expression of α1 and δ, individual-dependent presence of α3, α6, and γ2, and PHA activated expression of β3 [318]. It would be prudent to compare the primer efficiency between our primers and those used by the two publications.

6.3.4 CONCLUSIONS

The expression pattern of $GABA_AR$ in Jurkat E6-1 cells and murine splenocytes represent a poor model of healthy human T-lymphocytes. Furthermore the regulation of mRNA expression after activation of the cells with PMA/PHA or Ova had opposing effects. The characterization of human immune cells after activation has yet to be consistently reported, and would appear to be dependent on the individual [318], creating further complications in choosing a representative cellular model. Primary human cells, preferably those of an invidual with atopy, would represent the optimal paradigm and an easy method of isolation has been performed and described herein.

As mentioned in the discussion, there has been inconsistency in the reports of $GABA_AR$ expression in not only hPBMCs and murine splenocytes but also immortalized cell lines. Studies into the $GABA_AR$ expression in Jurkat cells, for example, one group found expression of α1, α3, α4, α6, β1, β2, β3, γ2, ε, and θ [325] while yet another study reported expression of α1, α3, α5, α6, β1, β2, β3, φ, and ρ2 [298]. Interestingly, the supplier of the cells for these two groups were the same (European Collection of Cell Cultures, ECACC) which should be assumed to be nearly identical cell samples. In addition to the mixed reports on $GABA_AR$ expression, TSPO expression in Jurkat cells has also experienced similar controversy. TSPO expression has been considered a significant

biological marker for cancerogenesis in some tissues and is abundantly expressed in most malignant cells [441-443]. Some studies have considered Jurkat cells as completely lacking expression of the TSPO and have used it as a negative control to show high specificity of TSPO-specific ligands. However, several studies have observed expression of TSPO in Jurkat E6-1 cells, under the pseudonym PBR [438], due to its upregulation in tumors and cancer cell lines and the role it appears to play in apoptosis regulation [444]. Interestingly, it would appear that the Jurkat TSPO receptors, detected using immunoblot, have only a low affinity for classical TSPO ligands which explains its late discovery [445]. Jurkat cells may then be a better model for testing BZDs as the binding affinity is lower than the normal TSPO receptor and would theoretically cause less interference. Whether this modified affinity is due to the previously unaccounted for presence of the $GABA_AR$ detected in our tests is still unknown but may explain the different characterization as most other TSPO receptors were studied in cancerous cell lines rather than both cancerous and an immunological cell.

The slow increase in response to repetitive doses of GABA is an incredibly unusual and appears to be a unique feature of these immune cells. The differences in response from neuronal $GABA_AR$ is tantamount to differences in receptor subunit composition, subtype populations, unknown subtype variants, and/or surface expression. The slower rise and decay of the lymphocytic $GABA_AR$ was also observed in whole-cell patch-clamp recordings of macrophages [317]. In addition, only 7 of the 10 macrophages patched elicited a GABA-evoked current. Which would suggest that, despite cell type, individual cells may have a varying level of expression of $GABA_AR$. The currents diminished in

amplitude with repeated application of GABA, resembling desensitization or possibly endocytosis [446].

A study of human activated T cells vs naïve T cells shows that there is differential expression of the $GABA_AR$ subunits. One study observed expression of α1, α4, β2, β3, γ1, and δ in resting cells and upon activation the β2 and γ1 were lost [323]. A similar finding was reported with THP-1 cells where α1, α4, β2, γ1, and δ subunits were reported while study of primary human monocytes only detected the presence of the β2 subunit [447].

Importantly, it cannot be ignored that any immunosuppressive effect observed from BZD may be the result of TSPO binding. Functional peripheral benzodiazepine receptor TSPO, has been observed to have immunosuppressive activity. TSPO expression has been observed in order of abundance in the following cells types: monocytes [448], polymorphonuclear neutrophils [449], B-cells, natural killer cells [448], $CD4^+$, $CD8^+$ [449], platelets, and erythrocytes [448]. In addition, murine splenocytes have also exhibited ligand binding, particularly in the macrophage population but also in the T-cells which show 5-fold lower binding [450]. It has also been observed that murine lymph node and spleen cells experienced inhibition of T and B cell stimulation by benzodiazepines [451]. In addition, when diazepam and clonazepam were administered to pregnant mice, the offspring had significant inhibition of T and B cell proliferative response [452]. This effect was enhanced using TSPO selective agonist Ro5-4864 [453]. Yet another study of long-term treatment with BZDs in rats has observed an increase of the percentage of T-lymphocytes and decrease in B lymphocytes and increase in corticosterone serum levels. Long-term treatment with BZDs decreased the number of apoptotic and necrotic cells [454]. BZDs have also been shown to decrease mast cell and TNF-α production as well as suppress the activation of the IL-6 [455-457]. TSPO receptors are present locally

in the lung [458] and expression has been shown to be affected by cigarette smoke [459], suggesting that inhalation of therapeutics would have an effect. Interestingly, most recently there has been interest in developing drugs selective for TSPO. XBD173 appears to be an excellent non-sedative anxiolytic and antipanic agent that has high selectivity for TSPO.

A study of TSPO specific PK11195 and Ro5-4864, mixed type diazepam, and $GABA_AR$ specific clonazepam; has found that these compounds all inhibited mitogen-driven proliferation of B- and T-cells in vitro as well as the suppression of IL-2 and IL-2 receptor expression [451]. However, clonazepam was around half as active and Ro15-1788 failed to antagonize the actions of both diazepam and clonazepam which may suggest an altogether new receptor type that is both distinct from TSPO and from $GABA_AR$. Interestingly, FG7142 and DMCM, anxiogenic inverse agonists, have also been observed to induced severe immunosuppression in rodents within 24hr [78]. One study has also reported the differential effect of two $GABA_AR$ modulators alprazolam and clonazepam on the immune systems of stressed adult male rats. The study found that both compounds increased neutrophil count while decreasing lymphocytes, anti-SRBC titer, and IL-2 level. Alprazolam was more effective than clonazepam and the toxic effects were exacerbated by stress. Stress is generally considered to be immunosuppressive and increases susceptibility to infections and cancer [460,461]. Clonazepam has also been found to bind strongly to TSPO receptors in rat aortic smooth muscles compared to other BZDs, concentrated in mitochondria [462]. Taking this evidence into account, dually targeting both $GABA_AR$ and TSPO may present a more effective means of asthma treatment than targeting either of them individually.

Intriguingly, after injury, TSPO has a significant increase in expression suggesting that it is involved in the neuroimmunological response. TSPO has been used as a biomarker of brain

damage and neurodegeneration since expression levels under normal conditions are low [463]. The selective expression of TSPO to locally damaged regions has been observed in Alzheimer's, frontotemporal dementia, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's [463]. They have also been found in mast cells and macrophages [464-466]. In addition, those with generalized anxiety disorder, social anxiety, PTSD, and panic disorder have reported reduced TSPO expression [463]. High affinity phenylpurine TSPO ligand XBD173 was able to alleviate panic attack in rodents and humans with no sedative properties [467]. Administration has had anxiolytic and anticonflict actions and is correlated with increases in allopregnanolone, pregnenolone, progesterone and THDOC [468].

However, caution should be taken in targeting the TSPO protein as evidenced from alpidem, which was an approved TSPO ligand in 1991 France to treat anxiety. This compound caused severe and sometimes lethal liver damage and was withdrawn by 1994 [469]. Despite this, recently another TSPO specific compound called AC-5216 has been under review for its antianxiety and antidepressant-like effects [470].

There is also the possibility that the α4 found expressed in the immune system are not assembled in the α4β3γ2 or the α4β3δ. It has been reported that a significant part of native $GABA_AR$ containing α4 does not contain either the γ or the δ subunit [471,472].

Evidence that targeting the GABAergic system in immune cells has shown promise for the treatment of asthma. Oral GABA decreases IgE levels in Ova sensitized Balb/c mice and exhibited an increase in Th1 associated IFN-γ and a decrease in Th2 associated IL-4 [473]. This is particularly significant for allergic responses which are associated with IgE production and a Th2 response.

In conclusion, the highly variable levels and plasticity of expression would suggest that there is a dynamic regulation of the receptor subunit which is dependent on species, state of activation, cell type, and the individual. Although we have ascertained that GABA is endogenously produced, it has yet to be determined whether GABA is also exogenously secreted in human cells. If GABA is secreted then this would further prove that the neurotransmitter serves a signaling role that may allow T-cells to communicate not only among themselves but also with neuronal CNS. Furthermore, it would be interesting to determine whether the amount of GABA that is endogenously produced increases or decreases after activation and differentiation; as well as the effect that BZDs might have on the quantities of GABA produced. Elucidating this mechanism of possible autocrine/paracrine signaling may give valuable insight into understanding how coordination and cross-talk occurs between the immune and nervous systems.

6.2 Implications of Findings

The CNS is segregated from the rest of the body with the existence of the Blood-Brain Barrier (BBB) that facilitates ion balance, nutrient transport and blocks the entrance of harmful molecules. The BBB was first discovered after a series of landmark experiments. In 1885, Paul Ehrlich injected water-soluble dye into the circulatory system which stained all organs except the brain and spinal cord [474]. Though at first, he mistakenly attributed this finding to the nervous tissues' low affinity to the dye [475]. Ehrlich's student Edwin Goldman later injected trypan blue directly into the cerebrospinal fluid and successfully stained the brain but did not enter the periphery [476]. It was later found that basic aniline dyes crossed the BBB but acidic aniline dyes did not. An extensive review was published of the permeabilities which resulted in the discovery that passage through the barrier was dependent on electrochemical properties. Compounds with a positive or no charge at blood pH are capable of passing but those with a negative charge are

impermeable [477]. Further study on the matter found more complex results that took into account the molecular weight, size, binding affinities, dissociation constants, lipid solubility, and charge [478].

The central nervous system was once previously considered to exist completely separately from the peripheral immune system. Typically, white blood cells in lymph nodes detect foreign pathogens, causing an immune response. These lymphatic vessels were never detected in the brain and thus it was assumed that the brain was entirely disconnected. However, the recent discovery of lymphatic vasculature in the CNS has led to a revolution in our understanding of neuroimmunology and neurodegenerative diseases associated with immune system dysfunction [479]. The necessary connection between the immune system and central nervous system would necessitate a balancing act to avoid overstimulation and inflammation in the brain; making communication between the two systems vital. How these two systems communicate has yet to be discovered but the presence of neurotransmitters in the immune system and their ability to mediate immunological response may be a hint to this mystery.

It has long been understood that the CNS regulates the innate immune response, controlling cytokine production in the spleen. The action potential is transmitted by the vagus nerve to the spleen by release of the neurotransmitter acetylcholine [480]. When this regulation is unrestrained, the cytokine cascades can be lethal, leading to sepsis [481]. In addition, the newly discovered lymphatic vessels may play a role in neurological diseases with a strong immunological element such as MS, autism, and Alzheimer's disease. Another study, by Jonathan Kipnis, has suggested that some compound exists that is released from the injured CNS and is transmitted to deep cervical

lymph nodes through lymphatic vessels where it activates the immune system [482]. This may explain the presence of similar receptor ion channels which might aid in the cellular signaling.

Interestingly, comorbity of asthma with psychological disorders is common with anxiety occurring in between 16-52% and mood disorders in 14-41% [483]. However, whether asthma causes psychological problems or if psychological problems cause asthma has yet to be determined [484]. Perhaps this is why, historically, asthma was seen as a psychological illness.

A major question that has yet to be fully addressed is what is the role the immune system plays in psychiatric disorders? There are multiple observations that link immune dysfunction with depression: patients with depression have a higher incidence of immune abnormalities, depression is a common side effect of cytokine therapy, IL-1 administration induces depression-like sickness behavior, activation of the hypothalamic-pituitary adrenal axis (HPAA) with some cytokines, other cytokines activate the brain serotonergic systems. All of these points will be discussed in order below.

Immune dysfunction has been observed in patients with depression for over a century. The earliest studies found that depression weakened the immune function in patients [485] but recent studies have found the opposite effect of depressed patients having heightened immune activation [486]. A review by Kronfol [487] notes that major depression is associated by positive acute-phase proteins and lower plasma concentrations of negative acute-phase proteins which is indicative of an inflammatory state. It has been suggested [488] that chronic depression is associated with chronic inflammation. This is consistent with reports that depressed patients have elevated concentrations of prostaglandins and cytokines [487,489].

Cytokine therapy has been used in the treatment of a variety of medical conditions including hepatitis C, multiple sclerosis, infections, leukemia, kaposi's sarcoma, melanoma, myeloma, renal carcinoma and other forms of cancer. Cytokines are divided into two groups: the pro-inflammatory and the anti-inflammatory. The proinflammatory cytokines include the IL-1, IL-6, and TNF which work to attract immune cells to the site of infection or injury and activate them to respond. The anti-inflammatory cytokines include the IL-10 and IL-13 which inhibit the synthesis of pro-inflammatory cytokines. The most common cytokines in these therapies utilize IFNα, IFNβ, IFNγ, and IL-2. Depression is most often associated with the treatment using IFNα and IL-2, it has also been observed with IFNβ but interestingly not with IFNγ [490,491]. Administration of IFNα, IL-1 and IL-6 have been shown to effect brain serotonergic systems which have been implicated in major depressive disorder. A recent meta-analysis on the immunology of depressed patients was performed and found that major depression is associated with overall leukocytosis, increased CD4/CD8 ratios, increase in the circulating haptoglobin, prostaglandin E_2, and IL-6 concentrations and reduced natural killer (NK)-cell cytotoxicity. It is interesting to note that the paper did not find consistent elevation of IL-1, the sole cytokine that induces depression-like behavior in animals, in depressed patients [492].

Sickness behavior is the observed behavioral changes that occur during the course of an infection. The changes include lethargy, depression, anxiety, loss of appetite [493], sleepiness [494], hyperalgesia [495], reduced grooming [496], and lowered abilities to concentrate [497]. Sickness behavior can be induced in animals through the administration of endotoxin lipopolysaccharide (LPS) or the cytokine interleukin-1 (IL-1).

HPAA activation is one of the most consistent biological markers for depression (50-70% occurrence rate). So the fact that IL-1 potently activates the HPAA appeared to be further evidence of this theory [498].

Most drugs used to treat depression inhibit serotonin reuptake despite lack of evidence that abnormalities in 5-HT cause depression [499]. Theoretically, if low serotonin levels caused depression, antidepressants should rapidly alleviate symptoms. However, serotonin reuptake inhibitors can require more than a month to achieve efficacy [500]. Despite these quandaries, serotonin reuptake inhibitors do alleviate depressed mood in many individuals. Some researchers have posited that the drugs increase neurogenesis and alleviation of depression may be a result of the long-term effects of the increased neuronal population [501,502]. Recent estimates show that current antidepressants available on the market only work in 60% of patients [503]. Thus continued pursuit of research into antidepressants is necessary.

Accumulating evidence for the linkage between depression and immunological dysfunction has been increasing. Raz Yirmiya was the first psychobiologist to liken that the symptom markers of major depressive disorder and sickness behavior are identical. In an experiment using rats treated with cytokines, he found that they were less sensitive to the rewarding properties of a saccharin solution and sexually-active partner [504]. This was able to be treated using chronic administration of antidepressant drugs. This has led to the theory known as "the cytokine hypothesis of depression" and proposes that depression could be the result of cytokine secretion due to activation or dysfunction of the immune system [504-507]. Another version of the hypothesis, found that depression was associated with increased secretion of cytokines (particularly IL-1) by macrophages and that this process specifically is the cause of depression [508]. This theory, it would

appear, has been elevated to the level of folklore status in the research community [486]. Yet another version of the theory proposes that immune activation in the periphery induces the production of cytokines and their receptors in the brain [509]. The activation of this response is believed to be initiated through stimulation of the vagus nerve which translates through the brain stem and into the forebrain [510,511].

However, it has yet to be determined as to whether cytokines are a cause of major depressive disorder or the cause. And although depression has received most of the attention, anxiety has also been linked to inflammation. An elevated level of Il-6 is observed in people with anxiety independent of depression-like symptoms. This has been purported to be a possible explanation as to why anxiety increases the risk of other inflammatory conditions [512]. Mice overexpressing IL-6 or TNF display an anxiogenic phenotype [513,514] and knockout of INF-γ enhances anxiety-like behavior in rodents [515]. In addition, the inflammatory state in schizophrenia is also associated with increases in prostaglandin E_2 and increase in cyclooxygenase-2 (COX-2) expression. Infection of pregnant mothers later resulting in offspring who developed schizophrenia has also often been reported [516,517]. Even in animal models, an immune activation of the mother during the second trimester of pregnancy led to schizophrenia-like symptoms in the offspring [518]. Another study found that increased IL-8 levels in human mothers was associated with a heightened risk of offspring developing schizophrenia [516]. There is also a fivefold increase in risk of developing psychoses if the infection invades the CNS in early childhood [516,519-522]. Another study has observed that unmedicated schizophrenia patients have a significantly higher number of monocytes than healthy controls [523]. Although, the same problem appears with this theory as it is unknown whether anxiety causes inflammation or inflammation causes anxiety.

Inflammation is also observed in age-related neurodegenerative diseases, such as Parkinson's disease (PD). Such diseases are characterized by loss of certain neuronal subpopulations, nigral dopaminergic neurons in PD, and the inflammatory response from the soluble factors secreted from injured neurons during degeneration appears to be an important factor in disease progression [524]. Chronic activation of microglia has been implicated in neurodegenerative disorders [525] as the brain ages, the amount of proinflammatory cytokines CD80, CD86 and ICAM-1 rises [526] while the levels of anti-inflammatory cytokines IL-10 and IL14 drops [527]. In addition, with age comes the deterioration of the BBB ultimately leading to cognitive decline and dementia [528,529].

An interesting phenomenon worthy of noting is the ability of psychological stress to trigger or exacerbate clinical symptoms in patients with asthma. This ability of stress in the mind, effecting the immune system is well documented. Children with asthma who experience an acute negative life event (death of a close family member) have a 2-fold increased risk of subsequent asthma attack while children with acute and chronic stress have a 3-fold increase in risk of attack in the two weeks following the acute event [530]. Studies with asthmatic college students during periods of high stress (final exam period) found that around final exams, challenge with the allergens they were sensitized to resulted in a higher number of eosinophils in both the sputum and blood [531] . A similar study within high school students found that there is a reduction in Th-1 cytokines IFN-g and IL-2 but an increase in pro-inflammatory Th-2 cytokine IL-6 [532].

It has been theorized that the way stress amplifies the immune response involves the activation of the HPA axis. The process first begins when the hypothalamus secretes corticotropin-releasing hormone (CRH). This triggers a release of adrenocroticotropin hormone (ACTH) in the

anterior pituitary gland. ACTH signals the zona fasciculate to stimulate the production of cortisol which binds to glucocorticoid receptor. The glucocorticoid receptor has been shown to regulate the expression of anti-inflammatory IL4, IL-5, and IL-13 on T-lymphocytes after activation from an allergen, leading to a shift to Th2-mediated immunity. Allergic diseases, such as asthma, rhinitis, aczema, and IgE-mediated food allergy, are characterized by a dominant Th2 response [533]. The HPA axis can be potently activated in situations that necessitate high levels of social evaluation which elicit self-conscious emotions like shame which produces ACTH and cortisol [534]. In children who experience both chronic and acute stress, a 5.5 fold reduction in glucocorticoid receptor mRNA was observed [535] suggesting that the down regulation leads to glucocorticoid resistance. Stress episodes before the development of the disease may then increase the susceptibility of the individual [536]. It has been found that the presence of high doses of cortisol bias the immune system towards excessive Th-2 cytokine response which may result in severe and prolonged symptoms following exposure to a trigger [328]. Thus, the effects of GCs and β2 agonists appear beneficial in the short-term, the long-term effects might perpetuate the increased vulnerability of the patient to allergens. In fact, in vitro and in vivo studies have found that GCs and β2 agonists potentiate IgE production [537,538].

Adrenergic receptors expression levels also appear to be effected by psychological stress. These receptors present on T- and B-cells regulate the expression of IL-4, IL-5, IL-13, histamine release by activated mast cells, and recruitment and activation of eosinophils in the airways. In children experiencing both acute and chronic stress, there was a 9.5-fold reduction in mRNA levels of the β2-adrenergic receptor [535] making the B2 agonist drugs less effective.

Another interesting effect of stress effecting the immune system comes from the observation that during pregnancy, Th-1 related diseases such as RA and MS remit but exacerbate postpartum. It was found that during pregnancy there is an increase in secretion of cortisol, norepinephrine, and 1,25-dihydroxyvitamin D3 and a reduction in monocytic production of Th1 type proinflammatory cytokines IL-12 and TNF-α. During pregnancy, cytokine production skews toward the Th2 type with an increase in IL4 and IL10 in particular [539]

The relationship between the immune system and the central nervous system is a still evolving field of study. Despite being segrated from the rest of the body by the BBB, the CNS still needs to be monitored for infection and/or trauma. Recent advances have observed how peripheral leukocytes such as $CD4^+$ cells gain critical surface molecules which allow them to traverse the BBB, rendering them capable of accessing the CNS to participate in surveillance and clearance of antigens [540]. A break-down in neural-immune interactions can result in unchecked inflammation in the CNS such as the autoimune demyelinating disorder, multiple sclerosis.

Some things to take into consideration for the future. The observed effect of anesthetics such as BZDs on ventilary control has observed that in the use of an animal model; rodents required 10-fold higher dosage to achieve the equivalent respiratory depression observed in humans [541]. Furthermore, human, mouse and rats have a differential expression of $GABA_AR$ in T-lymphocytes. One study found that 5, 8, and 13 different $GABA_A$ subunit isoforms in human, mouse, and rat $CD4^+$and $CD8^+$ T cells, respectively. The BZD sensitive γ2 subunit was only detected in mice which may make mouse studies untranslatable to human.

An i.p. injection has direct access to the vagal terminals in the peritoneum, is taken up by the lymphatic system and transported to the mesenteric lymph nodes, and then to the liver. Because

of this route, compounds are exposed to degradative enzymes and clear the liver, after which, it enters the bloodstream and is transported to the heart. In contrast, a substance injected via i.v. rapidly diffuses to the heart and then to the lungs, before distributing through the rest of the body [542]. The efficient and rapid diffusion of inhaled particles from the lungs into systemic circulation has been well studied [543] and thus makes an inhaled therapeutic an attractive option.

Animal models have been indispensable to understanding and developing treatments for asthma, however these models ignore the complex facets of the disease. Access to patient tissues would greatly increase our understanding of the disease.

In addition to asthma, there are many other immunological opportunites for designing new therapeutics through targeting $GABA_AR$.

$GABA_AR$ has been implicated in having an active role in the pathogenesis of psoriasis. Recent studies have found that there is a marked increase in the expressed GABA ligand and $GABA_AR$ in the involved skin of psoriatic patients [440]. There have also been reports that treatment with GABA analogues such as gabapentin and pregabalin have led to improvement in psoriasis [544]. In addition, oral pregabalin has been shown to ameliorate chronic polycythemia vera-associated pruritus [545] and uremic pruritus [546,547]

In another study, a cell line P815 which are mast cells with mastocytoma were found to contain α1 and α2 subtypes while H9 cells, which are T-cells, were found to contain α1, α4, and β1 subunits. In these two cell lines, GABA appeared to modulate cytotoxicity of immune cells. The presence of GABA and GAD in these cell types may be a clue about autoimmune diseases such as stiff-man syndrome in which patients develop antibodies to GAD [548].

In addition, low doses of GABA were able to dramatically inhibit the development of proinflammatory Th1 responses and disease progression in non-obese prediabetic type 1 diabetes (NOD) mice [322]. This may be a result of an observed arrest in T-cell receptor (TCR)-mediated T-cell cycle progression by GABA [322]. The arrest stalled cells in the G_0/G_1 phases but did not lead to cell apoptosis. This study also found that naïve T cells and activated T cells from the mice expressed α1, α2, β1, β2, and δ. Activation also resulted in the strong expression of γ3, the induction of which may suggest a specialized function in activated T cells.

GABA also appears to play a role in autoimmune disorders. Multiple Sclerosis (MS) patients have a decrease in GABA serum levels during relapse [549] and in the mouse model of rheumatoid arthritis and obesity, orally administered GABA down-regulates the inflammatory response [362,550]. It could be that the decrease in GABA serum is due to low production in the

Table 16. Summary of the studies performed on GABA in the immune system. References (Ref.) are from 1-14 are found in the text.

Species	Cell type	$GABA_AR$ subunit mRNA	$GABA_AR$ subunit protein	Components	Ref.
Rat	$CD4^+$, $CD8^+$ T cell	α1, α2, α3, α4, α6, β3, γ1, δ, ρ1, ρ2			313
Mouse	$CD4^+$ T cell from NOD mice	α1, α2, β1, β2, γ3, δ			311
	Peritoneal macrophage	α1, α2, β3, δ	α1		353
	Splenocytes from $GAT1^{+/+}$ and $^{-/-}$ mice	α1, α2, α5, β1, β2, δ, γ1, γ3		GAT1	2
	Macrophage, DC, and $CD4^+$ T cells	β1, ε		GABA, GAD65, GABA-T, GAT-2	306
Human	Monocyte	α1			314
	Monocyte	β2			436
	Neutrophil			GAD65/67	308
	$CD4^+$ T cell	α1, α3, β2	α1		314
	$CD8^+$ T cell	β2	α1		314
	Irradiated B cell	α1, α3, β2	α1		314
	PBMC	α1, α3, α4, β2, β3, γ2, δ, ε	α1		314
	PBMC macrophage			GABA	310
	PBMC T lymphocyte	α1, α3, α6, β3, γ2, δ, ρ2		GABA, GAD67, VIAAT, GABA-T, GAT1, GAT2	307
Cell lines	$CD4^+$ H9 T cell	α1, α4, β1			533
	CD^+ T Jurkat J6 cell	α1, α3, α4, α6, β1, β2, β3, γ2, ε, θ	α1		314
	HL60 cell		α1		314
	Monocytic THP-1 cell	α4, β2, γ1, δ			536
	Mouse EAE $CD4^+$ T cells	α1, α4, β2, β3, γ1, δ			312
	Mouse RAW 264.7 macrophage			GABA	310

immune system and thus low inhibition of T cell activity, leading to higher levels of cytokine

production and thus inflammation.

The role of GABA, and neurotransmitters in general, in the immune system and immune-related diseases is still under study. A summary of studies performed on GABA in the immune system is shown in Table 16 [2,317-319,321-325,364,447,548,551]. Future work is necessary as the cross-talk between the immune and nervous system present the opportunity for the creation of new strategies for treating immunological diseases by targeting neurotransmitter receptors.

APPENDIX A

Sequences

pCI_Lab gabra1 (α1)

TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
TATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCAT
GTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAA
TTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGG
TAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGT
ATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGC
CCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGA
CCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCAT
GGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGG
GATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATC
AACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGT
AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGAT
CACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTGC
TTCTGACACAACAGTCTCGAACTTAAGCTGCAGAAGTTGGTCGTGAGGCACTGGGCA
GGTAAGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGT
CGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCC
ACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGTTCAATTACAGCTCTTAAGGCTA
GAGTACTTAATACGACTCACTATAGGCTAGCCTCGAGAATTCGGCACGAGGGGGCC
CCGAGCTGGACAAGCCCGTGATGAAGAAAAGTCGGGGTCTCTCTGACTATCTTTGGG
CCTGGACCCTCATTCTGAGCACTCTCTCGGGAAGAAGCTATGGACAGCCCTCCCAAG
ATGAACTTAAGGACAACACCACTGTCTTCACGAGGATTTTGGACCGACTGCTGGATG
GTTATGACAATCGTCTGAGACCAGGCTTGGGAGAGCGTGTAACTGAAGTGAAGACG
GACATCTTTGTCACCAGTTTCGGACCCGTGTCAGACCACGATATGGAATATACAATA
GATGTGTTTTTCCGCCAAAGCTGGAAGGATGAAAGATTAAAATTCAAAGGACCCAT
GACAGTGCTCCGGCTGAACAACCTGATGGCCAGTAAAATCTGGACTCCAGATACATT
TTTCCACAATGGAAAAAAGTCTGTGGCCCACAACATGACCATGCCCAATAAACTCCT
GCGTATCACAGAGGATGGCACACTGCTGTACACCATGAGGTTGACTGTGAGAGCCG
AATGCCCCATGCACTTAGAAGACTTTCCCATGGATGCCCATGCCTGCCCACTAAAAT
TTGGGAGCTATGCTTATACAAGAGCAGAAGTTGTCTATGAGTGGACAAGGGAGCCA
GCCCGCTCAGTGGTTGTGGCAGAAGATGGGTCACGTTTAAACCAGTATGACCTTCTT
GGGCAAACAGTTGACTCTGGAATTGTTCAGTCCAGTACTGGAGAATATGTGGTTATG
ACGACTCACTTTCACTTGAAGAGAAAAATCGGCTACTTTGTTATTCAAACATATCTG
CCATGCATAATGACAGTCATTCTCTCCCAAGTCTCCTTCTGGCTTAACAGAGAGTCA
GTACCAGCAAGAACTGTCTTTGGAGTGACGACCGTTCTGACCATGACAACCTTGAGT
ATCAGTGCCAGAAATTCCCTCCCAAAGGTGGCTTATGCAACGGCCATGGACTGGTTT
ATTGCAGTGTGCTATGCCTTCGTGTTCTCGGCTCTGATTGAGTTTGCCACAGTAAACT
ATTTCACCAAGAGAGGGTATGCGTGGGATGGCAAAAGCGTGGTTCCAGAAAAGCCA
AAGAAAGTGAAGGATCCTCTCATTAAGAAAAACAACACATATGCTCCTACAGCAAC
CAGCTATACCCCTAACTTAGCCAGGGGTGACCCCGGCTTGGCCACTATTGCTAAAAG

TGCGACCATAGAACCGAAAGAAGTCAAGCCTGAGACAAAACCGCCAGAACCCAAG
AAAACCTTTAACAGCGTCAGCAAAATCGACCGACTGTCAAGAATAGCCTTTCCGCTG
CTATTTGGAATCTTTAACTTAGTCTATTGGGCCACGTATTTAAACAGAGAGCCTCAG
CTAAAAGCCCCCACACCCCATCAATAGGTTCTTTTAGTCGTATTCTGTTGTTCAGTCC
TCTGCACTGAGAATCGCTTTCTGTTCTCAACGCAGTGATTCCTGTCTGCCTTACTGCC
TCTGTCTTAAAAGAATTCACGCGTGGTACCTCTAGAGTCGACCCGGGCGGCCGCTTC
GAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAG
TGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTA
TAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTC
AGGGGGAGATGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTAAA
ATCGATAAGGATCCGGGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTC
CCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAG
CGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAG
CGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGT
CAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTC
GACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAG
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AAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTT
GCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAA
TTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATC
TGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCG
CATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCT
TGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATG
TGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGAT
ACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGC
ACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAA
ATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAA
GGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATT
TTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGA
TCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCT
TGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCT
ATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT
ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTAC
GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACA
CTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTT
TGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAAT
GAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAAC
GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAAT
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CTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCA
TTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGG
GGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCA
CTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATT
TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCAT
GACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAA

GATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACA
AAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTT
TTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTG
TAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCT
CTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGG
TTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGG
TTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTAC
AGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTAT
CCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAA
ACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATT
TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCT
TTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGGCTCGACAGATCT

pCI_Lab gabra2 (α2)

TCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
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GTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAA
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TAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGA
CGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGT
ATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGC
CCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGA
CCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCAT
GGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGG
GATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATC
AACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGT
AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGAT
CACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTGC
TTCTGACACAACAGTCTCGAACTTAAGCTGCAGAAGTTGGTCGTGAGGCACTGGGCA
GGTAAGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGT
CGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCC
ACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGTTCAATTACAGCTCTTAAGGCTA
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CURRICULUM VITAE

NINA Y. YUAN
Department of Chemistry and Biochemistry
University of Wisconsin-Milwaukee
Milwaukee Institute for Drug Discovery

EDUCATION

University of Wisconsin-Milwaukee
PhD in Chemistry-Biochemistry Division (August 2016)
BS in Biochemistry (2011)

EXPERIENCE

UWM CHEMISTRY DEPARTMENT *(A Tier 1 Research University in the Carnegie Classification of Institutions of Higher Education)*—Milwaukee, WI

Graduate Research Assistant, 2012 to Present

Research Project Highlights:

- Development of 6 Stable Recombinant Cell Lines Containing $GABA_A$ Receptor Subtypes with long (>10 passages) term stability to determine subtype selectivity of drugs which can be utilized in large scale drug screening assays.

- Utilization of neuronal iPSCs for drug screening on automated patch-clamp to provide the overall response from a native population of mixed $GABA_AR$.

- Development and optimization of high-throughput fluorescence-based assay for $GABA_A$ receptor in multiwell plate format using an eYFP on the Tecan Infinite M1000 which can be used in parallel with the stable recombinant cell lines.

- The study of $GABA_AR$ found natively in white blood cells and characterization of their electrophysiological and kinetic properties contributed to $2 Million collaborative grant from the NIH to develop new asthma therapy with the Milwaukee Institute for Drug Discovery (MIDD).

Graduate Teaching Assistant, 2011 to 2014

- Instructed twenty-four different discussions and laboratory classes ranging in rudimentary to advanced chemistry course.

- Excellent reviews given through anonymous student surveys.

UWM-RESEARCH FOUNDATION *(non-profit corporation to support research and innovation at UWM by providing funding for scholarships and grants and fostering corporate partnership)* –Milwaukee, WI

Graduate Student Ambassador, 2014 to Present

- Assessed the commercial potential and patentable technologies of research performed by graduate students across the UWM campus through personal interviews.

- Organized events for graduate students to tour industry partners and present their research to the broader graduate student population.

AWARDS, HONORS, ELECTED POSITIONS

- *Sosnovsky Award for Excellence in Graduate Research* **2016** University of Wisconsin-Milwaukee, WI
- *Chemistry Alumni Award* **2016** University of Wisconsin-Milwaukee, WI
- *GLCACS Student Research Presentation Award* **2015** GLCACS 19th Annual Conference: Northwestern University, IL
- *Graduate Student Council (2014-2015)* Representative of UWM graduate students: Milwaukee, WI
- *Keith Hall Award Excellence in Graduate Research* **2015** University of Wisconsin-Milwaukee, WI
- *Gordon Research Travel Award* **2014** Ion Channels Conference: Mt. Holyoke, MA
- *Mentorship Travel Award* **2014** University of Wisconsin-Milwaukee, WI
- *New Graduate Student Mentor* **2014** Mentor in UWM Chemistry Program: Milwaukee, WI
- *Keith Hall Award Excellence in Graduate Research* **2013** University of Wisconsin-Milwaukee, WI
- *Chancellor's Graduate Fellowship* **2011-2014** University of Wisconsin-Milwaukee, WI
- *Society of Applied Spectroscopy Travel Award* **2012** ACS 243rd National Meeting: San Diego, CA
- *UWM Graduate Student Travel Award* **2012** ACS 243rd National Meeting: San Diego, CA
- *SURF Poster Award* **2011** University of Wisconsin-Milwaukee, WI
- *SURF Research Funding* **2010-2011** University of Wisconsin-Milwaukee, WI

SKILLS AND TECHNIQUES

Specialization

- Ion channels electrophysiology using cutting edge technology in automated patch-clamp
- High-throughput assay development

Biochemical

- Mammalian cell cultures
  - HEK293T
  - iPSCs
  - Jurkat E6-1
  - HL-60
  - THP-1

- HepG2
- Murine Splenocytes

- Cloning
  - Transfections
  - Transformation
  - DNA-RNA extraction, purification, analysis
  - Stable cell line generation
- Animal handling certification for mice
  - Rotorod
  - Drug dosing
  - Human tumor xenografts
- Protein expression
  - Purification
  - Analysis

Analytical

- IonFlux16
- Assay development and troubleshooting
- High throughput screening
- Tecan M1000
- Microsoft Excel
- GraphPad Prism
- Fluorescence microscopy
- Fluorescence polarization assay
- Fluorescence based toxicity assays
- qRT-PCR Thermocycler
- SDS-PAGE gel
- Agarose gel
- NMR

PUBLICATIONS

1. *Characterization of GABA(A) Receptor Ligands with Automated Patch-Clamp Using Human Neurons Derived From Pluripotent Stem Cells.* **Yuan, N. Y**.; Poe, M. M.; Witzigmann, C.; Cook, J. M.; Stafford, D. C.; Arnold, L. A., *Journal of Pharmacological and Toxicological Methods* **2016 Pending**

2. *Development of GABAA Receptor Subtype-Selective Imidazobenzodiazepines as Novel Asthma treatments.* Forkuo, G.; Guthrie, M. L.; **Yuan, N. Y**.; Nieman, A. M.; Kodali, R.; Jahan, R.; Yocum, T. G.; Stephen, M.; Poe, M. M.; Li, G.; Yu, O.; Hartzler, B.; Zahn, N.; Emala, C. W.; Stafford, D. C.; Cook, J. M.; Arnold, L. A. *Molecular Pharmaceutics* **2016 Pending**

3. *A New Pharmacological Approach for Asthma through Tissue-Specific Modulation of GABA(A) Receptor.* Arnold, L. A.; Forkuo, G. S.; Nieman, A. M; Yu, O.B.; Guthrie, M. L.; **Yuan, N. Y**.; Kodali, R.; Jahan, R.; Emala, C. W.; Cook, J. M.; Stafford, D. C.; Grayson, M. H., *J Allergy Clin Immunol* **2016**. 137(2)

4. *Antitumor Activity of 3-Indolylmethanamines 31B and PS121912*. Guthrie, M. L.; Sidhu, P.S.; Hill, E.K.; Horan, T.C.; Nandhikonda, P.; Teske, K. A.; **Yuan, N. Y**.; Sidorko, M.; Kodali, R.; Cook, J. M.; Han, L.; Silvaggi, N. R.; Bikle, D. D.; Moore, R. G.; Singh, R. K.; Arnold, L. A., *Anticancer Res*, **2015**. 35(11): p. 6001-7

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6. *Identification of VDR Antagonists among Nuclear Receptor Ligands Using Virtual Screening.* Teske, K.; Nanhikonda, P.; Bogart, J. W.; Feleke, B.; Sidhu, P.; **Yuan, N. Y**.; Preston, J.; Goy, R.; Han, L.; Silvaggi, N. R.; Singh, R. K.; Bikle, D. D.; Cook, J. M.; Arnold, L. A., *Nuclear Receptor Research* **2014,** *1*, 1-8.

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12. *Transiently Transfected Cell Lines for GABA Receptor Screening*. **Nina Yuan**. Application Note Fluxion Biosciences, **2011**.

POSTER AND ORAL PRESENTATIONS

1. *Electrophysiological Assay of $GABA_A$ Receptor: Roles in Neuropharmacology & Immunology*, **Nina Y. Yuan**, Dr. James M. Cook, Dr. Alexander Arnold. UWM Chemistry and Biochemistry Research Symposium, Poster presentation, May **2016**.

2. *Electrophysiological Assay of GABAA Receptor: Roles in Neuropharmacology & Immunology*, **Nina Y. Yuan.** Northwestern Feinberg School of Medicine, Oral presentation, April **2016**.

3. *Electrophysiological Assay of GABAA Receptor: Roles in Neuropharmacology & Immunology*, **Nina Y. Yuan**. Great Lakes Chapter Chinese American Chemical Society, Oral presentation, May **2015**. Winner of GLCACS Student Research Presentation Award.

4. *Electrophysiological Assay of $GABA_A$ Receptor: Roles in Neuropharmacology & Immunology*, **Nina Y. Yuan**, Michael Poe, Chris Witzigmann, Dr. James M. Cook, Dr. Alexander Arnold. UWM Chemistry and Biochemistry Research Symposium, Poster presentation, April **2015**. Winner of 1st place presentation Keith Hall Award for Excellence in Graduate Research.

5. *Development of High-Throughput Assays to Identify New Alpha Subtype-Selective $GABA_A$ Receptor Modulators to Treat Anxiety and Depression Disorders*, Gordon Research Conference: Ion Channels (GRS), Oral Presentation, July **2014.**

6. *Development of High-Throughput Assays to Identify New Alpha Subtype-Selective $GABA_A$ Receptor Modulators to Treat Anxiety and Depression Disorders*, **Nina Y. Yuan**, Michael M. Poe, Chris Witzigmann, Dr. James M. Cook, Dr. Alexander Arnold. Gordon Research Conference: Ion Channels (GRC), Poster Presentation, July **2014.**

7. *Development of High-Throughput Assays to Identify New Alpha Subtype-Selective $GABA_A$ Receptor Modulators to Treat Anxiety and Depression Disorders*, **Nina Y. Yuan**, Michael M. Poe, Chris Witzigmann, Dr. James M. Cook, Dr. Alexander Arnold. Gordon Research Conference: Ion Channels (GRS), Poster Presentation, July **2014**

8. *Automated Patch Clamp: High-Throughput Electrophysiological Assay for Neuropharmacology Research in Mammalian Cells*, **Nina Y. Yuan.** UWM Neuroscience Workshop, Oral Presentation, July **2014.**

9. *Development of High-Throughput Assays to Identify New Alpha Subtype-Selective $GABA_A$ Receptor Modulators to Treat Anxiety and Depression Disorders*, **Nina Y. Yuan**, Michael M. Poe, Dr. James M. Cook, Dr. Alexander Arnold. UWM Chemistry and Biochemistry Research Symposium, Poster presentation, April **2014.**

10. *Identifying New Treatments for the Brain: Electrophysiological Assay and Generation of a Stable Recombinant Cell Line*, **Nina Y. Yuan**, Michael M. Poe, Dr. James M. Cook, Dr. Alexander Arnold. Yao Yuan Biotech/Pharma International Symposium, Poster presentation, April **2013**.

11. *Identifying New Treatments for the Brain: Anxiolytic Neurochemistry and Electrophysiology*, **Nina Y. Yuan**, Michael M. Poe, Dr. James M. Cook, Dr. Alexander Arnold. UWM Chemistry and Biochemistry Research Symposium, Poster presentation, April **2013**. Winner of 1st place presentation Keith Hall Award for Excellence in Graduate Research.

12. *Uppers, Downers, All Arounders: Identifying New Treatments for the Human Brain*, UWM Chemistry and Biochemistry Research Symposium and Recruiting, Oral presentation, April **2013**.

13. *Benzodiazepine Specificity of $GABA_A$ Receptor Subtypes: High-Throughput Electrophysiological Assay with Transiently Transfected Cells,* **Nina Y. Yuan**, Michael M. Poe, Dr. James M. Cook, Dr.

Alexander Arnold. ACS 243rd National Meeting, San Diego, CA, Poster presentation, March 24-30, **2012**.

14. *Benzodiazpine Specificity of $GABA_A$ Receptor Subtypes*, **Nina Y. Yuan**, Dr. James M. Cook, Dr. Alexander Arnold. UWM Chemistry and Biochemistry Research Symposium, Poster presentation, Spring **2011**.

RESEARCH PROJECTS

The Study of $GABA_AR$ Found Natively in White Blood Cells and their Pharmacological Properties. Interpretation of Significance in Disease Models, Prevention, and Treatment

- Study of Jurkat E6-1 cells have revealed the presence of the $GABA_AR$ in T-lymphocytes. These cells are responsive to GABA and their analog muscimol. In addition, the kinetic profile of these receptors differ from those found in our recombinant cell lines. These receptors are characterized by slow saturation kinetics. Splenocytes analyzed via patch-clamp also exhibit exceptionally high responses to the application of $GABA_AR$ ligands and immunocytochemistry has shown significant quantities of the receptor expressed on T and B lymphocytes and macrophages.

Development of 6 Stable Recombinant Cell Lines Containing $GABA_A$ Receptor Subtypes

- The generated cell lines show high expression of the proteins and expected electrophysiological response. Immunocytochemistry shows uniform expression of the receptors in the generated cell lines. Stability of expression over 15 passages has been confirmed by qRT-PCR.

Construction of 6 Single Plasmid Systems for $GABA_A$ Receptor subtypes

- Design of plasmids containing all three necessary components for a functional $GABA_AR$ (α, β, γ) with intention for genetically engineering stable mammalian cell lines using PhiC31 integrase gene and Cytomegalovirus immediate-early promoter to achieve high levels of expression in a HEK293T cell line.

iCell Human Neuron Evaluation and $GABA_A$ Receptor Quantification via RT-PCR and Automated Patch Clamp

- The use of human neurons derived from pluripotent stem cells, coupled with automated patch clamp, were assessed to determine relative efficiency and quality of data yield. In order to find this, cells were evaluated proteomically and genomically through their electrophysiological profile and their mRNA levels. Cells performed excellently on automated patch clamp. We concluded that screening compounds on recombinant cell lines to determine subtype selectivity in parallel with human-induced pluripotent stem cell neurons can provide a valuable and unique

perspective on the effects of $GABA_AR$ ligands. It was determined that coupling these two techniques can provide an excellent representation of the complexity arising from multiple subtypes expressed on a single cell.

High-Throughput Fluorescence-Based Assay for $GABA_A$ Receptor

- Using an Enhanced Yellow Florescent Protein which quenches in the presence of chloride ions, cells were transfected and the assay was optimized for use in a 96 and 384-well plate with our stable recombinant cell lines. Measurements were optimized to use a Tecan M1000.

Methylglyoxal/Glyoxalase 1 Anxiety Project

- Determination whether methylglyoxal acts as an agonist towards the $GABA_A$ Receptor. Transiently transfected cells containing the $\alpha1\beta3\gamma2$ were assayed and resulted in a strong hyperpolarizing effect with a dose response curve. This proves that a Glo1 inhibitor is a potential candidate for modulation to treat anxiety.

Pharmaceutical Company Proprietary Compound Testing-Collaboration

- A short collaboration with a pharmaceutical company, testing their subtype selective compound to determine if the deuterated form changes its pharmaceutical properties.

HZ-166 Testing Cook Compound

- The project involved determination of instrument ability to measure specific subunit selectivity of alpha subtype-selective drugs. This was accomplished by testing receptor subtypes containing $\alpha1$-6, $\beta3$, and $\gamma2$ with a novel benzodiazepine-based compound provided by Dr. James M. Cook (UWM) dubbed HZ-166. Measurements taken reveal similar results as those previously published using manual patch-clamp methods.

Automated Patch Clamp IonFlux Assay Development and Troubleshooting

- The objective of this study was the establishment of an automated (IonFlux, Fluxion) patch-clamp assay capable of measuring the electrophysiological response of benzodiazepine-bassed compounds to the $GABA_A$ receptor in transiently transfected mammalian (HEK293T) cells.

Determination of binding of inhibitors to Vitamin D Receptor-Coregulator

- VDR, involved in cell proliferation and differentiation, is a pharmaceutical target for treatment of a variety of disorders with a concentration on cancer. Disruption of the transcription process with co-regulator inhibitors are a potential method to combat cancer progression and development. This project involved performing in vivo study performed on mice and in vitro determination of binding behavior of small molecule to protein using dialysis and subsequent fluorescence polarization to identify whether molecules were reversible or irreversible inhibitors. This task also included the expression and purification of the VDR protein with high purity and functionality.

MENTORSHIP

Amanda Nieman (Fall 2015-Current) Graduate Student

Benjamin Hartzler (Spring 2016-Current) Undergraduate Student

Joshua Preston (Fall 2013-Fall 2015) Undergraduate Student

Nicholas Dimitri Nassif (Fall 2012-Spring 2013) Undergraduate Student

Robin Goy (Summer 2012-Spring 2013) Undergraduate Student

Chinedum Kevin (Fall 2011-Summer 2012) Undergraduate Student

MEMBERSHIPS AND ELECTED POSITIONS

• *American Chemical Society (2011-Current)*	Graduate Student Membership
• *Graduate Student Council (2014-2015)*	Representative of graduate student population in UWM Chemistry Department
• *New Graduate Student Mentor (2014)*	Mentor in UWM Chemistry