Appendix 1

Guidelines for future $GABA_AR$ Researchers

Intent: This Appendix is intended to be of aid to members of the Dougherty lab that wish to work on the $GABA_AR$ in the future. The observations collected here are based on my experiences and discussion with others working on the $GABA_AR$ from different labs. The conjectures are not necessarily rigorously tested and therefore are not included elsewhere in this thesis. However, there is no need for someone else to hit the same bumps in the road. All data herein are limited to those collected using the α_1, β_{2S}, and γ_{2L} human $GABA_AR$ genes subcloned into the pGEMHE vector and obtained from Sarah Lummis (Department of Biochemistry, University of Cambridge, United Kingdom).

A1.1 $\alpha_1\beta_2$ versus $\alpha_1\beta_2\gamma_2$ $GABA_AR$

The $\alpha_1\beta_2\gamma_2$ is the most abundant $GABA_AR$ in the mammalian brain. As such, it is the subject to much research. It is well-established, however, that when trying to express these receptors in a heterologous expression, such as *Xenopus laevis* oocytes, that you can also express $\alpha_1\beta_2$ $GABA_AR$. I found this finding to be true. Literature reports indicate the $\alpha_1\beta_2\gamma_2$ and the $\alpha_1\beta_2$ $GABA_AR$ differ in several ways including single-channel conductance, desensitization kinetics, deactivation kinetics, voltage dependence, and Zn^{2+} inhibition.[1]

I found that for wild type and conventional mutations, the overall mRNA mix for $\alpha_1\beta_2$ $GABA_AR$ needed to be 10 times more concentrated than for $\alpha_1\beta_2\gamma_2$ to achieve similarly sized macroscopic currents. This difference is likely due in part to the lower single-channel conductance of the $\alpha_1\beta_2$ $GABA_AR$ and partially due to lower surface

expression. Like $\alpha_1\beta_2\gamma_2$ $GABA_AR$, the $\alpha_1\beta_2$ $GABA_AR$ has two α and two β subunits, but the identity of the 5th subunit is still unknown. Concatamer studies from different groups have offered compelling electrophyiological evidence that the 5th subunit is both α_2 and β.[3] Given the ambiguity in these results, it was necessary to reassure myself that varying the mRNA ratios did not alter the EC_{50} or Hill coefficient of the $\alpha_1\beta_{2S}$ $GABA_AR$ (Figure A1.1, Table A1.1). Unlike the αβ $GABA_AR$, the αβγ $GABA_AR$ is highly sensitive to the mRNA ratios used, as discussed in the following sections.

Table A1.1 EC_{50} values for αβ only receptors

ratio	EC_{50} (μM)	n_H	I_{max}
1:1 αβ	1.5 ± 0.1	1.17 ± 0.08	-21 ± 4
1:6 αβ	1.6 ± 0.1	1.33 ± 0.08	-9 ± 2
6:1 αβ	2.1 ± 0.1	1.24 ± 0.08	-16 ± 4

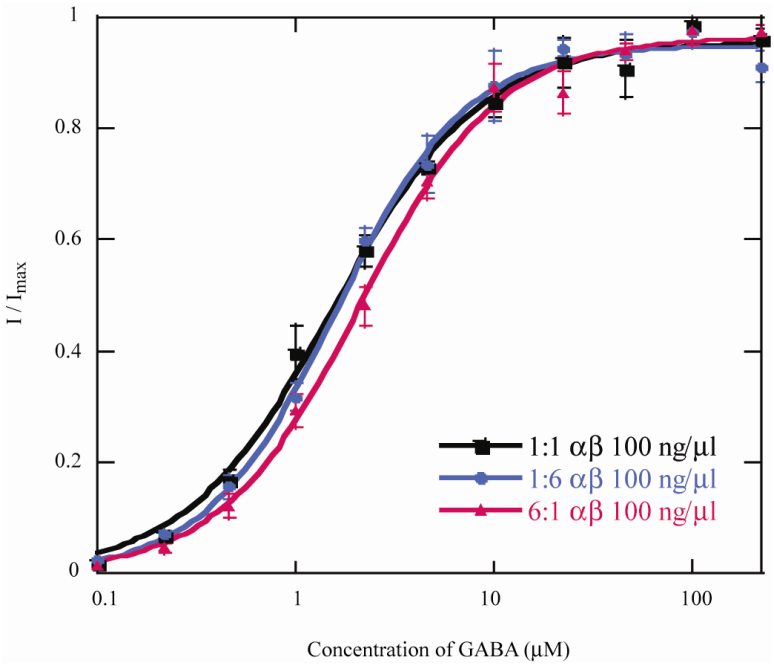

Figure A1.1 The GABA dose response relationship for wild type αβ $GABA_AR$ does not vary when the mRNA ratios are varied.

The αβ $GABA_AR$ is not physiologically relevant, but is often studied in place of the αβγ $GABA_AR$ because it is difficult to ensure the γ subunit is completely incorporated. Zn^{2+} block of the receptors has been reported as a means to determine the purity of the receptor population. The αβ $GABA_AR$ is reported to be sensitive to blockade by Zn^{2+} while the αβγ $GABA_AR$ is not. In principle then, application of Zn^{2+} with no corresponding decrease in macroscopic current would indicate a pure population of αβγ receptors. In my experience, pure populations of αβ $GABA_ARs$ could be blocked up to 70-90% with 10 mM Zn^{2+} however pure populations of αβγ $GABA_ARs$ showed variable blockade by the cation, sometimes up to 50%. Additionally, mixed populations of receptors behaved more similarly to the αβγ $GABA_AR$ than to the expectations based on a mixed population. As such, I concluded that the Zn^{2+} block test was not sufficient to determine a pure population of receptors. At more recent conferences, I have commented on this observation to members of the Czjakowski lab (Department of Physiology, University of Wisconsin, Madison) and they have seen similar behavior, substantiating the claim that this test is not useful experimentally. Indeed, recent papers on the $GABA_AR$ no longer mention Zn^{2+} block to differentiate between receptor subtypes.

A1.2 αβγ $GABA_AR$: mRNA Ratio

A1.2.1 Linearization Sites

When the gene of interest is contained within the pGEMHE vector, Nhe1 is most often used to linearize the DNA prior to *in vivo* transcription. Nhe1 was used to linearize both the α_1 and γ_{2L} genes. The Nhe1 cut site for these two genes was located after both the terminus of the gene and the poly-A tail within the vector. The β_{2S} gene, however,

contains a Nhe1 site within the gene, therefore an alternate restriction enzyme must be chosen. Initially, I chose the enzyme Spe1 which has a cut site after the gene terminus but before the poly-A tail. Capped mRNA made from the linearized DNA yielded functional $GABA_AR$ with the anticipated pharmacology in *Xenopus laevis* oocytes. In the more recent months, however, the entire lab has experienced lower expression levels and considerable effort on my part was put into increase expression of the $GABA_AR$ so I could finish some suppression experiments. In my quest, I found (through conversations with Kiowa Bower and Fraser Moss) that the poly-A tail of the mRNA helps stabilize the mRNA *in vivo*. As such, I tried Sph1, another restriction enzyme, when linearizing the β_{2S} DNA. The Sph1 restriction site is located after both the gene terminus and the poly-A tail. I found that for both the Spe1 derived and Sph1 derived capped mRNA, the ratios of mRNA for αβ receptors was 1:1 for wild type, though macroscopic currents were larger for oocytes injected with the Sph1-derived mRNA.

A1.2.2 βγ $GABA_AR$

A common problem when working with the αβγ $GABA_AR$ is that a mixed population of αβ and αβγ $GABA_AR$ can result. This problem is typically dealt with by using an excess of γ mRNA. When I initially attempted this using the DNA constructs originally provided by Neurion Pharmaceuticals (Pasadena, CA), I found that the more γ mRNA I used, the higher the EC_{50} and the lower the Hill coefficient. This eventually led me to try all combinations of the $GABA_A$ subunits. I found that in addition to expression from α/β mRNA mix, I also saw GABA-induced currents from β/γ mRNA mixes. I had hoped that the pGEMHE DNA constructs from Sarah Lummis would not have this same problem, but I did explicitly test for the problem and found that there was significant

GABA-induced current. More interestingly, I found that lower concentrations of 2:1 βγ mRNA resulted in higher levels of expression (Table A1.2, Figure A1.2). To me, these results suggest something endogenous to the oocyte is able to assemble with these subunits. These studies used β_{2S} linearized with Spe1, however, I did test for 2:1 βγ currents using β_{2S} linearized with Sph1 and found significant GABA-induced currents. In all cases, the βγ $GABA_A$Rs had hill coefficients less than 1, providing a defining characteristic.

Table A1.2 EC_{50} values for oocytes injected with βγ mRNA

ratio	EC_{50} (μM)	n_H	I_{max}	N
2:1 βγ (dil)	380 ± 50	0.69 ± 0.04	-1.2 ± 0.2	4
2:1 βγ (conc)	140 ± 10	0.95 ± 0.05	-0.55 ± 0.05	5

I_{max} is reported in μA. Dilute (dil) mRNA mix had a total mRNA concentration of 0.25 μg/μl while the concentrated (conc) mRNA mix had a total mRNA concentration of 1 μg/μl. The mRNA mix was mixed 1:1 (by volume) with water immediately prior to oocyte injection.

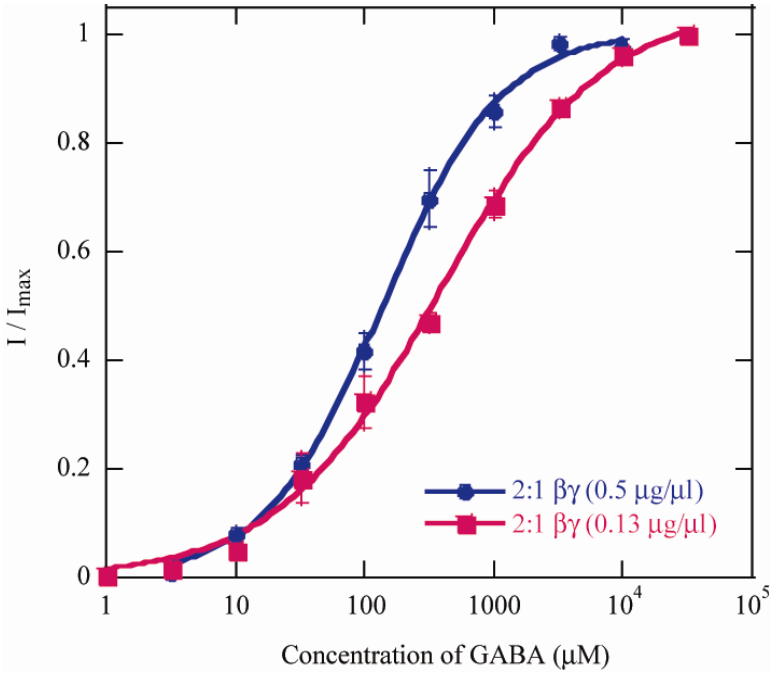

Figure A1.2 Dose response relationship for βγ $GABA_A$R

A1.2.3 αβγ mRNA Ratio

To determine the correct ratio of mRNA needed to produce a pure population of αβγ $GABA_A$Rs, I varied the relative amounts and then determined the GABA EC_{50} and Hill coefficient as well as the flurazepam potentiation, for each ratio. Literature values indicated the αβ $GABA_A$R has a significantly lower EC_{50} than the αβγ $GABA_A$R but similar Hill coefficients. Additionally the αβ $GABA_A$R is insensitive to benzodiazepine potentiation. The complicating factor is that overexpressing the γ subunit to a greater extent could lead to a mixed population of αβγ and βγ receptors. However, the βγ receptors were also determined to be relatively insensitive ($P < 0.7$) to benzodiazepines. Therefore, I reasoned that the ratio giving an EC_{50} greater than that of αβ $GABA_A$Rs (3 μM) and less than that of βγ $GABA_A$Rs (120 μM), with a hill coefficient of at least 1.2 (indicating a pure population), and maximal flurazepam (FLZM) potentiation would indicate the purest population of αβγ $GABA_A$R. The results for both β_{2S} linearized with Spe1 and with Sph1 are summarized in Table A1.3 and Figure A1.3.

Table A1.3 Results of various αβγ mRNA ratios on GABA EC_{50} and FLZM potentiation

αβγ	β_{2S} – Spe1				β_{2S} – Sph1			
Ratio	EC_{50}	n_H	I_{max}	P	EC_{50}	n_H	I_{max}	P
2:2:1	44 ± 1	1.3	-3.2 ± 0.8	2.6 ± 0.3	11.2 ± 0.5	1.4	10 ± 1	1.5 ± 0.3
1:1:1	47 ± 3	1.7	-9 ± 2	2.3 ± 0.1				
1:1:3	110 ± 10	0.96	-3 ± 1	2.0 ± 0.2	19.2 ± 0.5	1.4	8 ± 1	2.0 ± 0.3
1:1:5	110 ± 10	1.1	-3.4 ± 0.9	2.5 ± 0.5	39.3 ± 0.8	1.4	8.6 ± 0.9	1.7 ± 0.3
1:1:8	70 ±10	1.1	-1.5 ± 0.7		53 ± 2	1.1	5.5 ± 0.6	1.4 ± 0.1
1:1:10					49 ± 1	1.2	4.6 ± 0.7	1.6 ± 0.2

EC_{50} is reported in μM, I_{max} in μA. FLZM potentiation experiments were carried out at $EC_{5\text{-}10}$ of GABA (Spe1) and $EC_{15\text{-}10}$ (Sph1) using 1 μM FLZM.

For the β_{2S} linearized with Spe1, a 2:2:1 mRNA ratio was selected. Although the 1:1:1 ratio gave a higher Hill coefficient and I_{max} value, the error bars were also greater,

indicating more variability in the data. The 2:2:1 mRNA ratio gave the greatest potentiation and GABA EC_{50} and Hill coefficients consistent with literature values. FLZM potentiation for the β_{2S} linearized with Sph1 was lower in these experiments than previously seen with Spe1. I attribute this to the higher relative concentration of GABA used. The 1:1:3 mRNA ratio had the highest potentiation value but was not chosen as the standard because of the larger error bars for the GABA dose response relationship. The 1:1:5 ratio had a high hill coefficient and higher potentiation than all but the 1:1:3 ratio. Additionally the values obtained for the 1:1:5 ratio are consistent with the literature. Therefore 2:2:1 was used for wild type and conventional mutants for β_{2S} mRNA made from DNA linearized with Spe1 and 1:1:5 was used for wild type and conventional mutants for β_{2S} mRNA made from DNA linearized with Sph1.

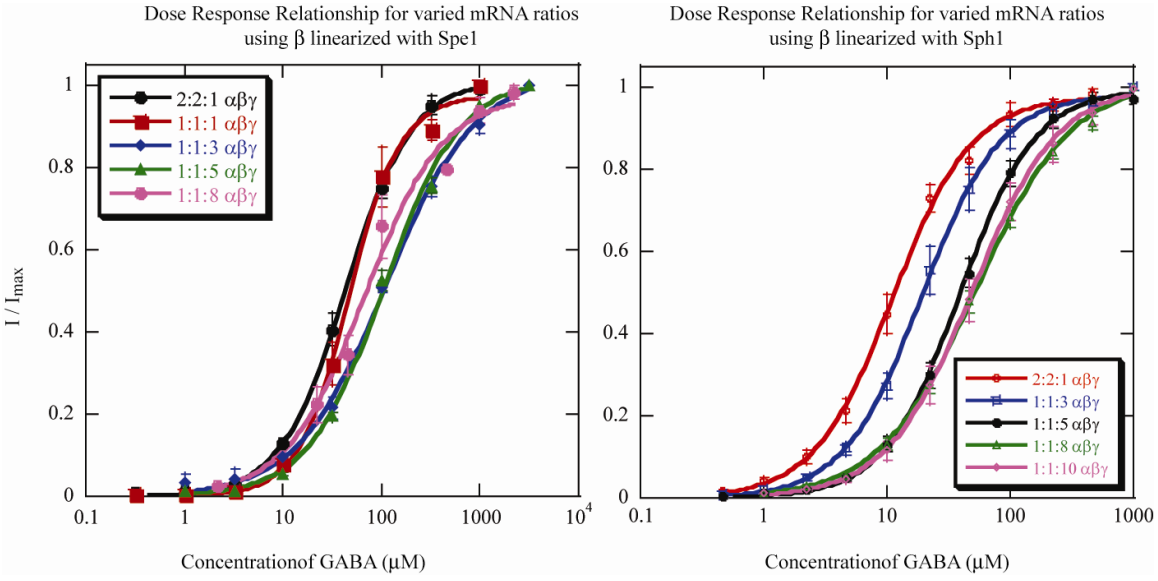

Figure A1.3 GABA dose response relationships for various mRNA ratios of the αβγ $GABA_AR$. *Left*: mRNA made from β_{2S} DNA linearized with Spe1. *Right*: mRNA made from β_{2S} DNA linearized with Sph1

A1.3 Unnatural Amino Acid Incorporation

For suppression experiments, I found the standard 5-fold excess of the subunit containing the TAG mutation to be sufficient for expression. Thus for suppression in the α subunit, I used a 5:1 mRNA mix for the αβ $GABA_AR$ and 10:2:1 (β_{2S}-Spe1) or 5:1:5 (β_{2S}-Sph1) for the αβγ receptor. Since injections of βγ respond to GABA, there are always significant read-through and re-aminoacylation currents when suppressing in the α subunit. These control experiments will yield receptors that are pharmacologically different from wild type and the unnatural amino acid. Alterring the mRNA ratio used for different unnatural amino acids may help with these problems, however I have not investigated this possibility thoroughly.

A1.4 Recommendations

My recommendation to future $GABA_AR$ experimenters is to use only β_{2S} mRNA made from DNA linearized with Sph1. Generally this mRNA led to higher expression and less variability. When mutating in the α and β subunits, it is worthwhile to first figure out the affect of the mutation in the αβ $GABA_AR$. In my experience, the GABA EC_{50} shifts in the same direction for both the αβ $GABA_AR$ and the αβγ $GABA_AR$. Additionally, there are fewer complicating factors with the αβ $GABA_AR$, thus these are useful intitial experiments to give a sense of what to expect in the αβγ $GABA_AR$.

BZD potentiation can be used to determine if the γ subunit is incorporated – but this test is not reliable if the mutation may affect BZD potentiation. I tried using fluorescent antibody labeling and TIRF microscopy to determine whether or not the γ subunit was completely incorporated. However the results were ambiguous. This may

be due to the primary antibodies not binding tightly enough or to nonspecific labeling by the secondary antibody. In either case, I do not recommend this method to detect surface expression.

When doing experiments, I recommend using the 1 ml plastic 96 well plates for everything except the benzodizapines. The BZDs are sticky and can be absorbed into the plastic of the 96 well plates, thereby altering the concentration in the solution. Therefore, it is best to use the glass-coated plates for your drug solutions. It is also necessary to make the BZD solutions in glass bottles or tubes (no flacon tubes). Similarly, the BZDs are hard to wash off the receptors and out of the Opus chambers. Thus, you should do BZDs after you finish the GABA dose response experiments because trace amounts of BZDs left on the Opus will affect your GABA EC_{50} values. After using the BZDs, wash the Opus by running 10% DMSO (in water) through pump B for at least 10 minutes at 1 ml/min, followed by Millipore water for the same amount of time. The glass-coated plates need to be washed with 70% isopropanol and then three times with deionized water at the end of the recording session.

Based on the pentobarbital structure, it seems likely that pentobarbital will be prone to crystallization, therefore the pentobarbital solutions should be kept at room temperature and if possible kept stirring constantly to prevent crystallization. I also found it helpful when trying to compare two things using pentobarbital, it's a good idea to run the experiments in parallel as this will control for any crystallization that has taken place.

GABA solutions should be made immediately prior to recording. Weigh out 30-50 mg of GABA (on the balance in the Chemistry lab) in a 50 ml falcon tube, then

calculate the volume of ND96 necessary to make a 10 mM solution. This is the stock solution. GABA is soluble up to 100 mM, thus higher concentrations are possible. In my experience, mutations where 10 mM GABA was needed have also had quite low Hill coefficients and tend not to truly turn over. Therefore I recommend using higher concentrations of GABA only if there is a high enough (>1.1) Hill coefficient or no response at concentrations < 50 μM.

Despite the complications of working with the $GABA_AR$, I hope the lab will continue to work with them as they have many allosteric modulators including barbiturates, anesthetics, and benzodiazepines. The mechanisms of action of these modulators are not well understood thus there are many experiments to be done. Additionally, there are benefits to working with the $GABA_AR$. For starters, you can record in ND96 with calcium which means the oocytes tend to be healthier. While longer Opus runs mean fewer runs can be performed during a given recording session, it also means other experiments can be performed while the Opus is running, which can be immensely useful.

A1.5 Opus Protocols

Table A1.4 Fluidics profiles for the OpusXpress

Profile	Initial Rate	Initial Duration	Subsequent Rate
1	0.1 ml/min	0 sec	2 ml/min
2	0.1 ml/min	0 sec	2 ml/min
3	4 ml/min	30 sec	3 ml/min

All experiments were carried out at -60 mV. The OpusXpress has at least three profiles for fluidics, defined in Table A1.4. In the subsequent sections, I have given the protocol name and then outlined the Opus protocols and setup for each experiment type.

A1.5.1 GABA EC_{50} Protocol

Table A1.5 GABA EC_{50} Protocol

Time	Application	Source	Fluidics
30 sec	ND96	B	Profile 1
30 sec	GABA	Drug plate	Profile 2
285 sec	ND96	B	Profile 3

Repeat for each dose.

This protocol is named "GABA_30sapp_285swash" and requires only pump B and the drug plates. ND96 with calcium is in pump B and the drug plates contain varying concentrations of GABA. I generally use three concentrations of GABA per order of magnitude (1, 2.2, 4.6, etc.). When determining the dose response relationship, I first apply a "high" concentration of GABA (usually 10 μM for αβ receptors and 100 μM for αβγ receptors), then the concentrations for the dose response relationship from low to high concentration and finally a zero concentration. The high dose at the beginning "wakes up" the receptors according to Sarah Lummis. The data varies less when I adhere to this rule. The zero dose at the end should not give current, thereby ensuring the previous responses were accurate. For wild type αβ $GABA_AR$ I use GABA concentrations from 0.1-100 μM GABA to determine the dose-response relationship. For wild type αβγ $GABA_ARs$ I use 0.46-1000 μM GABA.

A1.5.2 BZD Potentiation Protocol

This protocol is called "GABA_BZD_no prewash" and uses only pump B and the drug plates. For the BZD potentiation experiments, I repeat the EC_{5-10} dose of GABA three times and then average the responses and calculate the percent standard error. If the percent standard error is less than or equal to 10, the current is stable and the oocyte can be used to calculate the potentiation. Note that the last wash step is not saved as a

clampfit file since it is just a wash step. For multiple BZD potentiation runs, start with the experiments using the lowest concentrations of GABA and work up to the ones using the highest concentrations. The ten minute wash between BZD application and the beginning of the next run is sufficient to remove all the BZD. Remember that for BZD experiments you need to use glass (not plastic) for the drug solutions and drug plates and need to clean the opus (10% DMSO) and drug plates (70% isopropanol) specially.

Table A1.6 BZD Potentiation Protocol

Time	Application	Source	Rate
*			
30 sec	ND96	B	Profile 1
30 sec	GABA	Drug Plate	Profile 2
287 sec	ND96	B	Profile 3
*	*Repeat * to * 4 times with a high [GABA] the first time and EC_{5-10} GABA the next 3 times.*		
30 sec	ND96	B	Profile 1
30 sec	EC_{5-10} GABA + BZD	Drug Plate	Profile 2
241 sec	ND96	B	Profile 3
360 sec	ND96	B	Profile 3

A1.5.3 BZD $EC_{50,P}$ Protocol

Table A1.7 BZD $EC_{50,P}$ Protocol

Time	Application	Source	Rate
30 sec	ND96	B	Profile 1
30 sec	High dose of GABA	Drug Plate	Profile 2
285 sec	ND96	B	Profile 3
*			
30 sec	ND96	B	Profile 1
30 sec	EC_{5-10} GABA	A	Profile 1
30 sec	EC_{5-10} GABA + BZD	Drug Plate	Profile 2
279 sec	ND96	B	Profile 3
*	*Repeat * to * for all concentrations of BZD.*		
30 sec	ND96	B	Profile 1
30 sec	ND96	Drug Plate	Profile 2
285 sec	ND96	B	Profile 3

This is a modified version of the GABA EC_{50} protocol and is called "GABA_BZD_EC50." The set-up uses ND96 with calcium on Buffer B, EC_{5-10} GABA in ND96 with calcium as Buffer A and the drug plates contain EC_{5-10} GABA and the BZD unless otherwise specified. The protocol begins by simply applying a high dose of GABA as normally done to "wake-up" the receptors. The second part of the protocol is where the BZD dose-response relationship is determined. I use 0 μM BZD for my lowest dose and then increase the concentration (1, 3.2, 10, 3, etc.). BZD have nM EC_{50} values and only one binding site, so Hill coefficients are between 0.8 and 1.1. I usually start with 1 nM and go as high as 10 μM for FLZM. Diazepam (which we also have in the drug cabinet) is supposed to have a slightly lower EC_{50}.

There are two ways to analyze this data. The first is to take the average of all the EC_{5-10} peak currents and then subtract this value from the peak current for each of the BZD doses. This method works especially well if the GABA response does not drift. The second method is to use the GABA response right before the BZD application as the baseline for the BZD peak current. In general, the data from the BZD $EC_{50,P}$ takes longer to analyze than for a GABA EC_{50} as each oocyte usually needs to be analyzed one at a time. Additionally BZD solutions must be made for each concentration of GABA used. Again, don't forget to use glass-coated drug plates and glass tubes and to wash the Opus and drug plates carefully.

A1.5.4 Pentobarbital EC_{50} Protocol

Use the same protocol as the GABA EC_{50} but use pentobarbital instead of GABA. Pentobarbital has a Hill coefficient between 2 and 3 so the dose response curve is much steeper and therefore a smaller range of concentrations are needed. The PB EC_{50} for the

wild type αβ $GABA_AR$ is around 1.4 mM. I have not used PB with the αβγ $GABA_AR$. Evidence indicates that PB binds to the transmembrane region of the β subunit,[4-6] thus it is possible the PB EC_{50} is similar for both the αβ and αβγ receptors. My data (Chapter 6) suggest PB is a full agonist at the wild type αβ receptor, therefore PB can be used to determine whether GABA has become a partial agonist for a given mutation. The caveat with PB is that in addition to activating the $GABA_AR$, it also blocks the channel, thus the rebound current is used to determine the "peak current" at blocking concentrations of PB.

A1.6 Wild type tTraces

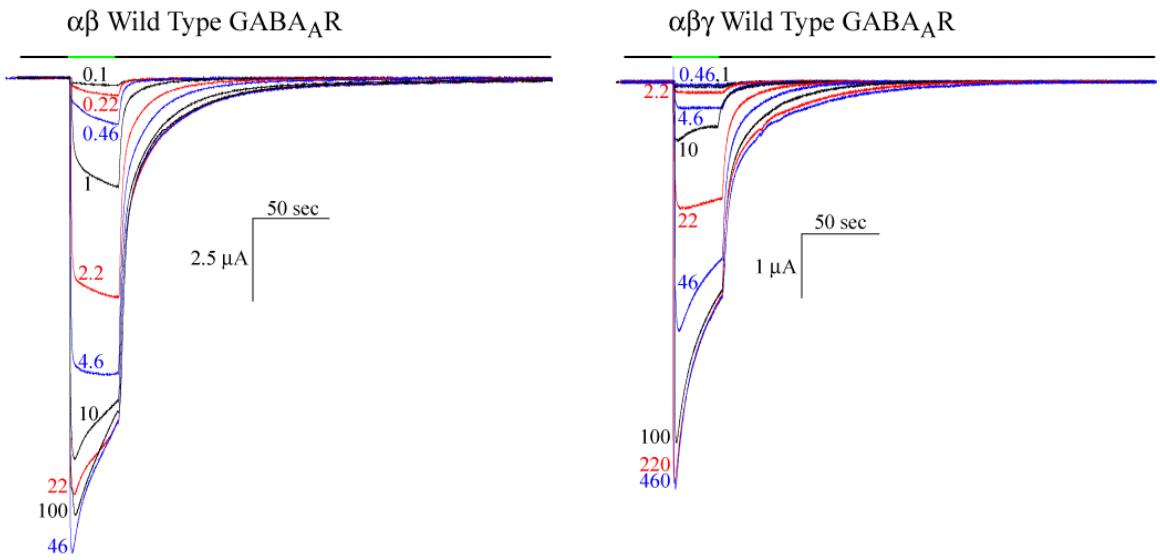

Figure A1.4 GABA traces of the wild type $GABA_AR$. Concentrations are given in µM. The αβγ (*right*) has more desensitization than the αβ (*left*) receptor. *Left*: For the oocyte shown here, the αβ $GABA_AR$ has an EC_{50} of 2.4 µM, a hill coefficient of 1.3, and I_{max}=-12.8 µA. *Right*: For the oocyte shown here, the αβγ $GABA_AR$ has an EC_{50} of 33 µM, a hill coefficient of 1.7, and I_{max}=-7.8 µA. Black bars denote ND96 and green bars denote application of GABA.

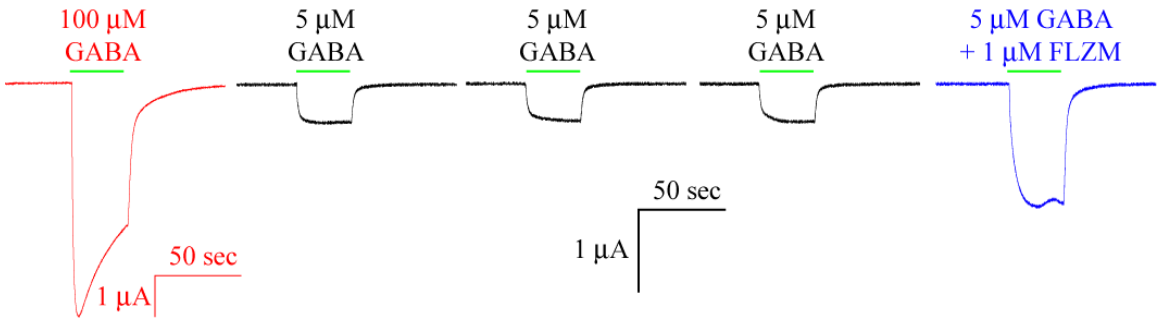

Figure A1.5 Sample traces from a FLZM potentiation experiment. This oocyte contained wild type αβγ $GABA_AR$ and had a FLZM potentiation of 2.18. A high dose of GABA is applied first (red, separate scale bars), then three doses of $EC_{5\text{-}10}$ GABA (black) and finally $EC_{5\text{-}10}$ GABA with 1 µM FLZM (blue). The average and standard error of the black traces is used to determine if the GABA response is stable. Green bars denote drug application. *Note*: Part of the washout has been removed from the trace to fit all traces on one line.

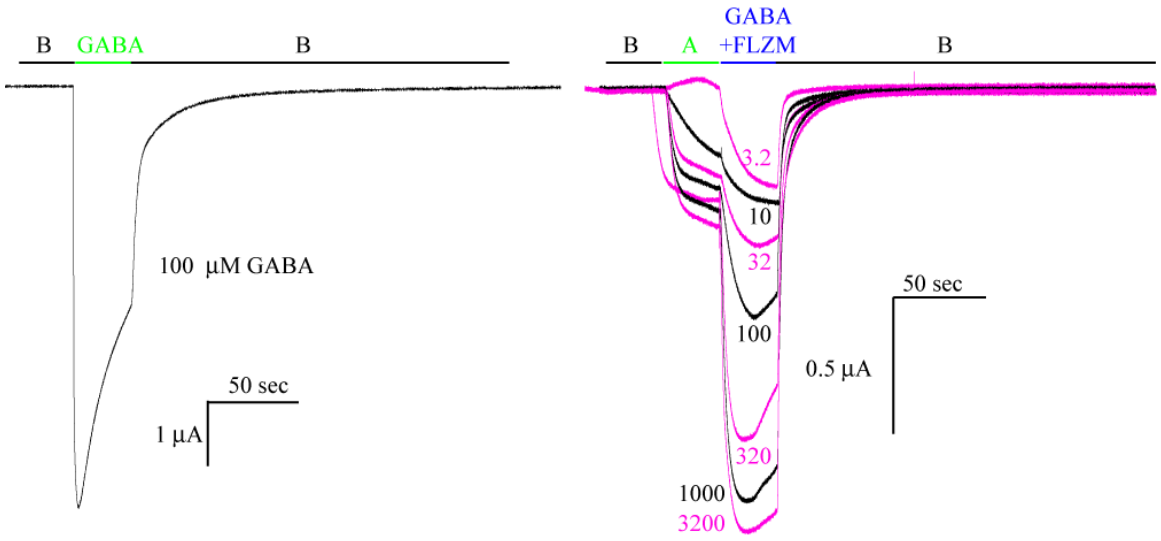

Figure A1.6 Sample traces from a FLZM EC_{50} experiment. *Left*: A high dose of just GABA is applied first to "wake-up" the receptors. Black horizontal bars at the top denote ND96 while the green bars denote GABA application. *Right*: Determination of FLZM dose-response relationship. Initially ND96 is applied (black bar), then $EC_{5\text{-}10}$ of GABA (green) is washed on through pump A, followed by application of $EC_{5\text{-}10}$ GABA and FLZM (blue bar), and finally the drugs are washed off with ND96 (black bar). In this case, the GABA induced current (under the green bar) was not stable. Concnetrations of FLZM are provided in nM. For the wild type αβγ receptors shown here, $EC_{5\text{-}10}$ is 5 µM GABA.

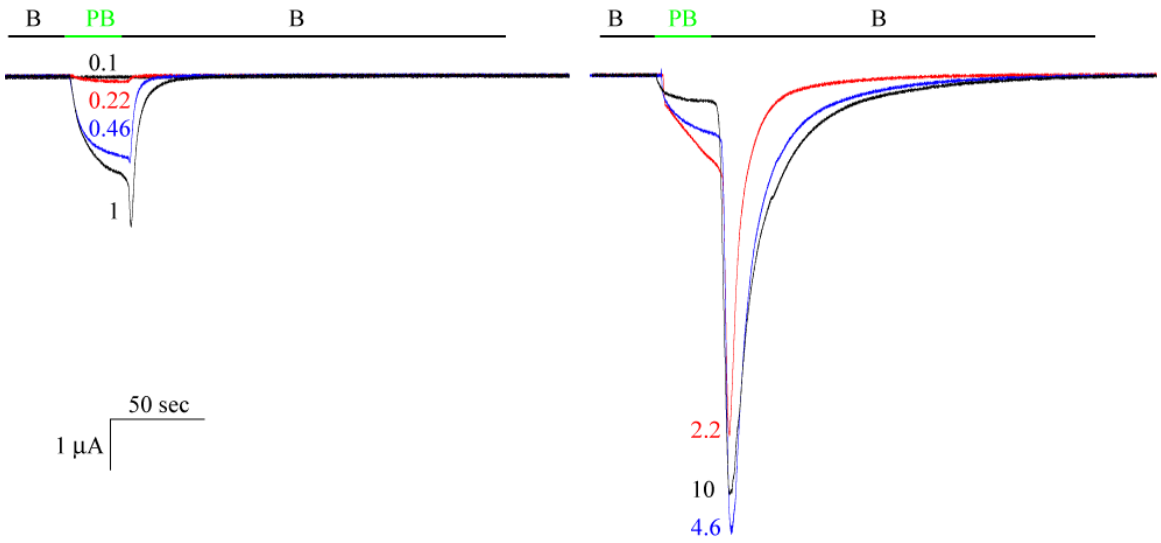

Figure A1.7 Sample pentobarbital traces for wild type αβ $GABA_AR$. Concentrations are given in mM. For [PB] > 0.46 mM, there is significant rebound current which is used as the peak response. Black horizontal bars (B) represent ND96 and green bars represent drug application.

A1.7 References

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